Induction of Quinone Reductase NQO1 by Resveratrol in Human K562 Cells Involves the Antioxidant Response Element ARE and is Accompanied by Nuclear Translocation of Transcription Factor Nrf2

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Abstract: The phytochemical resveratrol has been reported to induce NQO1, an enzyme involved in detoxification reactions, by as yet undetermined mechanisms. Using K562 cells as a model, we showed that 25-50 μM resveratrol increased NQO1 that peaked at 24-48 h. A 2.5-fold rise in NQO1 protein levels was accompanied by a comparable elevation in mRNA copy number and a 3- to 5-fold increase in NQO1 enzymatic activity. Fluorescent microscopic analysis in combination with transfection experiments with plasmids harboring different segments of the 5'-flanking region of NQO1 gene linked to a reporter provided evidence that the modulation of NQO1 gene expression by resveratrol involved the antioxidant response element ARE, accompanied by an increase in the state of phosphorylation of transcription factor Nrf2 and its re-distribution to the nucleus. This change in cellular localization of Nrf2 may be linked to resveratrol-elicited disruption of the Nrf2-Keap1 complex in the cytosol, followed by the translocation of Nrf2 to the nucleus where it locates the ARE-containing 5'-promoter region of NQO1 leading to its transcriptional activation.

Key Words: Resveratrol, quinone reductase 1, K562 cells, transcriptional control, transcriptional factor Nrf2, antioxidant response element ARE.

INTRODUCTION

The great majority of cancer in humans are considered attributable to environmental factors [1], which can include carcinogens, mutagens and genetic modulators and promoters from dietary sources [2, 3]. Counteracting the potentially damaging effects of food-derived components is the wide array of other agents contained in the diet capable of lowering cancer risks. These include dietary fiber, vitamins, minerals [4] and additionally, also phytochemicals from fruits and vegetables [5-12].

Resveratrol (3,5,4'-trihydroxystilbene) is a grape-derived polyphenol with claimed health benefits equal to and perhaps even surpassing those ascribed to the consumption of fruit and vegetable rich diets. Although a plethora of disease fighting activities has been described for resveratrol [13-22], interest in this chemical appears to largely lie in its reported ability to protect against coronary heart disease and cancer, both considered major causes of morbidity and mortality in developed countries [23-26]. In 1997, Pezzuto and coworkers made the seminal observation that resveratrol conferred protection against the stages of initiation, promotion and progression of carcinogenesis [27]. Since then, several explanations, ranging from antioxidant, anti-inflammatory, free radical scavenging, and signal modulatory activities have been proposed for the chemopreventive and other biological effects of resveratrol [8, 14, 24]. In addition, resveratrol has been reported to significantly affect the expression of genes including quinone reductase NQO1, previously known as DT-diaphorase [27-31].

NQO1 is an FAD^+-containing, dicoumarol-sensitive cytosolic protein that uses either NADPH or NADH as cofactor to catalyze two-electron reduction of quinones, which effectively circumvents one electron reduction-dependent formation of semiquinone radicals and hence potentially, generation of reactive oxygen species ROS [32-38]. NQO1 also functions as a nitroreductase as evidenced by its ability to use substrates such as dinitropyrenes and nitrophenylaziridines [39-41]. NQO1 is present abundantly in tissues requiring protection from oxidative damage [42], and is also significantly over expressed in tumor cells as compared to the tumor-adjacent normal counterpart [43-45]. As a key phase II detoxifying enzyme, NQO1 has been studied largely in the context of metabolism of drugs in humans [46-50]. Not surprisingly therefore, genetic polymorphism of NQO1 that renders loss of its enzymatic properties can compromise individuals' ability and capacity to metabolize xenobiotics, thus putting them at an increase in risk for diseases associated with exposure to environmental chemicals, such as, pediatric leukemias [51-53], urological malignancies [54], basal cell carcinomas and myeloid leukemia [55, 56]. In addition, mice with ablated NQO1 show increased susceptibility to BaP- or 7,12-dimethylbenz (a)anthracene-induced skin carcinogenesis [57, 58]. These observations underscore the interest to identify agents capable of inducing NQO1 as well as to investigate mechanisms that underlie NQO1 gene expression and regulation.

Resveratrol has been reported to induce phase II enzymes including NQO1 [27, 28, 59, 60] by as yet incompletely

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elucidated mechanisms. In this study, we investigated control of NQO1 gene expression by resveratrol using the human erythroleukemia K562 cell line as a model. These cells have previously been used to determine the effects of resveratrol on inhibition of cell proliferation and differentiation [61-63], and modulation of apoptosis [64, 65] and gene expression [62, 66]. We showed that 25-50 μM resveratrol increased NQO1 expression that peaked at 24-48 h. A 2.5-fold elevation in NQO1 protein levels was accompanied by a corresponding rise in its mRNA copy number and a greater than 5-fold increase in NQO1 activity. By combining fluorescent microscopic analysis with transfection experiments employing plasmids that contained different 5'-promoter regions of NQO1 linked to a reporter gene, we observed that modulation of NQO1 gene expression by resveratrol involved the ARE, concomitant with or accompanied by a change in the cellular localization of the transcription factor Nrf2, which, as a member of the "cap 'n' collar" family of basic leucine zipper trans-acting factors [67, 68], has been shown to participate in the regulation of phase II detoxifying genes [69, 70] as well as combating oxidative stress [71]. Our results suggest that resveratrol increases NQO1 expression by disrupting the cytosolically-localized Nrf2-Keap1 complex, facilitating the release of Nrf2 and its subsequent translocation into the nucleus, where it locates the 5'-promoter element of NQO1 containing the ARE to result in its transcription activation.

MATERIALS AND METHODS

Materials and Cell Cultures

K562 cells were obtained from the American Tissue Culture Collection and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and harvested at the indicated time. Resveratrol was purchased from Sigma Chemical Co. or LKT Laboratories, Inc, USA. Primary antibodies for Nrf2, Keap1, actin, histone H1, and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

NQO1 Assay

Control and resveratrol-treated cells were harvested. Cell pellets were washed twice with cold PBS and stored at -80°C until assayed. To prepare cell-free extracts, the frozen cell samples were suspended in lysis buffer (1% NP40, 1% aprotinin in PBS, $1x10^6$ cells/0.5-1.0 ml) and disrupted by gentle homogenization. The homogenate was centrifuged at 13,000 rpm for 3-5 min, and the supernatant was removed and used for total protein concentration determination by using the Coomassie (Bradford) protein assay kit from Pierce. Lysates were diluted with buffer to give equal protein concentrations and then used to assay NQO1 activity. This involved adding 20 μg protein to a solution containing 100 mM Tris-HCl, pH 8.5, 500 μM NADH, 10 μM menadione, and 0.3 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) with and without addition of 100 μM dicoumarol. The assays were performed at 37°C and terminated by the addition of 0.02 ml 20% SDS. NQO1 activity was measured as menadione-coupled reduction of MTT, in which differences in OD_{610} measurements of samples not containing dicoumarol compared to identically incubated samples with added dicoumarol were calculated. Since absorbance differences were found to be linear for at least 10 minutes, a single reading at 5 min was used to calculate NQO1 activity.

Western Blot Analysis

Whole cell lysates were prepared by suspending cells in lysis buffer (50 μl/10^6 cells) containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol (DTT), 0.5% Nonidet P-40, and 5% glycerol supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μl/ml of the protease inhibitor cocktail obtained from Sigma Chemical. Cells were lysed by three freeze-thaw cycles. The extracts were centrifuged and the clear supernatants were stored in aliquots at -80°C. Extracts from control and treated cells (10-30 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with the respective primary and secondary antibodies. Specific immunoreactive bands were detected by enhanced chemiluminescence or using color reactions. To reprobe blots, the membranes were first incubated for 10–30 min at 50°C in buffer containing 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS. Intensity of the specific immunoreactive bands on the membranes was quantified by densitometry.

Preparation of Nuclear Extracts

Nuclear extracts were prepared with kit purchased from Active Motif (InVitrogen, Carlsbad, CA), as per instructions provided in the manufacturer's manual. Briefly, K562 cells ($6x10^6$) were washed twice with cold PBS containing phosphatase inhibitors, resuspended in 0.25 ml hypotonic buffer, and transferred to a pre-chilled microcentrifuge tube. After 15 min incubation on ice, 25 μl detergent was added and the samples were vigorously vortexed for 10 seconds, followed by 30 s centrifugation at 14,000 × g in a refrigerated microcentrifuge. After the cytoplasmic fraction was removed, the nuclear pellet was resuspended in 50 μl complete lysis buffer supplemented with protease inhibitors containing 10 mM DTT, and incubated on ice for 30 min with intermittent vortexing. The solubilized nuclear extract was obtained by centrifugation at 14,000 × g for 10 min, and was stored in aliquots at -80°C.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts prepared from control and 8 h, 50 μM resveratrol treated cells as described above, were used for EMSA [72, 73]. The ARE oligodeoxynucleotides used for EMSA followed published sequence [74]:

5'-ctcGCAGTCACAGTGACTCAGCAGAATCTgc-3'

Consensus double-stranded ARE was annealed by incubating at 95°C for 3 min, followed by 60 min cooling at room temperature in the presence of STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and 5'-end labeled in a reaction containing 3.75 pmol ARE oligomer, 25 μCi [γ-^{32}P] ATP (3000 Ci/mmol, Amersham Biosciences) and T4 polynucleotide kinase (Promega). For

gel shift analysis, 10 µg of nuclear extract from control and resveratrol-treated cells was incubated with the ^{32}P-labeled oligomer. To determine specificity of complex formation, 100-fold excess unlabeled ARE oligomer containing the consensus or site-modified mutated ARE sequence was first incubated with the nuclear extracts for 10 min, followed by the addition of the labeled ARE. Binding involved 20-60 min incubation at room temperature in buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol and 50 µg/ml poly(dI-dC)·poly(dI-dC). The samples were electrophorezed on a native 4.5% polyacrylamide gel using 1 X-Tris glycine buffer. Formation of ARE:protein complexes was identified by autoradiography of the dried gel.

RNA Isolation and Quantitative Real-Time PCR

Total cellular RNA was isolated from control and resveratrol treated K562 cells at different time points, using TRIzol reagent (InVitrogen) according to protocols provided by the manufacturer. DNA-free (Ambion, Austin, TX) was used to remove DNA contamination. RNA purity and quantitation was based on agarose gel electrophoresis, and $A_{260/280}$ absorbance ratio. First strand cDNA synthesis used 2 µl total RNA incubated at 42°C, for 50 min with Superscript RNase H$^-$ reverse transcriptase (InVitrogen). A 10-fold diluted cDNA served as the template for PCR. To amplify and quantitate the human NQO1 gene, PCR was performed using the Lightcycler PCR system (Roche Diagnostics, Mannheim, Germany), in glass capillaries in a final volume of 20 µl which contained 1x Lightcycler master mix, 3 mM $MgCl_2$, 1 µM of each set of primers, and 2 µl of cDNA template or external standard template.

Primers for amplifying NQO1 were 5'-GGCTGAAC AAAAGAAGCTGG-3' and 5'-CGGAAGGGTCCTTTGT CATA-3'. As controls, 28S rRNA expression was also measured using 5'-TACTGATGATGTGTTGTTGCCA-3' and 5'-GGTCGTCTACGAATGGTTTAGC-3' as primers. Amplification of NQO1 consisted of heating samples at 95°C for 30 s followed by 40 cycles of heating at 20°C/s to 95°C with a 1-s hold, cooling at 20°C/s to 55°C with a 1-s hold, and heating at 20°C/s to 72°C with a 10-s hold. A melting curve was generated by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 60°C, and slowly heating at 0.2°C/s to 95°C, with fluorescence collection at 0.2°C intervals.

Human NQO1 fragment was amplified by conventional PCR and cloned into PCR 2.1-TOPO vector (InVitrogen) for transformation in TOPO10 competent cells. The plasmid DNA containing human NQO1 was purified using the Mini Plasmid Extraction Kit (Qiagen, Valencia, CA). Real-time PCR was performed using the Light Cycler DNA Master SYBR Green 1 kit according to provided instructions. Tenfold serial dilutions of the purified plasmid DNA containing 100 to 10^7 copies of NQO1 in 2 µl were used as standards to calculate NQO1 mRNA copy number in K562 cells. The data obtained were analyzed using the Lightcycler software provided by the manufacturer. Only log-linear portion of amplification was chosen for analysis. The background fluorescence was removed by setting a noise band, and a standard curve was prepared by plotting the crossing point (C_t) versus the log of copy number based on standards included in each run. Copy numbers for the experimental samples were calculated by comparing the crossing points of the samples with those of the standards. Experiments were performed in duplicate for each data point.

Construction of NQO1 Promoter-Reporter Gene Plasmids and Secreted form of Human Placental Alkaline Phosphatase (SEAP) Assay

To identify the transcriptional elements responsible for induction of NQO1 by resveratrol, NQO1 promoter-reporter gene constructs containing different upstream regions of the NQO1 promoter were generated. DNA fragments containing the human NQO1 gene promoter region were PCR-amplified from genomic DNA isolated from K562 cells. Primer sense sequences were respectively: 5'-cggggtaccTCAGGTGATC CACCCACCTC-3' (positions -787/-768) for generating pSEAP2-787; and (2) 5'-cggggtaccCTGGACTCTCTTGG GACGAC-3' (positions -364/-345) for generating pSEAP2-364. In both primer sets, the lower case sequence showed add-on Kpn I restriction site. Antisense primers for both reactions were, respectively, 5'-ccgctcgaGAGTCGCGTGT GTAGTGCACG-3' (positions +1/-20) with lower case showing an add-on Xho I restriction site. The PCR amplified fragments were digested with Kpn I and Xho I, and then cloned into pSEAP2 plasmid (BD Bioscience Clontech, USA) to generate constructs pSEAP2-hNQO1-787 and pSEAP2-hNQO1-364, respectively.

Reporter SEAP assays were performed using the following procedure. K562 cells (1×10^6 /ml, total, 2ml) were transfected using 10 µl Lipofectamine (Invitrogen, Carlsbad, CA) and 5 µg of plasmid DNA containing 4 µg of SEAP reporter from either pSEAP2-hNQO1-787 or pSEAP2-hNQO1-364, and 1 µg of pEGFP as control to normalize the efficiency of transfection. Four h after transfection, identical aliquots of transfected cells were added to separate wells of a 24-well plate, followed by different doses of resveratrol. Control and resveratrol-treated cells were incubated for another 48 h, at which time media were removed and used to measure the secreted SEAP activity using protocol provided by the manufacturer (BD GreatEscAPe^{TM} SEAP). In addition to pEGTP, SEAP activities were also normalized for efficiency of transfection by cell number.

Fluorescence Microscopy Analysis

K562 cells were harvested, washed with phosphate-buffered saline (PBS), added to slides and fixed in methanol for 5 min at room temperature. Fixed cells were treated with 0.1% Triton X-100 in PBS for 5 min and washed three times with ice-cold PBS. After blocking with 2.0% bovine serum albumin (BSA) in PBS for 30 min on ice, cells were incubated overnight at 4°C with rabbit polyclonal Nrf2 antibody (Santa Cruz) diluted 1:400 in 2% BSA solution. The Nrf2-reacted cells were washed with PBS, and incubated with FITC-conjugated anti-rabbit IgG, 4°C for 1 h in the dark. FITC-labeled cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI, 1 µg/ml; Molecular Probe) for 5 min. Fluorescence microscopy was performed on a Nikon microscope and images were captured using a digital camera (Optronics). The images were processed using Image-Pro Plus software.

RESULTS

Induction of NQO1 Expression by Resveratrol in K562 Cells

To determine effects of resveratrol on NQO1, K562 cells were treated with different concentrations of resveratrol (up to 50 μM) for 48 h. NQO1 mRNA copy number was quantitated using real-time PCR analysis (Fig. **1A**). These results showed a 2- to 5-fold dose-dependent increase in NQO1 mRNA expression. Next, effects of resveratrol on enzyme activity were studied using menadione-coupled reduction of tetrazolium dye. Results in Fig. **1 B** demonstrated that NQO1 was increased by resveratrol; extracts from 25-50 μM treated cells showed a 3-fold stimulation of enzyme activity, which corresponded to a significant 1.5- to 1.6-fold induction in NQO1 protein level (Fig. **1C**), and suggested that the activity change involved an increase in *de novo* synthesis of the NQO1 protein. A time-course study using 25 μM resveratrol (panels D-F) showed that the induction of NQO1 mRNA peaked at 24 h (panel D), whereas changes in its enzyme activity and protein levels reached maximum at 48 h (panel E and F).

Resveratrol Activates the NQO1 Gene at the Transcriptional Level

To further investigate mechanism of induction of NQO1 by resveratrol, reporter gene assays were performed. To estimate the relative strength of the upstream promoters of the NQO1 gene, plasmids containing various 5'-flanking region of NQO1 promoter linked to a SEAP reporter were constructed. Accuracy of the constructs was verified by direct sequencing of the recombinants. Plasmid DNA isolated from various recombinants was used for transient transfection studies in K562 cells. These experiments confirmed that the entire upstream region including the sequences harboring the ARE-responsive element (pSEAP2-hNQO1-787) is required for regulating NQO1 transcription. The importance of this upstream region was supported by the observation that pSEAP2-hNQO1-364 was devoid of activity, and that sequences containing only the ARE

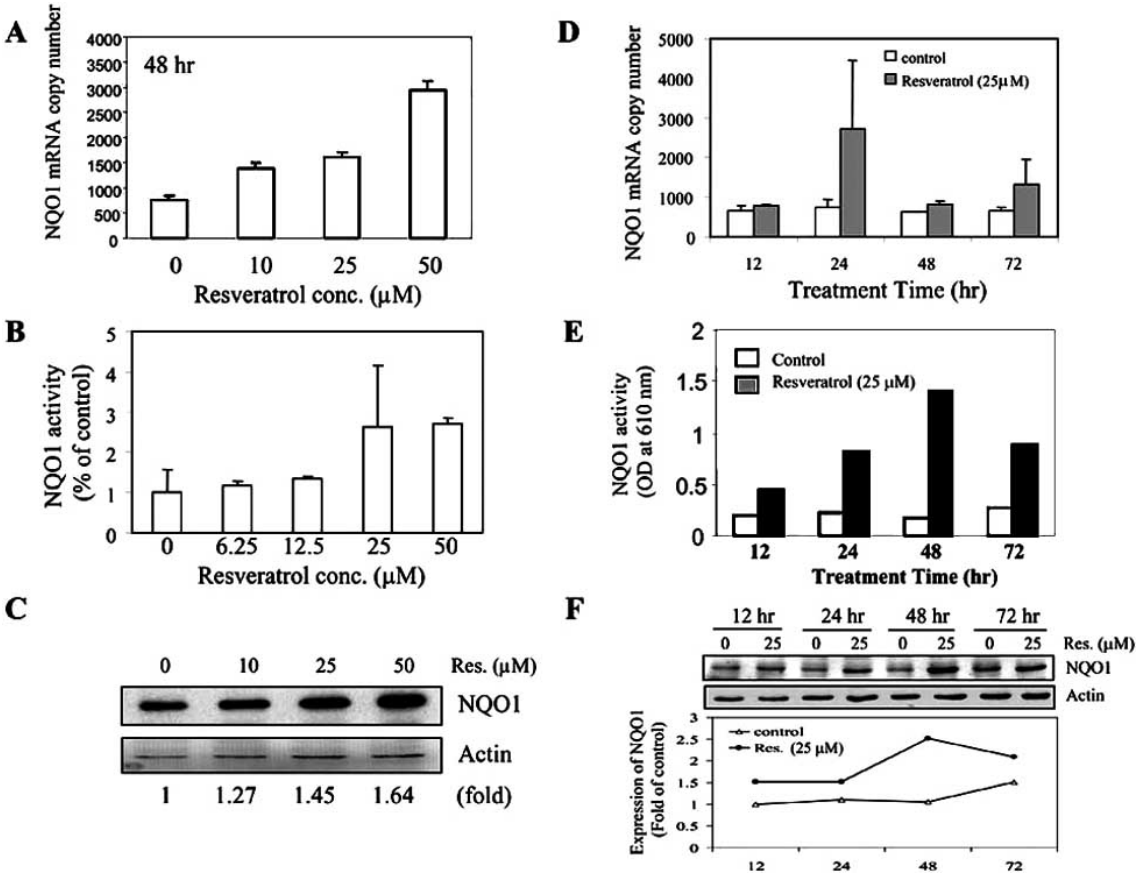

Fig. (1). Dose- and time-dependent induction of NQO1 by resveratrol in K562 human erythroleukemia cells. Cells were treated for 12-72 h (as indicated in the panels below) with various concentrations (0-50 μM) of resveratrol. ***Panel A.*** NQO1 mRNA copy number as determined by real-time PCR in control and resveratrol-treated K562 cells. The results were averaged from two separate experiments. ***Panel B.*** Changes in NQO1 activity in response to 48 h of treatment with different doses of resveratrol. The post-mitochondrial fractions were prepared and assayed for NQO1, as detailed in Materials and Methods. The results were averaged from three separate experiments with assays performed in triplicate each time. ***Panel C.*** Western blot analysis showing the effects of resveratrol on NQO1 protein level changes. ***Panels D-F.*** Temporal sequence of changes in NQO1 mRNA, activity, and protein resulting from treatment by 25 μM resveratrol. Cells were treated with 0.1% DMSO or 25 μM resveratrol, and harvested at the times indicated. Assays in Panels D-F were identical to those used in panels A-C.

(pSEAP2-ARE) or its mutated variant (pSEAP2-mARE) were also incapable of supporting reporter gene expression (Fig. **2A**). To test the effects of resveratrol on transcription activation of NQO1, reporter gene activity was measured in cells transfected with plasmid (pSEAP2-hNQO1-787), with and without further treatment with increasing doses of resveratrol. As shown in Fig. **2B**, resveratrol elicited a 3- to 4-fold increase in SEAP reporter activity.

Induction of NQO1 by resveratrol is associated with time-dependent change in expression and cellular location of transcription factor Nrf2

Recent studies have shown that transcription factor Nrf2 plays a central role in the transcriptional activation of phase II enzymes [75-77]. We therefore tested the possible involvement of Nrf2 in regulation of NQO1 by resveratrol. First, western blot analysis was performed to determine the relative accumulation of Nrf2 in the nucleus and cytosol in cells treated by resveratrol. Probing the blot with anti-Nrf2 antibody revealed that Nrf2 level in the nucleus increased as early as 8 h after resveratrol treatment, while its level in the cytoplasm remained relatively unchanged. Importantly, the increase in the nuclear fraction was accompanied by appearance of phosphorylated-Nrf2 (Fig. **3A**). These results suggest that Nrf2 translocated into nucleus following treatment by resveratrol. Immunofluorescence studies further showed that the staining pattern of anti-Nrf2 merged with that of DAPI only in resveratrol treated and not control cells, in support of the interpretation that Nrf2 became localized in the nuclei, as a result of treatment with the phytochemical (Fig. **3B**). Immunoblot analysis further demonstrated that resveratrol elicited time-dependent increases in Nrf2 up to 48 h, which were accompanied by corresponding decline in Keap 1 levels (Fig. **4A** and **4B**). In cells treated for 72 h with resveratrol, a noticeable reduction in the expression of Nrf2 and Keap1 was observed. RT-PCR analysis, however, failed to show change in Nrf2 mRNA expression in resveratrol-treated cells (Fig. **4C**).

We also tested the ability nuclear extracts prepared from control and resveratrol-treated K562 cells to form specific DNA:protein complexes using electrophoretic mobility shift analysis (Fig. **5**). Nuclear extracts of control and treated cells were incubated with human NQO1-ARE consensus sequence end labeled with [γ-^{32}P] ATP. Specificity of complex formation was ascertained by the inclusion of a 100-fold excess of cognate oligomer in the assay mixture, as well as using a mutated ARE oligonucleotide sequence. The slowest migrating complex (identified by arrow) was effectively competed by the inclusion of an excess of cognate ARE oligomer and not the mutated ARE oligomer (compare lane 5 with lane 6, panel A, Fig. **5**). Further-more, the complex failed to form when radiolabeled mutated ARE sequence was

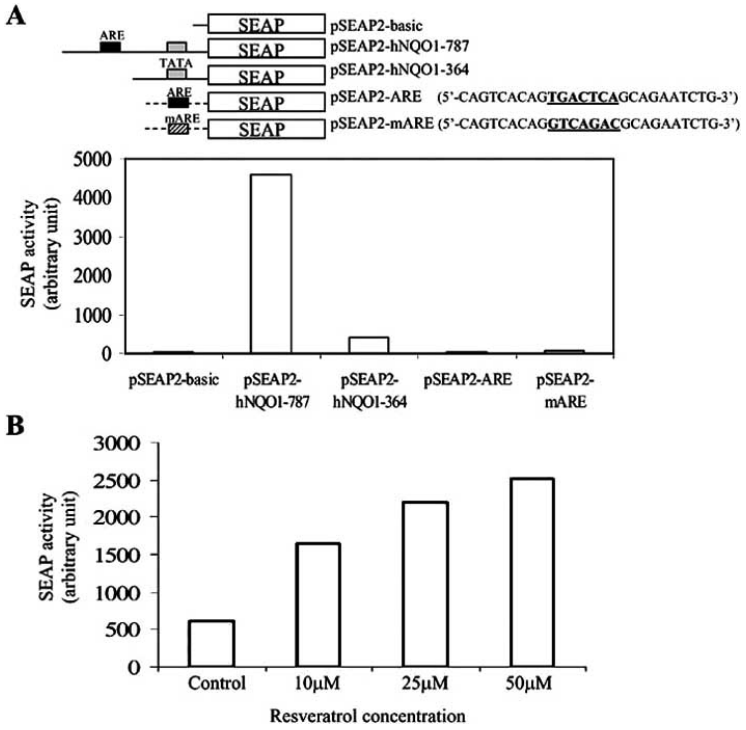

Fig. (2). Human NQO1 (hNQO1)-SEAP assay to quantitate transcriptional activation of NQO1 by resveratrol. ***Panel A.*** NQO1 promoter analysis. Various constructs involving 5'-flanking regions of the NQO1 gene linked to SEAP reporter were used to define the promoter regions required for induction of NQO1. These experiments confirmed that entire 5'-flanking region used in the present studies (up to -787), which contains the ARE-responsive element is required for activation of NQO1. ***Panel B.*** Effects of resveratrol on induction of NQO1 as revealed by SEAP activity utilizing the pSEAP2-hNQO1-787 construct. K562 cells were transiently transfected with plasmid prepared from the reporter gene, as described in Materials and Methods. The transfected cells were additionally incubated for 48 h with carrier (DMSO, 0.1%) or various concentrations of resveratrol.

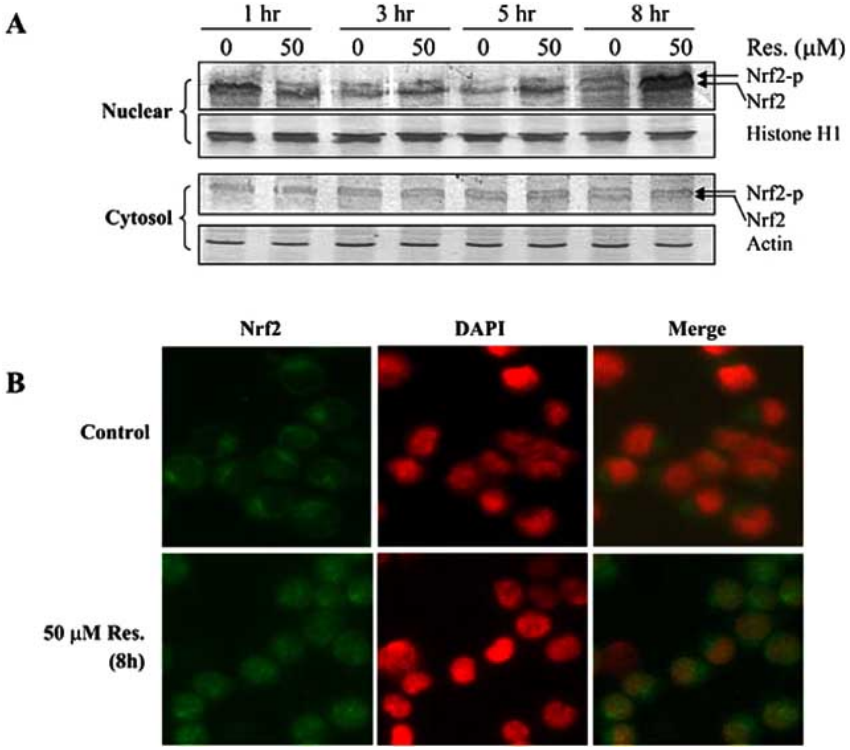

Fig. (3). Time-dependent increases on the expression and nuclear translocation of transcription factor Nrf2 in resveratrol-treated cells. K562 cells were treated with vehicle (DMSO, 0.1%) or 50 μM resveratrol, and harvested at the indicated times. Extracts were separated into the nuclear and cytosolic fractions, as described in Materials and Methods. ***Panel A.*** Equal amount of proteins (10-30 μg) from the separated fractions were analyzed for expression of Nrf2 by western blot analysis, using histone H1 and actin as loading controls for the nuclear and cytosolic extracts, respectively. The data shown are representative of two separate experiments with similar results. ***Panel B.*** Immunofluorescence analysis shows that resveratrol induces nuclear translocation of Nrf2. The merge shows that in resveratrol-treated cells, the FITC-labeled green fluorescence from Nrf2 is co-localized with 4',6-diamidino-2-phenylindole (DAPI) staining (red) used to reveal presence of the cell nuclei.

incubated with nuclear extracts (lane 7, panel A, Fig. **5**). Interestingly, no appreciable increase in the formation of this complex occurred using resveratrol-treated nuclear extracts (compare lane 4 with lane 3, panel A, Fig. **5**). To test whether Nrf2 might be part of the DNA complex formed, anti-Nrf2 was added to the nuclear extract prior to addition of labeled ARE. As control, anti-NQO2 was used. Although a slight reduction in DNA complex was found by addition of anti-Nrf2 (compare lane 2 with lane 3, panel B, Fig. **5**), no obvious super-shifted complex was readily identified in these studies. Since the ARE consensus sequence also contains an AP1 site, gel mobility shift was also performed using end-labeled AP1. A complex was clearly shown (lane 2, panel C, Fig. **5**). Interestingly, complex formation was increased by 30-40% using nuclear extracts derived from 8 h, 50 μM resveratrol-treated cells (lane 3, panel C, Fig. **5**). The specificity of this complex was demonstrated by its competition using excess unlabeled AP1 (lane 4, panel C, Fig. **5**).

DISCUSSION

NQO1, classically considered as a cytoprotective phase II enzyme, has undergone an expansion in cellular functions, as illustrated by recent reports documenting that NQO1-knockout animals show an increase in propensity for skin carcinogenesis [57, 58, 78] and display greater sensitivity towards cytotoxic quinones [79]. NQO1 provides an excellent model to investigate the regulation of gene expression by diet-derived chemopreventive agents for several reasons. First, NQO1 is an enzyme that is highly responsive to environmental and chemical stimuli, especially to monofunctional inducers [33, 80, 81]. NQO1 is induced by xenobiotics, antioxidants, oxidants, heavy metals, UV light, and ionizing radiations [48, 50, 82-85]. Such an increase in NQO1 expression could, in principle, elevate cellular levels of NAD^+ and potentially an increase in cellular capacity to more efficiently repair damages on DNA by the enzyme poly(ADP-ribose) polymerase [86-88]. Second, as demonstrated in this report, regulation of NQO1 by resveratrol appears to occur by a mechanism involving the nuclear translocation of Nrf2, and interaction with the ARE. Therefore, further characterizing this mechanism might provide new information on the regulation of gene expression by chemopreventive agents. Third, as a multitasking protein [46], NQO1 is mechanistically linked to the bioreductive activation of a number of prodrugs with potent chemotherapeutic activities [89-93]. For example, mitomycin C, a quinone-containing prodrug, is activated by NQO1 to reactive metabolites that crosslink DNA [94, 95]. Since a hypoxic environment prevails in solid tumors and

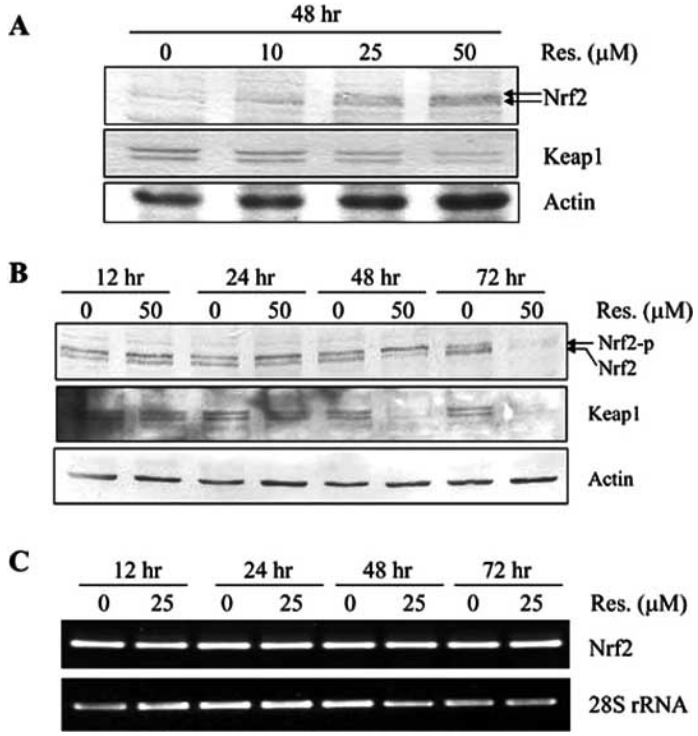

Fig. (4). Concentration and time dependence of induction of Nrf2 and Keap1 by resveratrol in K562 cells. ***Panel A.*** K562 cells were treated with vehicle (DMSO, 0.1%) or different concentrations of resveratrol for 48 h. Equal amounts of proteins (10-30 μg) from whole cell lysates were analyzed for Nrf2 and Keap1 expression by immunoblot analysis, using actin as the loading control. ***Panel B.*** Cells were treated for 50 μM resveratrol for different times and expression of Nrf2 and Keap1 determined as described in panel A. ***Panel C.*** Determination of effects of resveratrol on changes in Nrf2 mRNA levels by semi-quantitative RT-PCR analyses, with expression of 28S rRNA as loading control.

favors reductive metabolism [96-100], and because high levels of NQO1 expression is also found in tumors, induced expression of NQO1 may be exploited to complement these features for the design of chemicals that require their eventual conversion to active and potent drugs by NQO1 [101, 102]. In support of this concept, it is notable that NQO1 inducers such as 1,2-dithiole-3-thione (D3T) and oltipraz, are already being tested *in vitro* and in the clinic for chemoprevention and to enhance patient's response to bioreductive agents [76, 103]. We propose that induction of NQO1 by resveratrol suggest that this grape-derived phytochemical could be considered as a bioreductive agent enhancer.

Results of this investigation show that the addition of resveratrol to K562 cells induces an increase in expression of the NQO1 gene by a mechanism which involves the activation of its transcription, leading to the subsequent increase in NQO1 mRNA and protein levels (Fig. **1**). The concentrations (≥ 25 μM) of resveratrol required to elicit the observed NQO1 gene response are similar to the dose range shown for chemoprevention and cardioprotection, suggesting that the ability of resveratrol to induce NQO1 may be closely related to its observed biological effects.

Reporter gene expression assays localized the resveratrol-mediated induction of NQO1 to the entire promoter region harboring the ARE (Fig. **2**), which is known to be the cis-acting element recognized by several trans-acting factors including Nrf2 [75-77, 103-105]. The involvement of Nrf2 in trans-activation of NQO1 gene transcription, in response to resveratrol, is supported by immunoblot data showing that an increase in its presence in the nucleus occurred earlier than the subsequent increase in NQO1 transcription (Fig. **3**). The resveratrol-mediated nuclear translocation of Nrf2 was also supported by fluorescent microscopy analysis showing convergence of signals corresponding to nuclear staining by DAPI with that reflecting the presence of Nrf2. To better understand transcriptional activation of NQO1 gene by resveratrol, nuclear extracts were prepared from control and resveratrol-treated cells, and binding to radiolabeled ARE was studied. Although these studies revealed genuine ARE complexes, as supported by competition using excess unlabeled ARE and not mutated ARE, and also inability to form complexes using mutated ARE, they failed to show an increase in the ARE complex with nuclear extracts prepared from resveratrol-treated cells (Fig. **5**, panel A). Attempts to show the requirement of Nrf2 for ARE-mediated complex formation, as would be expected by anti-NRF2 induced super-shifted complex formation, also were unsuccessful (Fig. **5**, panel B). A number of explanations may be offered for the observed results. First, Nrf2 may be necessary but not sufficient for transcriptional activation of NQO1 by resveratrol. Binding of Nrf2 to ARE does not involve its formation as homodimers [106], indicating that its transcriptional competence and commitment requires heterodimerization with other proteins. A cadre of proteins, including

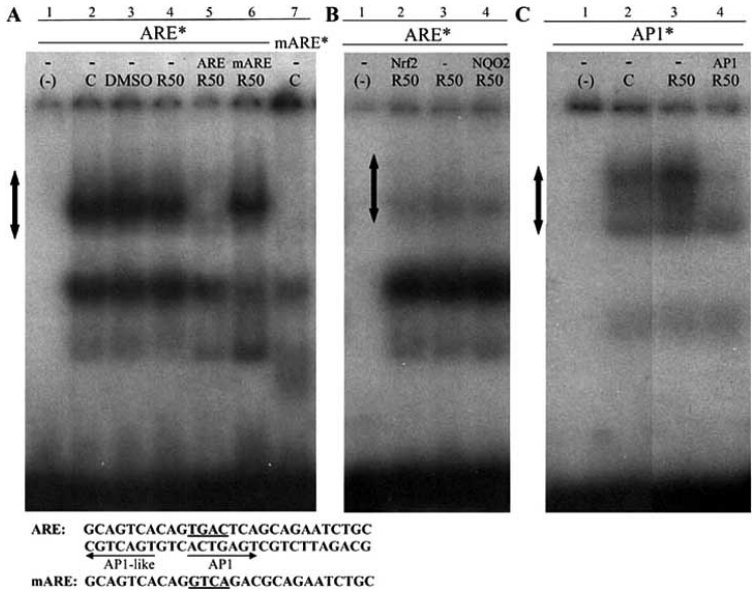

Fig. (5). Electrophoretic mobility shift assay (EMSA) to determine effects of resveratrol on DNA:protein complex formation. Gel mobility shift assays were performed as described in Materials and Methods. ***Panel A.*** Nuclear protein extracts prepared from control and cells treated for 8 h with 50 μM resveratrol were used for EMSA. Human NQO1 ARE was 5'-end labeled with [γ-^{32}P]ATP. For gel shift analysis, labeled ARE was incubated with 10 μg nuclear extract, in the presence of 50 μg/ml poly(dI-dC)·poly(dI-dC) (final concentration), with and without the addition of 100-fold excess unlabeled competitor ARE DNA or ARE mutated DNA. Lane 1, unbound radiolabeled DNA; lanes 2-4, nuclear extracts prepared from control (lane 2), cells treated with carrier amounts of DMSO (lane 3) and cells treated with 50 μM resveratrol for 8 h (lane 4); lanes 5 and 6, identical to lane 4 except that 100-fold cognate ARE oligomer (lane 5) or mutated ARE oligomer (lane 6) was also included; lane 7, gel shift assays using 5'-end-labeled mutated ARE. The ARE specific complex is identified by the vertical arrow in the autoradiogram. Notably, this complex was almost completely competed with 100-fold cognate ARE oligomer (compare lane 5 with lane 4) and not mutated ARE oligomer (compare lane 6 with lane 4). Further, the complex did not form when end-labeled mutated ARE oligomer was used (lane 7). ***Panel B.*** Lane 1, unbound radiolabeled DNA; lanes 2-4, gel mobility assays using nuclear extracts prepared from 8 h, 50 μM resveratrol treated cells, with (lane 2) and without (lane 3) addition of 3 μg of anti-Nrf2 (H-300 from Santa Cruz), or anti-NQO2 (lane 4). The arrow showed a slight reduction in complex formation using anti-Nrf2; however, no obvious super-shifted complex was readily identified. ***Panel C.*** Gel mobility shift assays using [γ-^{32}P]ATP 5'-end-labeled AP1 sequences (from Promega) and nuclear extracts prepared from control and 8 h, 50 μM resveratrol-treated cells. Lane 1, unbound radiolabeled DNA; lanes 2, nuclear extracts prepared from 0.1% DMSO treated control cells; lanes 3-4, nuclear extracts from resveratrol-treated cells with (lane 4) and without (lane 3) addition of 100-fold cognate AP1 oligomer.

c-fos, Fra-1, Nrf1, ARE-BP-1, and the Jun and small and large Maf protein families, have been identified as partners of Nrf2 in transcription complex formation [69, 75, 107, 108]. Members of the Maf protein family have been shown to act both as trans-activators and as repressor of genes under the control of Nrf2. In the case of heterodimerization of Nrf2 with c-Jun, participation by unknown cytosolic factors has been reported [109]. It is noteworthy that in transcription induction of NQO1 mediated by nitric oxide, only Nrf2 and small maf proteins (but not any other transcription factor mentioned above) were detected in the nuclear proteins that bound to NQO1-ARE following treatment by nitric oxide [110]. These findings raise the possibility that one or more of the same regulatory subtleties in transcription might also apply to induction of NQO1 gene expression by resveratrol. A second possibility for our lack of ability to detect increased transcription complex formation with ARE using resveratrol-treated nuclear extracts may lie in the nature and dynamics of increased phosphorylated Nrf2. Nrf2 is known to be phosphorylated by a number of protein kinases [80, 111]. The best characterized example is its phosphorylation at S40 by protein kinase C, which has been shown to facilitate the release of Nrf2 from Keap 1 and translocation to the nucleus [112]. However, gel mobility shift analysis subsequently revealed that Nrf2/MafK interaction with the ARE is independent of PKC phosphorylation [112, 113], suggesting that modification by phosphorylation of Nrf2 is more closely coupled to its stability and/or release from Keap1 rather than its functionality, as measured by ARE-dependent complex formation [70, 111, 113]. Since resveratrol has been reported to potently inhibit activities of several forms of protein kinase C [114], we might expect that treatment by resveratrol would actually lower PKC-mediated phosphorylation of Nrf2. Thus, it seems unlikely that increased phosphorylation of Nrf2 is mediated by PKC. A plausible candidate for the observed effects is ERK. We have previously reported that resveratrol increased phosphorylated ERK levels, probably by increased protein stability [115]. Moreover, inhibition of ERK has been shown to inhibit binding of Nrf2 and induction of human γ-

glutamylcysteine synthetase genes [116, 117]. It is also conceivable that the failure to observe increased complex formation in nuclear extracts of resveratrol-treated cells may merely reflect the need for nuclear translocation of Nrf2 as well as other nuclear proteins that have half-lives considerably shorter than Nrf2 and whose turnover may be simultaneously stimulated during treatment by resveratrol. Additional studies will be needed to fill in the absent molecular details and to validate some of the aforedescribed possibilities.

An additional observation with possible significant implications resulting from this investigation relates to time course studies analyzing changes in expression of Nrf2 and Keap 1; reproducibly, we observed that prolonged treatment by resveratrol increased the turnover of both proteins. For example, Keap1 expression was marked reduced after 48 h of treatment by resveratrol. Similarly, 72 h resveratrol-treated samples showed precipitous decline in Nrf2 levels (Fig. **4B**). In this context, it is interesting to note that resveratrol has been reported to increase the turnover of secreted and intracellular amyloid-beta (Aβ) peptides produced from different cell lines – an effect which could be prevented by several selective proteasome inhibitors and by siRNA-directed silencing of the proteasome subunit beta5 [118]. Accelerated degradation of Nrf2 in resveratrol-treated cells could provide a self-limiting mechanism of induction of NQO1 by this phytochemical and perhaps other chemo-preventive agents as well. This built-in feature to limit the effects of chemopreventive agents may be highly desirable as sustained increases in Nrf2 and Keap1 might have untoward consequences, such as hyperkeratosis in the esophagus and forestomach [119, 120]. In the context of NQO1, its sustained overexpression resulting from continuous activation of Nrf2 could result in tolerance to benzene and induce resistance to degradation by p53 hot-spot mutants.

The mechanism by which resveratrol elicits increased translocation of Nrf2 remains to be elucidated. Data presented in this study show that increased nuclear translocation of Nrf2 (Figs. **3** and **4**) was accompanied by reduced levels of Keap 1. How do these cellular changes result from treatment by resveratrol? We propose that resveratrol acts by first interacting with specific membrane receptors. This possibility is supported by HepG2 cell studies showing that resveratrol is taken up into cells in part by an active transport mechanism [121], and also data reporting that resveratrol inhibits uptake of glucose and dehydroascorbic acid in human transformed myelocytic cells [122]. What follows from binding of resveratrol to membrane receptors? In our previous studies using cultured bovine pulmonary endothelial cells, we have shown, through choice of selective signaling inhibitors, that the resveratrol-induced cellular phenotype change was linked to actin microfilaments and microtubules [115]. Resveratrol (30 μM) has been reported to induce the clustering of Fas and its redistribution into cholesterol and sphingolipid-rich plasma membrane rafts in colon cancer cells [123]. It is notable that both the lipid and protein constituents of rafts are able to communicate with the cytoskeleton, in addition to serving as a cellular link for surface receptors, signaling molecules and adaptor proteins [124]. There is also evidence to suggest that lipid rafts play a role in actin reorganization [125-127]. Together, these studies suggest a model of control of NQO1 gene expression by resveratrol, as depicted in Fig. **6**. In this model, we propose resveratrol acts by first interacting with membrane of target cells, affecting the plasma lipid rafts in a way that elicits signaling events as well as modulate the actin

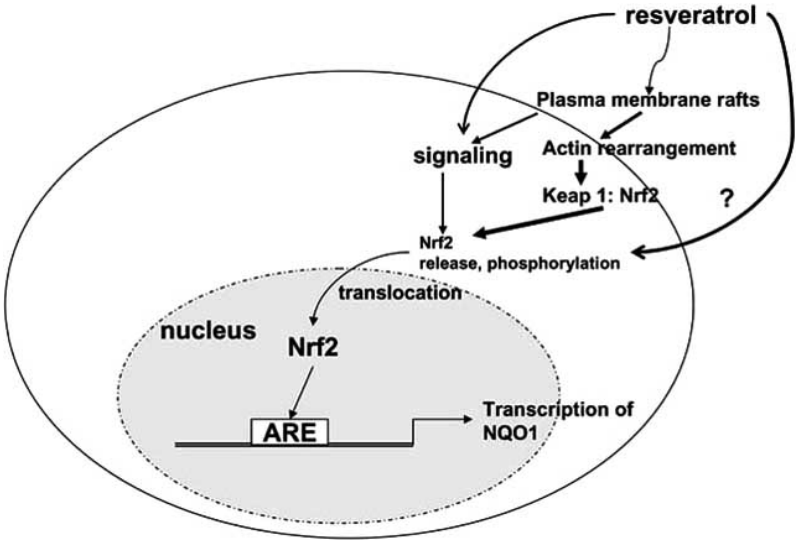

Fig. (6). A model for transcriptional regulation of NQO1 by resveratrol. In this model, resveratrol is postulated to act by first interacting with membrane of target cells. This interaction directly or indirectly perturbs the plasma membrane lipid rafts in ways that elicit signaling events and concurrently, also induces a rearrangement in the actin cytoskeletal architecture. Reorganization of the actin is hypothesized to affect the actin-tethered Keap1:Nrf2 complex, disrupting their interaction and resulting in release of Nrf2. The released Nrf2 becomes phosphorylated *via* signaling pathways concurrently affected by resveratrol. Subsequently, phosphorylated Nrf2 is translocated to the nucleus where it interacts with other nuclear proteins to locate the ARE and other DNA elements present at the 5'-flanking region of NQO1, to induce the increased transcription of NQO1.

cytoskeletal rearrangement. Reorgani-zation of the actin is hypothesized to affect interaction between Keap1 and Nrf2, resulting release of the latter, which may serve as a target for phosphorylation by one of several signaling pathways simultaneously affected by resveratrol. Once Nrf2 becomes phosphorylated, it is translocated to the nucleus where it interacts with other nuclear proteins to locate the ARE and other DNA elements present at the 5'-flanking region of NQO1, the end result being an increase in transcription of NQO1. Details of this model are continuing to be explored in ongoing studies in our laboratory.

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