Crystal Structure of Quinone Reductase 2 in Complex with Resveratrol[†,‡]

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ABSTRACT: Resveratrol has been shown to have chemopreventive, cardioprotective, and antiaging properties. Here, we report that resveratrol is a potent inhibitor of quinone reductase 2 (QR2) activity in vitro with a dissociation constant of 35 nM and show that it specifically binds to the deep active-site cleft of QR2 using high-resolution structural analysis. All three resveratrol hydroxyl groups form hydrogen bonds with amino acids from QR2, anchoring a flat resveratrol molecule in parallel with the isoalloxazine ring of FAD. The unique active-site pocket in QR2 could potentially bind other natural polyphenols such as flavonoids, as proven by the high affinity exhibited by quercetin toward QR2. K562 cells with QR2 expression suppressed by RNAi showed similar properties as resveratrol-treated cells in their resistance to quinone toxicity. Furthermore, the QR2 knockdown K562 cells exhibit increased antioxidant and detoxification enzyme expression and reduced proliferation rates. These observations could imply that the chemopreventive and cardioprotective properties of resveratrol are possibly the results of QR2 activity inhibition, which in turn, up-regulates the expression of cellular antioxidant enzymes and cellular resistance to oxidative stress.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a phytopolyphenol that occurs in grapes and a variety of medicinal plants. Because of its abundance in grapes, it is present in significant amount in grape products such as grape juice and wines, particularly red wine (*1*). The revealing of resveratrol as a cancer chemopreventive agent in 1997 (*2*) sparked extensive research on its chemopreventive properties (*3*). Resveratrol has been tested on a variety of cancers in animal model studies. As in the case of the 1997 study, Jang et al. (*2*) demonstrated in a mouse model that resveratrol is effective in prevention of DMBA- (dimethylbenzanthracene) and TPA- (tetradecanoylphorbol-13-acetate) induced skin cancer. Application of 1, 5, 10, or 25 μM of resveratrol with TPA twice a week for 18 weeks reduced the number of skin tumors per mouse by 68, 81, 76, or 98%, respectively, and the percentage of mice with tumors was lowered by 50, 63, 63, or 88%. In mouse models of colon cancer and small intestinal cancer, resveratrol prevents cancer formation with 100 and 70% efficacy, respectively (*4*, *5*).

Resveratrol has been suggested to block the multistep process of carcinogenesis, namely, tumor initiation, promotion, and progression (*3*). Despite extensive investigation, no clear molecular basis for the actions of resveratrol has emerged. A variety of hypotheses have been proposed to explain its functions: antioxidant effects, proapoptotic effects, cell-cycle regulation, inhibition of protein kinases, regulation of NFκB and IκB, and modulation of estrogen effects (*3*, *6*). As a polyphenol, resveratrol will evidently exhibit reductive properties and consequently will scavenge reactive oxygen species (ROS, such as H_2O_2, HO•, and O^{2-}) (*7*). In addition, resveratrol is also a moderate inhibitor of some oxidative and antioxidative enzymes, such as lipooxygenase, peroxidases, catalases, and others (*8*). Resveratrol has also been shown to inhibit free-radical formation induced by lipopolysaccharide (LPS) and phorbol esters (*9*). However, these studies used artificially high concentrations of resveratrol, and their relevance to the true biological targets of resveratrol is unclear.

The observations that are believed to be relevant to cancer prevention are the reported up-regulation of antioxidant and detoxification enzymes by resveratrol (*10*, *11*) such as catalase, quinone reductase 1 (QR1),[1] and glutathione-*S*-transferase (GST) (*12*). The increased enzymatic activities will protect cellular components from oxidative damage as the results of environmental oxidative insult or reactive metabolites, which can cause carcinogenesis (*13*−*15*). In fact, induction of QR1 in vitro has been used as a method to identify potential prostate cancer-preventive agents (*16*). A variety of natural products, including natural polyphenols, such as flavonoids (quercetin) (*17*) and chalconoids (*18*), also induce antioxidant and detoxification enzyme expression. A comparison of the structures of these flavonoids and chalconoids with resveratrol reveal that they share some apparent chemical similarities with two phenolate rings similarly

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[‡] The atomic coordinates and structure factors (PDB code 1SG0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[1] Abbreviations: QR1, quinone reductase 1; QR2, quinone reductase 2, GST, glutathione-*S*-transferase.

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spaced and oriented. These similarities might indicate a shared mechanism in their actions.

Recently, we identified quinone reductase 2 (QR2) as a potential target of resveratrol function through an affinity chromatographic method using a resveratrol-affinity column. The structure of QR2 in complex with resveratrol reveals that resveratrol specifically binds to the active-site cleft of QR2. The unique active-site pocket in QR2 could potentially bind other natural polyphenols, such as flavonoids and chalcone derivatives, as proven by the high affinity exhibited by quercetin toward QR2, which could indicate a shared mechanism in their chemopreventive properties. Using the RNAi method, we also have established a stable K562 cell line (K562RNAi) with QR2 expression suppressed to undetectable levels judged by Western blot analysis. K562 cells with QR2 expression suppressed by RNAi showed similar properties as resveratrol-treated cells in their resistance to quinone toxicity. Furthermore, the QR2 RNAi knockdown K562 cells exhibit increased antioxidant and detoxification enzyme expression and reduced proliferation rates, similar to cells treated with resveratrol reported in the literature (*6*, *11*).

MATERIALS AND METHODS

Chemicals, Reagents, and Antibodies. Resveratrol, quercetin, *N*-(2-hydroxylethylnicotinamide), and anti-actin antibody were from Sigma. QR2 antibody was raised in rabbits immunized with full-length recombinant human QR2 protein. All other chemicals were of the highest purity commercially available. Reduction of *N*-(2-hydroxylethylnicotinamide) to *N*-(2-hydroxylethyldihydronicotinamide) was carried out just before use by sodium hydrosulfite (*19*).

Protein Methods. To produce recombinant QR2, the coding region of QR2 cDNA fragments was amplified by PCR and cloned into pET23d vector (Novagen) between *Nco*I and *Xho*I sites with the first Met as part of the *Nco*I sequence and the native termination codon before the *Xho*I site. The insert was verified by DNA sequencing. The plasmid of the full-length protein of QR2 without any change in amino acids is transformed into B834 *Escherichia coli* strain (Novagen). The recombinant protein was purified by passing through DEAE Sepharose, Superdex 75 (Pharmacia), and Mono-Q column (Pharmacia) sequentially to obtain the homogeneous protein samples. Protein concentration was determined by BioRad protein assay kit according to the protocol of the manufacturer. Crystals of the native protein as well as the complex with resveratrol were all grown in conditions containing 0.1 M HEPES (pH 7.0), 10 μM FAD, and 1.2−2.0 M ammonium sulfate.

QR2 Refolding in the Presence of Different Metal Ions. Recombinant QR2 (5 mg) was dissolved in 10 mL of 7 M guanidine hydrochloride (50 mM Tris and 0.5 mM DTT at pH 8.5) solution. The resulting solution was passed through a 5-mL metal-chelating column pre-equilibrated with the same buffered guanidine hydrochloride solution. A total of 2 mL each of the resulting protein solution was diluted into 100 mL of refolding buffer (50 mM Tris and 50 μM FAD) containing 0.5 mM different metal ions (Zn^{2+}, Mn^{2+}, Fe^{3+}, or Cu^{2+}) or without metal ions. Resulting proteins from each refolding condition were purified by mono-Q column (Pharmacia).

Crystallographic Analysis. All diffraction data were collected at 100 K using a CCD detector at beamline 19BM of Advanced Photon Source (APS), Argonne National Laboratories. Raw data were processed using the HKL2000 software (*20*). Crystals of the native QR2 and QR2−resveratrol complex all belong to the $P2_12_12_1$ space group. The cell dimensions of all of the crystals are nearly identical, and consequently the complex structures were directly refined with the native structure previously solved by another laboratory (*21*) (1qr2 for apoprotein and 2QR2 for the QR2−menadione complex; Protein Data Bank) with CNS (*22*), and the density of resveratrol was clearly shown after Fourier-difference transformation. Figures were prepared using PDBviewer (*23*) and rendered with POV-ray (*24*) software packages.

Generation of QR2 Knockdown Cell Line by RNAi. The Ambion's "siRNA Target Finder and Design Tool" was used for the identification of an appropriate target sequence in QR2 mRNA. Two target sequences have been chosen following a BLAST search analysis to confirm the uniqueness of the selected sequences. Eventually, the construct targeted the sequence in the exon III of QR2 mRNA that has been chosen to create the stable K562 subline. K562 cells were transfected with pSilencer 3.1-HI hygrovector (Ambion) bearing QR2−RNAi (corresponding oligos: 5′-gatcccgaatgtggctgtagatgaattcaagagattcatctacagccacattctttttggaaa and 5′-agcttttccaaaaaagaatgtggctgtagatgaatctcttgaattcatctacagccacattcgg) using FuGene 6 Transfection Reagent (Roche) according to the protocol of the manufacturer. A total of 24 h after transfection, conditioned medium RPMI 1640 with 10% fetal bovine serum (Invitrogen) was changed for fresh media containing 200 μg/mL Hygromycin B (Invitrogen). As a negative control, a flask of mock-transfected K562 cells, i.e., transfected with salmon sperm DNA, was grown under selective pressure. The cells were allowed to grow under selection for approximately 40 days, changing media every 4 days, before cells were completely eliminated from the negative control flask and visibly enriched in the RNAi-transfected flask. Western blot analysis revealed nearly 100% suppression of QR2 expression in transfected cells, and the stable cell line is termed K562RNAi cells. The K562RNAi cells were then maintained in normal growth media.

Affinity Characterization by Fluorescence Quenching. For the fluorescence-quenching experiments, two to three concentrations of protein solutions, from 100−500 nM in pH 8.5 buffer (50 mM Tris, 140 mM NaCl, and 0.1% Tween 20) were used, respectively. Samples with increasing concentrations of a specific polyphenol but constant concentrations of QR2 were prepared. Similar samples were prepared containing the corresponding polyphenols without QR2 as controls. The fluorescence was measured at an emission of 340 nm with an excitation wavelength of 280 nm with a Varian fluorescence spectrophotometer. The fluorescence of the samples with QR2 subtracted by that of the samples without QR2 is used as the fluorescence of the QR2−polyphenol complex. The obtained values were globally fitted to an exhaustive binding model (*25*) with the software Prism.

Enzymatic Assays. QR2 activity was assayed according to Zhao et al. (*19*). The enzyme activity was determined spectrophotometrically at either 37 or 25 °C in pH 8.5 buffer (50 mM Tris, 140 mM NaCl, and 0.1% Tween 20) with

menadione as the substrate and 1-(2-hydroxyethyl)dihydronicotinamide as the cosubstrate. The reaction was initiated by addition of 2−8 ng of QR2, and the catalysis was monitored at a wavelength of 360 nm with a Unicam UV−vis spectrophotometer. The apparent K_m values for the cosubstrate in the presence of respective concentrations of resveratrol were obtained with the integrated Henri equation (*26*). The K_i was obtained by plotting the apparent K_m values versus the resveratrol concentration.

Menadione Toxicity Assays. Menadione toxicity was measured by cell-survival assays. A defined number of cells were incubated with defined concentrations of menadione in the presence or absence of other reagents (such as resveratrol) in a cell-culture incubator for 4 h. After the cells were changed to normal growth media, they were allowed to recover for 24 h. The number of living cells was analyzed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay according to the protocol of the manufacturer (Roche). All assays were conducted at least 3 times.

Antioxidant and Phase II Detoxification Enzyme Assays. All of the cells were grown under identical conditions alongside each other for a defined period of time. These cells included wild-type K562 cells and K562 RNAi cells. The pelleted cells were lysed by sonication in modified RIPA buffer with a complete protease inhibitor cocktail (Roche) (20 mM HEPES at pH 7.5, 140 mM NaCl, 1% Triton X-100, and 5% glycerol). After centrifugation, the protein contents in the supernatants were normalized by BioRad protein assays. The enzymatic activity assays were performed as described (*27*) with modification, except the total NAD(P)H-dependent quinone reductase assays, which were adopted from Chen et al. (*28*). Briefly, glucose-6-phosphate dehydrogenase activity is measured by the reduction of NADP. The reaction buffer of 0.4 mL consisted of 0.1 M HEPES (pH 7.6), 0.1 mM NADP, 0.8 mM glucose-6-phosphate, and 8 mM $MgCl_2$, and the reaction was initiated by addition of 40 μg of total cell lysate protein and quenched with the addition of SDS to the final concentration of 2% from a 20% stock. The changes in absorbance were recorded at 340 nm, and the enzyme activity was calculated as nmol of NADP reduced min^{-1} mg of $protein^{-1}$, using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Glutathione reductase activity was measured by the oxidation of NADPH. The reaction mixture consisted of 0.1 M HEPES (pH 7.6), 0.5 mM EDTA, 1.0 mM oxidized glutathione, and 0.1 mM NADPH. The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm and was calculated as nmol of NADPH oxidized min^{-1} mg of $protein^{-1}$, using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

The catalase activity was measured by the decomposition of hydrogen peroxide. Specifically, the assay mixture consisted of 0.1 M HEPES (pH 7.0) and 0.019 M hydrogen peroxide. The catalase activity was calculated following decomposition of hydrogen peroxide measured as a decrease in absorbance at 240 nm, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

GST activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Specifically, the reaction mixture consisted of 0.1 M MES (pH 6.5), 1.0 mM reduced glutathione, and 1.0 mM CDNB. The reaction was initiated and terminated similarly as mentioned above. The changes in absorbance were recorded at 340 nm, and enzyme activity was calculated as nmol of CDNB conjugate formed min^{-1} mg of $protein^{-1}$, using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

The total NADH-dependent quinone reductase activity was assayed by the reduction of menadione and subsequent reduction of MTT. The assay mixture (0.4 mL) contained 0.1 M Tris (pH 8.5), 120 μM NADH, 10 μM menadione, 0.3 mg/mL MTT, and 0.1% Tween 20. The activity was determined spectrophotometrically by measuring the reduction of MTT at 570 nm, using a molar extinction coefficient of 17×10^3 M^{-1} cm^{-1} at 25 °C.

All of the assays were conducted at least 3 times and reproduced with two separate lysates.

RESULTS

Potent Inhibition of QR2 Activity by Resveratrol. To investigate the biological targets of resveratrol function, we constructed a resveratrol-affinity column, in which resveratrol was immobilized on a solid matrix by cross linking to an epoxy-activated agarose resin. When prostate cancer cell-line (PC3) lysates and leukemia cell-line (K562) lysates were analyzed with the resveratrol-affinity column, QR2 was the major protein bound specifically to this column (will be published elsewhere). QR2 (also called NQO2, NRH:quinone oxireductase) was first described in 1961 (*29*), but its in vivo biological function is still a mystery. Its homologous protein, QR1 (also termed DT-diaphorase and NQO1), on the other hand, has been well-characterized (*12*) and catalyzes the obligatory two-electron reduction of quinones with NADH or NADPH as the electron donors. In contrast, QR2 cannot use the phosphorylated dihydronicotinamides as cosubstrates, and it has been shown that QR2 can use the reduced N-substituted nicotinamide analogues as cosubstrates in vitro (*30*), with the in vivo cosubstrate yet to be defined.

Upon identification of QR2 as the major protein bound to resveratrol-affinity column, we analyzed the effect of resveratrol on the enzymatic activity of QR2. Using N-hydroxyethyldihydronicotinamide as the cosubstrate and menadione as the substrate, resveratrol was shown to be a potent inhibitor of QR2 enzymatic activity (Figure 1A). The QR2 catalysis is indicated by the decrease of UV absorbance at the wavelength of 360 nm, which corresponds to the consumption of the dihydronicotinamide. Further analysis of the inhibitory properties reveals that resveratrol is a competitive inhibitor in respect to dihydronicotinamide with a K_i value of 50 nM. To determine the true affinity of resveratrol to QR2, we monitored quenching of the intrinsic protein fluorescence. Addition of resveratrol partially quenched the fluorescence of tryptophan when monitored at a wavelength of 340 nm (excitation wavelength of 280 nm). Using this method, we have determined that resveratrol binds to QR2 with a K_d of 34 ± 15 nM (Figure 1B). With this high affinity, QR2 is by far the highest affinity target of resveratrol. The projected concentration attainable (less than a few micromolar) (*31*) from the consumption of grape products such as red wine is also in the range that will exhibit robust inhibition of QR2 activity, while most of the earlier in vitro studies used artificially higher concentrations to obtain various biological effects.

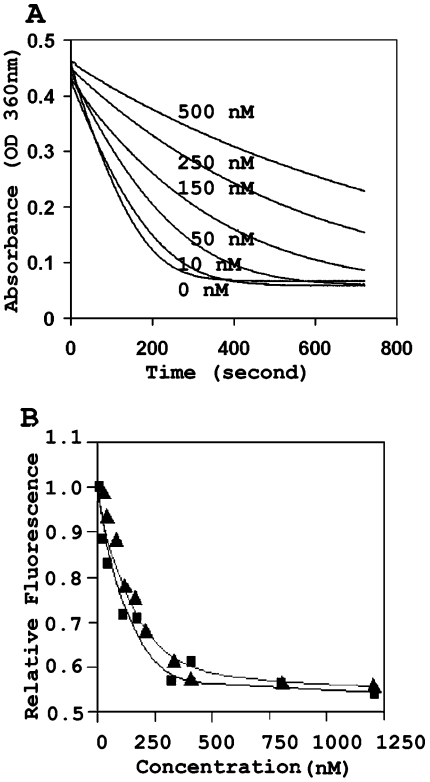

FIGURE 1: Resveratrol exhibits high affinity toward QR2 and inhibits QR2 activity. (A) QR2 enzymatic activity is strongly inhibited by the presence of resveratrol. Five concentrations of resveratrol (10−500 nM, labeled on the corresponding curve) were used to analyze the inhibition of QR2 activity. (B) Titration of the tryptophan fluorescence of QR2 by resveratrol. The fluorescence of two concentrations of QR2 (100 nM, ■; and 200 nM, ▲) was measured with increasing concentrations of resveratrol. The K_d is 35 ± 15 nM, obtained by globally fitting the data to an exhaustive titration model. The correlation coefficient is 0.996.

Table 1: QR2−Resveratrol Structure Refinement Statistics

data collection statistics	QR2−resveratrol
space group	$P2_12_12_1$
unit cell (Å)	$a = 83.33$, $b = 106.37$, $c = 56.98$
resolution (Å)	50.0−1.5
completeness (%; $I/\sigma \geq 0$)[a]	99.0 (98.5)
average I/σ	32.7
R_{merge} (%)[b]	5.8 (30.8)
refinement statistics	
resolution range (Å)	50−1.5
R factor[c]/R_{free} (%)	21.4/23.4
rmsd bond length	0.007
rmsd angles	1.7
mean B factor value	16.9

[a] Values for the highest resolution shell are given in parentheses. [b] $R_{merge} = \Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle$, where I and $\langle I\rangle$ are the measured and averaged intensities of multiple measurements of the same reflection. The summation is over all of the observed reflections. [c] R factor $= \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where F_o denotes the observed structure factor amplitude and F_c denotes the structure factor calculated from the model.

Structure of the QR2−Resveratrol Complex. We have solved the crystal structure of QR2 in complex with resveratrol to a 1.5 Å resolution (Table 1 for crystallographic statistics) by the molecular replacement method with the apoprotein structure solved by another group earlier (*20*). The overall structure of QR2 in the complex is indistinguishable with the apoprotein structure (*21*). An unmistakable density of resveratrol was observed at the active site in the QR2−resveratrol complex (Figure 2A), sitting parallel to the isoalloxazine ring of the bound cofactor FAD. The bound resveratrol adopts a perfectly flat conformation (Figures 2C and 3), binds deep into the QR2 dimeric interface, and fits into a deep hydrophobic cleft. The side chains of Y132′, F178′, F126′, M164′, and C121′ from one of the two protomers form one side of the hydrophobic cleft, whereas the other side of the cleft is formed by side chains from Y155 and F106 from the other protomer in addition to the isoalloxazine ring of FAD. The side chain of W105 forms the bottom of the hydrophobic cleft, wedged between the two phenolate rings of resveratrol. We surmise that the binding of ligand or substrates will quench the fluorescence of this tryptophan. On one end of the cleft resides side chains of N161 and hydroxyl groups from Y155 and Y132. These groups form a relatively hydrophilic surface, whereas at the other end of the cavity, the hydroxyl group of T71′ and main-chain carbonyls from G68 and D117 all point toward the cavity. All of these features indicate a unique cavity, in which three sides are very hydrophobic, but both ends have numerous moieties accessible for hydrogen-bond formation. The forth side is accessible to the solvent (Figure 3). The cavity is deep and narrow, and resveratrol seems to fit perfectly. In contrast to resveratrol, the substrate menadione occupies about two-thirds of the active-site cavity (*21*). The snugness of the fit of resveratrol into the cleft suggests that longer molecules or those with a nonflat conformation will not fit into the cavity. This explains why QR1 inhibitors such as dicumarol and cibacron blue do not potently inhibit QR2 activity (*19*).

Whereas the two benzene rings of resveratrol interact with many neighboring residues through hydrophobic and van der Waals interactions, all of the three hydroxyl groups form hydrogen bonds with the amino acids of QR2 (Figure 2C). The 4′-hydroxyl group forms two tight hydrogen bonds through two ordered water molecules with two QR2 main-chain carbonyl oxygens, D117′ and T71′. The 5-hydroxyl group forms a hydrogen bond with the side chain of N161, a residue that is not conserved between QR1 and QR2. The nonconservation of this residue may be important to differentiate between QR1 and QR2 in their catalytic activities and substrate preference, because H161 among QR1s is conserved and suggested to be involved in the catalysis of the quinone reduction (*32*). The hydrogen bond between the side chain of N161 and 5-hydroxyl group of resveratrol also provides the anchor point for resveratrol binding, because it seals a very hydrophobic pocket from the solvent. The 3-hydroxyl group forms a strong hydrogen bond with the carbonyl oxygen of G174′ (2.75 Å). All three hydrogen bonds anchor a flat resveratrol in parallel with the isoalloxazine ring of FAD, with a distance of 3.5 Å. Thus, the three hydroxyl groups of resveratrol provide not only enthalpy for the high-affinity binding to QR2 but also maintain the flat conformation of resveratrol.

A close examination of the active site in QR2 reveals a 17-Å long and 7-Å wide cavity that is a perfect fit for a *trans*-stilbene structure (Figure 3). Because of its narrow width, this cavity can only accommodate molecules that can adopt flat conformations. A number of classes of natural polyphenols, including *trans*-stilbene derivatives, flavonoids, chalcones, and 3-phenylcoumarins (Figure 4), all share the two aromatic ring structures and can adopt flat conforma-

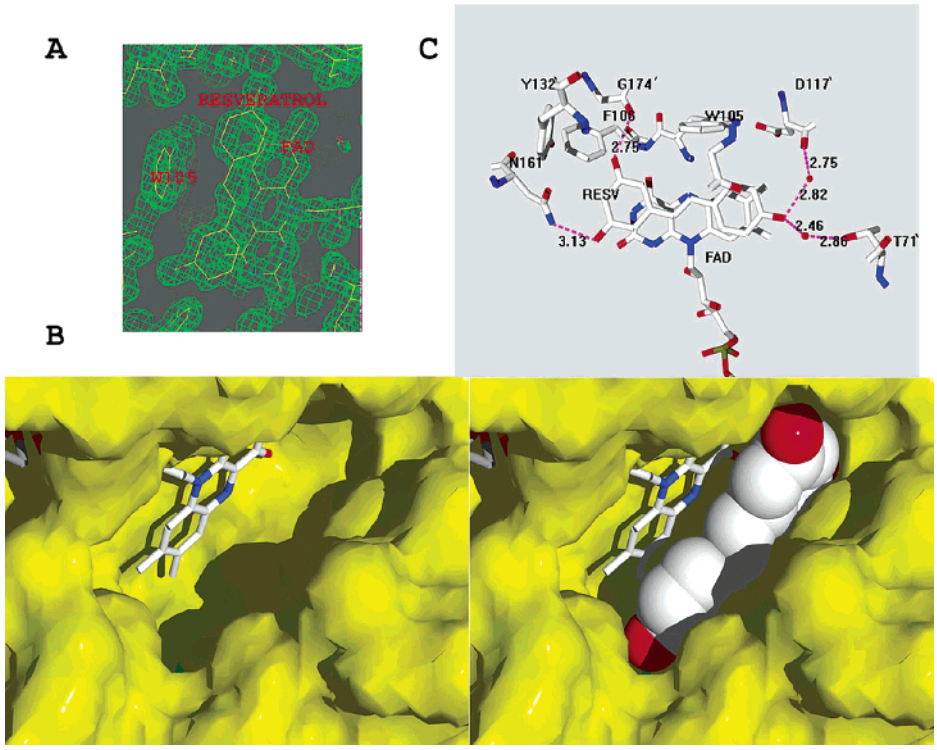

FIGURE 2: Structure of the QR2−resveratrol complex. (A) Electron density ($2F_o - F_c$, 1.5σ cutoff) covering part of the active site. Resveratrol, FAD, and W105 are labeled. (B) Two views of the resveratrol-binding site are shown in a surface representation shaded according to the cavity. The left panel shows the cavity occupied by resveratrol in the QR2−resveratrol complex, while the right panel shows the fit of resveratrol (ball representation) to the cavity. The cofactor FAD is represented as a stick model. (C) Hydrogen-bond network between resveratrol and QR2. All three hydroxyl groups on resveratrol form hydrogen bonds with side chains of amino acids from QR2. The distance labeled on the hydrogen bond is in angstroms.

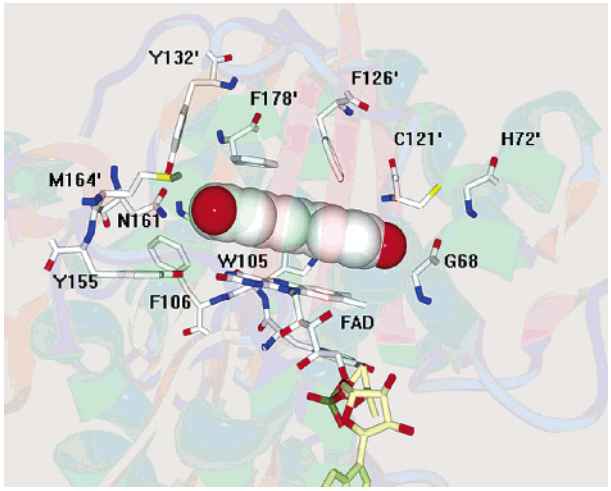

FIGURE 3: Resveratrol-binding cavity. The 17-Å long and 7-Å wide cavity is formed by hydrophobic side chains from QR2 and the isoalloxazine ring of FAD on two sides and the side chain of tryptophan 105 at the bottom. The cavity can presumably accommodate other polyphenols.

tions. These two aromatic rings are superimposable when those molecules are aligned. The similar lengths and hydrophobicity could be reasonably postulated to fit into the active-site cavity of QR2. The relatively hydrophilic ends of the cavity provide hydrogen-bonding partners for potential hydroxyl groups on the polyphenols. Evidently, proper positioning of these hydroxyl groups is essential for high-affinity binding, as in the case of resveratrol. This leads to the hypothesis that some of these molecules are potentially potent inhibitors of QR2 activity as well. One of the

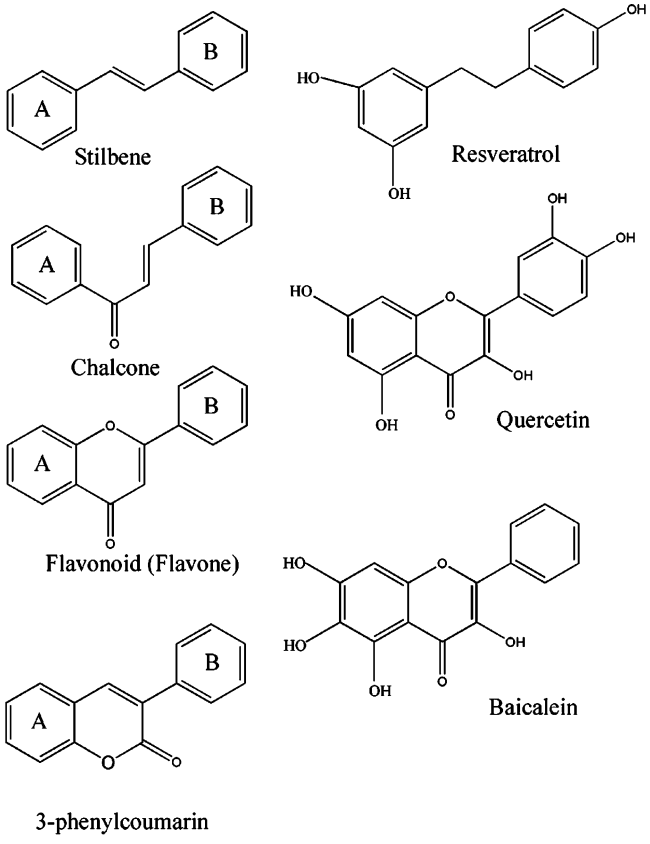

FIGURE 4: Structural comparison of natural polyphenols. Natural polyphenols, such as chalcone derivatives, flavonoids, and 3-phenylcoumarin derivatives, share the basic two-ring structures (A and B) similar to resveratrol.

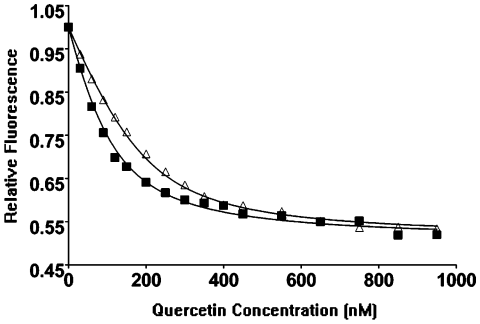

FIGURE 5: Affinity determination of quercetin toward QR2. The fluorescence of two concentrations of QR2 (200 nM, ■; and 400 nM, △) was measured in the presence of increasing concentrations of quercetin. The K_d obtained is 50.0 ± 5.4 nM with a correlation coefficient of 0.9965.

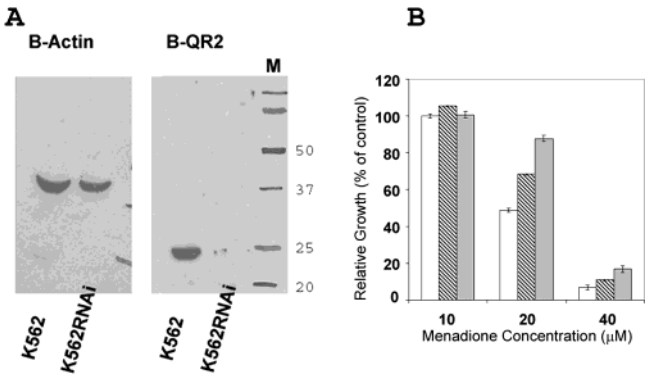

FIGURE 6: Increased resistance of resveratrol-treated and QR2 knockdown K562 cells. (A) Western blot analysis of QR2 expression in wild-type K562 and K562RNAi cells. The expression of QR2 in K562RNAi cells was undetectable in Western blot analysis (right panel) using QR2-specific antibodies. After stripping off the antibodies, the same membrane was blotted with anti-actin antibody as the loading control (left panel). (B) K562 cells untreated (white bars) and treated with 5 μM resveratrol (hatched bars), and K562RNAi (shaded bars) cells were treated with menadione (10, 20, and 40 μM) for 4 h. The number of cells that survived was analyzed by MTT assays (Roche).

flavonoids, quercetin, exhibits a K_d value of 50 nM toward QR2 when assayed by the tryptophan fluorescence quenching method (Figure 5) and shows potent inhibition of the QR2 enzymatic activity (data not shown), whereas flavone and baicalein, close analogues of quercetin, showed no inhibitory activities toward QR2, which further indicates the importance of the properly positioned hydroxyl groups. Furthermore, some of the chemopreventive properties shared among these compounds may be arisen from the common mechanism of QR2 inhibition, such as quercetin and 4,2,4′-trihydroxychalcone, which are all reported to have cancer-preventive properties (*17*, *18*).

Similar Properties of Cells with QR2 Knockdown and Cells Treated with Resveratrol. In this study, we compared the cellular resistance to menadione toxicity of resveratrol (5 μM) treated cells with those of the QR2-expression-suppressed cells, because metabolizing of quinones such as menadione can generate oxidative reactive oxygen species (*14*), and oxidative stress is suggested to be a major pathway in carcinogenesis (*14*, *15*, *33*). We have established a stable K562 cell line with QR2-expression suppressed by RNAi to undetectable levels judged by Western blot analysis (Figure 6A). The new cell line (K562RNAi) is viable and can be cultured under identical conditions to those of wild-type K562 cells. In the presence of 20 μM menadione in the culture media for 4 h, the number of viable cell was reduced to 50% for the wild-type K562 cell, while close to 90% of the K562RNAi cells survived and 70% of cells pretreated with 5 μM resveratrol survived under identical conditions, which lay between these of the wild-type and K562RNAi cells (Figure 6B). The similarity in the resistance to menadione toxicity between the cells treated with resveratrol and cells with QR2-expression suppressed indicates a possible link between those two events, because we have shown that resveratrol is a potent inhibitor of QR2 activity. The resistance to menadione toxicity was also observed in the QR2 gene knockout animals (*34*), which further validates our observation. This is in sharp contrast to QR1 gene knockout animals, which exhibit increased sensitivity to quinone toxicity (*35*). Those data indicate that the biological roles of QR1 and QR2 are very different, albeit their high sequence and structural homologies. The enhanced resistance to oxidative stress could be the first step in the chemoprevention (*15*, *36*).

Another property of resveratrol is the antiproliferative effect on a variety of cancer cell lines (*3*), as we have also observed with K562 cells. In the presence of 5 μM resveratrol, the doubling time of K562 cells increased from 26 h for the wild type to 35 h, whereas the doubling time of K562RNAi cells has increased to 40 h under identical growth conditions (data not shown).

The resistance to the quinone toxicity for cells treated with resveratrol and other natural polyphenols has been attributed to the increased expression of antioxidant and phase-II metabolizing enzymes (*10*), such as catalase, GST, and quinone reductases (*11*, *27*, *37*). Resveratrol treatment of cells resulted in the up-regulation of catalase, QR1, and glutathione reductase, and the extent of up-regulation depends on the concentration and time duration, from 10 to 20% to a few folds when analyzed on cardiac H9C2 cells (*11*). These enzymes can convert reactive oxidative chemicals to non-oxidative chemicals and thus protect cellular components, especially DNA, from damage (*15*). To understand the increased resistance to quinone toxicity of K562RNAi cells, we analyzed five antioxidant and phase-II metabolizing enzymes in the total cell lysates of wild-type K562 cells and K562RNAi cells (Table 2). The enzymes analyzed were glutathione reductase, glucose-6-phosphate dehydrogenase, catalase, GST, and total NAD(P)H-dependent quinone reductase (not necessarily QR1 alone), which are enzymes that protect cellular components from oxidative damage and consequently carcinogenesis (*38*). We observed that K562-RNAi cells showed markedly increased catalase activities, moderate but not significant increases of the total NAD(P)H-dependent quinone reductase activities, and GST activities. There is no change in the glucose-6-phosphate dehydrogenase activities, which provided us with an excellent internal assay control.

DISCUSSION

Resveratrol Is a Potent Inhibitor of QR2 Enzymatic Activity. Resveratrol has been reported to affect multiple proteins, including kinases such PKC and numerous oxygenases and reductases (*6*). Resveratrol was reported to inhibit ribonucleotide reductase with IC_{50} of 4−10 μM (*39*); it

Table 2: Antioxidant Enzymatic Activity Analysis[a]

enzyme activities	cell type	enzyme activity	percentage of wild type
glutathione reductase	wild type	40.7 ± 0.4	
(nmol of NADPH oxidized min^{-1} mg of $protein^{-1}$)	RNAi	38.7 ± 0.7	95
glucose-6-phosphate dehydrogenase	wild type	106.6 ± 0.8	
(nmol of NADP reduced min^{-1} mg of $protein^{-1}$)	RNAi	106.2 ± 1.5	100
catalase	wild type	5428.9 ± 72.2	
(nmol of H_2O_2 consumed min^{-1} mg of $protein^{-1}$)	RNAi	8360.1 ± 61.9[b]	154
GST activity	wild type	31.8 ± 0.5	
(nmol of CDNB formed min^{-1} mg of $protein^{-1}$)	RNAi	35.2 ± 0.6[c]	111
quinone reductase activity	wild type	24.1 ± 0.4	
(nmol of MTT formed min^{-1} mg of $protein^{-1}$)	RNAi	26.0 ± 0.5[d]	108

[a] Data represent mean ± standard error. [b] Statistically significant versus wild-type group; $p < 0.001$. [c] Statistically significant versus wild-type group; $p < 0.005$. [d] Statistically significant versus wild-type group; $p < 0.05$.

inhibits PKC activity with an IC_{50} of about 2 μM. Resveratrol was also reported to be an inhibitor of lipoxygenase, polyphenol oxidase, peroxidase, and superoxide dismutase with K_i values ranging from 10 to 400 μM. In the first report of chemopreventive properties of resveratrol, the authors attributed the functions of resveratrol to the inhibition of cyclooxygenase activity with IC_{50} of about 15 μM (*2*). Evidently, high concentrations of resveratrol will affect a variety of protein targets. However, the relevant biological targets should exhibit much higher affinity, because the consumption of grape products such as red wine could only supply modest concentrations and the amount of resveratrol in red wine was reported to be in the range from 0 to 30 μM (*40*). Thus, the biological target of resveratrol function that can explain the "French Paradox" (*41*) could not be these proteins identified so far, because they require resveratrol concentrations much higher than can be attained in the consumption of wine products to exert any effect. We could not rule out the possibility that other chemicals in grape products exerted the cardioprotective effect; however, the present study demonstrates that resveratrol is a much more potent inhibitor of QR2 enzymatic activity, with a dissociation constant of 35 nM. The concentrations attainable from the consumption of grape products should exhibit robust inhibition of QR2 enzymatic activities.

QR2 has also been identified as one of the bound proteins in a number of affinity matrix studies, including quinoline derivatives (*42*), bisindolylmaleimide-type protein kinase C inhibitors (*43*), and melatonin derivatives (*44*). Recently, it was reported that those quinoline derivatives bind to QR2 with an apparent affinity of about 1 μM (*45*). Even though these compounds exhibit much lower affinity toward QR2 than resveratrol, it is still noteworthy that a broad spectrum of compounds could potentially affect QR2 enzymatic activity.

QR2 Could Be the Common Target of the Chemopreventive Polyphenols. A variety of natural polyphenols have been suggested to have chemopreventive properties, including stilbene derivatives (resveratrol) (*46*), flavonoids (quercetin and apigenin) (*47*), chalcone derivatives (*18*), and some coumarin derivatives. All those compounds share a common structural feature in which two phenolate rings are spaced similarly, as also observed by Howitz et al. (*48*). Therefore, it is a feasible hypothesis that these polyphenols could work through a common target (or targets) to exert their chemopreventive functions, because their shared chemical features could be more than a coincidence. The structure of QR2 in complex with resveratrol reveals that QR2 possesses a unique active-site cleft that can accommodate a *trans*-stilbene structure, which is shared among these classes of natural polyphenols: a *trans*-stilbene structure that can adopt a flat conformation. It is tempting to suggest that human QR2 is a sensor molecule for some natural ligands, polyphenols in particular, and the inhibition of QR2 activity could modulate the reductive potential of cells and hence the cellular resistance to oxidative stress. We have tested a number of related compounds, including quercetin and baicalein. Quercetin is shown to be a strong inhibitor of QR2 enzymatic activity, with similar affinity toward QR2 as resveratrol, consistent with an earlier report (*30*), whereas baicalein, a strong QR1 enzymatic activity inhibitor (*49*), does not inhibit QR2 activity significantly. Also, flavone itself does not inhibit QR2 enzymatic activity. This reinforces the notion that the hydrogen bonds between resveratrol and amino acids from QR2 are critical for the affinity and specificity, and the variation of the positioning of the hydroxyl groups will determine the affinity and specificity toward certain protein targets. Certainly, the various health benefits of natural polyphenols could be attributed to their effects on different molecular targets, as in the case of lifespan regulation, which was reported to be the result of sirtuin activation (*48*).

Implication of the Catalytic Mechanism. The catalytic mechanism of QR2 and QR1 is likely very similar, a classic ping-pong mechanism (*45*). FAD is reduced by accepting a hydride from the reduced nicotinamide to form the $FADH_2$−enzyme complex, and subsequently, nicotinamide is released from the active site. The quinone is then bound to the vacated active site and subsequently accepts a hydride from $FADH_2$, completing the obligatory two-electron transfer from reduced nicotinamide to quinone (*32*, *45*). In the case of QR1, Y155 is involved in the catalysis in the protonation of the O2F oxygen (FAD) during catalysis, which is further assisted by a neighboring histidine residue (H161). Tyrosine 155 is conserved between QR1 and QR2; however, histidine 161 in QR1 is not conserved, which is replaced by an asparagine residue in QR2. The involvement of N161 in QR2 catalysis is not clear, because it could not stabilize a developing negative charge as a histidine does.

It was suggested that the metal ion, zinc ion, which is unique to QR2, might be involved in the catalysis (*19*). Zhao et al. also suggested that the endogenous protein may use a copper ion instead of a zinc ion as in the recombinant protein, and the copper ion could potentially be involved in the electron transfer process such as in a number of other reductases (rebredoxin and cytochromes). However, the involvement of the metal ion is not clear, even though there

is a possible bonding network that can connect the metal ion to the catalytic active site. The side chains involved include residues H172, Y132, N161, and Y155. H172 is directly coordinating the metal ion while also forming a hydrogen bond with the hydroxyl group of Y132. Most likely, the metal ion is required for the stability of the structure only, because we tested a number of metal ions in a refolding protocol to identify the optimum metal ion for the catalysis. After QR2 denaturation with 7 M guanidine hydrochloride, the denatured protein solution was passed through a chelating column to get rid of any possible metal ions. We tried to refold the denatured protein in solutions containing different metal ions. The denatured QR2 can refold in the presence of Fe^{3+}, Mn^{2+}, or Zn^{2+} ions, and the resulting proteins exhibited near identical activity in the reduction of menadione to the purified recombinant protein, which contains Zn^{2+} ion. However, the protein cannot refold into the active form in the absence of any metal ions in the refolding buffer. Consistent with our studies, Kwiek et al. recently reported that the QR2 enzyme purified from human red-blood cells contained partially zinc and partially copper ions (*45*).

Antioxidant Enzyme Expression Regulation and Cellular Implications. Much evidence supports the notion that induction of the phase-II response is an efficient strategy for reducing the risk of a variety of diseases (*12*, *50*). The induction goes through the transcription activation of the antioxidant response element (ARE) upon binding of the leucine zipper transcription factor Nrf2 (*51*, *52*). Gene knockout animal studies, including Nrf2, GST, and QR1, firmly support the hypothesis that Nrf2 is involved in the regulation of detoxification genes and the roles of those detoxification proteins involving in the prevention of carcinogenesis (*53*−*57*). Furthermore, the human population polymorphic for certain phase-II enzymes are more susceptible to chemical toxicity and carcinogenesis (*58*−*63*).

Inducers of phase-II genes can be divided into two classes: electrophiles and some natural polyphenols. The electrophiles are a diverse class of chemicals that are chemically reactive and can modify sulfhydryl groups by alkylation, oxidation, or reduction (*64*). Even though these inducers enhance the expression of an array of detoxification enzymes, such as QR1, GST, catalase, and others, they do not induce the expression of QR2 protein, further differentiating the biological functions of QR2 from QR1 (*34*).

The recent work by Wakabayasi and co-workers indicated the mechanism of detoxification enzyme induction by electrophiles (*64*). Under basal conditions, Keap1, a recently identified protein associated with the actin cytoskeleton, binds very tightly to Nrf2, anchors this transcription factor in the cytoplasm, and targets it for ubiquitination and proteasome degradation, thereby repressing the ability of Nrf2 to induce phase-II genes (*52*, *65*−*69*). Inducers disrupt the Keap1−Nrf2 complex by modifying cysteine residues in Keap1, allowing Nrf2 to translocate to the nucleus where, in heterodimeric combinations with other basic leucine zipper proteins, it binds to AREs of phase-II genes and accelerates their transcription (*64*).

However, the mechanism of induction of detoxification enzymes by natural polyphenols, a class of antioxidants, is still to be understood. We propose that there might be endogenous electrophiles that could modify the cysteine residues of Keap1 and thus regulate the expression of detoxification enzymes. These endogenous electrophiles can be further regulated by QR2 through reduction. Thus, inhibition of QR2 would increase the concentration of the endogenous electrophiles and consequently they will modify Keap1 and thus up-regulate the expression of these antioxidant enzymes. Evidently, this is purely speculative, and much more work needs to be done to elucidate the biological functions of QR2.

Altogether, our results suggest that QR2 is a novel target of polyphenol function. Resveratrol and other natural polyphenols such as quercetin are potent inhibitors of QR2 enzymatic activity, and we hypothesize that the inhibition of QR2 activity induces the up-regulation of antioxidant enzymes, even though the mechanism is yet to be elucidated. The up-regulation of antioxidant enzymes in turn increases cellular resistance to oxidants such as menadione and further protects cellular components from oxidation-related damage.

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