Resveratrol Prevention of Oxidative Stress Damage to Lens Epithelial Cell Cultures Is Mediated by Forkhead Box O Activity

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PURPOSE. To evaluate the potential role that FoxO transcription factors play in modulating resveratrol's protective effects against oxidative stress in lens epithelial cells.

METHODS. Primary human or porcine lens epithelial cells (LECs) were treated with resveratrol (RES) 25 μ M and incubated under either physiologic (5%) or chronic hyperoxic (40%) oxygen conditions. Acute oxidative stress was applied using 600 μ M H_2O_2 . Changes in expression of FoxO1A, FoxO3A, and FoxO4 were analyzed. The production of intracellular reactive oxygen species (iROS), SA- β -galactosidase (SA- β -gal) activity, and autofluorescence (AF) was assessed by flow cytometry. SiRNAs of FoxO1A, FoxO3A, and FoxO4 were used to study the roles that these transcription factors play in resveratrol's protective effects against cell death induced by oxidative stress.

RESULTS. RES incubation under 40% oxygen increased the expression of FoxO1A, FoxO3A, and FoxO4. RES also increases mitochondrial membrane potential under 5% and/or 40% O_2 conditions and significantly decreased iROS, SA- β -gal, and AF normally induced by hyperoxic conditions. While RES had a mild pro-apoptotic effect in nonstressed cells, it significantly prevented apoptosis induced by H_2O_2 stress. SiRNA inhibition of FoxO1A, FoxO3A, and FoxO4 not only led to loss of the anti-apoptotic effects of RES in stressed cells but actually exhibited a mild pro-apoptotic effect.

CONCLUSIONS. RES exerts a protective effect against oxidative damage in LEC cultures. The levels of expression of FoxO1A, FoxO3A, and FoxO4 appear to play a central role in determining the pro- or anti-apoptotic effects of RES. This has implications for future studies on oxidative stress-related lenticular disorders such as cataract formation. (*Invest Ophthalmol Vis Sci.* 2011;52:4395–4401) DOI:10.1167/iov.10-6652

Cataract development has a strong relationship to increasing age in both humans and animals.^{1–4} There is considerable evidence to support the concept that oxidative stress and the generation of reactive oxygen species (ROS) can accelerate cataract development through damage to lens epithe-

lial and fiber cells.^{5–8} Caloric restriction (CR) has been shown to induce alterations in both the endogenous generation of ROS and the activation of protective mechanisms against oxidative damage.⁹ CR has also been shown to delay cataract formation in both mice and rats^{3,9} and to extend the lifespan of several species.^{10–12} The specific mechanisms by which CR exerts such effects on cataract progression, oxidative stress, and lifespan are not completely understood. However, there is experimental evidence suggesting that some of the effects of CR are mediated by the silent information regulator (Sirt1) and downstream activation of several members of the Forkhead box O (FoxO) gene family.¹³

The FoxO genes encode a family of transcription factors that modulate the expression of genes involved in the cellular response to oxidative stress, DNA repair, and apoptosis.¹⁴ Activation of FoxO transcription factors can promote stress resistance by binding to the promoters of genes such as manganese superoxide dismutase and catalase.^{15,16} This gene family plays a central role in oxidative stress response and is an important mediator of hematopoietic stem cell resistance to physiologic oxidative stress.¹⁷

The naturally occurring polyphenol resveratrol (RES) is an activator of SIRT1 and the FoxO transcription factors and has been shown to mimic some of the effects of CR including decreased production of ROS and increased protection against oxidative stress. RES has been shown to suppress selenite-induced oxidative stress and cataract formation in rats¹⁸ and together with proanthocyanidin extract can reduce significantly ROS production in canine lens epithelial cells.¹⁹ Furthermore, the activation Jun-N-terminal kinase (JNK) plays an important role in cell death signaling.²⁰ RES also inhibits H_2O_2 -induced JNK phosphorylation in HLEB-3,²¹ which may be one mechanism to prevent cell death. Therefore, we choose also to analyze the effects of JNK inhibition in our model system. However, RES is known to have both pro- and anti-apoptotic effects that may be cell and context dependent. In other words, like many biological processes, the effects of a specific molecule can be different depending on the cellular background or environment. Currently, it is not known what factors may influence the disparate effects of RES on apoptosis. Here we evaluate the potential protective effects of RES under chronic oxidative stress conditions and investigate the role that FoxO transcription factors play on this molecule's effects in lens epithelial cells. We used both porcine and human primary lens epithelial cell cultures for our studies because of the limited availability of human tissue.

METHODS

Cell Cultures

All studies were conducted in accordance with the Declaration of Helsinki and ARVO animal statement. Primary LEC cultures were de-

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Supported by NIH K23 EY014019, core Grants EY01894, EY016228, EY05722, and Research to Prevent Blindness Sybil B. Harrington Scholar Award.

Submitted for publication September 29, 2010; revised January 10 and February 1, 2011; accepted February 1, 2011.

Disclosure: G. Li, None; C. Luna, None; I.D. Navarro, None; D.L. Epstein, None; W. Huang, None; P. Gonzalez, None; P. Challa, None

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veloped from lenses extracted from freshly obtained enucleated porcine eyes or postmortem human eyes obtained from the New York Eye Bank within 7 days postmortem according to the tenants of the Declaration of Helsinki. Briefly, extracted lenses (free of any adherent tissues) were rinsed with medium 199 (GIBCO BRL, Carlsbad, CA), before careful removal of the lens capsules. Using a pair of fine forceps, the outer cortical layer (approximately 2 mm thickness), together with its attached lens epithelium, was carefully peeled out. The outer cortical lens tissue was then minced and digested in medium 199 containing 1.5 mg/mL collagenase IV and 0.2 mg/mL porcine albumin at 37°C for 60 minutes. At the end of the digestion, the contents were centrifuged at 100g for 10 minutes at 22°C, and the cell pellet was suspended in Dulbecco's modified Eagle medium (DMEM) containing 20% fetal bovine serum (FBS), penicillin (100 Units/mL), streptomycin (100 µg/mL), and gentamicin (20 µg/mL), and then plated on plastic Petri plates coated with 2% gelatin. Cell cultures were grown at 37°C and under 5% CO₂. Primary human or porcine LEC cultures generated using this procedure (second and third passages) were used throughout this study.

Oxidative Stress Model and Treatment of RES and JNK Inhibitor

Primary human or porcine LEC cells were incubated under either physiologic (5%) or hyperoxic (40%) oxygen conditions and treated with and without RES 25 µM in fresh culture medium daily for 8 days. The production of intracellular reactive oxidative species (iROS), SA- β -galactosidase activity (SA- β -gal), autofluorescence (AF), and mitochondrial membrane potential (JC-1 staining) was assessed by flow cytometry. All studies were performed in triplicate.

Primary human lens epithelial cells (HLECs) were harvested from cadaveric donor tissue and grown until confluence as described before. The influence of the JNK inhibitor SP600125 (Sigma, St. Louis, MO) and RES were studied in triplicate using an acute oxidative stress model. Human lens cells were treated with 25 µM RES or 10 µM JNK inhibitor or both for 18 hours. Controls were treated with neither agent but with vehicle control. The cultures were then treated with 600 µM H₂O₂ for 2 hours, and cytotoxicity was measured using an assay kit (MultiTox-Fluor Multiplex Cytotoxicity Assay Kit; Promega, Madison, WI) according to the manufacturer's instructions.

Assay of iROS

The production of iROS was measured by 2',7'-dichlorofluorescein oxidation as described previously with a brief modification.²² Briefly, 10 mM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was dissolved in methanol and was diluted 500-fold in Hank's Buffered Salt Solution to give a 20 µM concentration of H2DCFDA. H₂O₂ treated and control cells in 96-well plates were treated with inhibitors and their specific vehicles for 1 hour, and then exposed to H2DCFDA for an additional 1 hour. After incubation, the fluorescence was read at the 485 nm excitation and 530 nm emission on a fluorescence plate reader.

Flow Cytometry Analysis of Senescence-Associated- β -Galactosidase (SA- β -gal) and Senescence-Associated AF

SA- β -gal was assayed by staining cells with fluorescein-di- β -D-galactopyranoside (FDG; Molecular Probes, Carlsbad, CA). FDG (5 mg) was dissolved in 76 µL of DMSO/ethanol (1:1) and H₂O/DMSO/ethanol (8:1:1) to make the final FDG stock solution (20 mM). FDG working solution was 2 mM by dilution of stock solution in PBS. Briefly, cells in 12-well plates were trypsinized and spun 100g for 5 minutes at RT. After washing with PBS, cells were loaded with 30 µL of PBS plus 30 µL of a FDG working solution. After a 1 minute incubation at 37°C, the suspension was diluted 10-fold with ice cold PBS. The samples were kept on ice for 30 to 60 minutes until flow cytometry analysis was performed using an excitation and emission frequency of 485 and 535 nm, respectively. Senescence-associated AF was performed

without staining cells, and flow cytometry measurements were set at 563–607 nm.

Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)

Mitochondrial membrane potential was monitored in cells loaded with 2 µM of JC-1 dye according to the manufacturer instructions. JC-1 is a positively charged fluorescent compound, which is taken up by mitochondria in proportion to the inner mitochondrial membrane potential. When a critical concentration is reached, JC-1 monomers (fluorescent green) form aggregates (fluorescent red). Briefly, cells in 6-well plates treated with or without H₂O₂ were trypsinized and pelleted by a centrifuge. The cells were then washed with PBS once, loaded with JC-1 in 1 mL of PBS to final concentration of 2 µM, and incubated at 37°C and 5% CO₂ for 15 minutes. The cells were pelleted and washed again with PBS and resuspended in 200 µL of PBS. The cells were analyzed on a flow cytometer using a 488 nm excitation and appropriate emission filters for Alexa Fluor 488 dye and R-phycoerythrin.

Cytotoxicity Assays

Cytotoxicity was measured with the assay kit (MultiTox-Fluor Multiplex Cytotoxicity Assay Kit) according to the manufacturer's instructions. Briefly, cells were plated in 96-well plates after nucleofection of FoxO siRNA. RES 25 µM was used to treat the cells at 24 hours after transfection, and an additional 24 hours later. The cells were further treated with H₂O₂ 600 µM for 4 hours in an assay reagent (MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent) in an equal volume, were then added to each well, mixed briefly on an orbital shaker, and then incubated for 30 minutes at 37°C. Fluorescence was then measured as follows: cytotoxicity: excitation, ~485 nm; emission, ~520 nm. The assays were performed in triplicate.

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated (RNeasy kit; Qiagen, Valencia, CA). RNA yields were measured using fluorescent dye (RiboGreen; Molecular Probes, Eugene, OR). First-strand cDNA was synthesized from total RNA (1 µg) by reverse transcription using oligodT and reverse transcriptase (Superscript II; Invitrogen, Carlsbad, CA). Quantitative polymerase chain reactions (Q-PCR) were performed in a 20 µL mixture that contained 1 µL of the cDNA preparation and 1× supermix solution (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA), using the following PCR parameters: 95°C for 5 minutes followed by 50 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. The fluorescence threshold value (C_T) was calculated using system software (iCycle; Bio-Rad). The absence of nonspecific products was confirmed by both the analysis of the melt curves and by electrophoresis in 3% Super AcrylAgarose gels. β -Actin was used as an internal standard of mRNA expression. The specific primer pairs used to amplify genes were the following: human FoxO1A (NM_002015)—For 5'-TCC CAC ACA GTG TCA AGA CAA CGA, Rev 5'-ACT GCT TCT CTC AGT TCC TGC TGT; human FoxO3A (NM_001455)—For 5'-ACT TCA AGG ATA AGG GCG ACA GCA, Rev 5'-AGA GCT CTT GCC AGT TCC CTC ATT; human FoxO4 (NM_005938)—For 5'-AAG CCA AGA CAG AAT GCC TCA GGA, Rev 5'-TGA CTC AGG GAT CTG GCT CAA AGT; β -actin (NM_001101.3)—For 5'-CCT CGC CTT TGC CGA TCC G, Rev: 5'-GCC GGA GCC GTT GTC GAC G.

Western Blot

Human or porcine LEC from each well was washed twice with ice-cold PBS and harvested in 100 µL lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 1× protein inhibitor cocktail [Roche, Indianapolis, IN]; 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM NaF) with a cell scraper and incubated on ice for 15 minutes. Cells were disrupted by sonication (Microson Ultrasonic XL2000; Misonix, Farmingdale, NY) equipped with a 2.4

FIGURE 1. Reduction of cellular senescence markers by RES in porcine LEC cells. Porcine LEC cells in passage 2–3 were plated in six-well plates. When the cells reached confluency, the cells were incubated in 5% O₂ or 40% O₂ and treated with RES 25 μ M or vehicle control once a day for 8 days. Cells were then harvested, and the induction of iROS (A), mitochondrial membrane potential (B), SA- β -gal (C), and AF (D) was determined by staining the cells with H2DCFDA, JC-1, FDG, or no staining, respectively, and then analyzed by flow cytometry. The data represent the mean of percentage changes $\pm$ SD, $n = 3$. ** $P < 0.01$; *** $P < 0.001$ compared to their corresponding controls. # $P < 0.05$; ### $P < 0.001$ compared to 5% O₂.

mm microprobe (Misonix) at setting three for five pulses. The sample was then centrifuged at 14,000g for 30 minutes at 4°C, and supernatant was stored at –80°C until use. Proteins of the extracts were separated by 10% SDS-PAGE gels and electrotransferred to a PVDF membrane. After blocking with 5% nonfat dry milk in 0.01 M Tris-HCl (pH 8.0), 0.2% Tween 20 for 1 hour, membranes were incubated overnight at 4°C with a rabbit polyclonal FoxOs antibodies (1:1000). Primary antibodies against FoxOs were purchased from Cell Signaling Technology (Danvers, MA). After treatment with anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (1:5000; Pierce Biotechnology, Rockford, IL), immunoreactive bands were visualized by chemiluminescence (ECL Plus; GE Health Care, Piscataway, NJ) and

membranes exposed to light film (BioMax MR; Kodak, Rochester, NY). Membranes were reprobed with antitubulin β antibody (Sigma) for protein loading control.

Inhibition of FoxO Expression by siRNAs

Nucleofection of human LEC cells was performed according to the manufacturer's instructions with some modifications (Nucleofector; Amaxa, Gaithersburg, MD). The human LEC cells 5×10^5 cells were resuspended in 100 μ L of endothelial nucleofection solution (Amaxa). FoxO siRNAs (Applied Biosystems, Foster City, CA) 120 pmol was added per 5×10^5 cells, and the samples were transferred into

FIGURE 2. RES with chronic oxidative stress increased the expression of the FoxO genes. Human and porcine LEC cells were cultured in six-well plates. When the cells reached confluency, the cells were incubated in 5% O₂ or 40% O₂ and treated with RES 25 μ M or vehicle control in fresh medium once a day for 8 days. Total proteins were extracted and Western blot analysis conducted with antibodies against FoxO1A, FoxO1A3A, and FoxO1A4. The expression of the FoxOs were normalized by β -tubulin. (A) Immunoblot of human LEC cells; (B) densitometry analysis normalized by β -tubulin of (A); (C) immunoblot of porcine LEC cells; (D) densitometry analysis of (C) normalized by β -tubulin. * $P < 0.05$ compared to both 5% and 40% O₂ group, $N = 3$ from three individual samples; (E) light microscopy images of human LEC cells exposed to hyperoxic conditions; (F) porcine cells exposed to oxidative stress with and without RES. The figures are representative results from three experiments. RES prevents hyperoxic-induced changes in porcine lens epithelial cells.

certified cuvettes (Amaya) by using programs T13. The same programs were used for transfection of negative siRNA as transfection control. DMEM medium with 20% FBS was warmed to 37°C, and 500 μ L was added immediately after transfection to each cuvette and the human LEC cells were collected. After 10 minutes of recovery at 37°C, the cells were dispensed in the wells of a 96-well plate containing 100 μ L prewarmed LEC culture medium, and the plate was incubated at 37°C. The culture medium was changed with fresh medium 24 hours after transfection.

Statistical Analysis

The data were presented as the mean $\pm$ SD. For multiple comparisons of groups, ANOVA was used. Statistical significance between groups was assessed by paired or unpaired Student's *t*-test, with Bonferroni's correction. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Protective Effect of RES against Oxidative Stress

RES treatment of primary porcine LEC cultures under 40% O₂ chronic oxidative stress conditions demonstrates decreased intracellular ROS production (69% reduction) and increased mitochondrial membrane potential (32.4% increase). This demonstrates that RES has a protective effect against chronic oxidative stress (see Figs. 1A and 1B). Next, the effect of RES on measurable cellular senescence markers was evaluated. RES

shows a significant decrease in both senescence-associated β -galactosidase activity (Sa- β -gal) and AF. Accumulation of lipofuscin was measured via flow cytometry. Sa- β -gal was decreased by 74.7% and AF by 44%. These data demonstrate decreased cellular senescence and hence increased cell survival by RES and underscores its protective effects against oxidative stress (see Figs. 1C and 1D).

FoxO1A, FoxO1A3A, and FoxO1A4 Expression with RES and Chronic Oxidative Stress

Human and porcine LEC cultures were incubated under 5% and 40% O₂ conditions with and without RES and Western blot analysis performed to analyze FoxO1A, FoxO3A, and FoxO4 expression. Hyperoxia (40% O₂) slightly increased the expression of the FoxO3A and FoxO4, but not FoxO1A. RES treatment under 5% and 40% O₂ conditions also increased FoxO expression relative to untreated controls. However, only the RES-treated 40% O₂ group showed significant increase in the expression of FoxO1A, FoxO3A, and FoxO4 (see Figs. 2A–D). The cells exposed to hyperoxic conditions displayed characteristic morphologic changes associated with cellular senescence. These changes include enlarged size and autofluorescence granules in the perinuclear region in both human and porcine LEC cultures. RES prevents hyperoxic induced changes in porcine lens epithelial cells (Fig. 2E–F).

FIGURE 3. Protective effects of RES and JNK inhibitor against acute oxidative damage. Human LEC cells were cultured in 96-well plates. When the cells reached confluency, the cells were treated with RES 25 μ M, JNK inhibitor (SP600125 10 μ M), or both for 18 hours. Controls were treated with neither agent but vehicle control. The cultures were then treated with 600 μ M H₂O₂ for 2 hours, and cytotoxicity was determined using an assay kit (MultiTox-Fluor Multiplex Cytotoxicity Assay kit). (A) The data represent the mean of percentage changes $\pm$ SD, $n = 3$ –5. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to their corresponding controls. ### $P < 0.001$ compared to control without H₂O₂ treatment; (B) light microscopy images of Con, RES, RES plus JNK inhibitor, and corresponding H₂O₂-treated cultures. The figures are representative results from three experiments. Both RES and JNK inhibition are protective against acute oxidative damage.

Effects of RES and JNK Inhibition on Cell Viability under Acute Oxidative Stress

Human LEC cultures were incubated with RES, JNK inhibitor SP600125, or both agents for 18 hours. Controls received vehicle. There was an 18% increase in measured cytotoxicity with RES alone and a 27% increase with RES and JNK inhibition. Acute oxidative stress was induced with a 2 hour exposure to 600 μ M H₂O₂ and cytotoxicity measured. H₂O₂ treatment alone increased cytotoxicity by 69.2% ($P < 0.001$) over non-H₂O₂ treatment controls. The H₂O₂ exposure in the presence of the JNK inhibitor or RES alone resulted in an insignificant change in cytotoxicity over baseline control. However, the JNK inhibitor resulted in a 36% decrease relative to H₂O₂ treatment ($P < 0.001$), while RES resulted in a greater decrease of 41.8% ($P < 0.001$). H₂O₂ exposure in the presence of the JNK inhibitor and RES together resulted in the largest decrease in cytotoxicity of 51.2% ($P < 0.001$). Of note, when used together, the two agents resulted in cytotoxicity below even that of untreated controls ($P < 0.01$). Therefore, both JNK inhibition and RES protect against oxidative-induced cytotoxicity of human LEC cultures but have some untreated cytotoxic effects (see Fig. 3). Morphologic analysis demonstrates similar protective phenomena. As shown in figure 3B, H₂O₂ is cytotoxic to human LEC cells; RES partially retards H₂O₂-induced cytotoxicity. Treatment with both RES and a JNK inhibitor simultaneously results in the least observable cytotoxicity.

RES's Protective Effect against Oxidative Stress Occurs through the FoxO Pathway

FoxO1A, FoxO3A, and FoxO4 siRNAs were transfected into human lens epithelial cell cultures using the Amaxa transfection system. Each of the three FoxO siRNAs were effective in decreasing their respective FoxO gene expression by approximately 80% relative to control scramble siRNAs ($P < 0.001$) (see Fig. 4A). Next, respective FoxO siRNAs were similarly transfected into human lens epithelial cell cultures, and the cultures exposed to either 600 μ M H₂O₂ for 2 hours or incubated with RES for 12 hours followed by a 2 hour exposure to 600 μ M H₂O₂. Cytotoxicity was then measured relative to control administration of a scramble siRNA. As anticipated, H₂O₂ administration results in increased cytotoxicity relative to controls in all groups. However, the protective effect of RES is seen only in the control scramble siRNA group. The protective effect of RES is lost when FoxO1A, FoxO3A, and FoxO4 expression are decreased with their respective siRNAs ($P < 0.001$). Due to the overlapping nature of each of the FoxO actions, the highest amount of cytotoxicity is seen when all three siRNAs are transfected together ($P < 0.001$) (see Fig. 4B).

DISCUSSION

Here we demonstrate that RES is protective against oxidative damage in lens epithelial cells. Oxidative stress has been im-

FIGURE 4. FoxOs are critical for RES protection against oxidative stress. Human LEC cells were transfected with specific siRNAs of FoxO1A, FoxO3A, FoxO4, or the mixtures of all three siRNA. Controls were transfected with same amount of scramble siRNA for both individual siRNA and mixture. One day after transfection, cells were either harvested for realtime RT Q-PCR (A) or treated with RES 25 μ M or vehicle control for 18 hours, followed by acute stress exposure using 600 μ M H₂O₂ for 2 hours. Cytotoxicity was measured as previously outlined (B). The data represent the mean of percentage changes $\pm$ SD, $n = 3-5$. *** $P < 0.001$ compared to their individual controls without H₂O₂ treatment. # $P < 0.05$, ### $P < 0.001$ compared to their individual controls with H₂O₂ treatment.

plicated as a major factor in cataract development, and the FoxO pathway has emerged as a major convergence point in the cellular response to oxidative stress. We have successfully demonstrated that RES increases human primary lens epithelial cell culture survival under acute (600 μ M H₂O₂) oxidative stress conditions (Fig. 3). RES also decreases the production of cellular senescence markers (Fig. 1).

Previous studies have shown that chemical compounds including RES have the potential to increase lifespan in yeast,²³ worms,^{24–26} flies,²⁴ fish,²⁷ and mice on a high-fat diet.²⁸ In our study, we show that RES dramatically reduced cellular senescence markers under chronic oxidative stress in human lens cells, which is consistent with a protective effect and may help explain part of the mechanism of extending lifespan.

We show that RES increase the expression of FoxO1A, FoxO3A, and FoxO4 under chronic oxidative stress conditions (Fig. 2). This response is consistent with a homeostatic response to protect lens epithelial cells. Baur et al.²⁸ found that RES extended lifespan in a high-fat diet mouse model and that it appeared to be dependent on activation of Sir2. It was further demonstrated that the Sir2 homolog SIRT1 controls the cellular response to stress by regulating the FoxO family.²⁹ SIRT1 and the FoxO transcription factor FoxO3 form a complex in cells in response to oxidative stress. In our study, we found that RES increased expression of FoxO3A and also enhanced the expression of FoxO1A and FoxO4 under chronic oxidative stress conditions, suggesting that FoxO1A and FoxO4 may also be involved in the protective effects of RES (Fig. 2). This is further supported by the finding that knockdown of individual FoxOs by FoxO siRNAs decreases the protective effects of RES against acute oxidative stress. A triple knockdown of all three FoxOs demonstrates the greatest cytotoxic effect that we measured in this study (Fig. 4B). In other words, the knockdown of FoxO1A, FoxO3A, and FoxO4 expression abolishes resveratrol's protective effect against acute oxidative damage to human LEC cells. Furthermore, it has been shown that FoxO-deficient mice (FoxO1, FoxO3A, and FoxO4) exhibit an increase in ROS generation and changes in the expression of ROS-associated genes in hematopoietic stem cell populations.¹⁷ Although RES increased the expression of the antioxidants (SOD-1, catalase, and HO-1)²¹, which exert protective effects, RES can also influence other genes that have markedly different effects. RES can induce a concentration-dependent inhibition of cell growth by inhibiting DNA synthesis, decreasing the number of viable cells, and increasing the activity of executioner caspases 3 and 7.³⁰ Therefore, RES appears to have context-dependent effects. Furthermore, since RES increases FoxO expression under oxidative stress conditions and when this effect is functionally decreased with specific siRNAs, we see a small pro-apoptotic effect. This observation is potentially relevant to understanding why RES can exert opposite effects on cell survival in different cell types and conditions. This context-dependent effect may explain the observation of increased RES toxicity in nonstressed cells, while RES prevents cell death in stressed cells by upregulating FoxOs. Although the overexpression of Sir2 in *Drosophila* has been shown to promote caspase-dependent (but p53-independent) apoptosis that is mediated by JNK,³¹ the pro-apoptotic effects of RES in nonstressed LECs could not be prevented by inactivation of JNK. Other mechanisms such as JNK-independent activation of BIM appear more likely to be involved in the induction of apoptosis by RES in nonstressed cells.³² Although cell death in H₂O₂-treated cells could be partially prevented by inhibition of JNK, RES effects were additive, suggesting that RES was preventing cell death not only by inhibition of JNK but also through other mechanisms (Fig. 3). Our results also showed that each of the three FoxOs tested were needed for the protective effects of RES against H₂O₂-induced cell death (Fig.

4B), which suggests that the observed upregulation of FoxOs mediated by RES might play a critical role in its protective effects.

In conclusion, our results demonstrate that RES has the potential to protect LECs from oxidative damage and that the levels of FoxO expression appear to be critical in determining whether RES acts as a pro- or anti-apoptotic agent. Moreover, this effect appears to be independent of the JNK pathway. Molecules that affect the expression of FoxO1A, FoxO3A, and FoxO4 have the potential to modulate the effects of RES between pro- and anti-apoptotic states. Such molecules may have the potential to treat disorders associated with oxidative stress damage such as cataracts.

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