Effect of resveratrol and zinc on intracellular zinc status in normal human prostate epithelial cells

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Zhang JJ, Wu M, Schoene NW, Cheng WH, Wang TT, Alshatwi AA, Alsaif M, Lei KY. Effect of resveratrol and zinc on intracellular zinc status in normal human prostate epithelial cells. Am J Physiol Cell Physiol 297: C632–C644, 2009. First published June 24, 2009; doi:10.1152/ajpcell.00139.2009.—To evaluate the influence of resveratrol on cellular zinc status, normal human prostate epithelial (NHP/E) cells were treated with resveratrol (0, 0.5, 1, 2.5, 5, and 10 μM) and zinc (0, 4, 16, and 32 μM, representing zinc-deficient [ZD], zinc-normal [ZN], zinc-adequate [ZA], and zinc-supplemented [ZS] conditions, respectively). A progressive reduction in cell growth was observed in cells treated with increasing amounts of resveratrol (2.5–10 μM). Resveratrol at 5 and 10 μM resulted in a dramatic increase in cellular total zinc concentration, especially in ZS cells. Flow cytometry indicated that 10 μM resveratrol induced arrest of the cell cycle at the G2/M phase in association with the observed cell growth inhibition. Data from an in vitro experiment using zinquin as an indicator of intracellular free Zn(II) status demonstrated complex interactions between resveratrol and Zn(II). Fluorescence spectrophotometry and fluorescence microscopic analyses revealed that intracellular free labile zinc was progressively elevated from nearly twofold in ZS cells with no resveratrol to multifold in ZA and ZS cells with 10 μM resveratrol compared with the corresponding ZN cells. Furthermore, increases in cellular zinc status were associated with elevated levels of reactive oxygen species and senescence, as evidenced by morphological and histochemical changes in cells treated with 2.5 or 10 μM resveratrol, especially in ZA and ZS cells. Taken together, the interaction between resveratrol and zinc in NHP/E cells increases total cellular zinc and intracellular free labile zinc status and, subsequently, reactive oxygen species production and senescence. Nutrient interaction; zinquin

Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin synthesized in the fruits of a wide variety of plants (e.g., grapes, peanuts, and mulberries) in response to environmental stress, injury, UV irradiation, and fungal infection (20). Relevant to its widely considered cardiovascular protective properties (31), resveratrol has been shown to exert anti-inflammatory (23), antiangiogenic (8, 38), and antimutagenic (39) effects, as well as to function as an estrogen receptor agonist (17). Resveratrol has also been identified as an antioxidant (25, 28) that can quench highly reactive free radicals via donation of a hydrogen atom for formation of less reactive phenoxy radicals (27). This interception of free radicals by resveratrol suppresses destructive self-propagating chain reactions, such as lipid peroxidation, thereby sparing α-tocopherol. In addition, phenolic compounds may suppress free radical reactions via chelation of catalytic metal ions, notably iron and copper (7, 22). Indeed, the inhibition of LDL oxidation by resveratrol (15) was attributed to resveratrol’s ability to chelate copper ions (5). Resveratrol was established as the most potent chelator of copper, but not iron.

In the United States and Europe, resveratrol is commercially available as a nutraceutical, ranging from 50 μg to 100 mg per dosage. Oral intakes of resveratrol at 50 mg/kg body wt in rats can result in plasma resveratrol concentration as high as 10 μM (26). A recent human study reported that single-dose intakes of up to 5 g of resveratrol resulted in peak plasma concentration of 2.4 μM at 1.5 h after intake (6). Moreover, peak levels of two monoglucuronides and resveratrol-3-sulfate were three- and eightfold higher, respectively. These levels are close to the systemic concentration of ~5 μM that has been suggested by mechanistic in vitro cellular studies to be required to elicit bioactivities relevant to chemoprevention (9, 13, 18, 23, 32, 34).

However, many studies have reported potential harmful prooxidant properties of polyphenolic compounds at high concentrations in the presence of metal ions. Resveratrol at high doses was found to induce DNA breaks in a manner involving formation of a resveratrol-Cu(II) complex, resulting in reduction of copper ions and induction of reactive oxygen species (ROS) (1, 16). Resveratrol and other polyphenolic compounds have been shown to induce apoptotic cell death more effectively in various cancer cell lines than in normal cells (9). The cytotoxic effect has been proposed to involve mobilization of copper ions and their subsequent prooxidant actions. Resveratrol in the presence of Cu(II) has also been confirmed to cause DNA degradation in peripheral lymphocytes derived from human blood (3). Moreover, inhibition of DNA degradation in lymphocytes by scavengers of reactive oxygen and by neocuproine, a Cu(I)-specific sequester, demonstrated that the DNA breakage is induced by ROS derived from reduction of Cu(II)-Cu(I) by these polyphenols, including resveratrol (4). Therefore, multiple lines of evidence have established a possible interaction between copper and resveratrol.

Zinc is an integral component of a wide variety of enzymes, transcription factors, and other functional proteins, where it exerts specific actions over a wide range of physiological processes, such as growth, development, and functioning of the endocrine, immune, and nervous systems, and may contribute to the development of tumors (29, 40, 41). Zinc has also been established as an antiapoptotic factor, with an antiapoptotic mechanism that minimizes ROS-induced oxidative damage to

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cellular organelles (36). However, it has been shown that, although under physiological conditions zinc can suppress apoptosis caused by zinc deficiency, zinc supplementation at very high concentrations may trigger cell death by apoptosis or necrosis in some cells (36). Therefore, the maintenance of intracellular zinc homeostasis is a critical process in all living cells.

In view of the structural similarity between zinc and copper, as well as the observation that resveratrol administration markedly increased plasma zinc and copper status in adult rats (24), we hypothesize that resveratrol supplementation will alter zinc homeostasis and trigger intracellular zinc deposition. At low mineral status, resveratrol will enhance zinc status by increasing cellular zinc uptake; however, at high mineral status, high resveratrol intake may cause excessive mineral deposition. To test this hypothesis, we designed an in vitro study of six levels of resveratrol and four levels of zinc to determine the interrelationship of resveratrol with zinc in normal human prostate epithelial (NHPrE) cells. NHPrE cells were chosen, because 1) normal prostate glands are able to accumulate 20 times more zinc than other soft organs, such as liver and kidney; 2) the glandular epithelial cells in the peripheral zone of the prostate accumulate the highest levels of total intracellular zinc; and 3) very limited data are available on the influence of resveratrol on normal human prostate cells or other normal human cell types. Data generated in this study will contribute to the understanding of the nutritional interactions between resveratrol and zinc and aid in the setting of dietary reference intakes and tolerable upper limits for resveratrol and zinc.

MATERIALS AND METHODS

Materials. Resveratrol, ZnSO₄ (1 M) solution, zinquin, and zinquin ethyl ester were purchased from Sigma-Aldrich (St. Louis, MO). Resveratrol, zinquin, and zinquin ethyl ester were dissolved in DMSO at 20, 25, and 25 mM, respectively, and stored at −20°C.

Experimental design. A factorial design involving six levels of trans-resveratrol (0, 0.5, 1, 2.5, 5, and 10 μM; from none to the high end of plasma levels attainable by oral consumption in humans) and four levels of zinc (<0.4, 4, 16, and 32 μM, which represent zinc deficiency (ZD), zinc level in most culture media (ZN), adequate level of plasma zinc (ZA), and the high end of plasma level of zinc attainable by oral supplementation in humans (ZS), respectively (19)) was used in the investigation of the effect of zinc and resveratrol on the proliferation and intracellular zinc content of NHPrE and HepG2 cells. A simplified experimental design involving four levels of zinc and two or three levels of resveratrol was used in intracellular free labile zinc assay, as well as real-time PCR.

Cell culture. NHPrE cells directly isolated from a 29-yr-old and a 21-yr-old male Caucasian donor were purchased from Lonza (Walkersville, MD). The cells are normal human cells that have not been immortalized in any way and are guaranteed for ≥16 population doublings before senescence. All NHPrE cells used in our experiments started at passage 3, equivalent to about six population doublings. Data reported in this study were obtained from the NHPrE cells from the 29-yr-old donor for the sake of consistency. NHPrE cells from the 21-yr-old donor were used to repeat key experiments to validate the results. NHPrE cells were plated at 2,500 cells/cm² in tissue culture dishes containing prostate epithelial cell growth medium (PREGM) supplemented with 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 μg/ml human recombinant epidermal growth factor, and 6.5 ng/ml triiodothyronine (as growth supplements) without antibiotics and cultured at 37°C in a 5% CO₂ incubator. Endotoxin-free (<0.005 endotoxin units/ml) media were used. The media were changed at day 1 and subsequently every 48 h. Cells were grown to 80% confluence for 6 days and subcultured using 1:8 trypsin-EDTA at passage 3 for experimental treatment. A zinc-free prostate epithelial basal medium (PREGM without zinc), in which the manufacturer had omitted ZnSO₄, was used as ZD medium and supplemented with necessary growth components. Zinc content was determined to be <1 μM, as detected by flame atomic absorption spectrophotometry. For the other zinc treatment groups, zinc was added to the media as ZnSO₄, so that the only difference between these media was the zinc concentration. The ZN, ZA, and ZS media contained 4, 16, and 32 μM ZnSO₄, respectively. The ZA group was used to represent human plasma zinc levels, and the ZS group was used to represent the high end of plasma zinc levels attainable by oral supplementation in humans. Resveratrol in 0.05% DMSO was added to each zinc medium to generate 0, 0.5, 1, 2.5, 5, and 10 μM resveratrol. ZN medium without resveratrol was used as the control group for comparison with other treatments. Cells were cultured overnight in normal PREGM before their respective treatment media was changed.

HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured at 25,000 cells/cm² in DMEM supplemented with 10% FBS and appropriate antibiotics. Chelex 100 ion-exchange resin (Bio-Rad, Hercules, CA) was used to deplete zinc from FBS. Briefly, the resin was neutralized to physiological pH with 0.25 M HEPES (pH 7.4) and then mixed with FBS at a ratio of 1:4 at 4°C for 2 h. The Chelex 100 resin was then separated from FBS by centrifugation followed by filtration through a 0.22-μm filter for sterilization and removal of residual Chelex 100 resin. Cheled FBS has a zinc concentration <1.0 μM, as determined by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). The basal DMEM with 10% chelated FBS containing <0.1 μM zinc was termed ZD medium. For the other zinc treatment groups (ZN, ZA, and ZS), zinc was added to the media in the form of ZnSO₄ (see above). Resveratrol in DMSO was added to each level of zinc medium to generate 0, 0.5, 1, 2.5, 5, and 10 μM resveratrol. ZN medium without resveratrol was used as the control group for comparison with other treatments. Cells were cultured overnight in normal DMEM before exposure to their respective treatment media was changed.

Cell proliferation and total intracellular zinc assay. Aliquots (100 μl) of the collected samples were used for total cell count determinations by the NucleoCounter using the Nucleocasette kit and NucleoView software (New Brunswick Scientific, Edison, NJ) according to the manufacturer’s protocol. Briefly, all cells were lysed and then stabilized with buffers supplied by the vendor. These samples were introduced into Nucleocassettes preloaded with propidium iodide and then scanned by the NucleoCounter. The NucleoCounter features an integrated fluorescence microscope and charge-coupled device camera designed to count cells by detecting signals from the fluorescent dye, propidium iodide, which intercalates to DNA in the nuclei of the lysed cells. The remaining cells were then resuspended in 1.5 ml of PBS and subjected to two 30-s periods of sonication on ice. Cellular zinc content was measured by flame atomic absorption spectrophotometry (model 5000, Perkin Elmer, Norwalk, CT) using standard curves of 0.05–1.0 ppm generated with certified zinc reference solutions (Fisher Scientific, Fair Lawn, NJ), as previously described (30). In addition, the certified zinc solutions were compared with Bovine Liver Standard Reference (US Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. Cellular zinc was calculated as nanomoles per 10⁶ cells.

Cell cycle analysis. Subconfluent cells were treated in different culture media as described above for 3 days and then harvested at just below 70% confluence, washed with cold PBS, and processed for cell cycle analysis. Briefly, 1.5 × 10⁶ cells were aliquoted in a 50-ml polypropylene centrifuge tube, washed once in 3 ml of PBS (without calcium or magnesium), and resuspended in 1.5 ml of PBS. To these
resuspended cells, 15 ml of 70% ethanol were added, the tubes were capped, and their contents were mixed gently by vortex. Cells were stored at 4°C for 2–4 h or overnight and then labeled with propidium iodide. Data files representing 10^6 cellular events were acquired using CellQuest software operating a FACScalibur cytometer (Becton Dickinson, San Jose, CA). Percent distributions of the DNA content of the samples in G0/G1, S, and G2/M phases of the cell cycle were determined using modeling software (ModFit LT, version 3.0, Verity Software House, Topsham, ME). The presence of cells in the sub-G0/G1 phase was used as a measure of apoptosis of the cells; phases of the cell cycle were determined using calibration standards, and LinearFlow Green (Molecular Probes, Carlsbad, CA) and DNA QC Particle kit (Becton Dickinson) were used to verify instrument performance.

In vitro assay for zinc-resveratrol interaction. In experiment 1, 100 μl of PBS containing the full combination of zinc concentrations (0, 2, 4, 6, 8, 16, 24, and 32 μM) in the form of ZnSO4 and resveratrol (0, 5, 10, or 20 μM) were aliquoted into a 96-well plate in triplicates and then mixed with 25 μM zinquin, a membrane-permeable fluorescent zinc probe specific for Zn(II).

In experiment 2, 100 μl of PBS containing the full combination of zinc concentrations (0, 2, 4, 6, 8, 16, 24, and 32 μM final concentration) and zinquin (5 or 25 μM) were aliquoted into a 96-well plate in triplicates and then mixed with (10 μM) or without resveratrol.

The plates were incubated in 37°C for 5 min before the fluorescence signal from each well was quantified at 340-nm excitation and 460-nm emission using a Genios Pro spectrophotometer (Tecan Group, Männedorf, Switzerland).

Determination of labile level of free intracellular zinc. Free intracellular zinc was measured to determine whether increases in resveratrol in the medium would lead to elevations in free intracellular Zn(II), which has been suggested to depress cell proliferation, but not induce apoptosis, in normal cells. Zinquin ethyl ester was used to quantify the level of free labile intracellular Zn(II) by spectrophotometry using a method adopted from Zalewski et al. (43) and Coyle et al. (12) with slight modification. At the end of the treatment, 100 μl of suspended cells at 1 × 10^6/ml were transferred to a 96-well optical plate (Nunc). Zinquin was added to the cells at a final concentration of 25 μM in PBS, and the cells were incubated for 30 min before fluorescence was measured at 340-nm excitation and 460-nm emission in a Genios Pro spectrophotometer. The background fluorescence of unloaded cells (caused by autofluorescence and light scattering) was subtracted to provide the zinquin-dependent fluorescence.

Quantitative real-time PCR. Treated cells were washed three times in PBS and then lysed in the plate with TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction according to the manufacturer’s instruction. The isolated total RNA was quantified by spectrophotometry using a SPECTRAmax Plus spectrophotometer ( Molecular Devices, Sunnyvale, CA), and 1 μg of total RNA was used for cDNA synthesis in a 20-μl system with an AffinityScript multiple-temperature cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instruction. A sample of 0.5 μl of the 20-μl cDNA product was used in a 25-μl real-time PCR system as template and amplified by Taqman PCR Master Mix [Applied Biosystems (ABI), Carlsbad, CA] according to the manufacturer’s instruction. Primer and probe sets for real-time PCR for metal-responsive transcription factor 1 (MTF-1) probe assay ID Hs00232306_m1) and GAPDH (assay ID Hs99999905_m1) were purchased from the Taqman Gene Expression Assays of ABI, and the assays were carried out according to the manufacturer’s instruction. The primer and probe sets for detection of total metallothionein (MT) were synthesized by ABI as a custom order, because no single common amplicon region was found for the MT isoforms most highly expressed in human mononuclear cells (MT-1H, -1H-like, -1G, -1L, -1E, -1A, and -2) (11). A combination of two forward primers, two reverse primers, and two Taqman probes was used to amplify all the MT isoforms in one single assay. For this assay, the concentration of each of the four primers was 450 nM and the concentration of each of the two probes was 125 nM. The primers/probes for MTF-1, total MT, and GAPDH are shown in Table 1. Quantitative real-time PCR was performed with a polymerase-activating step of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min and, finally, 95°C for 15 s. The real-time fluorescence signal was detected and quantified by a sequence detection system (PRISM 7000, ABI).

Cell morphology and fluorescence microscopic imaging. Cells were cultured in Nunc Lab-Tek II chamber slides (8 wells) and treated as described above for 3 days. Morphological images of live cells were obtained microscopically before further experimental procedures. Cells were washed three times in PBS and then fixed by addition of 3.7% formaldehyde to each well for 30 min at 37°C. The fixed cells were washed three times with PBS, incubated with 25 μM zinquin ethyl ester in PBS for 30 min at 37°C, and washed three times with PBS. A coverslip was mounted on the slide, and petroleum jelly was used to seal the edge of the coverslip to prevent evaporation. The slides were then placed under a fluorescence microscope (AxioObserver 100, Carl Zeiss, Oberkochen, Germany) for image acquisition. Filter cube set 49 for 4’,6-diamidino-2-phenylindole (365-nm excitation, 435-nm filter, 445-nm emission) was used to visualize fluorescence of individual cells. Photomicrographs for each treatment were obtained using the same magnification scale (×200) and exposure time (2 s) within 10 min to avoid errors from autofading of the fluorescence signal. The fluorescence of each cell in the captured images was then quantified using the Automeasure module of AxioVision (release 4.7). Values were calculated from ≥50 cells for each treatment and are expressed as means ± SE corrected for background fluorescence.

**Table 1. Sequences of primers and probes for quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>hMT1, 2</td>
<td>5' GCACCTCCTGCAAGAAAGCT 3'</td>
<td>5' TCGACCCCCACACGAGGAGGACAGG BHQ1 3'</td>
</tr>
<tr>
<td>hMT1, 2 Alt</td>
<td>5' TCGACCCCCACACGAGGAGGACAGG BHQ1 3'</td>
<td>5' GCACCTCCTGCAAGAAAGCT 3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5' GCCACCTGTCACGAGGACAGG BHQ1 3'</td>
<td>5' GCACCTCCTGCAAGAAAGCT 3'</td>
</tr>
</tbody>
</table>

**hMT, human metallothionein; MTF, metal-responsive transcription factor 1.**

*Primers sequences for Taqman gene expression assays are confidential information of Applied Biosystems (Carlsbad, CA); assay ID numbers are listed.*
Senescence assay. Senescence assay was conducted using a senescence detection kit (MBL, Woburn, MA) according to the manufacturer’s instructions. Briefly, NHPrE cells were cultured in 24-well plates at 6,000 cells/well and treated for 3 days. The culture medium was removed, and the cells were washed once with PBS. The cells were then fixed in 0.25 ml of fixative solution at room temperature for 10 min. The cells were washed again with PBS and stained in 250 μl of 1× staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) and 1× staining supplement for 12 h. Cells were then overlaid with 70% glycerol and observed under a microscope for development of blue color.

Statistical analysis. Data were analyzed with SAS 9.1.3 Windows software (SAS Institute, Cary, NC) and are presented as means ± SE. Student’s t-test between control and treated groups was applied in the cell cycle distribution analysis. A one-way ANOVA followed by post hoc test least significant differences was used to determine the statistical significance among treatments. The level of statistical significance was set at P < 0.05.

RESULTS

Resveratrol inhibits proliferation of NHPrE cells. Growth of NHPrE cells, as determined by the total number of cells per plate, was markedly depressed by resveratrol, but to a lesser extent by zinc (Fig. 1A). After 3 days in culture, treatment with resveratrol resulted in little or no reduction in cell growth until the concentration reached 2.5 μM, which resulted in an average 30% reduction of cell growth compared with no addition of resveratrol among all zinc treatments (Fig. 1A). Thereafter, a progressive reduction in cell growth was observed with the increase in resveratrol concentration to 5 and 10 μM among each zinc treatment. In general, there was little or no change in the pattern of cell growth reduction among zinc treatments, with the exception of lowered cell growth in ZD and ZS cells compared with ZN and ZA cells treated with 0 or 0.5 μM resveratrol. Similarly, slower cell growth has been observed in ZD and ZS than in ZN NHPrE cells in the absence of resveratrol (C. T. Han, unpublished observations). The same experiment was performed with NHPrE cells from the 21-year-old donor, and a similar pattern of responses was obtained (data not shown).

Resveratrol significantly enhances total zinc level in NHPrE cells. Little or no increase in cellular zinc level was observed until resveratrol reached 5 μM, compared with 0 μM resveratrol, among all zinc treatments. A further marked increase in cellular zinc level was observed at 10 μM resveratrol in each zinc condition. In the ZD, ZN, and ZA cells, a similar pattern of little or no change in cellular zinc status was observed without resveratrol with low resveratrol concentration (0, 0.5, and 1 μM). However, in the ZS cells, as the resveratrol concentration was increased to 1, 2.5, 5, and 10 μM, dramatic increases of total cellular zinc (2-, 3-, 5-, and 7.5-fold, respectively) were observed (Fig. 1B). The same experiment was performed with NHPrE cells from the 21-year-old donor, and a similar pattern of responses was obtained (data not shown).

Zinc, but not resveratrol, affects proliferation and intracellular zinc level of HepG2 cells. Since the liver is an important organ responsible for heavy metal metabolism and storage, an experiment similar to that carried out in NHPrE cells was conducted in HepG2 cells to investigate whether resveratrol and zinc would affect the proliferation and intracellular zinc content in HepG2 cells. Zinc was found to be the only factor that actually affected the proliferation of HepG2 cells significantly, with an ~20% reduction of the number of ZD cells and no significant change in the number of ZA and ZS cells compared with ZN cells (Fig. 2A). This observation confirmed previous findings from our laboratory (42). In addition, the intracellular total zinc level in HepG2 cells increased progressively as the zinc level of the media increased from ZD to ZS, as observed previously (42). Resveratrol appeared to have no overall effect on cell proliferation and intracellular zinc content in HepG2 cells, since in each zinc group, resveratrol treatments did not alter cell number and total zinc content. However, high resveratrol concentration (5 and 10 μM) tended to depress intracellular total zinc by ~20% in ZD cells, but not in ZN, ZA, and ZS cells (Fig. 2B).

Resveratrol does not influence apoptosis but inhibits proliferation of NHPrE cells by inducing cell cycle arrest at the G2/M phase. Cell cycle analyses were performed to examine the influence of resveratrol (0 or 10 μM) on progression

![Fig. 1. Effect of resveratrol (Res) and zinc on proliferation (A) and total intracellular zinc level (B) of normal human prostate epithelial (NHPrE) cells. NHPrE cells (25,000/well) were plated in 6-well plates, treated with resveratrol (0, 0.5, 1, 2.5, 5, and 10 μM) and zinc [zinc-deficient (ZD), zinc-normal (ZN), zinc-adequate (ZA), and zinc-supplemented (ZS) conditions] for 3 days, and harvested. Total cell count for each sample was measured with the NucleoCounter, and cellular zinc status was determined by atomic absorption spectrophotometry. Total intracellular zinc level is expressed in nanomoles of zinc per 10⁶ cells to account for differences in cell numbers between samples. Values are means ± SE from 3 separate experiments. Means with the same superscript (a–l) are not significantly different (P < 0.05).](https://www.ajp-cell.org)
through the cycle phases in the ZN NHPrE cells. Resveratrol at 10 μM caused a 1.5-fold increase in the percentage of cells in the G2/M phase (from 12% to 30%) compared with controls, indicating a arrest of the cell cycle at the G2/M phase (Fig. 3B).

No sign of apoptosis was detected in the control cells or cells treated with 10 μM resveratrol, as indicated by absence of DNA fragments in the sub-G0/G1 region (Fig. 3A, Apo).

Resveratrol chelates zinc in vitro. In the present study, the marked enhancement of total intracellular zinc content, especially in ZA and ZS cells, suggests that resveratrol may form a complex with Zn(II), and it is this complex that possibly transports most of the zinc into the cell through a channel separate from that used for free zinc ion trafficking. Thus, to address this issue, we designed an in vitro experimental system to include seven levels of Zn(II) (0, 2, 4, 8, 16, 24, and 32 μM) in the form of ZnSO4 and four levels of resveratrol (0, 5, 10, and 20 μM) Zn(II) (Fig. 4A). In addition, with different concentrations of zinquin (5 or 25 μM), there was a significant decrease in fluorescence at 10 μM resveratrol (Res10) compared with the 0 μM resveratrol control (Res0) at 4, 8, 14, 24, and 32 μM Zn(II) (Fig. 4B).

Resveratrol enhances the effect of zinc supplementation on intracellular free labile zinc level in NHPrE cells. The labile pool of intracellular zinc plays an important role in many cell activities. To investigate the effect of resveratrol on the intracellular labile zinc level, we used zinquin ethyl ester, which emits four times stronger fluorescence than zinquin (data not shown), to quantify the free labile zinc level per plate by spectrofluorometry (Fig. 5), as well as to visualize the free labile zinc pools within individual cells under a fluorescence microscope (Fig. 6). Zinquin ethyl ester-Zn(II)-associated fluorescence was found mainly in the secretory granules in the cytoplasm. Clear dark zones in the center of cells representing nuclei can be identified easily (Fig. 6A).

Spectrofluorometry (Fig. 5) and fluorescence microscopic imaging results (Fig. 6B) revealed that the medium zinc concentration and total intracellular zinc status positively affected intracellular free labile zinc levels. Resveratrol at 10 μM...
by resveratrol treatment, the abundance of MT and MTF-1 mRNA was measured to determine whether these messages were affected by resveratrol treatment. Interestingly, MT mRNA level was affected by cellular zinc status, but not by resveratrol treatment (Fig. 7A). In the absence of resveratrol treatment, the MT mRNA level was higher in ZS than in ZD and ZN cells (Fig. 7A). With 10 μM resveratrol, the abundance of MT mRNA was higher in ZS than in ZD, ZN, andZA cells. Moreover, there was no significant difference of MT mRNA abundance between treatments with and without resveratrol in all four zinc groups. In addition, resveratrol and zinc appeared to have little or no significant effect on the abundance of MTF-1 mRNA in NHPe cells (Fig. 7B).

Resveratrol induces senescence in NHPe cells through ROS generation. Although the NHPe cells treated with resveratrol and zinc were not apoptotic, proliferation was impaired, especially in cells treated with 10 μM resveratrol at all zinc levels, with only one population doubling in 3 days (Fig. 1A). The proliferation inhibition, but not apoptosis induction, effect of resveratrol was highly likely to be mediated by the enhanced ROS generation as a by-product of the interaction between resveratrol and transition metals, especially Zn(II). Therefore, the ROS levels of NHPe cells treated with three levels of resveratrol (0, 2.5, and 10 μM) and four levels of zinc were determined using the dye CM-H2DCFDA, a cell-permeable indicator for ROS. The increase of resveratrol concentration in culture medium significantly enhanced the cellular ROS level of NHPe cells, starting from 2.5 μM and reaching the highest level at 10 μM, at each zinc level after 1 day of incubation, as indicated by the resveratrol dose-dependent increase of fluorescence (Fig. 8). ROS levels appeared to be higher in resveratrol-treated ZA and ZS than ZD and ZN NHPe cells.

Morphological changes, such as flattened and wider shapes, larger volumes, and larger nuclei, were observed in resveratrol-treated NHPe cells, especially ZA and ZS cells compared with ZN and ZD cells (Fig. 9). Moreover, these cells were not as tolerant as the controls to the suboptimal culturing environ-

markedly increased intracellular free labile zinc status, as measured by spectrofluorometry, in ZA and ZS cells by 49% and 52%, respectively, compared with their corresponding controls with no resveratrol. In contrast, 2.5 μM resveratrol did not influence intracellular free labile zinc level in ZA and ZS cells. Moreover, in ZD and ZN cells, 2.5 and 10 μM resveratrol exerted no effect on intracellular free zinc level (Fig. 5). More than twofold increases in fluorescence were observed only in the ZS cells compared with other zinc groups treated with 0 or 2.5 μM resveratrol. In contrast, at 10 μM resveratrol, at least threefold increases in fluorescence were observed in ZA and ZS cells compared with respective groups treated with no resveratrol. Moreover, no difference in fluorescence was identified in ZD and ZN cells among the 0, 2.5, and 10 μM resveratrol treatments (Fig. 6B). Similar response patterns were identified in the results derived from both methods (Figs. 5 and 6B).

Effect of zinc and resveratrol on MT and MTF-1 mRNA abundance. MT, the major labile zinc-binding protein, has been reported to be upregulated by zinc supplementation and positively correlated with the total and labile free intracellular zinc levels ranging from deficient to adequate (12). In view of the marked increases in total cellular zinc...
Resveratrol-Zinc Interactions in NHPrE Cells

Fig. 6. A: subcellular localization of intracellular free labile zinc. Left: cells under normal setting (no fluorescence); right: cells under fluorescence settings. Arrows show location of nuclei. Image is representative of 3 separate experiments. B: effect of resveratrol and zinc on intracellular free labile zinc status by fluorescence microscopic imaging. NHPrE cells were cultured in Nunc Lab-Tek II chamber slides (8 wells), treated with resveratrol (0, 2.5, and 10 μM) and zinc (ZD, ZN, ZA, and ZS) for 3 days, fixed by 3.7% formaldehyde, and stained with 25 μM zinquin ethyl ester for 30 min at 37°C. Then cells were observed under a fluorescence microscope with 365-nm excitation and 445-nm emission and ×200 magnification. Mean fluorescence intensity of each cell in the captured images was quantified using the Automeasure module of AxiosVision. Values are means ± SE of background-subtracted fluorescence intensity from ≥50 cells in each treatment. Different superscripts indicate significant differences among groups (P < 0.05); treatments with the same superscript are not significantly different.

Fig. 7. Effect of resveratrol and zinc on total metallothionein (MT, A) and metal-responsive transcription factor-1 (MTF-1, B) mRNA abundance. NHPrE cells were cultured in 6-well plates, treated with resveratrol (0 and 10 μM) and zinc (ZD, ZN, ZA, and ZS) for 3 days, and harvested. Total RNA was extracted from cell samples, and 1 μg of extracted total RNA was reversely transcribed into cDNA. Quantitative real-time PCR analyses were performed in triplicates. Relative quantity was established with GAPDH as endogenous normalization control. mRNA level of ZN cells without resveratrol was set to 1. Values are means ± SE from 3 separate experiments. Means with the same superscript are not significantly different (P < 0.05).

Discussion

There have been many reports on the anticancer, antioxidant, and longevity activity of the health-promoting phytochemical resveratrol. In the US market, resveratrol has been commercially available in the form of nutraceutical supplements. However, there have been very few studies of the negative aspect of resveratrol, such as its prooxidant activity. A cell-based approach to examination of resveratrol supplementation in the context of nutrient interaction between phytochemicals and trace minerals has not been used until now. Our study was designed to address this issue by using resveratrol and zinc as an example to build a feasible and efficient model to investigate the interaction between phytochemical supplements and trace minerals. NHPrE cells were selected as our cell model system, because they represent normal prostate tissue and they accumulate high levels of zinc. Human hepatoblastoma HepG2 cells were used to represent physiological liver cells, because the cost of using normal human liver cells is economically not feasible.

Steady cell growth is typical for low-passage normal human prostate cells and is known to decline drastically as the passage number increases. Taking this into consideration, we have employed low-passage cells in the present study. NHPrE cells in the control groups (non-resveratrol-treated cells) from all zinc treatments had doubled more than three times at the end of 3 days of culture. Cell growth of NHPrE cells was significantly affected by resveratrol treatment, with >80% reduction with 10 μM resveratrol after 3 days of culture (Fig. 1A). In another study, which used the same NHPrE cells, resveratrol had no significant effect, NHPrE cells treated with 10 μM resveratrol and exposed to the “atmosphere” outside the incubator at room temperature for 15 min became detached and lost the ability to be recultured (data not shown).

Since the observed morphological changes of NHPrE cells matched those described for cells undergoing senescence, the experimental design used for the ROS experiment was used to determine zinc-resveratrol interactions on senescence in these cells. Senescence was measured with a detection kit designed to histochemically detect senescence, the experimental design used for the ROS experiment was replicated in all four zinc treatments. Increased zinc concentration appeared to induce enhanced senescence when the cells were treated with 2.5 or 10 μM resveratrol (Fig. 10). The strongest effect was observed in the ZA and ZS cells, with ~10% and 70% of these cells undergoing senescence when treated with 2.5 and 10 μM resveratrol, respectively.

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In the present study, zinc status only slightly inhibited cell growth of ZD and ZS NHPrE cells (Fig. 1A). A concurrently run study conducted in our laboratory partially supports our results. Using exactly the same cells without resveratrol treatment, Han et al. found that cell growth was reduced by 39% in ZD cells (unpublished observation). Most importantly, a slight 20% enhancement of cell growth with a low concentration of resveratrol (0.5 μM) was observed in the ZN cells (Fig. 1A). These results suggest that a low concentration of resveratrol might help improve cell proliferation and promote longevity, as reflected by the “French paradox” in a bigger picture (31).

The dramatic resveratrol-induced increase in total cellular zinc level in NHPrE cells suggested a possible interaction between resveratrol and zinc. Since the liver is a very important organ responsible for heavy metal metabolism and storage, we asked whether the response to resveratrol treatment would be the same for the prostate cells. To our surprise, cell proliferation and total cellular zinc content of

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the HepG2 liver cells used in our system were not affected by resveratrol treatment. Zinc deficiency exerted a modest (~20%) inhibition on the proliferation of HepG2 cells (Fig. 2A), consistent with our previous report (42). In addition, intracellular zinc content in HepG2 cells did not differ markedly among each zinc level, ranging from 1 to 2.3 nmol/10⁶ cells. When the intracellular zinc level between the HepG2 cells and the NHPRe cells was compared, we found only a 3-fold difference in the control group ZNR0 (4 μM zinc, 0 μM resveratrol), but a >10-fold difference in the ZNR10 (4 μM zinc, 10 μM resveratrol) and ZSR10 (32 μM zinc, 10 μM resveratrol) groups. These results again confirm the ability of prostate cells to accumulate 10-fold more zinc than cells from other organs, e.g., liver and kidney (10, 35), and suggest that the inhibition of cell proliferation in resveratrol-treated NHPRe cells may be related to the increase of total cellular zinc content.

Under normal circumstances, zinc uptake into the cells and its transport into and out of the intracellular organelles are accomplished by its transporters, which span the membranes and facilitate the movement of zinc (14). However, in our present study, in view of the marked increase of total intracellular zinc with the addition of resveratrol starting from 5 μM in ZD, ZN, and ZA cells and 1 μM in ZS cells, zinc uptake may not only rely on the zinc transporters that are located on the cell membrane under resveratrol treatment. Other channels may be activated by resveratrol treatment and allow entry of extracellular zinc. Since resveratrol has been shown to form a resveratrol-Cu(II) complex, resulting in the reduction of copper ions and the formation of ROS (1), the similarity between the atomic structures of zinc and copper leads us to suggest that resveratrol may chelate zinc to form a complex that passes the cell membrane through a channel specific for resveratrol.

Using zinquin and zinquin ethyl ester, we were able to establish the existence of the complex by showing the resveratrol dose-dependent quenching of the fluorescence emitted by the complex formed by zinquin and Zn(II) (Fig. 4). These results clearly indicate that resveratrol was able to compete for Zn(II) with zinquin, leading to less Zn(II)-

Fig. 9. Morphological change caused by resveratrol and zinc treatment in NHPRe cells. NHPRe cells were cultured in Nunc Lab-Tek II chamber slides (8 wells), treated with resveratrol (0, 2.5, and 10 μM) and zinc (ZD, ZN, ZA, and ZS) for 3 days, and examined under a microscope at ×200 magnification. Images are representative of 3 separate experiments.
The only possible explanation for this chelating effect of resveratrol for zinc is that resveratrol is able to form a complex with Zn(II), just as with Cu(II), which was accompanied by the generation of hydroxyl radical, as established by electron paramagnetic resonance (7a). This complex-forming and prooxidant property of resveratrol with copper was later established to be hydroxyl group dependent, since trans-stilbene without the hydroxyl groups was found to be inactive in Cu(II)-dependent, resveratrol-promoting DNA breakage (4).

Most of the total cellular zinc detected by atomic absorption spectrophotometry is very tightly bound to MT or other cellular proteins for various functions and is essentially nonexchangeable (12, 43). The other pool of intracellular zinc, which is a very small fraction, remains labile and is dynamically exchangeable with tightly bound and extracellular zinc pools. The intracellular labile zinc pool is metabolically important, because it responds to zinc deprivation or supplementation by decreasing or increasing its content, respectively (43). Inasmuch as resveratrol is able to increase total intracellular zinc dramatically, the intracellular free labile zinc content may be altered by the interaction of resveratrol and zinc in our cell system. Two methods were employed to address this issue: spectrofluorometry and fluorescence microscopic imaging. Both methods utilized the same chemical, i.e., zinquin ethyl ester, and are based on the same principle: the content of labile free zinc is proportional to the fluorescence emitted by the complex formed by zinquin ethyl ester and Zn(II). Zinquin ethyl ester, instead of zinquin, was chosen because of an ethyl ester in place of the 6-methoxy group of toluenesulfonamidoquinoline in the molecular structure of zinquin ethyl ester, which facilitates its retention in living cells and enhances its fluorescence. Using zinquin ethyl ester, we were able to identify the subcellular localization of free labile Zn(II) in the extranu-
clear secretory granule, which is consistent with the findings of two previous reports (33, 37). Results from both methods indicate that free labile zinc increases along with the extracellular zinc concentration, whereas resveratrol tends to increase the free labile zinc level in ZA and ZS cells but to decrease the free labile zinc level in ZN and ZD cells (not statistically significant; Figs. 5 and 6B). These findings suggest that resveratrol may enhance the effect of total intracellular zinc on intracellular free labile zinc content. The positive correlation of extracellular zinc, total cellular zinc, and intracellular free zinc, as well as MT mRNA (Figs. 1B, 5, 6B, and 7), observed in the present study was also reported in a rat hepatocyte study using both methods (12), which provided evidence in support of the results of our study.

Since all our experimental results favor the contention that resveratrol enhances zinc uptake by complex formation, we then asked the following question: Is the additional intracellular Zn(II) bound to MT, is it converted to free labile zinc, or does it remain coupled with resveratrol? To answer this question, we used a highly sensitive and reproducible method, quantitative real-time PCR, to assess the MT transcript levels in NHP'e cells treated with zinc and resveratrol. We found a twofold elevation of total MT mRNA levels in ZS cells but no change in ZD cells in our system (Fig. 7A). A 13-fold induction and a 1-fold suppression of MT-1 mRNA by 40 μM zinc supplementation and 10 μM N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) depletion, respectively, were detected by quantitative real-time PCR and reported by Cousins et al. (11). MT-1 is only one of many targets of our MT probes and primers that were designed to detect the transcript level of MT-1 mRNA by 40 μM zinc supplementation and 10 μM N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) depletion, respectively, were detected by quantitative real-time PCR and reported by Cousins et al. (11).

MT-1 is only one of many targets of our MT probes and primers that were designed to detect the transcript level of the MT isoforms MT1H, MT-1H-like, MT-1G, MT-1L, MT-1E, MT-1A, and MT2, which are the isoforms most abundantly expressed in human mononuclear cells. In another study that used the same MT probes, only twofold induction of total MT mRNA level by modest oral zinc supplementation (15 mg of zinc as ZnSO4 per day) for 10 days in leukocytes from human subjects was reported (2). Since this result was achieved under physiological conditions, just as in our experimental system, it is reasonable to believe that the expression of total MT mRNA is fairly stable and does not fluctuate much, regardless of zinc status, under physiological conditions, although some isoforms of MT, such as MT-1, do fluctuate. We also investigated the expression of MTF-1 mRNA, an upstream regulator of MT, under the influence of zinc and resveratrol. To our surprise, there was little or no change in the mRNA level of MTF-1 among the treatments. However, the MTF-1 mRNA abundance was significantly higher in the ZD cells treated with 10 μM resveratrol than in the ZN and ZS cells not treated with resveratrol, as well as the ZS cells treated with 10 μM resveratrol (Fig. 7B). A threefold induction of MTF-1 mRNA was reported in the 10 μM TPEN-induced zinc-deficient human THP-1 mononuclear cells (11). It appears that induction of zinc deficiency by medium zinc depletion is not as severe as that by TPEN chelation. However, the latter is not clinically practical, nor is it feasible.

On the other hand, the induction of cellular senescence (Fig. 10) provides strong support for the hypothesis that resveratrol enhances zinc uptake by complex formation. Formation of a resveratrol-Zn(II) complex is very likely to generate ROS, which could cause DNA damage. Senescence is one response to sublethal cellular stress. The increased percentage of cells undergoing senescence, along with increased zinc and resveratrol concentration, may be explained by increased ROS generation as the result of enhanced complex formation, as supported by the strong positive association between the percentage of senescent cells and the quantity of ROS generated within the cells (Fig. 8).

In summary, the results of the present study suggest a Zn(II)-resveratrol interaction. Because of the three parallel hydroxy groups in the molecular structure of resveratrol, resveratrol and Zn(II) are able to form complexes at a ratio of 1:1 or up to 1:1.5 in a dynamic equilibrium, depending on the availability of Zn(II) and resveratrol (Fig. 11). In ZD and ZN cells, in which intracellular zinc concentration is relatively low, the resveratrol-Zn(II) complex is likely to be formed at a ratio of 1:1, inasmuch as the binding capacity of resveratrol for Zn(II) would not be completely saturated. However, in ZA and ZS cells, the chelating capacity of resveratrol for Zn(II) is more likely to be saturated [resveratrol-Zn(II) ratio of 1:1.5] because of the increased availability of Zn(II). When the complexes formed by Zn(II) and resveratrol pass through the cell membrane and remain in the coupled form in the senescent, metabolically inactive cells, the unsaturated resveratrol under ZD and ZN conditions continues to chelate Zn(II) from the free labile zinc pool, which decreases labile free zinc. On the contrary, in ZA and ZS cells with an elevated level of total cellular zinc, a small portion of Zn(II) bound to the saturated resveratrol starts to dissociate from the complex to join the free labile zinc pool, resulting in a modest increase of free labile zinc in the presence of a dramatic increase of total cellular zinc content.

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