Antiproliferative Effects of Resveratrol and the Mediating Role of Resveratrol Targeting Protein NQO2 in Androgen Receptor-positive, Hormone-non-responsive CWR22Rv1 Cells

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Abstract. *Trans-resveratrol, a polyphenol present in red wines and various human foods, was first reported to exhibit chemopreventive properties based on studies using a mouse skin cancer model. Our laboratory and others subsequently demonstrated the antiprostate cancer (anti-CaP) activity of resveratrol, as evident in its suppression of cell proliferation, arrest of cell cycle progression, and induction of apoptosis in androgen-responsive LNCaP and androgen-non-responsive DU145 and PC-3 CaP cells. However, the molecular mechanism of action of resveratrol has not been tested in androgen receptor (AR)-positive hormone-non-responsive CWR22Rv1 cells, which mimic the transition stages of prostate carcinoma. In this study, we investigated the antiproliferative effects of resveratrol in the context of modulation of growth suppression and NF-κB expression as mediated by resveratrol targeting protein NQO2, using both control and NQO2 siRNA silenced CWR22Rv1 cells. Exposure to resveratrol resulted in a potent, dose-dependent inhibition of CWR22Rv1 proliferation, which was accompanied by a reduction in the expression of NF-κB p65. The suppression of NF-κB p65 expression was abrogated in NQO2 siRNA silenced CWR22Rv1 cells, suggesting that NQO2 is upstream of and integral to the regulation of NF-κB p65. To our knowledge, this study is the first to reveal that resveratrol targeting protein NQO2 plays a mediating role in resveratrol-induced changes of NF-κB p65, which may contribute to the anti-CaP activities elicited by resveratrol.*

Resveratrol is a grape-derived polyphenol shown to have diverse biological properties. The chemopreventive activity of resveratrol stems from the observation by Pezzuto and co-workers demonstrating that this dietary polyphenol inhibited events connected to tumor initiation, promotion, and progression (1). Numerous studies since then have confirmed its activity to suppress cell proliferation, induce apoptosis, block cell invasion, and inhibit angiogenesis in a wide variety of cell lines (2-8). Moreover, administration of resveratrol in the diet was found to suppress DMBA-induced mammary cancer in rats (9, 10) while oral ingestion of resveratrol reduced the incidence and tumor size of premalignant and malignant colon lesions (11, 12). These studies support the potential utility of resveratrol as an anticancer agent; however, the mechanism by which resveratrol elicits its chemopreventive effects remains incompletely understood. To fully grasp the role of resveratrol in chemoprevention, identification of cellular targets that might mediate the activity of resveratrol is imperative.

Attempts to isolate and characterize the cellular protein targets of resveratrol have been made using resveratrol affinity column combined with matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). This approach has resulted in the discovery of dihydronicotinamide riboside quinone reductase 2 (NQO2) as a resveratrol targeting protein, denoted as RTPs (13). NQO2 was first described in the early 1960s as a flavin adenine dinucleotide (FAD)-containing, metallo-oxidoreductase possibly belonging to the category of phase II detoxification enzymes (14, 15). NQO2 has been associated with several unanticipated, novel cellular activities in recent years. Detailed characterization of its enzymatic properties (15-17), together with the use of *NQO2* knockout animals (18, 19) and knockdown cell lines (20, 21), have suggested that NQO2 participates in the functioning of melatonin, control of cell number/size, coupling of signaling pathways, modulation of the tumor suppressor p53, and prevention of malignancy. Whether NQO2 may function as a mediator and/or facilitator of prostate cancer (CaP) chemoprevention by resveratrol remains to be determined. In this study, the possible participatory role of NQO2 in the antiproliferative activity elicited by resveratrol was investigated using CWR22Rv1 CaP cells.

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Materials and Methods

Reagents. Resveratrol was purchased from LKT laboratories (St Paul, MN, USA). Primary antibodies for NF-κB p65, NF-κB p50, IκB, bcl-2, bax, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal anti-NQO2 antibody was generated in rabbits by Cocalico Biologicals, Inc (Reamstown, PA, USA). Fetal calf serum, RPMI-1640, penicillin and streptomycin were purchased from Cellgro, Inc (Herndon, VA, USA). All other chemicals and solvents used were of analytical grade.

Cell culture and growth inhibition assay. Human CWR22Rv1 cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 supplemented with penicillin, streptomycin and 10% heat inactivated fetal calf serum as previously described (22-24). For treatment, cells were seeded at a density of 5×10^4 cells/ml. Resveratrol dissolved in dimethyl sulfoxide (DMSO) was added to the culture media to the final concentration specified. At the indicated times, control and treated cells were harvested. Cell count was performed using a hemocytometer and cell viability was determined by trypan blue exclusion (22-24). Harvested cells were washed twice with phosphate-buffered saline (PBS), and pellets were stored at –80˚C for additional biochemical and molecular analyses.

Cell cycle analysis. Cell cycle phase distribution was assayed by flow cytometry. Following 3-day treatment of CWR22Rv1 cells with different concentrations of resveratrol (0, 10, and 50 μM), cells were washed with PBS and stained with 1.0 μg/ml DAPI containing 100 mM NaCl, 2 mM $MgCl_2$ and 0.1% Triton® X-100 (Sigma Chemical Co., St. Louis, MO, USA) at pH 6.8, as described elsewhere (23, 25, 26). The DNA-specific DAPI fluorescence was excited with UV light emitting laser (Ni-Cad), and collected with appropriate filters in an ICP-22 (Ortho Diagnostic, Westwood, MA, USA) flow cytometer. MultiCycle software from Phoenix Flow Systems (San Diego, CA, USA) was used to deconvolute the cellular DNA content histograms to obtain quantification of the percentage of cells in the respective phases (G_1, S and G_2/M) of the cell cycle. Flow cytometry was also used to show cells undergoing apoptosis, evident by the appearance of the sub-G1 peak (23, 25, 26).

Studies involving small interfering RNA inhibition of NQO2 expression. Human CaP CWR22Rv1 cells were grown in monolayer cultures in six-well plates in RPMI-1640 supplemented with 10% fetal bovine serum. *NQO2* small interfering RNA (siRNA) was purchase from Dharmacon, Inc. (Lafayette, CO, USA). Cells were seeded at a density of 2×10^5 cells/ml overnight then transfected with *NQO2*-specific siRNA using DharmanFECT 3 transfection reagent following the procedure suggested in the manufacturer's protocol. For treatment, resveratrol was added to the culture media 24 h post transfection. Control and treated cells were harvested at 48 h after incubation with resveratrol. Cell count was performed using a hemocytometer and cell viability was determined by trypan blue exclusion (22-24). Harvested cells were washed twice with PBS, and pellets were stored at –80˚C for further analyses.

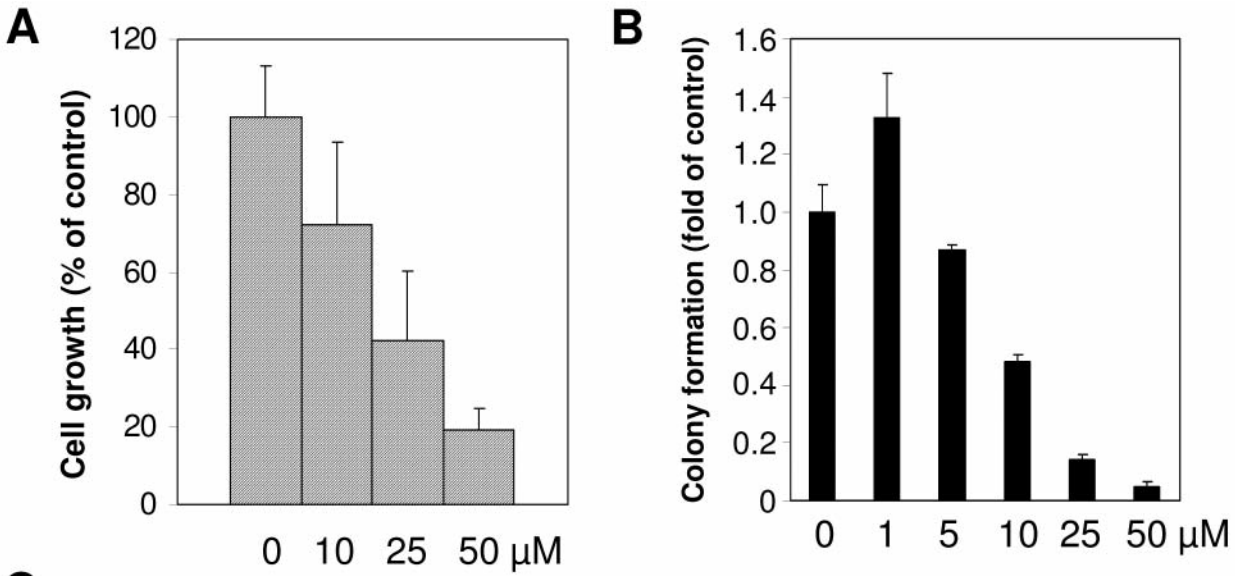
Preparation of whole cell extracts. For immunoblotting experiments, cells were collected by centrifugation and were lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton® X-100, 1% deoxycholate, 0.1 % SDS, 1 mM dithiothreitol and 10 μl/ml protease inhibitor cocktail). The extracts were centrifuged and the clear supernatants were stored in aliquots at –70˚C for further analysis. Protein content of cell lysates was determined by Coomassie protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard.

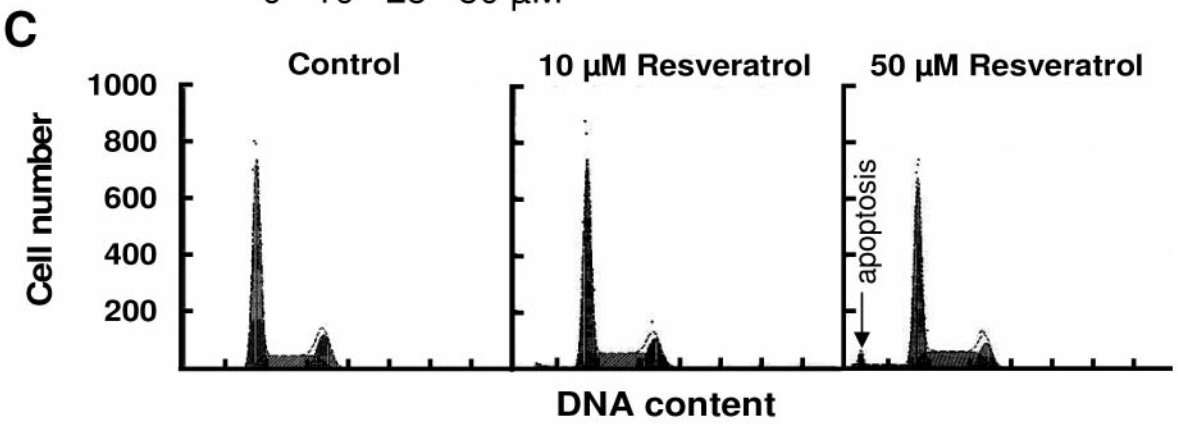
Immunoblotting. The aliquots of lysates (20 μg of protein) were boiled with sample buffer for 5 min, and resolved by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween 20) containing 3% nonfat dried milk overnight at 4˚C. The blots were incubated with various primary antibodies followed by incubation for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase in TBST. Actin expression was used as loading control. The intensity of the specific immunoreactive bands were detected by enhanced chemiluminescence (ECL), using the manufacturer's protocol (KPL, Inc., Gaithersburg, MD, USA) and quantified by densitometry and expressed as a ratio to actin, as previously described (24).

Results

Effect of resveratrol on CWR22Rv1 cell proliferation, colony formation and cell cycle distribution. To obtain information on whether resveratrol exerts a comparable effect on AR positive hormone-non-responsive cells, its effect on proliferation of CWR22Rv1 CaP cells was studied. Cells were incubated with increasing doses of resveratrol, and subjected to trypan blue exclusion assay to measure cell growth. Resveratrol treatment resulted in dose-dependent inhibition of cell proliferation, compared with controls. By 72 h, there was a ~30% , ~60% and 80% diminution of cell growth by 10, 25 and 50 μM resveratrol, respectively (Figure 1A). To confirm the growth modulatory effects of resveratrol, clonogenic assay were performed and the results were quantified based on spectrophotometric determination of cell-retained dye. The result in Figure 1B shows that the inhibition of colony formation occurred in a dose-dependent fashion at concentrations ≥5 μM resveratrol.

To probe further into the nature of growth inhibition by resveratrol in CWR22Rv1 cells, flow cytometric analysis was performed. In these experiments, CWR22Rv1 cells were treated with the vehicle alone (0.1% DMSO) or with 10 and 50 μM of resveratrol for 3 day. The distribution of cells in different phases of the cell cycle phase is presented as histograms and the percentage of cells in G_1-, S-, and G_2/M-phases were calculated and depicted in Figure 1C. The results showed that the percentage of cells in the G_1-phase was slightly reduced by resveratrol. This reduction was accompanied by a concomitant increase in S-phase cells and a decrease in G_2/M phase cells, suggesting that resveratrol prolonged the transition and/or accumulated cells in the S-phase of the cell cycle. In addition, cell cycle analysis revealed that resveratrol apparently induced apoptosis as evident by the appearance of the sub-G_1 fraction (Figure 1C); notably, the





%	Control	10 μM	50 μM
G_1	56.3	55.9	52.0
S	26.5	29.4	35.7
G_2/M	17.3	14.6	12.3
apoptosis	n.d.	1.7	6.7

Figure 1. *Effect of resveratrol on cell growth, colony formation and cell cycle phase distribution in CWR22Rv1 cells. A, Cells were treated with increasing concentrations of resveratrol (0, 10, 25 and 50 μM) and the cell numbers were determined at 72 h by counting using a hemocytometer. The bars show dose dependent growth suppression, expressed as a percentage of control (set as 100%) for the resveratrol-treated cells. B, Clonogenicity of CWR22Rv1 cells. Cells were plated, in triplicate, in 6-well tissue culture plates at a density of 2,000 cells/ml in the absence or presence of the indicated concentration of resveratrol. After 1 week, colonies were stained with 1.25% crystal violet, and quantified by extraction with 10% acetic acid and measuring the absorbance at 595 nm. Values are expressed as mean±SD for three experiments. C, Effects of resveratrol on the cell cycle phase distribution. Cells were treated with increasing concentrations of resveratrol (0, 10 and 50 μM) for 72 h and the effect on cell cycle distribution was analyzed by flow cytometry, and the percentage of cells in G_1-, S-, and G_2-phases were calculated and are represented as histograms. Cells with hypodiploid DNA content (sub-G_1) represent fractions undergoing apoptotic DNA fragmentation.*

percentage of apoptotic cells increased from non-detectable (n.d.) in control cells to 1.7% and 6.7% in 72 h, 10 and 50 μM resveratrol-treated cells, which partially contributed to the growth inhibitory effects of resveratrol (Figure 1A).*Silencing NQO2 affects resveratrol-induced CWR22Rv1 growth control.* To assess whether resveratrol targeting protein NQO2 may contribute to resveratrol-induced growth suppression, CWR22Rv1 cells were transfected with NQO2

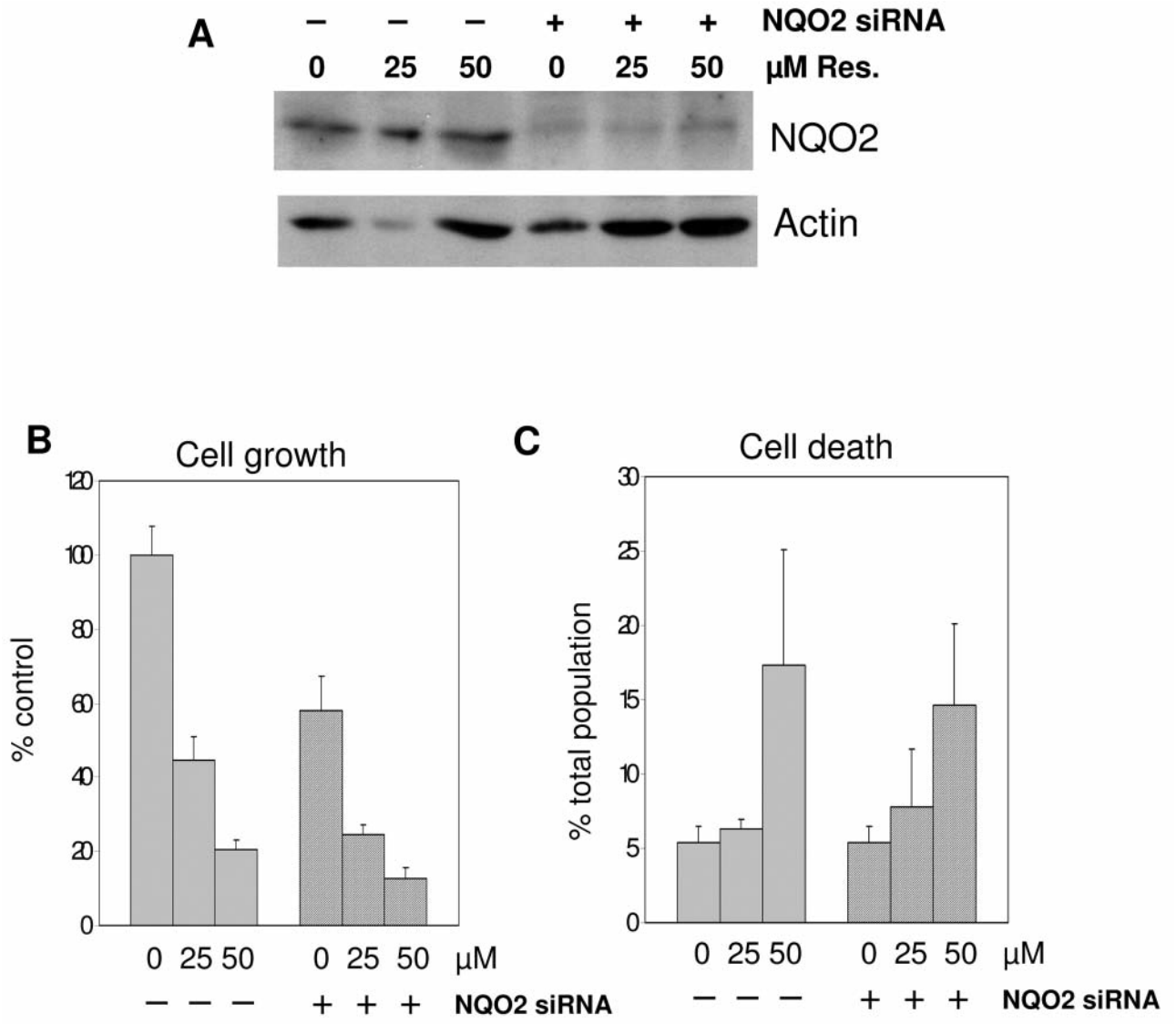


Figure 2. *Effect of silencing NQO2 on resveratrol-induced CWR22Rv1 growth control. A, Western blot analysis of the NQO2 protein in control and NQO2 siRNA-silenced CWR22Rv1 cells. Cells (2×10^5 cells/ml) were seeded overnight then transfected with 100 nM NQO2 siRNA. Twenty four hours post transfection, cells were treated with increasing concentrations of resveratrol (0, 25 and 50 μM) for 48 h. Cell lysates were prepared, separated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed for NQO2. The blots were stripped and reprobed with actin which served as loading control. B, Resveratrol induced growth control in control and NQO2 siRNA-silenced CWR22Rv1 cells. Cells were prepared and treated the same way as described previously in section A and the cell numbers were determined following 48 h resveratrol treatment by counting using a hemocytometer. The bars show dose-dependent growth suppression, expressed as a percentage of control (set as 100%) for the resveratrol-treated cells in control and NQO2 siRNA-silenced group. C, Cell viability was measured at the end of 48 h resveratrol treatment using the trypan blue dye exclusion assay. The percentage of dead cells was calculated and expressed as mean±SD for three experiments.*

siRNA. Western blot analysis showed success in application of NQO2 siRNA in silencing the basal expression of NQO2 as evident by the greatly reduced levels of NQO2 (Figure 2A). Resveratrol-induced growth responses were investigated in control and NQO2 siRNA-treated cells. Silencing of NQO2 by siRNA alone resulted in ~40% growth suppression in CWR22Rv1 cells (Figure 2B), while no growth modulatory affects were observed in controls treated with non-specific siRNAs (data not shown). In addition, treatment by resveratrol showed a more pronounced growth inhibition in NQO2 siRNA-treated cells compared to control cells (Figure 2B). These results suggest that resveratrol-induced growth suppression may be attributed to NQO2-dependent and -independent mechanisms. In contrast, no

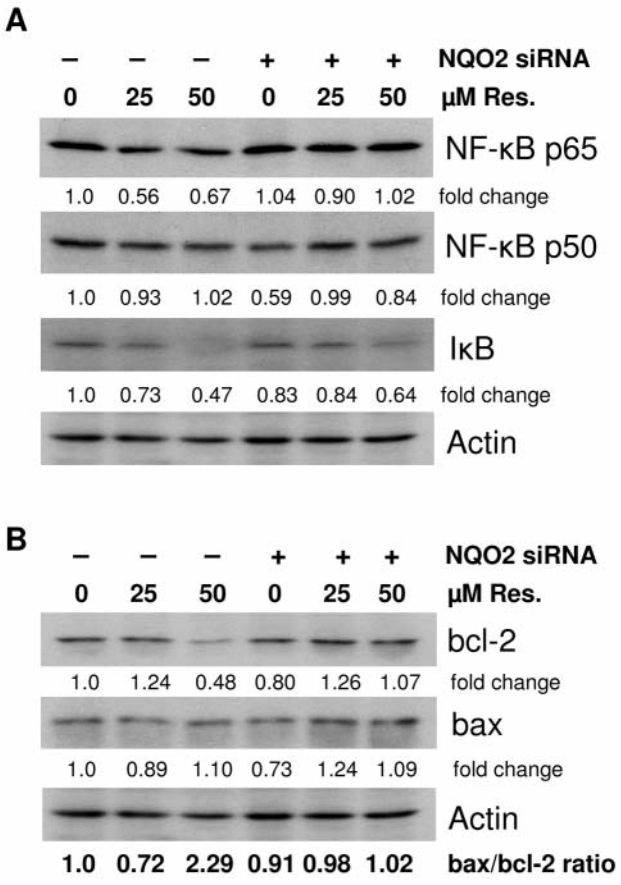


Figure 3. *Effect of silencing NQO2 on resveratrol-induced changes on the expression of NF-κB p65, p50, IκB, bcl-2 and bax in CWR22Rv1 cells. Cells (2×10^5 cells/ml) were seeded overnight then transfected with 100 nM NQO2 siRNA. Twenty four hours after transfection, cells were treated with increasing concentrations of resveratrol (0, 25 and 50 μM) for 48 h. Cell lysates were prepared, separated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. A, Western blot analysis was performed for NF-κB p65, p50 and IκB. The blots were stripped and reprobed with actin which served as loading control. B, The total protein expression level of bax and bcl-2 was also determined by Western blot analysis. The changes in the ratio of bax to bcl-2 was also calculated. In panel A and B, the intensity of the specific immunoreactive bands were quantified by densitometry and expressed as a fold difference against actin (loading control).*

apparent differences existed in effects of resveratrol on cell viability between control and NQO2 siRNA-treated cells, consistent with the notion that resveratrol-induced cell death is not related to the full expression of NQO2 (Figure 2C).

Requirement of NQO2 in resveratrol-mediated NF-κB changes and evidence showing NQO2 silencing abrogates the resveratrol-induced changes in bax/bcl2 ratio. NQO2 has been suggested to play a role in TNF-induced control of NF-κB expression. It is therefore of interest to determine whether NQO2 may exert a contributory or facilitory function in resveratrol mediated NF-κB changes. Accordingly, we assayed changes in NF-κB p65, NF-κB p50 and IκB expression in response to treatment by resveratrol, using both control and *NQO2*-knockdown CWR22Rv1 cells. Resveratrol treatments resulted in down regulation of NF-κB p65 and IκB expression (Figure 3A). Interestingly, effects of resveratrol on NF-κB p65 but not IκB were largely abrogated in NQO2 siRNA-silenced cells (Figure 3A). These results suggest that resveratrol-induced changes of NF-κB p65 and IκB expression utilized NQO-2-mediated and NQO2-independent mechanisms. There was no change in NF-κB p50 expression in either control or siRNA groups in response to resveratrol; surprisingly, however, silencing *NQO2* by siRNA alone resulted in ~40% reduction of NF-κB p50 expression (Figure 3A). These results suggest that NQO2 may play a role in the control of NF-κB p50 distinct from treatment by resveratrol, while NQO2 is apparently required in resveratrol-induced NF-κB p65 changes. Because bcl-2 plays an integral role in cell death and since NF-κB regulates the expression of antiapoptosis proteins including bcl-2, we further investigated the involvement of NQO2 in resveratrol-induced cell death by measuring the expression of bcl-2 and its corresponding apoptosis agonist, bax, in control and *NQO2* siRNA-treated cells. Western blot analysis clearly showed a dose-dependent suppression of bcl-2 expression, with only minimum changes in bax in resveratrol-treated CWR22Rv1 cells, as vividly illustrated by a marked increase in the bax-to-bcl-2 expression ratio (Figure 3B). Reduction of NQO2 expression by siRNA diminished resveratrol-induced changes in bax-to-bcl-2 expression ratio (Figure 3B). These results suggest that *NQO2* knockdown did not potentiate resveratrol-induced cell death, while NQO2 is required for resveratrol-mediated NF-κB-regulated bcl-2 expression.

Discussion

Resveratrol was found to inhibit proliferation of both hormone-dependent and hormone-refractory CaP cells (3, 27, 28). Previously, panels of CaP cell lines which mimic the early and late stage of prostate carcinoma (29-32) were used for testing the effects of resveratrol and results showed that resveratrol is an effective inhibitor of cell growth in both androgen-dependent (AD) and androgen-independent (AI) human CaP cells (2). Whether resveratrol is capable of impeding progression of prostate carcinoma from AD to androgen-refractory (HRPC) states remains unknown. In this study, CWR22Rv1 cells that mimic the transition of CaP from AD to HRPC were used to test the anti-CaP effect of resveratrol. We demonstrate for the first time that resveratrol also suppresses cell proliferation with a less pronounced effect on cell cycle and induction of apoptosis in

CWR22Rv1 cells. These results not only reinforce that resveratrol is an effective inhibitor of cell growth in general but also imply that it can interfere with the progression of CaP from the AD to HRPC states *in vitro*.

To further determine the possible chemopreventive potential of resveratrol, control and *NQO2* siRNA-silencing CWR22Rv1 cells were used to examine the role of resveratrol targeting protein NQO2 in resveratrol-mediated growth control. We found that silencing *NQO2* by siRNA exerted a growth inhibitory effect similar to treatment by resveratrol in CWR22Rv1 cells. In addition, silencing *NQO2* also sensitized the responsiveness of cells to resveratrol treatment, resulting in more potent growth inhibition in *NQO2* siRNA-treated cells compared to control cells (Figure 2B). Thus, these findings support the interpretation that resveratrol-induced growth control may be partially explained by a NQO2-dependent mechanism; thereby suggesting the need to further study the effects attributed to the interaction between resveratrol with NQO2 and/or NQO2 interacting proteins.

Growth-modulatory effects of resveratrol have been reported to be in part ascribed to changes in the expression of NF-κB. The studies described herein further examining the involvement of NQO2 in NF-κB mediated growth control by resveratrol showed that resveratrol inhibited IκB expression in both control and *NQO2* siRNA-treated cells, whereas resveratrol-inhibited NF-κB p65 expression in CWR22Rv1 was effectively abrogated in NQO2-silenced CWR22Rv1 cells. In addition, although resveratrol reduced the expression of NF-κB-dependent gene product bcl-2 in control CWR22Rv1 cells, no similar changes occurred in *NQO2* siRNA-silenced CWR22Rv1 cells. These data suggest that resveratrol-induced changes in NF-κB p65 are at least partially mediated through NQO2 and provide mechanistic support for the mediating role NQO2 plays in the anti-CaP activity of resveratrol.

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