Molecular Vision 2013; 19:2385-2392 <http://www.molvis.org/molvis/v19/2385>
Received 1 January 2013 | Accepted 21 November 2013 | Published 23 November 2013

Resveratrol inhibits proliferation of hypoxic choroidal vascular endothelial cells

Sankarathi Balaiya, Ravi K. Murthy, Kakarla V. Chalam

Department of Ophthalmology, University of Florida College of Medicine, Jacksonville, FL

Purpose: Resveratrol, a polyphenolic phytoalexin present in red wine, has a protective role against tumor-induced angiogenesis. Exudative age-related macular degeneration is characterized by hypoxia-induced choroidal vascular endothelial cell (CVEC) proliferation. In this study, we evaluated the effect of resveratrol on hypoxic CVECs and the underlying signaling pathways involved.

Methods: CVECs (RF/6A) after induction of hypoxia with cobalt chloride ($CoCl_2$, 200 µM) were exposed to increasing doses of resveratrol (2, 4, 6, 8, 10, and 12 µg/ml). Cell viability was measured with 4-[3-(4Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) colorimetric assay. The effect of resveratrol on hypoxia-induced vascular endothelial growth factor (VEGF) release was analyzed with enzyme-linked immunosorbent assay. The mechanistic pathway was further evaluated by analyzing phosphorylated stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) using immunoblot and cleaved caspase-3 with In-Cell enzyme-linked immunosorbent assay.

Results: Resveratrol inhibited hypoxic CVEC proliferation. Hypoxia-induced VEGF release (30.9±2.6 pg/ml) was inhibited in a dose-dependent fashion by 2, 4, 6, 8, 10, and 12 µg/ml resveratrol to 12.4±2.1, 11.0±1.9, 10.3±3.0, 7.5±1.9, 5.5±2.0, and 5.5±2.3 pg/ml, respectively. SAPK/JNK increased by 1.8-fold and 3.9-fold after treatment with 4 and 12 µg/ml resveratrol, respectively. Significant increase in caspase-3 levels was observed with 12 µg/ml resveratrol.

Conclusions: Our study demonstrates that resveratrol suppresses hypoxic CVEC proliferation through activation of the SAPK/JNK pathway. Resveratrol, a nutritional supplement and inhibitor of CVECs, may be a useful adjunct to current anti-VEGF therapy in wet age-related macular degeneration.

Age-related macular degeneration (AMD) is a leading cause of vision loss in the elderly population in the United States [1,2]. The wet exudative form of AMD results from hypoxia-mediated proliferation of choroidal vascular endothelial cells (CVECs). Hypoxia upregulates angiogenic factors such as vascular endothelial growth factor (VEGF) and forms a choroidal neovascular complex with consecutive vision loss [1,3].

Resveratrol (3, 4, 5-trihydroxy-trans-stilbene). a natural phytoalexin found in grapes, red wine, peanuts, and pines [4,5], prevents oxidative stress–induced DNA damage. Topical and systemic administration of resveratrol blocks tumor initiation, promotion, progression, and angiogenesis in various cancers [6-8] through downregulation of hypoxia inducible factors (HIFs) and VEGF [9,10]. In lung adenocarcinoma cells, the downstream effect of resveratrol is mediated through inactivation of stress-activated protein kinase/c-JUN N-terminal kinase (SAPK/JNK) phosphorylation [11]. The effect of resveratrol on proliferating CVECs is not known.

Correspondence to: K.V.Chalam, Department of Ophthalmology, University of Florida College of Medicine 580, W, 8th street, Tower-2, Jacksonville, FL, 32209; Phone: (904) 244-9361; FAX: (904) 244-9391; email: kchalam@jax.ufl.edu

In this study, we evaluated the effect of resveratrol on hypoxia-induced CVEC proliferation. We further studied the effect of resveratrol on hypoxia-induced VEGF release with CVECs and its effect on SAPK/JNK, a stress-related pathway.

METHODS

Cell culture: Choroidal vascular endothelial cells (RF/6A; CVECs; American Type Culture Collection, Manassas, VA) were cultured in MEM (minimal essential medium; Thermo Scientific, Logan, UT), and media were supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 µg/ml of streptomycin (Invitrogen). Cells were maintained at 37 °C in logarithmic scale in a 75 cm^2 cell culture flask in an incubator consisting of 95% air and 5% CO_2.

Hypoxic treatment: Hypoxic condition was induced in CVECs by exposing the cells to cobalt chloride ($CoCl_2$; Sigma-Aldrich, St. Louis, MO) as described below [3,12]. The induction was confirmed with cytotoxicity analysis.

We have previously reported that 200 µM $CoCl_2$ provides a non-lethal dose of hypoxia in CVECs [3]. In this study, 4×10^3 cells/well CVECs in complete media were seeded in 96-well culture plates and maintained for 48–72 h to reach 60%–80%

confluence. Cells were exposed to 200 µM $CoCl_2$ for 24 h, and cell viability was analyzed using 4-[3-(4Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) assay. WST-1, a colorimetric assay, measures cell viability based on the cleavage of tetrazolium salts to formazan by mitochondrial dehydrogenases in viable cells (Roche, Mannheim, Germany). After treatment, cells were incubated with WST-1 solution for 2 h at 37 °C. The plates were read at 440 nm with a reference wavelength at 630 nm using a multidetection microplate reader (BioTek Synergy HT, Winooski, VT).

Effect of resveratrol on hypoxic choroidal vascular endothelial cell viability: Semiconfluent cells were treated with 200 µM $CoCl_2$ and cotreated with resveratrol (Sigma-Aldrich) at increasing concentrations of 2, 4, 6, 8, 10, and 12 µg/ml for 24 h. The effect of the varying doses of resveratrol on cell viability after hypoxic insult was evaluated using WST-1 assay. Experiments were performed in triplicate to check for concordance.

Vascular endothelial growth factor enzyme-linked immunosorbent assay: We tested whether resveratrol was involved in inhibiting CVEC proliferation under hypoxic conditions by inhibiting VEGF release. VEGF levels were measured from the conditioned media using enzyme-linked immunosorbent assay (ELISA) after 1×10^4 CVEC were plated on a six-well culture plate and concurrent treatment with $CoCl_2$ and resveratrol for 24 h. Conditioned media were collected from each treatment condition. The concentration of VEGF in the conditioned media was measured with an ELISA kit (Invitrogen), according to the manufacturer's instructions. Experiments were performed in triplicate for concordant results. Three independent experiments were performed in triplicate for concordance.

Immunoblot analysis: To determine whether resveratrol inhibits hypoxic CVEC proliferation by altering HIF-1α, SIRT1, and SAPK/JNK proteins, CVECs were treated with 200 µM $CoCl_2$ and cotreated with 4 and 12 µg/ml resveratrol, under two separate experimental groups for 24 h. The two concentrations were chosen based on data from the WST-1 assay. Proteins were extracted from each treatment group using a protein isolation kit according to the manufacturer's protocol (Ambion, Invitrogen). Protein concentrations were evaluated using Bradford assay solution (Bio-Rad, Hercules, CA).

Twenty microgram protein samples were run on either 6% or 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes, which were blocked in PBS-Tween-20 (PBST) with 5% bovine serum albumin at 4 °C. The membranes were incubated with antiphospho-SAPK/JNK, antitotal SAPK/JNK (Cell Signaling Technology, Danvers, MA) at 1:1,000, anti-SIRT1(1:3,000; Upstate Biotechnology, Billerica, MA), and anti-HIF-1α (1:300; Santa Cruz Biotechnology, Dallas, TX) in PBS (0.138 M sodium chloride and 0.0027 M potassium chloride; pH 7.4; Sigma) at 4 °C overnight. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500; Upstate Biotechnology) served as a loading control. After washing with PBST, the blots were incubated with horseradish peroxidase-conjugated antirabbit immunoglobulin G antibodies at a 1:1,000 dilution for 1 h at room temperature. Blots were washed 3 times in PBST, and the proteins were detected with the enhanced chemiluminescence method. The intensity of the immunoreactive bands was quantified with densitometric analysis using ImageJ analysis software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at RSB). The protein levels were expressed either in optical density units (ODU) or in percentage.

Intracellular analysis of caspase-3 activity: We evaluated whether resveratrol regulates cell viability by the activation of caspase-3, a death protease that acts as a key mediator in mediating apoptosis, using In-Cell ELISA assay (Thermo Scientific). We seeded 4×10^3 cells/well in a 96-well microtiter plate and allowed them to attach for 48 h. We maintained three different treatment groups: group 1, hypoxic condition (200 µM concentration); group 2, hypoxia with resveratrol (200 µM+4 µg/ml); and group 3, hypoxia with resveratrol (200 µM+12 µg/ml). Cells without any treatment served as controls. Activation of caspase-3 was evaluated according to the manufacturer's instructions (Thermo Scientific). Briefly, following treatment, cells were fixed using 4% paraformaldehyde and permeabilized for 15 min using 1×surfact-amps X-100. Cell permeabilization was quenched using 30% H_2O_2 followed by the addition of blocking buffer for 30 min. Cells were incubated with caspase-3 at 1:500 at 4 °C overnight. After washing, they were incubated with antirabbit horseradish peroxidase conjugate for 30 min at room temperature followed by the addition of 3,3',5,5'-tetramethylbenzidine substrate. Cleaved caspase-3 protein levels were assessed by measuring the absorbance at 450 nm using a microplate reader. The results were compared against internal control α-tubulin (intensity of signal in absorbance units [AU]). The ratio of caspase-3 and cell number was calculated, normalized, and expressed in the figure. Three independent experiments were performed in triplicate for concordance.

Statistics: Results were expressed as mean±standard error of the mean (SEM). Means and standard deviations (SDs) were analyzed using GraphPad InStat software (GraphPad InStat3, LaJolla, CA). Means were compared using the ANOVA with

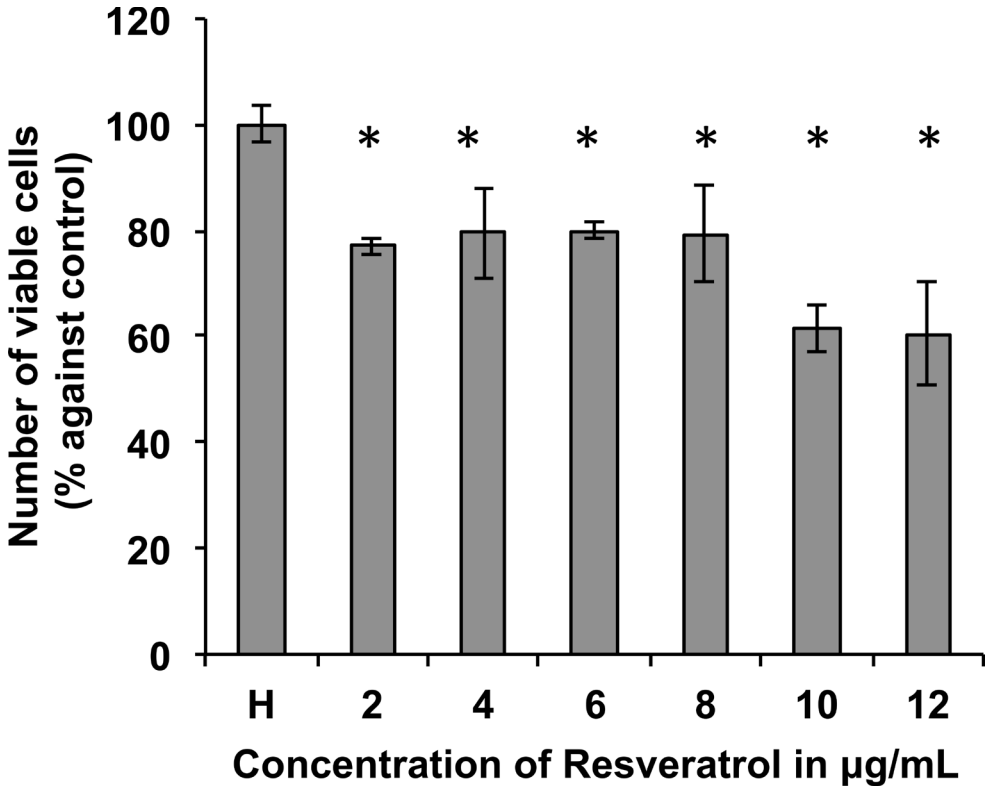

Figure 1. 4-[3-(4Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate assay (WST-1) showing changes in cell viability in the presence of hypoxia (200 µM) and resveratrol (2−12 µg/ml). The X-axis represents the concentration of resveratrol in µg/ml. The Y-axis represents the number of viable cells adjusted against the control and expressed as a percentage. H represents the hypoxia mimetic cobalt chloride treatment. Asterisks indicate significant difference compared with corresponding hypoxic cells (ANOVA with post hoc Tukey test). *p<0.05; n=8; error bars±standard error of mean (SEM).

post hoc Tukey test. Statistical significance was accepted for p values of less than 0.05.

RESULTS

Hypoxia-induced choroidal vascular endothelial cell proliferation: The results of the cell proliferation experiments were calculated as percentage and normalized against control. In a pilot study, we exposed CVECs to varying doses of cobalt chloride to induce hypoxia (100–700 µM). Hypoxia measured with cell viability and flow cytometry experiments was optimal at the 200 µM concentration of cobalt chloride treatment [3]. At this dose in this study, CVEC proliferation was 109.3±11.3% compared to untreated control.

Effect of resveratrol on hypoxia-induced choroidal vascular endothelial cells: Resveratrol inhibited cell proliferation at all doses studied in a statistically significant manner. After hypoxic insult, adding 2 µg/ml resveratrol inhibited proliferation to 77.0±1.6% and maintained the cell proliferation in an inhibitory stage to 79.5±8.6% after 4 µg/ml resveratrol was added. Choroidal endothelial cell viability stabilized at increased doses of resveratrol to 79.9±1.4% at 6 µg/ml and 79.2±9.1% at 8 µg/ml. These inhibitory effects of resveratrol on hypoxic CVECs was statistically significant ($p<0.05$). At higher doses of resveratrol at 10 and 12 µg/ml, the cell viability decreased further to 61.4±4.3% and 60.5±9.9%, respectively ($p<0.05$; Figure 1).

Vascular endothelial growth factor levels at hypoxia: VEGF levels from the conditioned media of untreated cells were 8.9±2.4 pg/ml, which increased to 30.9±2.6 pg/ml in hypoxic CVECs. In hypoxic CVECs, adding resveratrol induced a dose-dependent inhibition of VEGF, in a statistically significant manner at all doses studied. VEGF levels in the conditioned media were measured at 12.4±2.1, 11.0±1.9, and 10.3±3.0 pg/ml at 2, 4, and 6 µg/ml, respectively ($p<0.01$). At high doses of resveratrol, the decrease in VEGF levels stabilized to 7.5±1.9 pg/ml at 8 µg/ml. It decreased further to 5.5±2.0 pg/ml at 10 and 12 µg/ml resveratrol ($p<0.01$; Figure 2).

Resveratrol activates stress-activated protein kinase/c-Jun N-terminal kinase at hypoxia: To explore the temporal effects of resveratrol treatment on hypoxic CVECs, we evaluated the activation/phosphorylation of SAPK/JNK (a pathway that trigger during cellular stress) using immunoblot. We found that hypoxic CVECs phosphorylate the SAPK/JNK pathway (0.025 ODU) that increased to 1.8 fold (0.045 ODU) after the 4 µg/ml resveratrol treatment. The increased activation was statistically significant ($p<0.01$). As the added resveratrol increased to 12 µg/mL, hypoxic cells showed upregulation of

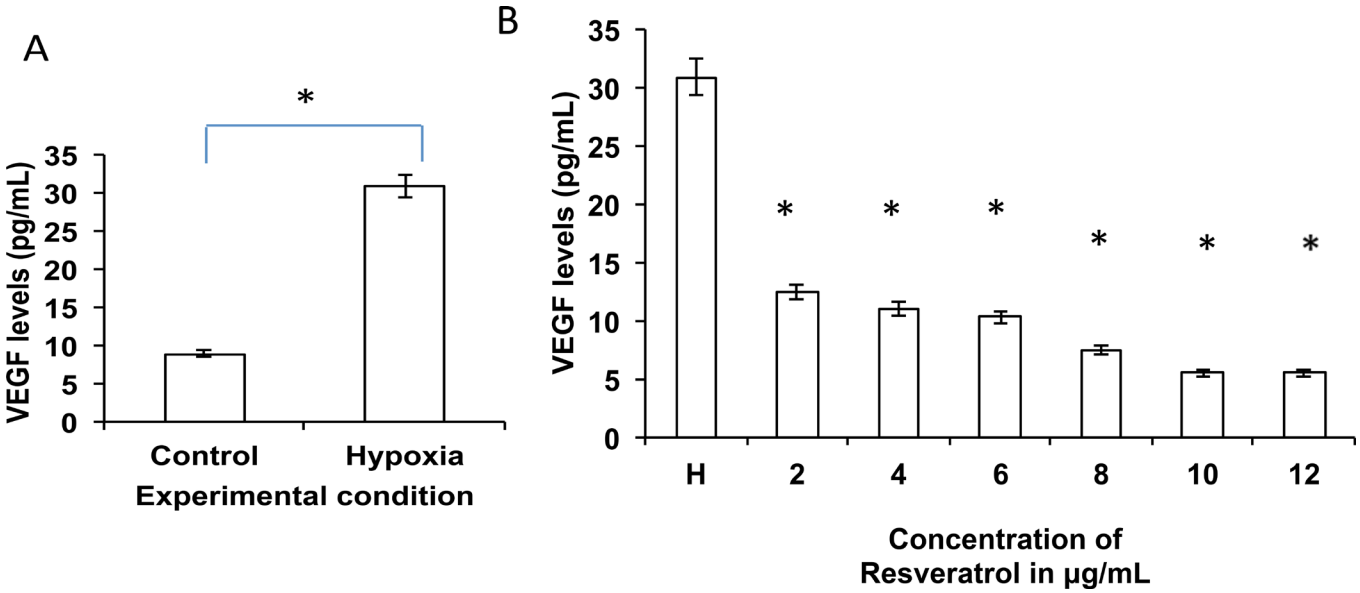

Figure 2. Resveratrol suppresses vascular endothelial growth factor levels in choroidal vascular endothelial cells at hypoxia. **A**: Basal levels of vascular endothelial growth factor (VEGF) in choroidal vascular endothelial cells (control) and after hypoxic induction using cobalt chloride. **B**: VEGF levels in hypoxic choroidal vascular endothelial cells after the treatment of increasing doses of resveratrol (2–12 µg/ml). The x-axis represents the treatment conditions. The y-axis represents the VEGF levels in pg/ml. 'R' represents the resveratrol treatment in escalated doses. A dose-dependent decrease in the VEGF level is noted. Asterisks indicate significant difference compared with corresponding hypoxic cells (*p<0.01; n=3; error bars±standard error of mean [SEM]).

SAPK/JNK (0.097 ODU) in a dose-dependent manner and were observed to be statistically significant. The increased level of activation at this dose was 3.9-fold higher when compared to hypoxic CVECs (p<0.01; Figure 3A,B).

Given the importance of SIRT1 in mediating the effects of resveratrol, observing SIRT1 levels in hypoxic CVECs is important. The levels of hypoxia-responsive protein SIRT1 decreased significantly at high levels (12 µg/mL) of resveratrol treatment (Figure 3C,D), while the transcriptional VEGF regulator HIF-1α decreased (Figure 3C) with the decrease in secreted VEGF (Figure 2).

Activated caspase-3/apoptosis evaluation: Compared to untreated cells (0.188±0.02 AU), hypoxia-challenged CVECs showed reduced activation of caspase-3 (0.179±0.005 AU). A similar observation was noted after 4 µg/ml resveratrol was added to the hypoxic cells (0.173±0.01AU). As the resveratrol dose increased to12 µg/ml, the activated caspase-3 levels increased to 1.1 fold (0.200±0.01AU) compared to the hypoxia-treated cells. This increased activation of caspase-3, though small, was statistically significant (p<0.05; Figure 4).

DISCUSSION

Age-related macular degeneration, a common cause of blindness after the age of 65, is characterized by either the presence of drusen (dry AMD) or VEGF-induced choroidal endothelial cell proliferation with associated leakage (wet exudative form of AMD) [13]. Hypoxia is a common initiator of angiogenesis in various ocular neovascular diseases such as AMD and plays a key role in VEGF release and induction of choroidal endothelial cell proliferation [14]. Molecular targets that inhibit hypoxia may play a key role in the progression of dry form to wet exudative AMD.

Resveratrol, a natural polyphenol exhibits various bioactivities, including antitumorigenic [7], antiangiogenic [15], neuroprotective [16], vasodilative [17], and antioxidative [18,19] functions. Topical and systemic administration of resveratrol blocks tumor initiation, promotion, and progression in various cancers [15,20]. Administering resveratrol promoted apoptosis in xenografted tumors in athymic nude mice [21,22]. Our study shows that resveratrol promotes apoptosis of proliferating choroidal endothelial cells and inhibits hypoxia-induced CVEC proliferation through activation of the SAPK/JNK pathway.

We induced hypoxia in CVECs to closely mimic in vivo active choroidal neovascularization. Our previous observations in a dose response analysis of cobalt chloride revealed

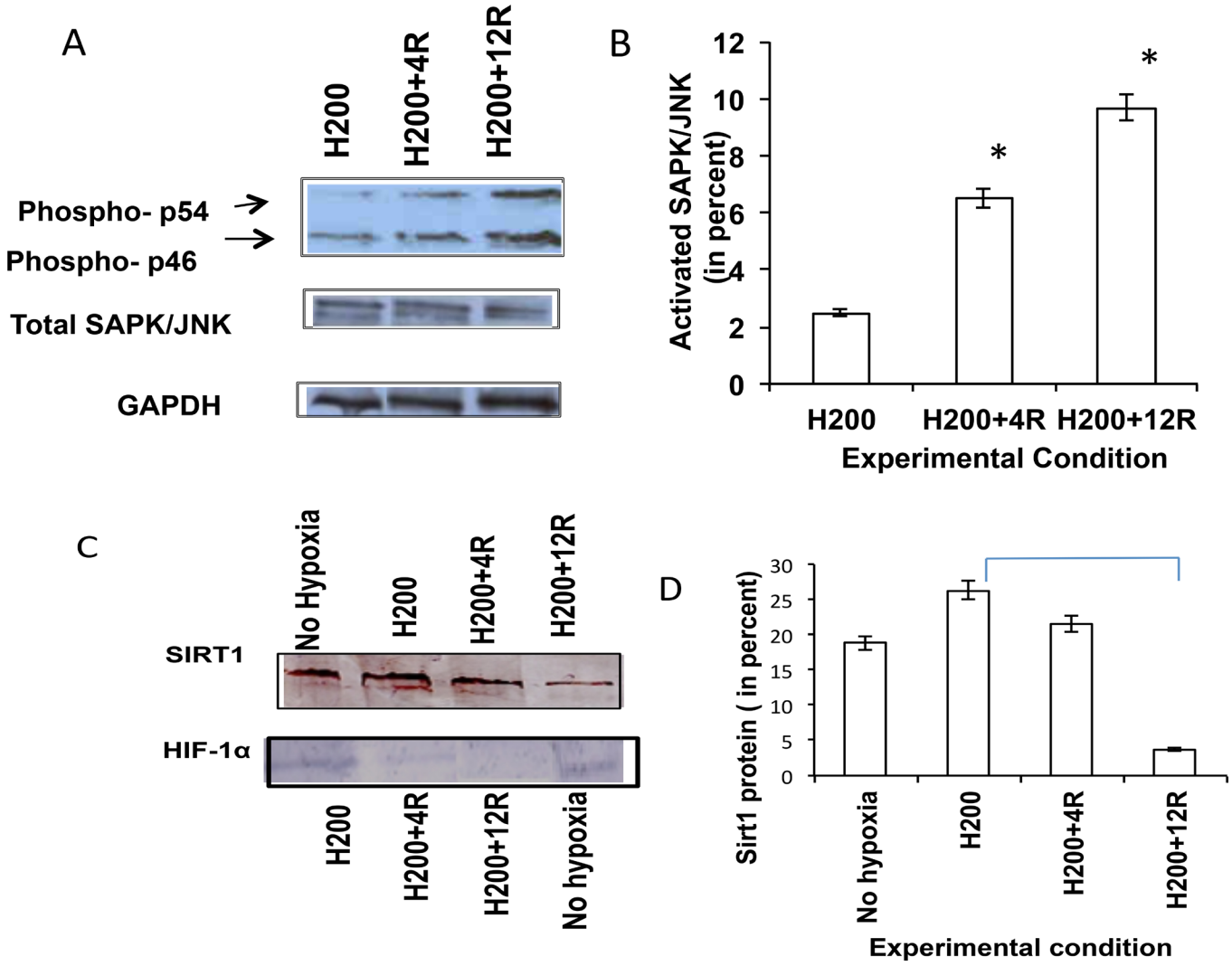

Figure 3. Effect of resveratrol on activation of stress-activated protein kinase/c-Jun N-terminal kinase in hypoxic choroidal vascular endothelial cells. **A**: Cells were treated with hypoxia mimetic cobalt chloride at 200 µM concentration ($CoCl_2$; H200) for 24 h and cotreated with resveratrol at 4 µg/ml (H200+4R) and 12 µg/ml (H200+12R) to evaluate the activated/phosphorylated stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK; top panel) and total SAPK/JNK (middle panel). The bottom panel shows the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Representative data were shown. **B**: Bars represent optical density units of quantified activated/phosphorylated SAPK/JNK levels in percent. H represents hypoxia mimetic cobalt chloride treatment. R represents resveratrol treatment. Asterisks indicate significant difference compared with corresponding hypoxic cells (*p<0.01). Data were analyzed by ANOVA with post hoc Tukey test and represented as error bars±standard error of mean (SEM; n=2). **C**: Choroidal vascular endothelial cells treated without/with hypoxia mimetic cobalt chloride at 200 µM concentration ($CoCl_2$; H200) for 24 h and cotreated with resveratrol at 4 µg/ml (H200+4R) and 12 µg/ml (H200+12R). Representative data were shown. **D**: Bars represent optical density units of quantified Sirt1 protein in percent. H represents hypoxia mimetic cobalt chloride treatment. R represents resveratrol treatment. Asterisks indicate significant difference compared with corresponding hypoxic cells (*p<0.01). Data were analyzed by ANOVA with post hoc Tukey test and represented as error bars±SEM (n=2).

optimal hypoxic response in CVECs at 200 µM concentration [12]. In this study, we used a 200 µM concentration of hypoxia mimetic $CoCl_2$ to analyze the effects of resveratrol in hypoxic CVECs. One of the major concerns of using resveratrol is the potential toxicity at high dosages. In most in vitro studies, resveratrol was used at concentrations ranging from 5 to 100 µmol/l [23]. In the present study, the resveratrol dosages ranged from 8.7 to 52.6 µM, comparable to those used in melanoma and endothelial cell culture studies [7]. Khan et al. observed dose-dependent inhibition of human microvascular endothelial cell proliferation after treatment with resveratrol. They observed cell migration inhibition after treatment of resveratrol for 20 h and identified 4 µg/ml as an optimal dose [24]. Therefore, in the current study, we chose the 24 h time

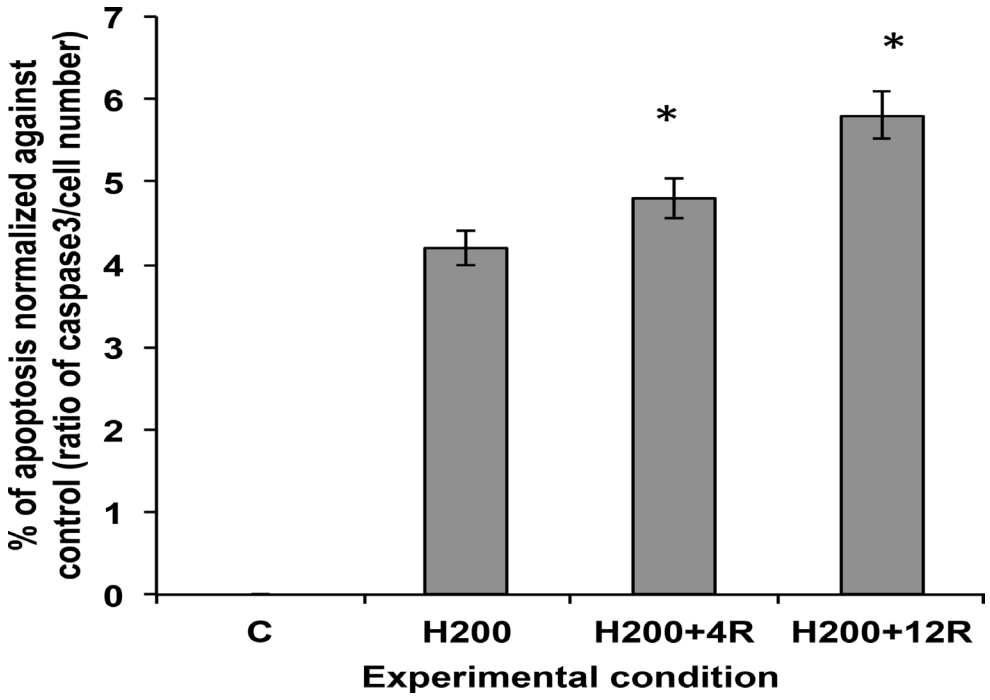

Figure 4. Evaluating the apoptosis or activation of caspase-3 activity at hypoxic condition (H200) and cotreatment of hypoxia with resveratrol at 4 µg/ml (H200+4R) and 12 µg/ml (H200+12R). X-axis represents the treatment conditions. Y-axis represents the cleaved caspase-3 in percent after the adjustment of caspase-3 with cell number. C represents control or untreated cells. H represents hypoxic condition. R represents resveratrol treatment. Data analysis: ANOVA with post hoc Tukey test. Asterisks indicate significant difference compared with corresponding hypoxic cells (*p<0.05; n=3; error bars±standard error of mean [SEM]).

point to observe the maximal inhibitory effect of resveratrol in CVECs. We also observed dose-dependent inhibition of cell survival in hypoxic CVECs.

Resveratrol inhibits HIF and VEGF expression in human ovarian cancer cells [25]. Growth factors and cytokines upregulate HIF expression mainly by enhancing the protein translation as well as by inhibiting its degradation [26] through the ubiquitin proteasomal pathway [27-30]. Additionally, an autocrine loop between hypoxia and growth factors may coexist in tumors, thus enhancing their angiogenic capability and new blood vessel growth. Our results suggest that resveratrol may act as a multifactorial antiangiogenic agent by downregulating the levels of HIF protein, thus suppressing the HIF-related proangiogenic factor, VEGF. In this study, CVECs under hypoxic conditions demonstrated an increase in VEGF levels in the media. Conditional media from resveratrol treated hypoxic CVECs showed a decrease in VEGF levels at all treatment doses. Our data also showed decreased levels of HIF-1α in parallel to decreased VEGF levels at resveratrol doses of 4 and 12 µg/ml. This further confirms that the antiangiogenic effects of resveratrol is mediated by a resveratrol-induced change in HIF-related VEGF protein.

Based on earlier observations, we chose to investigate further the effects of resveratrol at 4 µg/ml and 12 µg/ml concentrations [24]. Resveratrol induces apoptosis in human leukemia HL-60 cells through the JNK pathway [31]. Mitogen-activated protein kinases such as stress-activated protein kinase (SAPK)/JNK and p38 kinases are activated under metabolic and environmental stress and mediate apoptotic signals [32]. Resveratrol can induce apoptosis through c-Jun-NH_2-kinase and p53-dependent signaling pathways, in mouse JB6 epidermal cells [33], LNCaP [34], Hep G_2 [35], or a mutant p53 prostate cancer cell line, DU 145 [36], and human glioma U251 and U87 cells [37], suggesting that resveratrol modulates various intracellular signal transduction pathways, which often go awry during the course of angiogenesis. In this study, we found that exposing CVECs to hypoxia results in the activation of SAPK/JNKs, which increases significantly after resveratrol treatment. This implies that the mechanisms in which resveratrol mediate cell death in hypoxic CVECs observed is comparable with the reported studies in other model systems.

Resveratrol is a potent activator of SIRT1, and the effects are mediated by SIRT1 [24]. Decreased levels of SIRT1 at 12 µg/ml observed in our study confirm that resveratrol inhibits hypoxic CVEC proliferation through the SIRT1 pathway.

Resveratrol induces apoptotic cell death in several cell lines, including hormone-sensitive LNCaP prostate cells [38], hormone-insensitive DU 145 prostate cells [36], human B cell chronic leukemia cells [39], as well as several other human cancer cell lines such as MCF7, SW480, HCE7, and HL60 [40]. Jiang et al. reported that resveratrol-induced apoptosis requires the activation of caspase-3 and involves the

upregulation of Bax expression and activation of caspase-9 in U251 glioma cells [37]. Other studies revealed the activation of p53 for apoptosis [41], which indicates that multiple apoptotic signaling cascades may be activated by resveratrol, depending on the specific cell type and cellular environment. Resveratrol mediates apoptotic effect at a relatively higher concentration (100–300 µmol/L) [6] that support our observation of caspase-3. Our study on hypoxic CVECs showed activation of caspase-3 at 12 µg/ml resveratrol but not at 4 µg/ml resveratrol. However, our study also suggests that additional intracellular signaling pathways may have a possible role in resveratrol-induced apoptotic cell death process.

In conclusion, resveratrol, a natural antioxidant, inhibits proliferation of hypoxic CVECs through stopping VEGF secretion as well as promoting apoptosis, two different but complimentary mechanisms. Resveratrol, a compound found in many natural foods, may be a useful adjunct to current anti-VEGF therapy in managing exudative AMD.

REFERENCES

1. Coleman HR, Chan C, Ferris FL, Chew EY. Age-related macular degeneration. Lancet 2008; 372:1835-45. [PMID: 19027484].
2. National Advisory Eye Council (US). Vision Research: A National Plan 1994–1998. NIH publication no. 93–3186. Bethesda, MD:US Department of Health and Human Services; 1993.
3. Balaiya S, Khetpal V, Chalam KV. Hypoxia initiates sirtuin1-mediated vascular endothelial growth factor activation in choroidal endothelial cells through hypoxia inducible factor–2α. Mol Vis 2012; 18:114-20. [PMID: 22275802].
4. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, Kim AL. Resveratrol: A review of preclinical studies for human cancer prevention. Toxicol Appl Pharmacol 2007; 224:274-83. [PMID: 17306316].
5. Niles RM, McFarlanda M, Weimera MB, Redkarb A, Fub Y, Meadows GG. Resveratrol is a potent inducer of apoptosis in human melanoma cells. Cancer Lett 2003; 190:157-63. [PMID: 12565170].
6. Szende B, Tyihák E, Király-Véghely Z. Dose-dependent effect of resveratrol on proliferation and apoptosis in endothelial and tumor cell cultures. Exp Mol Med 2000; 32:88-92. [PMID: 10926121].
7. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of Resveratrol, a natural product derived from grapes. Science 1997; 275:218-20. [PMID: 8985016].
8. Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr 2001; 21:381-406. [PMID: 11375442].
9. Zhang Q, Tang X, Lu QY, Zhang ZF, Brown J, Le AD. Resveratrol inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1A and VEGF expression in human tongue squamous cell carcinoma and hepatoma cells. Mol Cancer Ther 2005; 4:1465-74. [PMID: 16227395].
10. Trapp V, Parmakhtiar B, Papazian V, Willmott L, Fruehauf JP. Anti-angiogenic effects of resveratrol mediated by decreased VEGF and increased TSP1 expression in melanoma-endothelial cell co-culture. Angiogenesis 2010; 13:305-15. [PMID: 20927579].
11. Yang YT, Weng C, Ho C, Yen G. Resveratrol analog-3,5,49-trimethoxy-trans-stilbene inhibits invasion of human lung adenocarcinoma cells by suppressing the MAPK pathway and decreasing matrix metalloproteinase-2 expression. Mol Nutr Food Res 2009; 53:407-16. [PMID: 19072741].
12. Zhang H, Fan H, Xu N, Duan X, Duan X, Liu B. Effect of cell cycle and radiosensitivity by CoCl2 induced hypoxia in Eca109 cells. Chinese-German J Clin Oncol 2008; 7:230-2. .
13. Balaiya S, Malyapa R, His W, Murthy RK, Chalam KV. Evaluation of proton beam radiation sensitivity of proliferating choroidal endothelial and retinal ganglion cells with clonogenic assay. Cutan Ocul Toxicol 2012; 31:14-9. [PMID: 21861774].
14. Stefánsson E, Geirsdóttir A, Sigurdsson H. Metabolic physiology in age related macular degeneration. Prog Retin Eye Res 2011; 30:72-80. [PMID: 20951826].
15. Bråkenhielm E, Cao R, Cao Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. FASEB J 2001; 15:1798-800. [PMID: 11481234].
16. Wang Q, Xu J, Rottinghaus GE, Simonyi A, Lubahn D, Sun GY, Sun AY. Resveratrol protects against global cerebral ischemic injury in gerbils. Brain Res 2002; 958:439-47. [PMID: 12470882].
17. Nagaoka T, Hein TW, Yoshida A, Kuo L. Resveratrol, a component of red wine, elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and potassium channels. Invest Ophthalmol Vis Sci 2007; 48:4232-9. [PMID: 17724212].
18. Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. Lancet 1993; 341:1103-4. [PMID: 8097009].
19. Mizutani K, Ikeda K, Kawai Y, Yamori Y. Protective effect of resveratrol on oxidative damage in male and female stroke-prone spontaneously hypertensive rats. Clin Exp Pharmacol Physiol 2001; 28:55-9. [PMID: 11153537].
20. Kundu JK, Surh YJ. Molecular basis of chemoprevention by resveratrol: NF-kB and AP-1 as potential targets. Mutat Res 2004; 555:65-80. [PMID: 15476852].
21. Garvin S, Ollinger K, Dabrosin C. Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts *in vivo*. Cancer Lett 2006; 231:113-22. [PMID: 16356836].

22. Sareen D, Darjatmoko SR, Albert DM, Polans AS. Mitochondria, calcium, and calpain are key mediators of resveratrol-induced apoptosis in breast cancer. Mol Pharmacol 2007; 72:1466-75. [PMID: 17848600].

23. Estrov Z, Shishodia S, Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M, Aggarwal BB. Resveratrol blocks interleukin-1beta-induced activation of the nuclear transcription factor NF-κB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. Blood 2003; 102:987-95. [PMID: 12689943].

24. Khan AA, Dace DS, Ryazanov AG, Kelly J, Apte RS. Resveratrol Regulates Pathologic Angiogenesis by a Eukaryotic Elongation Factor-2 Kinase-Regulated Pathway Am J Pathol 2010; 177:481-92. [PMID: 20472894].

25. Cao Z, Fang J, Xia C, Shi XL, Jiang BH. eds-3,4,5′-trihydroxystibene inhibits hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression in human ovarian cancer cells. Clin Cancer Res 2004; 10:5253-63. [PMID: 15297429].

26. Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Bu¨rgel T. Normoxic induction of the hypoxia-inducible factor 1a by insulin and interleukin-1h involves the phosphatidylinositol-3-kinase pathway. FEBS Lett 2002; 512:157-62. [PMID: 11852072].

27. Ivan M, Konda K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. HIF-1a targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001; 292:464-8. [PMID: 11292862].

28. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of HIF-1a to the von Hippel-Lindau ubiquitilation complex by O2-regulated prolyl hydroxylation. Science 2001; 292:468-72. [PMID: 11292861].

29. Salceda S, Caro J. Hypoxia-inducible factor-1a protein is rapidly degraded by the ubiquitin-proteosome system under normoxic conditions: its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997; 272:22642-7. [PMID: 9278421].

30. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1a is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 1998; 95:7987-92. [PMID: 9653127].

31. Su JL, Lin MT, Hong CC, Chang CC, Shiah SG, Wu CW, Chen ST, Chau YP, Kuo ML. Resveratrol induces FasL-related apoptosis through Cdc42 activation of ASK1/JNK-dependent signaling pathway in human leukemia HL-60 cells. Carcinogenesis 2005; 26:1-10. [PMID: 15217905].

32. Roduit R, Schorderet DF. MAP kinase pathways in UV-induced apoptosis of retinal pigment epithelium ARPE-19 cells. Apoptosis 2008; 13:343-53. [PMID: 18253836].

33. She QB, Huang C, Zhang Y, Dong Z. Involvement of c-jun NH(2)-terminal kinases in resveratrol-induced activation of p53 and apoptosis. Mol Carcinog 2002; 33:244-50. [PMID: 11933078].

34. Narayanan BA, Narayanan NK, Re GG, Nixon DW. Differential expression of genes induced by resveratrol in LNCaP cells: P53-mediated molecular targets. Int J Cancer 2003; 104:204-12. [PMID: 12569576].

35. Kuo PL, Chiang LC, Lin CC. Resveratrol-induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. Life Sci 2002; 72:23-34. [PMID: 12409142].

36. Lin HY, Shih A, Davis FB, Tang HY, Martino LJ, Bennett JA, Davis PJ. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. J Urol 2002; 168:748-55. [PMID: 12131363].

37. Jiang H, Zhang L, Kuo J, Kuo K, Gautam SC, Groc L, Rodriguez AI, Koubi D, Hunter TJ, Corcoran GB, Seidman MD, Levine RA. Resveratrol-induced apoptotic death in human U251 glioma cells. Mol Cancer Ther 2005; 4:554-61. [PMID: 15827328].

38. Morris GZ, Williams RL, Elliott MS, Beebe SJ. Resveratrol induces apoptosis in LNCaP cells and requires hydroxyl groups to decrease viability in LNCaP and DU 145 cells. Prostate 2002; 52:319-29. [PMID: 12210493].

39. Roman V, Billard C, Kern C, Ferry-Dumazet H, Izard JC, Mohammad R, Mossalayi DM, Kolb JP. Analysis of resveratrol-induced apoptosis in human B-cell chronic leukaemia. Br J Haematol 2002; 117:842-51. [PMID: 12060119].

40. Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res 2002; 8:893-903. [PMID: 11895924].

41. Huang C, Ma WY, Goranson A, Dong Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. Carcinogenesis 1999; 20:237-42. [PMID: 10069459].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 23 November 2013. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.