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Identification and purification of resveratrol targeting proteins using immobilized resveratrol affinity chromatography

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Abstract

The phytochemical resveratrol (*trans*-3,4',5-trihydroxystilbene) is a naturally occurring polyphenol with a plethora of health-beneficial properties, including a preventive role in cancer. We surmise that resveratrol may exert its diverse biological effects by interacting with specific target proteins, denoted RTPs. To test this possibility, resveratrol was immobilized on epoxy-activated agarose forming a resveratrol affinity column (RAC), which was used to detect and isolate RTPs. Distinct RTPs can be resolved on RAC by fractionation with increasing NaCl, followed by 1 mM ATP, and finally, with 1–2 mM resveratrol. A 22-kDa polypeptide, RTP-22, eluted with resveratrol was identified by MALDI-TOF MS and cloning/expression in *Escherichia coli*, as dihydronicotinamide riboside quinone reductase 2 (NQO2). The utility of RAC was additionally explored with extracts derived from different staging prostate cancer cells. NQO2 was most abundant in CWR22R v1, a model for prostate cancer transition from androgen-dependent to the hormone-refractory state, but was marginally expressed in JCA-1 cells as representing more advanced stage prostate cancer. These results provide evidence for the existence of distinctive RTPs in mammalian cells and that RAC is a facile approach to identify and purify RTPs.

Keywords

Resveratrol; Resveratrol targeting proteins; Immobilized resveratrol affinity columns; Prostate carcinogenesis

The relationship between diet/nutrition and the incidence and mortality of most human cancers is well supported by epidemiologic, in vitro, animal and, to a more limited extent, clinical studies [1–5]. To fully grasp the chemopreventive role of diet and nutrition, it is essential to identify specific bioactive ingredients and elucidate their mechanism of action [4,6–8]. Information of cellular targets that might mediate the activity of chemopreventive agents is also imperative.

Resveratrol is a grape-derived polyphenol shown to have diverse biological properties [9–13]. Although resveratrol has been historically studied as a cardioprotectant, recent studies have also focused on its chemopreventive activities, stemming from the observation by Pezzuto and co-workers [14] that this dietary polyphenol inhibited events connected to

tumor initiation, promotion, and progression. Mechanism of chemoprevention by resveratrol, however, remains incompletely defined.

We hypothesize that resveratrol exerts its diverse biological effects by interacting with specific targeting proteins, denoted RTPs, probably via its stilbenoid core and hydrophilic side groups. In support of this possibility, resveratrol has been reported to interact with receptors, notably estrogen receptors ER- α and ER- β , and aryl hydrocarbon receptor AhR [15–18]. Further, resveratrol also affects numerous enzymes and/or signaling molecules, such as, ribonucleotide reductase, mitochondrial proton F₀F₁-ATPase/ATP synthase, DNA polymerases α and δ , several kinases [19–29], and most recently NAD⁺-dependent histone deacetylase known as sirtuins [30].

To identify and purify RTPs, we have immobilized resveratrol on a solid matrix, forming a resveratrol affinity column (RAC). We have characterized the binding properties of RAC using HL-60 cell extracts. Sequential elution with high salt (0.35 M followed by 1.0 M NaCl), 1 mM ATP, and finally 1–2 mM resveratrol afforded displacement of distinct proteins showing various binding affinities for resveratrol. A 22-kDa polypeptide, named RTP-22 and eluted with resveratrol, has been identified as dihydronicotinamide riboside quinone reductase 2 (NQO2) by MALDI-TOF MS, and further confirmed by cloning.

RAC was used to ascertain quantitative changes of NQO2 in extracts from different staging prostate cancer cells. Expression of NQO2 was most pronounced in CWR22R v1, representing cells in transition between androgen-dependent and hormone-refractory states, and was least abundant in the JCA-1 cells, which were derived from more advanced prostate cancer. Our results support the interpretation that distinctive RTPs exist in mammalian cells, and that RAC provides a simple method for their identification and purification.

Materials and methods

Materials and methods

Epoxy-activated agarose resin (12 atom linker, 33 μ mol of epoxy group/ml of packed gel) was purchased from Sigma Chemical (St. Louis, MO). Resveratrol was obtained from Sigma Chemical or LKT Laboratories (St. Paul, MN). Stock of resveratrol (12.5 mM) was prepared in dimethyl sulfoxide (DMSO) and kept at -20°C . Biochemical and molecular biology grade chemicals and reagents were purchased from various commercial vendors. Large quantities of cultured HL-60 and K562 cells were obtained from National Cell Culture Center (NCCC, Minneapolis, MN).

Cell culture

Human HL-60, K562, LNCaP, PC-3, DU145, and CWR22R v1 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The JCA-1 cells were established in this institution from the primary cancer site of an individual showing poorly to moderately differentiated prostatic adenocarcinoma before hormonal therapy was administered [31–33]. Routinely, cells were cultured in RPMI-1640 media containing L-glutamine, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Cells were split once a week and media were changed every 3–4 days.

Preparation of immobilized resveratrol affinity column

One gram of epoxy-activated agarose was suspended in ice-cold water for 5 min and washed extensively to remove the preservatives. Resveratrol (23 mg) dissolved in 2.5 ml of 0.1 M NaOH was added to 1 ml of resuspended epoxy-activated agarose, followed by an overnight incubation at room temperature to allow chemical coupling of resveratrol to the resin. To

stop the reaction, 6 ml of 1 M sodium acetate buffer (pH 5.0) containing 1 mM dithiothreitol (DTT) was added to the mixture to neutralize unreacted epoxy groups and prevent further oxidation of resveratrol. Immobilized resveratrol resin was washed successively with 0.1 M sodium acetate, pH 5.0, containing 1 mM DTT and 70%, 30%, 10%, and 0% ethanol, respectively. Controls consisted of mock-treated beads (identical procedure except that no resveratrol was added) or beads immobilized with tyrosine as the ligand.

Fractionation of cytoplasmic extracts on RAC

Cultured mammalian cells were lysed by 3 freeze–thaw cycles with buffer containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM Mg(OAc)₂, 1 mM DTT, 0.5% NP-40, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µl/ml of the protease inhibitor cocktail from Sigma Chemical. Cell-free extracts were obtained by a 10-min centrifugation in a refrigerated microcentrifuge.

To characterize RAC (Fig. 1), 200 µl HL-60 cell extract containing 0.6–1.0 mg protein, or as indicated in the figure, was mixed with 50 µl control (mock-treated or tyrosine-linked) or resveratrol-immobilized agarose beads in a 1.5-ml Eppendorf tube. The tube was incubated overnight at 4 °C with mild tumbling. The gel slurry containing protein extract was loaded onto a minicolumn (from Pierce Chemical) and washed with 10–20 ml lysis buffer to remove unbound proteins. The column was next eluted 5–7 times, each time with 0.5 ml lysis buffer containing 0.35 M NaCl, and was followed by the same number of washing using 1 M NaCl supplemented buffer. Next, the column was equilibrated with the lysis buffer and eluted with 1 mM ATP. As the last step, the column was again re-equilibrated with the lysis buffer and eluted with 1–2 mM resveratrol dissolved in 2% DMSO.

Identification of RTP-22 from K562 cells by MALDI-TOF MS

Cytoplasmic extracts were prepared from K562 cells and fractionated as described (Fig. 1A). The middle silver-stained protein band corresponding to apparent molecular mass 22-kDa, designated RTP-22 of a 1 mM resveratrol-eluted triplet, was excised, reduced, carbamidomethylated, and digested with trypsin. The resulting peptide mixture was desalted and concentrated using Zip-Tip C18 micro-columns (Millipore), and applied to the MALDI target using solution phase nitrocellulose method, as described [34]. Peptide masses were determined using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) in reflector mode using standard instrument settings. Internal mass calibration was carried out using trypsin autodigestion products (842.51 and 2211.10 Da). For protein identification, peptide mass fingerprinting generated by MALDI-TOF MS was matched to the theoretical tryptic digests of proteins from the NCBI nonredundant protein database using the ProFound software (<http://prowl.rockefeller.edu/cgi-bin/ProFound>). The database search result showed a positive match (5 matched peptides with mass error <0.01 Da; amino acid sequence coverage 28%) with dihydronicotinamide riboside quinone reductase 2 (NQO2).

Cloning and expression of NQO2 (RTP-22)

The identification of RTP-22 as NQO2 provided information for its cloning, using NQO2 cDNA (MGC-12729) purchased from ATCC. The primers used in cloning were, respectively: 5'-AACCATGGCAGGTAAGAAAGTACTC (added *NcoI* site is underlined) and 5'-AACTCGAGTTATTGCCCGAAGTGCCAGTG (added *XhoI* site is underlined). Following digestion with appropriate restriction enzymes, the PCR product was ligated into pET23d expression vector (Novagen, Madison, WI) at the *NcoI* and *XhoI* sites. The pET23 vector with human NQO2 insert was transformed into *Escherichia coli* strain B834 (Novagen) for over-expression using 1 mM isopropylthiogalactoside (IPTG). Recombinant NQO2 was purified from bacterial extracts using DEAE–Sephacrose, Superdex 75, and

Mono-Q column (Pharmacia) chromatography. About 50 mg of purified NQO2 was obtained from 1 liter of bacteria culture.

Fractionation of cytoplasmic extracts from prostate cancer cells representing different stages of prostate carcinogenesis using RAC

Prostate cancer cells were lysed by repeated freeze–thaw cycles with lysis buffer and cytoplasmic extracts were obtained by centrifugation in a refrigerated microcentrifuge, as described above. Lysates (600 µg in 200 µl lysis buffer) from LNCaP, CWR22R v1, DU145, PC-3, and JCA-1 cells were individually applied to RAC. To demonstrate specificity of binding, extracts were also mixed with 1 mM resveratrol prior to binding to the affinity resin, in order to establish competition of retention of RTP-22. Proteins eluted by 1 mM resveratrol in the last step were analyzed by 10% SDS–PAGE and visualized by silver staining.

Results

Characterization of RAC

The overall goal of this study was to identify, isolate, and characterize cellular targets of resveratrol as an approach to gain information on its chemopreventive attributes. To capture and identify RTPs, we immobilized resveratrol onto epoxy-activated agarose beads. Nonspecific adsorption of resveratrol on column surfaces was removed by exhaustive washing, as detailed in Materials and methods. Chemical coupling of resveratrol evidently could occur at one or more of the three available hydroxyl groups (3, 5, 4'), resulting in a resveratrol-immobilized affinity column (RAC). Structure–function studies have shown that modifications at 3- or 5-hydroxyl groups do not significantly alter the biological activity of resveratrol [23,35,36], suggesting that immobilization at either positions is not likely to affect the binding of resveratrol to putative RTPs and that the majority of column-bound resveratrol should retain similar binding properties as resveratrol in solution. We therefore surmise that quantitative retention of RTPs on RAC is possible, provided that RAC is in relative excess to RTPs present in a given amount of lysate.

Using a fractionation procedure illustrated in Fig. 1A, we tested the binding properties of RAC with HL-60 cell extracts. Binding capacity of RAC was estimated by applying varying amounts of cytoplasmic extracts to a fixed volume of RAC (Fig. 1B) and also by varying the ratio of RAC to the lysate (Fig. 1C). Specificity and affinity of RAC was verified by determining the concentration of resveratrol required to effectively compete binding of RTPs to RAC (Fig. 1D) and the amount of resveratrol that quantitatively displaced RTP-22 from RAC (Fig. 1E). In each instance, HL-60 cell lysates were incubated with RAC and eluted, as described in Materials and methods. The eluted samples were analyzed by SDS–PAGE and visualized by silver staining. A number of proteins showed specific retention on RAC, the most prominent and consistent of which was a resveratrol-eluted protein migrating on SDS–PAGE with an apparent molecular weight of 22-kDa. This protein was designated RTP-22. Apparently, 50 µl RAC, prepared as described in Materials and methods, sufficed to quantitatively retain RTP-22 from up to 3 mg of HL-60 cell lysate (Figs. 1B and C). Moreover, binding of RTP-22 to RAC was effectively competed by 1 mM resveratrol (Fig. 1D), while 0.5 mM resveratrol effectively displaced RTP-22 from the affinity resin (Fig. 1E).

Screen of several cancer cell extracts using RAC showed abundant expression of RTP-22 in K562 cells (data not shown), which were chosen for further characterization of RTP-22. Extracts with and without preincubation with 1 mM resveratrol (to establish competition of binding of RTP-22 to RAC) was separately fractionated according to the scheme in Fig. 1A.

Fig. 2A compares the profile of proteins eluted with 1 mM ATP, and the ones eluted with resveratrol. Several larger molecular weight silver-stained protein bands were efficiently displaced from the column using ATP (Fig. 2A, lane 3), whereas a different set of proteins was eluted using resveratrol (Fig. 2A, lane 6). Notably, these silver-stained bands were absent in fractionation similarly carried out with tyrosine-immobilized beads (see lanes 2 and 5, Fig. 2A) or in mock-treated beads (data not shown). In both ATP and resveratrol eluates, several of the bound proteins were clearly competed by resveratrol, suggesting that binding to this polyphenol primarily determined their retention on RAC (compare lanes 3 and 4 in the case of ATP eluate and lanes 6 and 7 for resveratrol-eluted proteins, Fig. 2A). The most consistent resveratrol-eluted proteins comprised a triplet; the middle protein band was assigned RTP-22, as evidenced by its complete competition for binding by resveratrol and requirement for resveratrol for displacement from RAC. Notably, results in Fig. 2A also revealed that not all proteins eluted by ATP (compare lanes 3 and 4) or resveratrol (compare lane 6 with 7) were competed by resveratrol.

Silver-stained RTP-22 was excised from the gel and subjected to in-gel trypsin digestion to generate peptide fragments that were further determined by MALDITOF MS. This procedure generated signature patterns that were used in database searches to match predicted tryptic peptide masses of proteins with known identity, resulting in the identification of RTP-22 as NQO2 (Fig. 2B). The identity of RTP-22 as NQO2 was additionally verified by cloning and expression, as detailed in Materials and methods.

Differential expression of NQO2 in cells representing different stages of prostate carcinogenesis

We had previously demonstrated that resveratrol significantly suppressed growth of androgen responsive (AD) and hormone-refractory (AI) prostate cancer (CaP) cells, and that it also markedly reduced prostate specific antigen (PSA) expression [37,38]. Because of the slow natural history of CaP and the long latency associated with AD → AI transition, this form of malignancy offers an excellent model for testing whether chemopreventive agents such as resveratrol might confer protection in humans. To explore role, if any, NQO2 might have in CaP progression, RAC was used to fractionate extracts prepared from cells representing the early (LNCaP: androgen-dependent, AR-positive), intermediate, transition (CWR22R v1: androgen-independent, AR-positive), and late, hormone-refractory (DU145, PC-3, and JCA-1: androgen-independent, AR-negative) stages of prostate carcinogenesis. The CWR22R v1, as cells derived from androgen-insensitive tumors that recurred after the androgen-sensitive CWR22 xenograft, was induced into regression by androgen withdrawal, more closely resembling the situation in AD → AI progression [39,40]. Notably also are the JCA-1 cells as they were derived from the primary site of an individual prior to hormonal therapy and could therefore be a more appropriate proxy for primary human prostate carcinoma [31]. Results in Figs. 3A and B compared the expression of NQO2 (RTP-22) in these cell lines, as monitored by RAC (Figs. 3A and B). Comparison of the silver-stained protein profiles shows that RTP-22 was the major protein retained on RAC in all cell lines monitored, being most prominent in CWR22R v1 cells and least abundant in JCA-1 cells (Figs. 3A and B).

Discussion

To investigate the mechanism of chemoprevention by resveratrol, a recently identified grape-derived polyphenol, we have developed an innovative approach, named RAC, which has been shown to quantitatively capture cellular targeting proteins of resveratrol (Fig. 1). A unique feature of this strategy is that rather than espousing mechanistic themes linked to malignant traits, such as unrestricted proliferation, resistance to apoptosis, and propensity for metastasis that permeate much of the current chemoprevention scientific literature, RAC

relies on the specificity and affinity resveratrol displays towards distinct target proteins in responsive cells, and as such, RAC might reveal novel targets pertinent to the biological activities of resveratrol, and not ones merely restricted to its chemopreventive activity. A caveat of the RAC strategy is that while its affinity prefabrication feature offers a more focused analytical scope, it may also potentially bias against proteins with low to no binding affinity for resveratrol, yet could be of importance in its panoramic bioactivities, including chemoprevention.

Using RAC, we describe here the first experimental demonstration and purification of RTPs from cultured mammalian cell extracts. RAC offers a facile experimental procedure whereby fractionation of cytosol of cells on such a matrix selectively enriches distinct RTPs, including the one designated RTP-22. RTP-22 has been identified as quinone reductase type 2 (NQO2) using mass spectrometric analysis and cloning (Fig. 2). NQO2 expression shows substantial change in CaP cells mimicking different stages of prostate carcinogenesis, being most abundant in cells (CWR22R v1) representing AD $\rightarrow$ AI transition.

An assumed functional role of RTPs in prostate carcinogenesis would imply that RTPs derived from the currently treatable androgen-dependent form of CaP (using LNCaP as a proxy) could be qualitatively and quantitatively different from those obtained from the untreatable androgen-independent form of CaP (represented by PC-3). Such differences may have translational implications, e.g., to aid in the design of selective nutritional intervention strategies for different forms of CaP. A closer examination of the proteins eluted by resveratrol using extracts from LNCaP, CWR22R v1, DU145, PC-3, and JCA-1 cells revealed quantitative and qualitative differences in their respective RTP profiles (Fig. 3A). For example, a high molecular weight protein (>116-kDa) appears to be uniquely eluted from CWR22R v1 cell extracts, whereas several proteins migrating in the 66-kDa areas were observed in PC-3 extracts (Fig. 3A). At least 2–3 proteins, with 36- to 45-kDa molecular weights, were identified in JCA-1 cells. The nature and function of these proteins remain unknown; their qualitative presence in different staging CaP cells raises the possibility that they could find application both as biomarkers and as drug targets.

In conclusion, a facile method, named RAC, has been developed to analyze the diverse biological functions of resveratrol. Use of RAC has provided evidence for our hypothesis that there are specific cellular mediators of resveratrol and that RTPs may have a direct and indirect role in the mechanism of action of resveratrol, including chemoprevention. RAC is uniquely suited to enrich low abundance proteins, narrow the complexity of the sample being analyzed, and facilitate the rapid and accurate generation of RTP profiles. These attributes have applications in diverse biological settings: comparison between normal versus malignant states; between uninduced versus inducible experimental situations; and for elaboration of tissue specific and system biology specific responses to resveratrol. While our studies are in progress, Barnes and co-workers [41,42] reported the isolation and characterization of DING as a genistein targeting protein in human breast cancer, using an affinity chromatography approach with 2-carboxygenistein coupled to AH-Sepharose 4B.

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Fig. 1.

Purification of RTPs from RAC and characterization of RAC using HL-60 cells. (A) Purification strategy for the isolation of RTPs from RAC. In step 1, a cell lysate is passed over RAC or a mock-coupled or tyrosine-linked control column in parallel, and washed exhaustively with lysis buffer to remove nonspecific proteins. In step 2, the column is eluted stepwise eluted with 0.35 and 1 M NaCl. In step 3, column-bound proteins are eluted using 1 mM ATP. The last step involves elution with 1–2 mM resveratrol dissolved in 2% DMSO. (B–E) In the upper frames of (B–E), specificity and affinity of RAC were determined by varying amounts of lysate (B) and RAC (C), and also with respect to concentration of resveratrol required to effectively compete RTP binding to RAC (D) and for elution of RTPs from RAC (E). Lower frames of (B–E) represent data compiled from densitometric tracings of RTP-22 (identified by the arrow), based on analysis using 2–3 different preparations of RAC, with each preparation assayed in duplicate or triplet using HL-60 extracts.

Fig. 3. Changes in NQO2 (RTP-22) in cells representing different stages of prostate cancer, as assayed by RAC (A,B). (A) The comparison of silver-stained RTP profiles of different staging prostate cancer cell extracts fractionated according to the protocol in Fig. 1A and eluted with resveratrol, whereas (B) represents quantitation of densitometric tracings of NQO2 shown in (A).