Resveratrol at Anti-angiogenesis/Anticancer Concentrations Suppresses Protein Kinase G Signaling and Decreases IAPs Expression in HUVECs

JANICA C. WONG[1,3,4] and RONALD R. FISCUS[1,2,4]

[1]*Diabetes & Obesity Research Center, Alzheimer's & Parkinson's Disease Research Center, Cancer Research Center, Roseman Medical Education and Research Building at Summerlin Campus, Roseman University of Health Sciences, Las Vegas/Henderson, NV, U.S.A.;*
[2]*College of Medicine and College of Pharmacy, Roseman University of Health Sciences, Henderson, NV, U.S.A.;*
[3]*Department of Chemistry, University of Nevada Las Vegas, Las Vegas, NV, U.S.A.;*
[4]*Cancer Molecular Biology Section, Nevada Cancer Institute, Las Vegas, NV, U.S.A.*

Abstract. *Background: Resveratrol increases nitric oxide (NO) production via increased expression and activation of endothelial-form-NO-synthase (eNOS) in endothelial cells. However, the role of downstream cGMP/protein kinase G (PKG) signaling, a pathway activated by NO/eNOS, in pro- and anti-angiogenic effects of resveratrol is still unclear. Materials and Methods: Endogenous NO/cGMP/PKG pathway and downstream cell-survival proteins (Inhibitor of Apoptosis Proteins, IAPs) were studied in relation to pro- and anti-angiogenic effects of resveratrol in human umbilical vein endothelial cells (HUVECs). Results: Resveratrol at higher/anti-angiogenic concentrations inhibits HUVEC tube formation and cell migration/invasion (indices of angiogenesis). Resveratrol at lower concentrations stimulates proliferation and protects HUVECs against spontaneous apoptosis. 8-Br-cGMP, a direct activator of PKG, protects against pro-apoptotic effects of high-concentration resveratrol. Western blot analyses showed that anti-angiogenic concentrations of resveratrol suppress endogenous PKG kinase activity and decrease the expression of four cell-survival proteins, c-IAP1, c-IAP2, livin and XIAP. Conclusion: Resveratrol-induced anti-angiogenesis/pro-apoptosis induced suppression of PKG signaling and decreased expression of the cell-survival proteins c-IAP1, c-IAP2, livin and XIAP.*

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Correspondence to: Ronald R. Fiscus, Roseman University of Health Sciences, 11 Sunset Way, Henderson, Nevada, NV 89014. Tel: +1 702 4273139, Fax: +1 702 9685573, e-mail: rfiscus@roseman.edu

Key Words: Resveratrol, protein kinase G (PKG), angiogenesis, HUVECs, Inhibitor of Apoptosis Proteins (IAPs).

Resveratrol, a polyphenol found in grapes, berries, peanuts and red wine, is believed to have multiple beneficial effects on the human body. Extensive studies have shown that resveratrol can exert anticancer effects by regulating the cell cycle, cell proliferation, apoptosis, invasion and metastasis of cancer cells as well as by inhibiting tumor angiogenesis (*i.e.* inhibiting the formation of new blood vessels that provide extra blood flow to the growing tumor) (1).

Molecular targets of resveratrol are thought to be diverse, including cytokines, transcription factors, growth factors (*e.g.* VEGF, EGF) and apoptosis/cell death-regulating proteins (*e.g.* survivin, p53, Bcl-2) (1). Of particular interest are the kinase inhibitory effects of resveratrol. Specifically, resveratrol has been shown to inhibit the kinase (catalytic) activity of several PKC isoforms, the MAP-kinase ERK1/2 and Syk, a tyrosine protein kinase (1). However, it has not been reported whether resveratrol has effects on cyclic-GMP-dependent protein kinase (protein kinase G, PKG), a biologically- and clinically-important serine/threonine-kinase mediating many physiological and pharmacological effects of nitric oxide (NO), an important pro-angiogenic factor, or on downstream proteins regulated by PKG.

Angiogenesis is the process of generating new capillary blood vessels, which plays fundamental roles in both physiological (*e.g.* wound healing) and pathological processes (*e.g.* tumor angiogenesis, tumor growth and metastasis). NO has been shown to contribute to angiogenesis stimulated by vascular endothelial growth factor (VEGF), a pro-angiogenic factor secreted by many tumors (27). Numerous studies have shown that the soluble-guanylyl-cyclase (sGC)/cGMP/PKG-I signaling pathway mediates the stimulatory effects of VEGF and NO on endothelial cells, enhancing cell proliferation and migration resulting in angiogenesis (5, 13, 15, 17, 21). Interestingly,

although angiogenesis induced by mammalian VEGF, which involves activation of VEGFR-1/Flt1 receptors, depends on NO/cGMP/PKG signaling, the angiogenesis induced by VEGFR-2 receptors, activated by the viral form of VEGF, VEGF-E, appears independent of PKG (5).

Recently, we showed that the expression of three of the Inhibitor of Apoptosis Proteins (IAPs), c-IAP1, livin and survivin, are dependent on the NO/cGMP/PKG-Iα signaling pathway in non-small cell lung cancer (NSCLC) cells (24). NSCLC cells were found to possess hyperactivated PKG-Iα, which mediated the high-level expression of c-IAP1, livin and survivin, potentially contributing to the resistance to chemotherapeutic agents (chemoresistance) caused by the hyperactivated PKG-Iα. A similar role for PKG in promoting expression of cell-survival proteins, especially the IAPs, in endothelial cells may be responsible for the cytoprotective effects of low/physiological levels of NO and may play an important role in the effects of resveratrol on angiogenesis.

The aim of the present study was to investigate the role of the NO/cGMP/PKG-I pathway in regulating apoptosis/cell survival in HUVECs and determine whether the pro- and anti-angiogenic effects of resveratrol involve the NO/cGMP/PKG-I pathway and downstream expression of four cell-survival proteins, c-IAP1, c-IAP2, livin and XIAP.

Materials and Methods

Cell cultures and reagents. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with endothelial cell growth supplements (ECGS), 10% fetal bovine serum, streptomycin (50 μg/mL) and penicillin (50 units/mL) (Lonza, Walkersville, MD, USA). The cells were cultured at 37˚C in an atmosphere of 5% CO_2/95% air.

Assessment of apoptosis by Cell Death Detection ELISA Plus. The Cell Death Detection ELISAPLUS assay (Roche Applied Science, IN, USA) as described in our previous publication (24).

Assessment of cell proliferation by MTT assay. Proliferation was measured by the MTT assay (Roche Applied Science), following the protocol of the manufacturer.

Assessment of de novo DNA synthesis by BrdU-(5'Bromo-2-deoxyuridine) ELISA colorimetric assay. The rate of DNA synthesis was measured by BrdU ELISA assay (Roche Applied Science, Indianapolis, IN, USA), as described in our previous publication (25).

Protein extraction and western blotting using near-infrared fluorescence imaging. Protein extraction and western blotting was performed as described previously (24). Primary antibodies used were specific against PKG-Iα/β (1:1000), p-(ser-239)-VASP (1:500) (all from Cell Signaling Technology, Danvers, MA, USA), β-actin (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA), c-IAP1, c-IAP2, livin and XIAP (Chemicon, Billerica, MA, USA). Western blots were imaged and analyzed using Odyssey system from LI-COR (LI-COR Biosciences, Lincoln, NE, USA).

Matrigel-tube formation assay. Growth factor-reduced basement membrane matrix (BD Matrigel™ , BD Bioscience, San Jose, CA, USA) (200 μL) was added to each well of 24-well plates for 4 hours at 37˚C. HUVECs (70,000 cells) in 500 μL of DMEM with 10% FBS were added to each Matrigel-coated well with different concentrations of resveratrol. The cells were then incubated for 16 h at 37˚C in an atmosphere of 5% CO_2/95% air to allow tube formation.

In vitro cell migration (invasion) assay. The *in vitro* cell migration assay was performed as described previously (25).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted by the RNeasy plus mini-kit (Qiagen, Valencia, CA, USA). One μg of the total RNA was used for first-strand cDNA synthesis using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The specific primer pairs used were: PKG-Iα, F: 5'-AGAAGCGG CTGTCAGAGAAG-3' and R: 5'-TGGATCTGCGACAGCTCCAA-3' (product size 271 bp); PKG-Iβ, F: 5'-CACCTTGCGGGAT TTACAGT-3' and R: 5'-TGGATCTGCGACAGCTCCAA-3' (product size 378 bp); GAPDH, F" 5'-GAGTCAACGGATTTGGTCGTAT-3' and R: 5'-ATGGGT GGAATCATATTGGAAC-3' (product size 140 bp). A premixed PCR Master Mix (Promega, Madison, WI, USA) was used for efficient amplification of DNA templates. PCR cycle conditions were 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec for 35 cycles, with an initial denaturation at 94˚C for 2 min and a final extension of 10 min at 72˚C. Samples were run on 1.5% agarose gels at 100 V for 40 min.

NanoPro 100 system, an automated capillary-electrophoresis-based in-capillary nanofluidic immuno-detection system, used for ultrasensitive detection of proteins in biological samples. The methodology for the NanoPro 100 system (ProteinSimple, Santa Clara, CA, USA) has been extensively described in two recent book chapters published by our laboratory (10, 11). Briefly, cells were lysed with M-Per lysis buffer in the presence of a cocktail of protease and phosphatase inhibitors, following recommendations from the manufacturer ProteinSimple. Cell lysates (62.5 ng total protein/μL) were prepared in Premix G2 pH 5-8 (nested) separation gradient containing pI standards and protease inhibitors. Separation time was 50 min at 15 MilliWatts. Anti-PKG-Iα/β antibody (Cell Signaling Technology, Danvers, MA, USA), which recognizes both PKG-Iα and PKG-Iβ isoforms, was used as the primary antibody and anti-rabbit-HRP was used as the secondary antibody. The incubations were 110 and 55 min, respectively. Chemoluminescent reagents were automatically introduced into the capillaries of the NanoPro 100 system for generation of the chemoluminescent signals. Results are expressed as electropherograms, with peaks representing the targeted protein and its various isoforms and phosphoforms.

Statistical analysis. Statistical analysis was performed by one- or two-way ANOVA using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). Differences between experimental groups were determined by the Dunnett's *post-hoc* test.

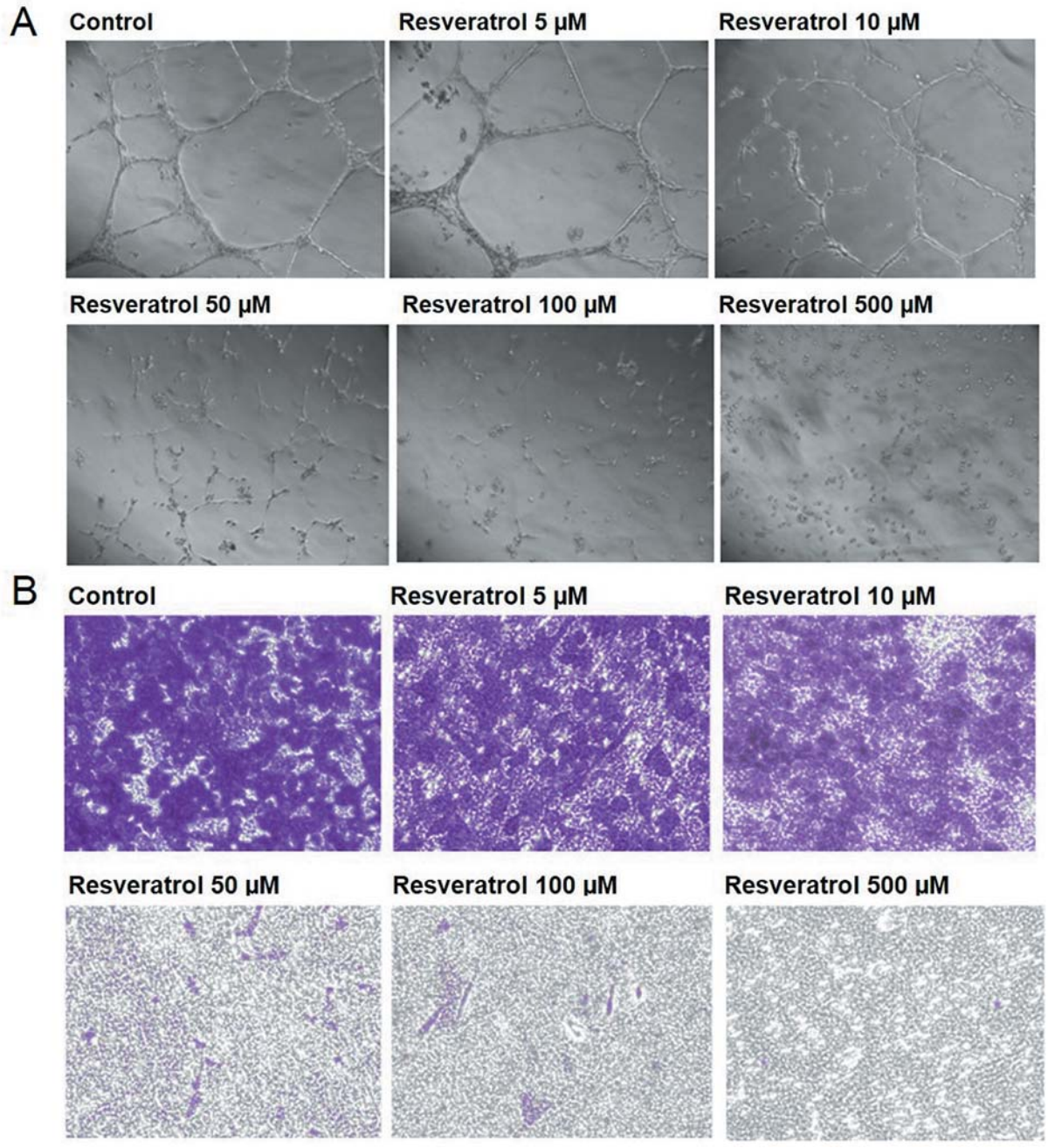

Figure 1. *Resveratrol inhibited tube formation and cell migration/invasion in HUVECs. A: Resveratrol inhibited tube formation in a concentration-dependent manner, causing almost complete inhibition at higher concentrations (50, 100 and 500 μM). B: Resveratrol at higher concentrations (50, 100 and 500 μM) inhibited cell migration/invasion of HUVECs. Cells that migrated to the bottom of transwell inserts were stained with crystal violet.*

Results

Resveratrol, at anti-angiogenic/anticancer concentrations, inhibits tube formation and cell migration/invasion of HUVECs. In the present study, HUVECs were seeded on matrigel-coated plates to determine the tube formation ability during exposure at different concentrations of resveratrol. Figure 1A shows that resveratrol at higher concentrations (50, 100 and 500 μM) inhibited tube formation of HUVECs. In addition, we assessed the cell migration ability of the cells, which also plays an important role in angiogenesis and sprouting of new blood vessels. Figure 1B shows the results from the *in vitro* cell

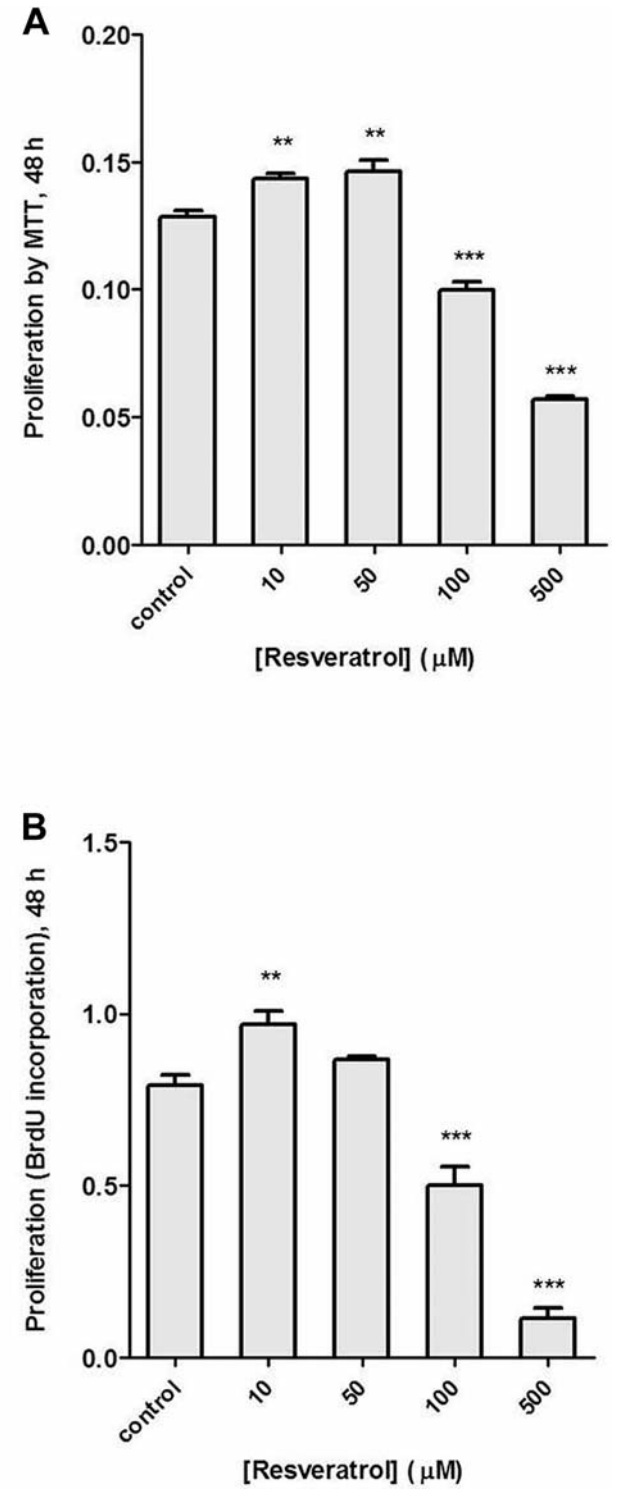

Figure 2. *Biphasic proliferation curves of HUVECs treated with resveratrol for 24 h. A: Resveratrol stimulated proliferation at lower concentrations, 10 μM and 50 μM (**p<0.01), but inhibited proliferation at higher concentrations, 100 μM and 500 μM (***p<0.001). B: Similar results were obtained using BrdU incorporation (de novo DNA synthesis). The data represent the mean±SEM of six observations per treatment group.*

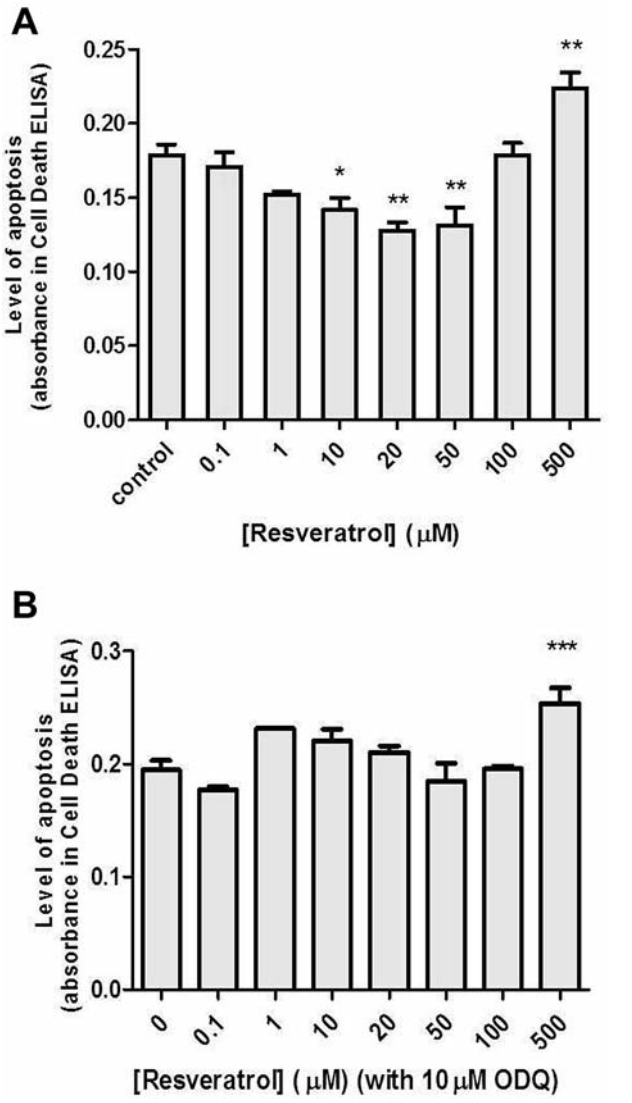

Figure 3. *Biphasic effects of resveratrol on apoptosis of HUVECs and potential involvement of endogenous NO. A: Resveratrol protected cells from apoptosis at lower concentrations (10 μM, 20 μM and 50 μM) and caused higher levels of apoptosis at the higher concentration (500 μM). B: Co-incubation of resveratrol with the soluble guanylyl cyclase inhibitor ODQ at 10 μM for 24 h completely blocked the cytoprotective effects of resveratrol at 10, 20 and 50 μM. The data represent the mean±SEM of four observations per treatment group. *p<0.05, **p<0.01, ***p<0.001.*

migration (invasion) assay. Similar to its effect on tube formation, resveratrol at higher concentrations (50, 100 and 500 μM) also inhibited cell migration/invasion of HUVECs. Therefore, our data demonstrated that resveratrol at higher concentrations (50, 100 and 500 μM) inhibits *in vitro* angiogenesis (*i.e.* tube formation and cell migration/invasion) of HUVECs.

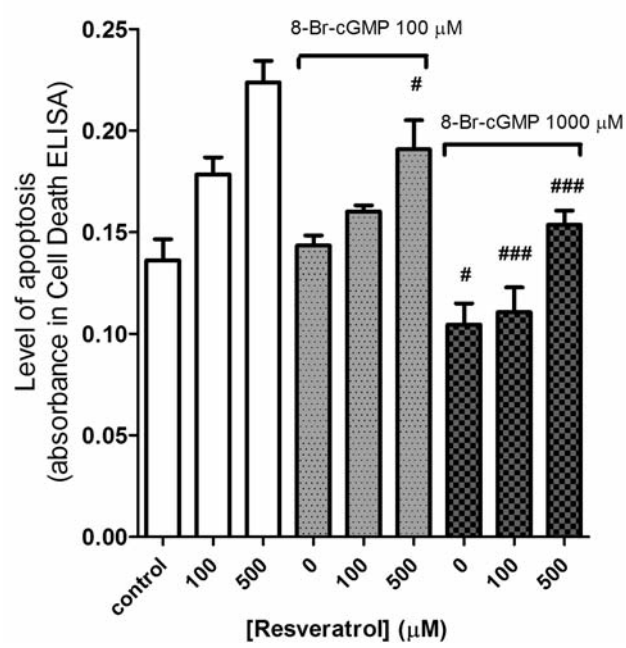

Figure 4. *Protection of HUVECs from resveratrol-induced apoptosis by 8-Br-cGMP. Co-incubation of 8-Br-cGMP (100 and 1000 μM) with resveratrol at the higher concentrations (100 μM and 500 μM) for 24 h significantly suppressed the induction of apoptosis. 8-Br-cGMP, a direct activator of PKG, at 100 μM inhibited apoptosis ($^{\#}p<0.05$) caused by 500 μM of resveratrol. 8-Br-cGMP at 1000 μM inhibited both basal/spontaneous apoptosis ($^{\#}p<0.05$) and resveratrol-induced apoptosis (100 μM and 500 μM, $^{\#\#\#}p<0.001$), compared to resveratrol alone (i.e. no 8-Br-cGMP). The data represent the mean±SEM of four observations per treatment group.*

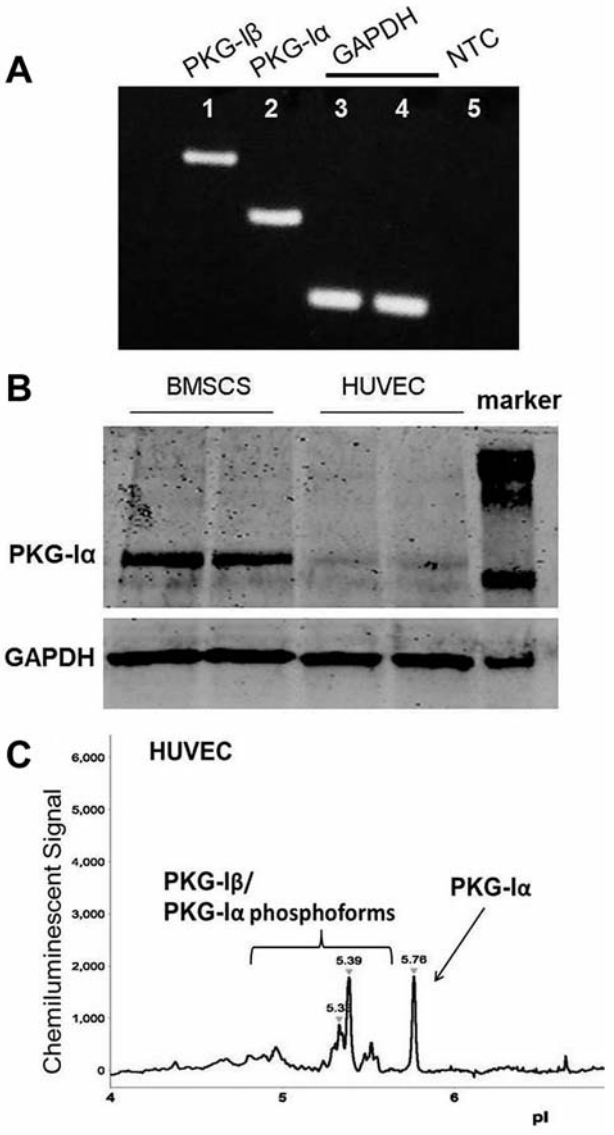

Figure 5. *PKG-I expression in HUVECs. A: RT-PCR result showing both PKG-Iα (lane 2) and PKG-Iβ (lane 1) mRNA expression in HUVECs. GAPDH (lanes 3 and 4) was used as the house-keeping control. NTC (lane 5), no template control. B: Western blot analysis (30 μg of total protein) showing PKG-Iα expression in OP9 bone marrow stromal cells (BMSCs) and HUVECs. C: NanoPro 100 electropherogram (30 ng of total protein) showing protein expressions of PKG-Iα, PKG-Iα phosphoforms and possibly PKG-Iβ.*

Biphasic effects of resveratrol on proliferation and apoptosis of HUVECs and potential involvement of endogenous NO. We further determined whether the same concentrations of resveratrol had any effect on the proliferation and apoptosis of HUVECs. Figure 2 shows that resveratrol, at various concentrations, has biphasic effects on proliferation of HUVECs, determined by both the MTT assay (A) and BrdU incorporation for *de novo* DNA synthesis (B). Resveratrol significantly stimulated proliferation at a low concentration (10 μM), but inhibited proliferation at higher concentrations (100 and 500 μM). Interestingly, resveratrol also had biphasic effects on the apoptosis of HUVECs. Figure 3 shows that resveratrol significantly protected HUVECs from apoptosis at lower concentrations (10, 20 and 50 μM) and caused increased levels of apoptosis at the highest concentration (500 μM).

Based on data from Förstermann's laboratory, showing that resveratrol stimulates the production of NO and upregulates eNOS activity (22, 26) and on our own previous data showing that endogenous NO, at lower/physiological levels (*i.e.* picomolar to very-low nanomolar levels), causes pro-survival/anti-apoptotic effects in many types of mammalian cells (19, 25), we hypothesize that anti-apoptotic effect of lower-concentration resveratrol involves low-level NO production and ability of NO at these low levels to activate soluble-guanlylyl-cyclase (sGC) and increase cGMP production. To test this hypothesis, we treated HUVECs with 10 μM of ODQ, a sGC

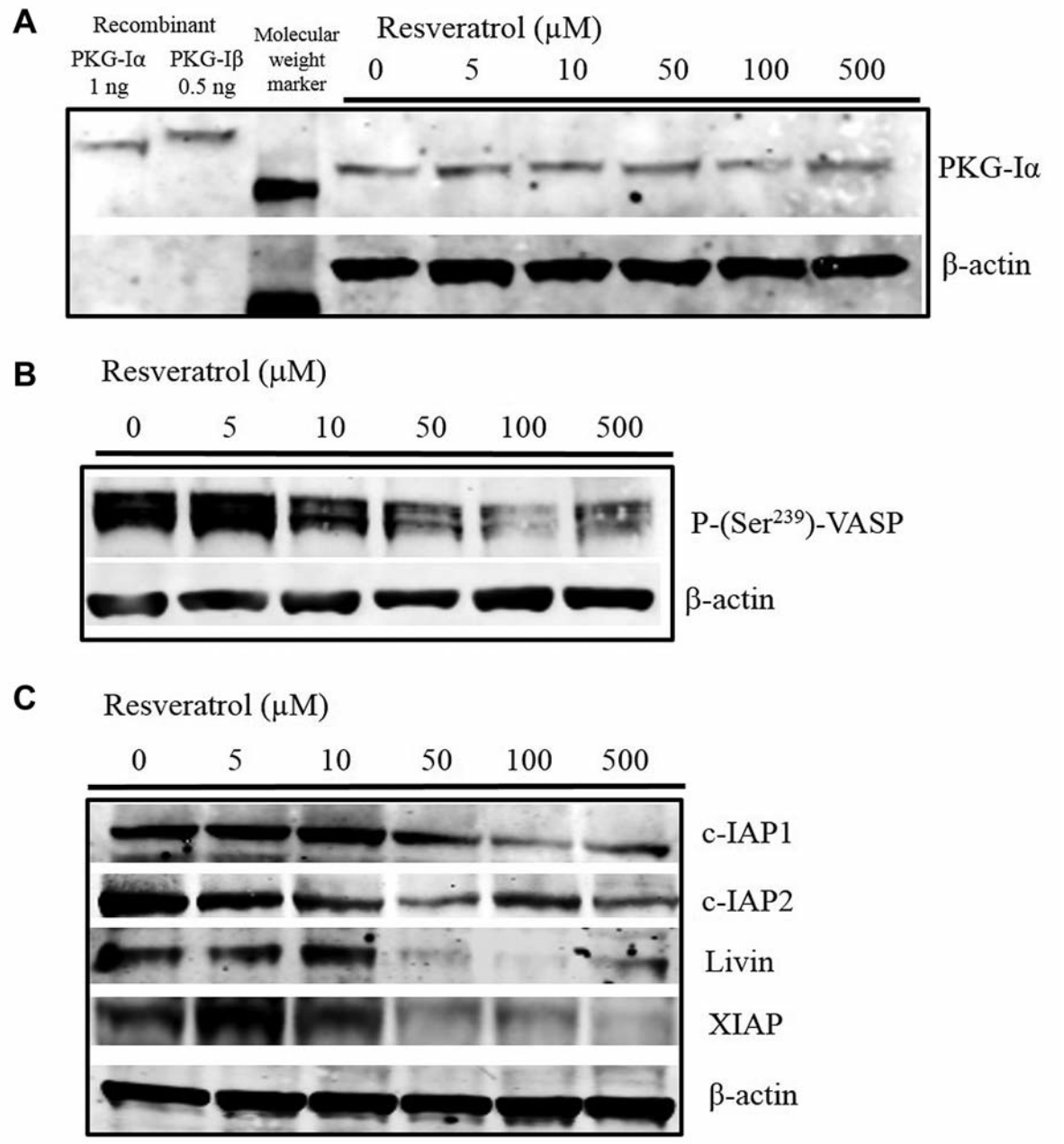

Figure 6. *A: Resveratrol did not alter the expression of PKG-I in HUVECs. Cells were exposed to resveratrol at the indicated concentrations for 24 h, with 50 μg of total cellular protein added in each well. Recombinant PKG-Iα (1 ng) and PKG-Iβ (0.5 ng) were used as positive controls. B: Western blots showing that resveratrol at the higher concentrations (50, 100 and 500 μM) dramatically suppressed VASP phosphorylation at Ser239, a measure of endogenous PKG-I kinase activity in HUVECs. C: Western blots showing that resveratrol at the higher concentrations (50, 100 and 500 μM) suppressed the protein expression of four Inhibitor of Apoptosis Proteins (IAPs), c-IAP1, c-IAP2, livin and XIAP, in HUVECs.*

blocker, used previously in our laboratory to effectively block accumulation of cGMP in several different mammalian cells (19, 24). Also, others have shown that ODQ at 10 μM is sufficient to block synthesis/accumulation of cGMP in cultured endothelial cells (2).

As seen in Figure 3B, ODQ, completely abolished the anti-apoptotic effects of lower-concentration resveratrol by inhibiting endogenous NO-induced activation of sGC and cGMP biosynthesis. These data suggest that anti-apoptotic effects of low-concentration resveratrol are mediated, at least

in part, by stimulation of endogenous NO production and elevation of cGMP levels, which would lead to enhanced kinase activity of PKG-Iα, a kinase involved in cytoprotection against apoptosis in other mammalian cells (24, 25).

Stimulation of endogenous PKG-I kinase activity with 8-bromo-cGMP protects against pro-apoptotic effects of higher-concentration resveratrol in HUVECs. To test whether endogenous PKG-I had a cytoprotective role in HUVECs exposed to higher/pro-apoptotic concentrations of resveratrol, we treated cells with 8-Br-cGMP (a cGMP analog that directly stimulates PKG-I activity) and pro-apoptotic concentrations of resveratrol (100 and 500 μM). Figure 4 shows that 8-Br-cGMP (100 and 1,000 μM) protected against pro-apoptotic effects of 100 and 500 μM resveratrol in HUVECs. In addition, when 8-Br-cGMP (1,000 μM) was used in the absence of resveratrol, spontaneous apoptosis of HUVECs was significantly lower. These data show that stimulation of endogenous PKG-I kinase activity has cytoprotective/anti-apoptotic effects in human endothelial cells, as shown in several other types of mammalian cells (9-12, 19, 24, 25).

Expression of PKG-I isoforms in HUVECs. It has been shown that the PKG-I protein is expressed in human endothelial cells of different origin, such as the human umbilical vein, aorta, artery and foreskin microvasculature (6, 15). However, the PKG-I antibodies that are used often recognize both PKG-Iα and PKG-Iβ isoforms, and none of the previous reports have clearly identified which (or both) of the PKG-I isoforms are expressed in HUVECs. Therefore, one of the aims of the current study was to determine whether both mRNA and protein expression levels of PKG-Iα and PKG-Iβ isoforms are expressed in HUVECs.

In addition to RT-PCR and western blot analysis, we used a novel ultrasensitive capillary electrophoresis (CE)-based "nano-proteomics" system, NanoPro 100 (ProteinSimple, Santa Clara, CA, USA). Our laboratory has recently developed new applications for NanoPro 100 to provide exquisitely-high sensitivity and phospho-protein resolving power, resulting in quantification of proteins with a sensitivity >100-times better than conventional western blot analysis (10, 11). This allows for identification of protein expression levels of lower abundance proteins that would be missed when using the less sensitive western blotting.

Figure 5 shows the results from RT-PCR (A), conventional western blot (B) and Nanopro 100 system (C), illustrating the expression of PKG-I isoforms. In Figure 5A, HUVECs express mRNA for both PKG-Iα (lane 2) and PKG-Iβ (lane 1). Interestingly, in Figure 5B, the western blot analyses show that HUVECs seemingly expressed only the PKG-Iα protein, with no evidence of a second band that would represent the presence of the PKG-Iβ isoform. Cell lysates of bone marrow-derived mesenchymal stem cells (OP9 cells) were used as a positive control for the expression of PKG-Iα, as previously reported by our laboratory (25). Figure 5C shows a NanoPro 100 electropherogram, illustrating that HUVECs not only expressed PKG-Iα, but also PKG-Iα phosphoforms and PKG-Iβ.

Effects of resveratrol on the protein expression of PKG-I, VASP phosphorylation at Ser239 and the downstream Inhibitor of Apoptosis Proteins (IAPs). We hypothesized that anti-angiogenic/pro-apoptotic effects of resveratrol at the higher concentrations are mediated, at least in part, through altering PKG-I expression levels and/or PKG-I kinase activity. Figure 6A shows that resveratrol did not alter PKG-I protein expression in HUVECs. Figure 6B also shows that resveratrol decreased VASP phosphorylation at Ser239, a biomarker commonly used as an indicator of endogenous PKG-I kinase activity. Although PKG-I expression levels appear unaltered, kinase activity of PKG-I (pVASP-Ser239) was dramatically inhibited by resveratrol at the anti-angiogenic/pro-apoptotic concentrations. Furthermore, in Figure 6C, resveratrol, at the same concentrations, dramatically decreased protein expression levels of four IAPs, c-IAP1, c-IAP2, livin and XIAP. These data suggest a possible downstream mechanism of resveratrol-induced apoptosis, *i.e*. resveratrol (>50 μM) may cause a decreased kinase activity of endogenous PKG-I and subsequent decreased expression of certain IAPs, the cell-survival proteins c-IAP1, c-IAP2, livin and XIAP, in HUVECs.

Discussion

HUVECs are a readily-available source of primary human vascular endothelial cells, isolated from normal human umbilical veins (16, 20). They have been well-characterized and, if grown under proper culturing conditions, can maintain many of the properties of human primary endothelial cells (14). Thus, HUVECs are commonly used for studying many of the endothelial processes *in vitro*, such as angiogenesis.

Our results show that resveratrol, at concentrations of 50, 100 and 500 μM, has anti-angiogenic effects, inhibiting tube formation and cell migration in HUVECs. These observations are consistent with a recent report using a resveratrol analog in VEGF-stimulated HUVECs and chick chorioallantoic membranes (CAMs) (4). Also, resveratrol has biphasic effects on angiogenesis in HUVECs, promoting angiogenesis and cell survival at lower concentrations (<50 μM) and inhibiting angiogenesis and causing cell death at higher concentrations (>50 μM). These observations are consistent with a previous report also using HUVECs (23). By comparison, vascular smooth muscle cells do not show a biphasic proliferative response

to resveratrol, showing only inhibition of cell proliferation at all concentrations. The authors report that resveratrol-induced anti-proliferative effects through estrogen receptor (ER)-dependent iNOS activation and production of high/toxic levels of NO (7). NO, at these high levels, is known to have cytotoxic effects, partly *via* its ability to combine with superoxide to form peroxynitrite, a toxic pro-oxidant (3, 9-12). However, from the data of the present study, it appears that cytotoxic/anti-proliferation effects of higher concentrations of resveratrol are independent of high-level NO and downstream activation of sGC, because the sGC inhibitor ODQ did not block resveratrol-induced apoptosis (Figure 3B). Instead, the cytotoxic/anti-proliferation effects of resveratrol appeared to be mediated through inhibition of the cytoprotective PKG-I kinase activity, as shown in Figure 6B.

Data from the present study show that higher/anti-angiogenic concentrations of resveratrol (50, 100 and 500 μM) cause both suppression of PKG-I kinase activity, as well as decreased expression of four IAPs (c-IAP1, c-IAP2, livin and XIAP) in HUVECs (Figure 6C). Our recent publication has shown that endogenous PKG-Iα kinase activity is necessary for maintaining higher levels of expression of certain IAPs, such as cIAP1 and livin (24), thus the suppression of PKG activity by resveratrol in human endothelial cells, shown in the present study, may be responsible for the decrease in c-IAP1 and livin expression.

A recent publication reported that resveratrol (100 μM) causes decreased expression of XIAP in another type of endothelial cells, bovine aortic endothelial cells (18), which is consistent with our observation that resveratrol at 50, 100 and 500 μM causes decreased expression of XIAP in HUVECs. Also, another recent publication has shown that piceatannol, a natural metabolite of resveratrol, enhances cisplatin sensitivity in ovarian cancer cells through an increase in XIAP degradation (resulting in decreased levels of XIAP) *via* a p53-dependent pathway (8).

Conclusion

Our data showed that the NO/cGMP/PKG-I signaling pathway plays an important role in protecting against spontaneous apoptosis in human endothelial cells and that the higher/anti-angiogenic concentrations of resveratrol cause suppression of this cytoprotective pathway. Down-regulation of PKG-I kinase activity and the downstream decreases in the IAPs (*i.e.* decreased protein expression levels of c-IAP1, c-IAP2, livin and XIAP) in HUVECs may be an important mechanism for the induction of apoptosis and inhibition of endothelial cell proliferation and tube formation (*i.e.* inhibition of angiogenesis) induced by resveratrol, which may be related to the overall anticancer effects of resveratrol.

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