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ORIGINAL RESEARCH

Effects of resveratrol on proliferation and apoptosis in rat ovarian theca-interstitial cells

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ABSTRACT: Polycystic ovary syndrome (PCOS) is characterized by ovarian dysfunction and associated with ovarian theca-interstitial (T-I) cell hyperplasia, hyperinsulinemia, systemic inflammation and oxidative stress. This *in vitro* study tested whether rat T-I cell growth with or without insulin can be altered by resveratrol, a natural polyphenol with anti-carcinogenic, anti-inflammatory, anti-proliferative and antioxidant properties. Rat T-I cells were cultured with and without resveratrol and/or insulin, and the effects on DNA synthesis, number of viable cells and markers of apoptosis were evaluated. Resveratrol alone induced a potent 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; these effects of resveratrol counteracted the pro-proliferative and anti-apoptotic effects of insulin. Immunofluorescence analysis of cells incubated with resveratrol showed concentration- and time-dependent morphological changes consistent with apoptosis. The present findings indicate that resveratrol promotes apoptosis to reduce rat T-I cell growth *in vitro* as well as inhibiting insulin-induced rat T-I cell growth. This suggests a possibility that resveratrol and/or mechanisms mediating its effect may be relevant to the development of novel treatments for PCOS, which is characterized by both excessive ovarian mesenchyma growth and hyperinsulinemia.

Key words: apoptosis / insulin / ovarian theca-interstitial cells / proliferation / resveratrol

Introduction

Theca-interstitial (T-I) cells play a crucial role in the regulation of ovarian function. Under pathological conditions such as polycystic ovary syndrome (PCOS), the ovaries are typically enlarged, with the hyperplasia of T-I cells associated with ovarian dysfunction (Hughesdon, 1982). Furthermore, excessive growth of the T-I compartment may be induced by stimuli such as oxidative stress and insulin. This concept is supported by our previous *in vitro* studies, whereby insulin and moderate oxidative stress stimulated the proliferation of T-I cells (Duleba *et al*., 1997a, b, 2004). In addition, insulin also protected T-I cells from apoptosis (Spaczynski *et al*., 2005). In contrast, antioxidants inhibited the growth of T-I cells (Duleba *et al*., 2004). More recently, statins, which are inhibitors of 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase, were found to reduce T-I cell growth by inhibiting the mitogen-activated protein kinase (MAPK) pathway (Izquierdo *et al*., 2004; Kwintkiewicz *et al*., 2006a, b; Rzepczynska *et al*., 2009). In clinical trials, statins have been shown to improve a broad range of clinical, endocrine and metabolic aspects of PCOS (Banaszewska *et al*., 2007; Sathyapalan *et al*., 2009).

The study of natural compounds with pharmacological activity, such as polyphenols, has become an emerging trend in nutritional and pharmacologic research. Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a natural polyphenol synthesized by plants as a phytoalexin that protects against ultraviolet radiation and fungal infection. It is found in high concentrations in grapes, berries, nuts and red wine with potentially beneficial anti-carcinogenic, anti-inflammatory, antioxidant and cardio-protective properties (Jiang *et al*., 2005). Resveratrol has also been shown to exhibit profound *in vitro* and *in vivo* growth-inhibitory and apoptosis-inducing activities in several cancer cell lines and animal models of carcinogenesis (Joe *et al*., 2002; Jiang *et al*., 2005). These properties of resveratrol have been linked to the inhibition of proliferation in association with cell cycle arrest and apoptotic cell death typically observed *in vitro* at concentrations in the range of 25–400 μM (Joe *et al*., 2002; Sun *et al*., 2006; van Ginkel *et al*., 2007). In addition, resveratrol also exerts its effects by interacting with multiple cellular targets and modulating various signal transduction pathways, including the inhibition of the MAPK pathway (Athar *et al*., 2009). In a recent study, dietary resveratrol supplementation has been shown to reduce hepatic HMG-CoA reductase mRNA expression in hamsters fed a high-fat diet (Cho *et al*., 2008). Hence, resveratrol may share with statins the ability to inhibit the mevalonate pathway.

In view of the parallels between the actions of statins and resveratrol, we proposed to study the effects of resveratrol on T-I cells. In this report, for the first time, we demonstrate that resveratrol inhibits proliferation, induces apoptosis and alters morphology of T-I cells.

Materials and Methods

Animals

Immature (25-day-old) female Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in an air-conditioned environment with a 12 h light: 12 h dark cycle. All animals received standard rat chow and water *ad libitum*. In order to stimulate folliculogenesis, the rats received daily injections of 17β-estradiol (1 mg/0.3 ml of sesame oil s.c.) for 3 days (from 28 to 30 days of age). At 31 days of age, the rats were anesthetized using ketamine and xylazine (i.p.) and sacrificed through intracardiac perfusion using 0.9% saline. All treatments and procedures were carried out in accordance with accepted standards of humane animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Cell culture and treatments

The ovaries were dissected and T-I cells purified through discontinuous Percoll gradient centrifugation as previously described (Magoffin and Erickson, 1988; Duleba *et al.*, 1997a, b). The purity of this cell preparation has been verified using immunocytochemical assays (Duleba *et al.*, 1997a, b). The cells were counted, with viability routinely in the 85–95% range. The purified T-I cells were cultured for up to 48 h at 37°C in an atmosphere of 5% CO_2 in humidified air, in serum-free McCoy's 5A medium (supplemented with antibiotics, 0.1% BSA and 2 mM L-glutamine). The cells were incubated without (control) or with resveratrol (30–100 μM) and/or insulin (30 nM). Since resveratrol is not soluble in purely aqueous solutions, it was initially dissolved in ethanol and subsequently diluted in culture media; the final concentration of ethanol in cultures was 0.1%. Cultures not exposed to resveratrol received an identical concentration of ethanol. All the above chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell proliferation assays

The purified T-I cells were incubated for 48 h in 96-well culture plates with or without individual additives. The extent of DNA synthesis was determined through a thymidine incorporation assay. Radiolabeled [^{3}H] thymidine (1 μCi/well) was added to the cells during the last 24 h of culture. At the end of the culture period, the cells were harvested with a multiwell cell harvester (PHD Harvester, Model 290; Cambridge Technology, Inc., Watertown, MA, USA). Radioactivity was measured in a liquid scintillation counter, Wallac 1409 (PerkinElmer, Shelton, CT, USA). Each treatment was carried out in at least eight replicates.

The total number of viable cells was estimated with the use of a CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA). This assay involves the conversion of resazurin to resorufin by metabolically active cells, resulting in the generation of a fluorescent product at the excitation wavelength 544 nm and the emission wavelength 590 nm that is proportional to the number of viable cells. Fluorescence was determined with the use of a microplate reader (Fluostar Omega, BMG, Durham, NC, USA). To validate the assay, a standard curve with a known number of cells was generated and a linear correlation was verified ($r^2 = 0.99$; $P < 0.001$).

Caspase-3/7 activity assay

The measurement of apoptosis in T-I cells was made using the Apo-ONE® Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI, USA), following the manufacturer's instructions. The cells were incubated overnight in 96-well culture plates, replaced with fresh media and then treated with various concentrations of resveratrol (30–100 μM) at different time points (3, 6, 12, 24 and 48 h) in the absence or presence of insulin (30 nM). Caspase-3/7 activity was measured in a microplate reader (Fluostar Omega, BMG, Durham, NC, USA) at excitation wavelength 485 nm and emission wavelength 520 nm.

TUNEL assay

The detection of DNA fragmentation in T-I cells was determined using the HT TiterTACS™ Assay kit (Trevigen, Gaithersburg, MD, USA), following the manufacturer's instructions. The cells were incubated overnight in 96-well culture plates, replaced with fresh media, and then treated with various concentrations of resveratrol (30–100 μM) for 48 h. Briefly, the cells were fixed with 3.7% buffered formaldehyde solution for 7 min, washed with PBS, permeabilized with 100% methanol for 20 min, washed twice with PBS, digested with proteinase K for 15 min, quenched with 3% hydrogen peroxide, washed with distilled water, labeled with the TdT reaction mix and incubated at 37°C for 1 h in a humidified chamber and the reaction terminated with stop buffer. Then the cells were incubated with Strep-HRP for 10 min, washed four times with PBS, followed by the addition of TACS-Sapphire™ substrate and the colorimetric reaction was stopped with 0.2 N HCl after 30 min. Negative controls were labeled without the TdT enzyme and positive controls were generated with TACS-Nuclease™ to create DNA breaks. The colorimetric reaction was measured in a microplate reader (Fluostar Omega, BMG, Durham, NC, USA) at absorbance 450 nm.

DAPI nuclear and F-actin staining

The T-I cells were treated with resveratrol (50 and 100 μM) at various time points (6, 12, 24 and 48 h) and then subjected to DAPI and F-actin staining to observe nuclear and cellular morphological changes. Approximately 16 000 T-I cells per well were seeded in duplicate in 8-well culture slides (BD Biosciences, Bedford, MA, USA). Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 30 min, washed three times with PBS, blocked with 1% BSA in PBS for 30 min, washed twice with PBS and stained with Texas red-phalloidin and DAPI (Molecular Probes, Carlsbad, CA, USA). Slides were then examined under an Olympus BX61 fluorescent microscope at 40× magnification (Olympus America, Melville, NY, USA). Apoptotic cells were morphologically defined by nuclear shrinkage and chromatin condensation or fragmentation, whereas cellular morphological features were defined by disrupted or disorganized F-actin filaments.

Statistical analysis

Statistical analysis was performed with JMP 7.0 software (SAS, Cary, NC, USA), using analysis of variance followed by *post hoc* pairwise comparisons using Bonferroni's correction. Baseline data are expressed as means (± SEM). Normality of distribution was assessed by the Shapiro–Wilk test. When appropriate, data were logarithmically transformed. A value of $P < 0.05$ was considered statistically significant.

Results

Resveratrol inhibits T-I DNA synthesis in a dose-dependent fashion

To determine whether resveratrol affects T-I cell proliferation, the extent of DNA synthesis was measured using a radiolabeled thymidine incorporation assay. As shown in Fig. 1, resveratrol significantly inhibited DNA synthesis in a dose-dependent manner compared with the

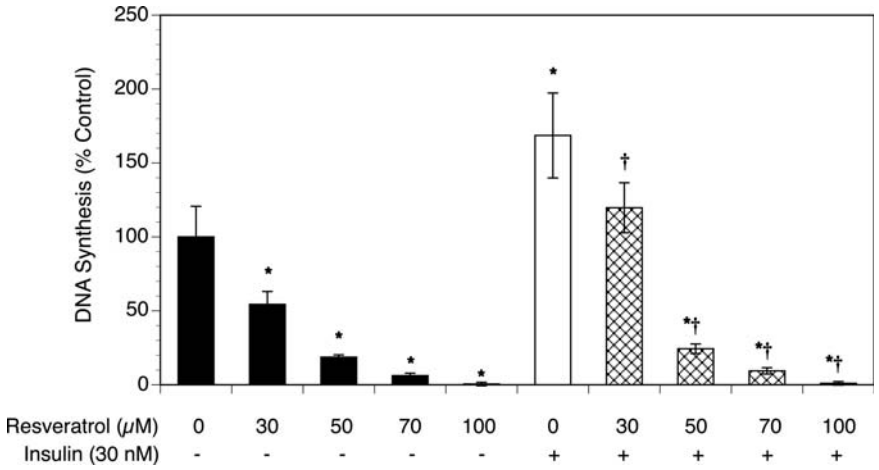

Figure 1 Effect of resveratrol (30–100 μM) on proliferation in the absence and presence of insulin (30 nM).

Ovarian T-1 cells were cultured for 48 h in chemically defined media. Proliferation was evaluated by determination of DNA synthesis (by thymidine incorporation). Each bar represents mean ± SEM ($N = 8$); * denotes means significantly different from control in the absence of insulin ($P < 0.001$); † denotes means significantly different from insulin alone ($P < 0.001$; applies only to comparison among cultures containing insulin).

control, reducing thymidine incorporation by 99% at the highest concentration of resveratrol ($P < 0.001$).

We have previously reported that insulin and moderate oxidative stress stimulated the proliferation of rat T-1 cells (Duleba *et al*., 1997a, b; Spaczynski *et al*., 2005; Kwintkiewicz *et al*., 2006a, b). In other biological systems, resveratrol has also been shown to increase insulin sensitivity and protect against insulin resistance (Baur *et al*., 2006; Lagouge *et al*., 2006). To evaluate whether resveratrol could modulate insulin-induced stimulation of cell proliferation, T-1 cells were treated without (control) or with insulin (30 nM) and/or resveratrol at a concentration between 30 and 100 μM. As shown in Fig. 1, insulin alone stimulated thymidine incorporation by 69% above control levels ($P < 0.01$). In the presence of insulin, resveratrol also induced a dose-dependent inhibition of DNA synthesis, virtually eliminating thymidine incorporation at the highest concentration of resveratrol ($P < 0.001$).

Resveratrol reduces the number of viable T-1 cells

As shown in Fig. 2, resveratrol treatment resulted in a dose-dependent decrease in the number of cells, with a 35% reduction in the viable cell number at the highest concentration of resveratrol ($P < 0.01$). Similarly, resveratrol induced a dose-dependent decrease of the viable cell number in the presence of insulin ($P < 0.05$). Notably, the effects of individual treatments on the total viable cell number are typically less pronounced than the effects on DNA synthesis, especially in short-term cultures. This difference is due to the fact that DNA synthesis is a more sensitive marker of change in the proliferation rate, although the total viable cell number represents the sum of both proliferating and non-proliferating cells.

Resveratrol-induced apoptosis is mediated by caspase activation

Resveratrol has been shown to induce apoptosis in various cell lines (Joe *et al*., 2002; Jiang *et al*., 2005). To determine whether the inhibitory effect of resveratrol on the growth of T-1 cells was, at least in part, due to the induction of apoptosis, caspase-3/7 activity was measured in the absence and presence of resveratrol at concentrations ranging from 30 to 100 μM and at various time points (3, 6, 12, 24 and 48 h). Figure 3 indicates that resveratrol induced a concentration-dependent increase in caspase-3/7 activation, as observed after 3 and 6 h of resveratrol treatment ($P < 0.005$). This caspase activation was no longer evident after 12, 24 or 48 h of exposure to resveratrol.

Our previous *in vitro* study revealed that insulin protected T-1 cells from apoptosis (Spaczynski *et al*., 2005). The ability of insulin to modulate resveratrol-induced apoptosis was also studied. Figure 4 demonstrates that insulin alone at all of the tested time points (3, 6, 12, 24 and 48 h) protected T-1 cells from caspase-3/7 activation ($P < 0.01$). However, resveratrol effectively counteracted the actions of insulin, as shown by a concentration-dependent increase in caspase-3/7 activity ($P < 0.01$).

Resveratrol induces DNA fragmentation

To further verify that resveratrol indeed induces apoptosis in T-1 cells, an additional method of apoptosis identification, the TUNEL assay, was used to detect DNA fragmentation. As shown in Fig. 5, resveratrol induced a dose-dependent increase in DNA fragmentation ($P < 0.001$).

Resveratrol induces morphological changes in T-1 cells

The effects of resveratrol on the nuclear morphology and cytoskeleton of T-1 cells were observed under a fluorescent microscope following DAPI staining of the nucleus and F-actin staining with Texas red-phalloidin. The morphological features of apoptosis include nuclear shrinkage with condensed chromatin and cytoskeleton degradation/fragmentation. As shown in Fig. 6A, untreated control cells had large oval-shaped nuclei, although cells exposed to resveratrol had small spherical nuclei with condensed chromatin. These effects were

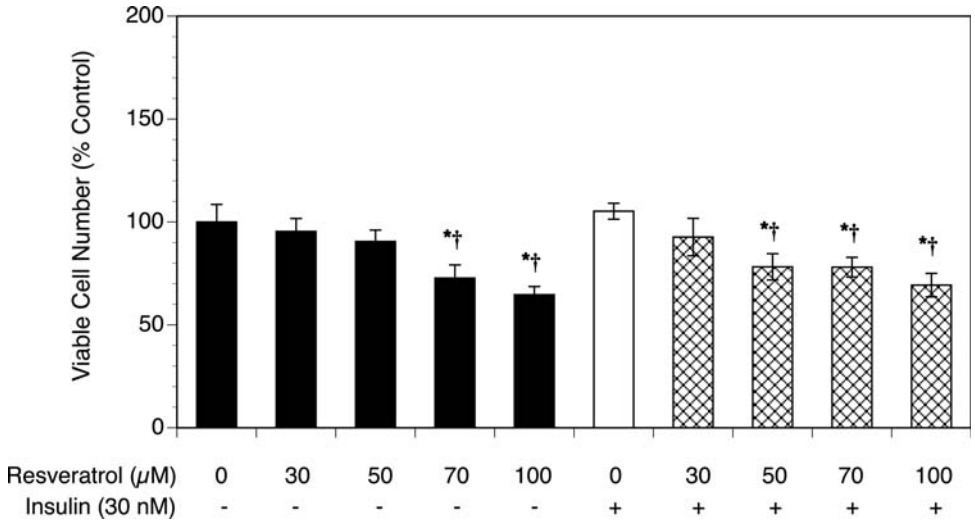

Figure 2 Effect of resveratrol (30–100 μM) on cell viability in the absence or presence of insulin (30 nM).

Ovarian T-I cells were cultured as described in Fig. 1. Proliferation was evaluated by estimation of the number of viable cells using MTS assay. Each bar represents mean ± SEM ($N = 8$); * denotes means significantly different from control in the absence of insulin ($P < 0.05$); † denotes means significantly different from insulin alone ($P < 0.05$; applies only to comparison among cultures containing insulin).

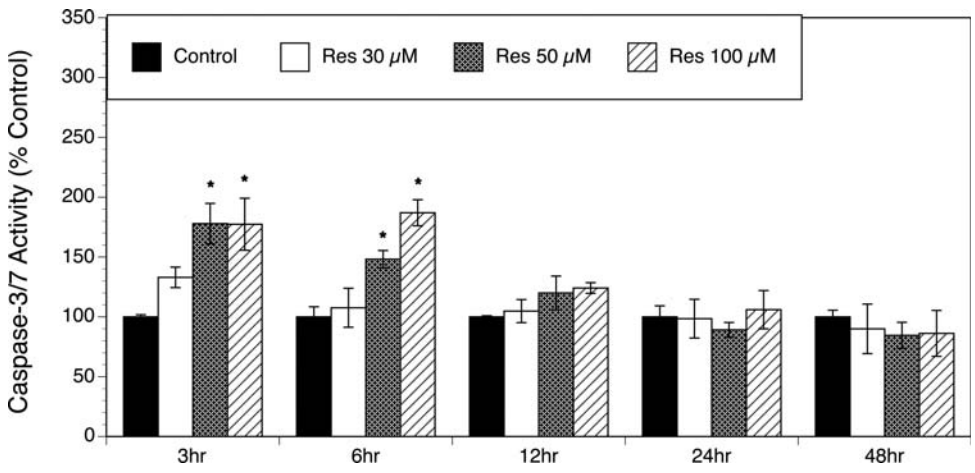

Figure 3 Effect of resveratrol (30–100 μM) on caspase-3/7 activation.

Ovarian T-I cells were exposed to resveratrol (Res) for 3, 6, 12, 24 and 48 h in chemically defined media. Caspase-3/7 activity was determined by the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA). Each bar represents mean ± SEM ($N = 4$); * denotes means significantly different from control ($P < 0.005$).

progressive, with the most prominent changes observed after 48 h of exposure to resveratrol. As can be seen in Fig. 6B, resveratrol treatment also resulted in progressive time- and concentration-dependent degradation of the F-actin cytoskeleton, compared with the untreated control cells, which displayed linear strands of actin filaments that traversed the entire length of the cells.

Discussion

The present study demonstrates that resveratrol inhibits the cell proliferation and promotes the apoptosis of ovarian T-I cells by: (i) limiting DNA synthesis and cell viability, (ii) increasing caspase-3/7 activity and DNA fragmentation and (iii) inducing nuclear and cytoskeletal morphological changes consistent with apoptosis. To our knowledge, this study is the first report evaluating the effects of resveratrol on ovarian T-I cells.

Over the past decade, resveratrol has emerged as a very promising natural compound with immense therapeutic potential. In several experimental models of carcinogenesis, resveratrol inhibits cancer initiation, promotion and progression (Jang *et al.*, 1997). However, to date, the studies evaluating the effects of resveratrol on ovarian function are limited, with data indicating that resveratrol exerts

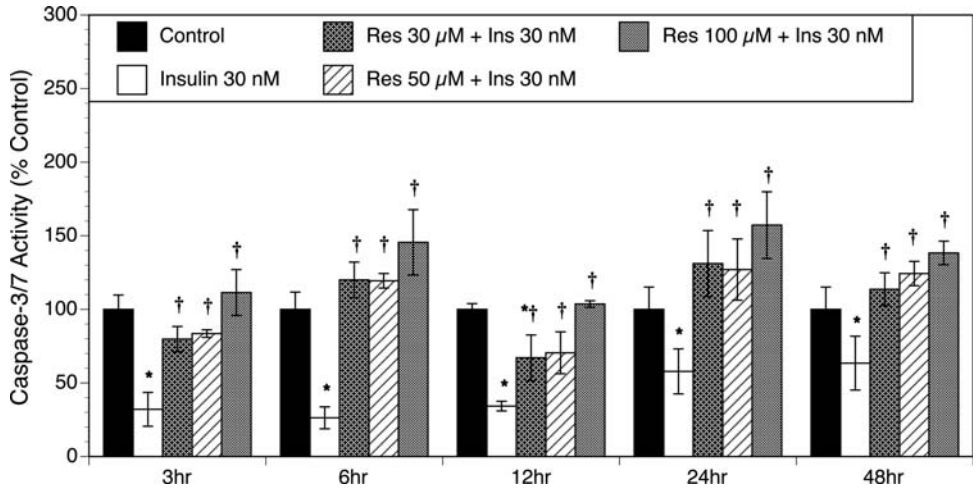

Figure 4 Effect of resveratrol (30–100 μM) on caspase-3/7 activity in the presence of insulin (30 nM).

Ovarian T-I cells were stimulated with 30 nM insulin (Ins) for 2 h and then incubated with different concentrations of resveratrol (Res) for 3, 6, 12, 24 and 48 h in chemically defined media. Caspase-3/7 activity was measured as indicated in Fig. 4. Each bar represents mean ± SEM ($N = 4$); * denotes means significantly different from control ($P < 0.01$); † denotes means significantly different from insulin alone ($P < 0.01$; applies only to comparison among cultures containing insulin).

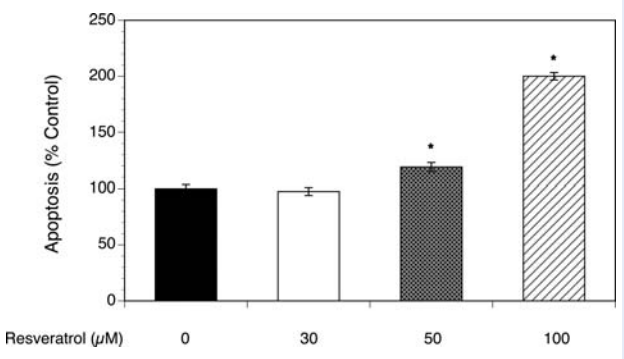

Figure 5 Effect of resveratrol (30–100 μM) on DNA fragmentation.

The detection of apoptosis through DNA fragmentation was determined by the terminal dUTP nick-end labeling (TUNEL) assay. Ovarian T-I cells were exposed to resveratrol (Res) for 48 h in chemically defined media, fixed and labeled according to the HT TiterTACS™ (Trevigen, Gaithersburg, MD, USA) protocol prior to colorimetric analysis. Each bar represents mean ± SEM ($N = 8$); * denotes means significantly different from control ($P < 0.001$).

predominantly estrogenic effects (Henry and Witt, 2002, 2006). Recent studies have indicated that resveratrol counteracts oxidative stress and exerts hepatoprotective effects against acute and chronic liver damage in rodents (Kasdallah-Grissa *et al*., 2007, Ajmo *et al*., 2008). Resveratrol effectively scavenges hydroxyls and superoxides and protects against lipid peroxidation in cell membranes and DNA damage caused by reactive oxygen species (Leonard *et al*., 2003). In addition, resveratrol has been demonstrated to increase intracellular antioxidant levels and phase II enzymes in cultured cardiomyocytes and aortic smooth muscle cells (Cao and Li, 2004; Li *et al*., 2006). Our previous studies have shown that moderate oxidative stress promotes the proliferation of T-I cells while antioxidants have the opposite effects (Duleba *et al*., 2004; Kwintkiewicz *et al*., 2006a, b). Hence, it is tempting to speculate that the effects of resveratrol observed in the present study may be, at least in part, due to its antioxidant properties.

More recently, reports on the potential for resveratrol to extend lifespan in cell culture and animal models have continued to generate scientific interest. It has been reported that resveratrol mimics caloric restriction and interferes with the aging process by activating sirtuins (Howitz *et al*., 2003). Sirtuins are a conserved family of NAD^+-dependent histone deacetylases involved in gene silencing processes related to aging and the promotion of cell survival. Indeed, resveratrol administration (and activation of SIRT1) has increased the lifespans of yeast (Howitz *et al*., 2003), fruit flies and worms (Wood *et al*., 2004), fish (Valenzano *et al*., 2006), and mice that are fed a high-calorie diet (Baur *et al*., 2006). However, not all actions of resveratrol are related to the activation of SIRT1. For example, resveratrol protects HepG2 cells from oxidative stress-induced apoptosis by mediating the downstream activation of AMP-activated protein kinase and poly(ADP-ribose) polymerase (Shin *et al*., 2009). In another study, resveratrol inhibited platelet-derived growth factor-stimulated proliferation of mesangial cells by inhibiting Akt and Erk1/2 independently of SIRT1 (Venkatesan *et al*., 2008). In several cell lines and primary rat hepatocytes, resveratrol also inhibited insulin-signaling pathways in a SIRT1-independent pathway (Zhang, 2006).

Furthermore, resveratrol has also gained prominence as a potent chemopreventive agent that interferes with signaling pathways regulating cell death and survival. Resveratrol has been reported to inhibit the proliferation of numerous human cancer cell lines, including MCF-7, HL60, SW480 and prostate LNCaP (Joe *et al*., 2002; Benitez *et al*., 2007), vascular smooth muscle cells (Mnjoyan and Fujise, 2003) and hepatic stellate cells (Souza *et al*., 2008). Similarly, our results show that resveratrol also dose-dependently inhibits cell proliferation by limiting DNA synthesis in T-I cells. The anti-proliferative activities of

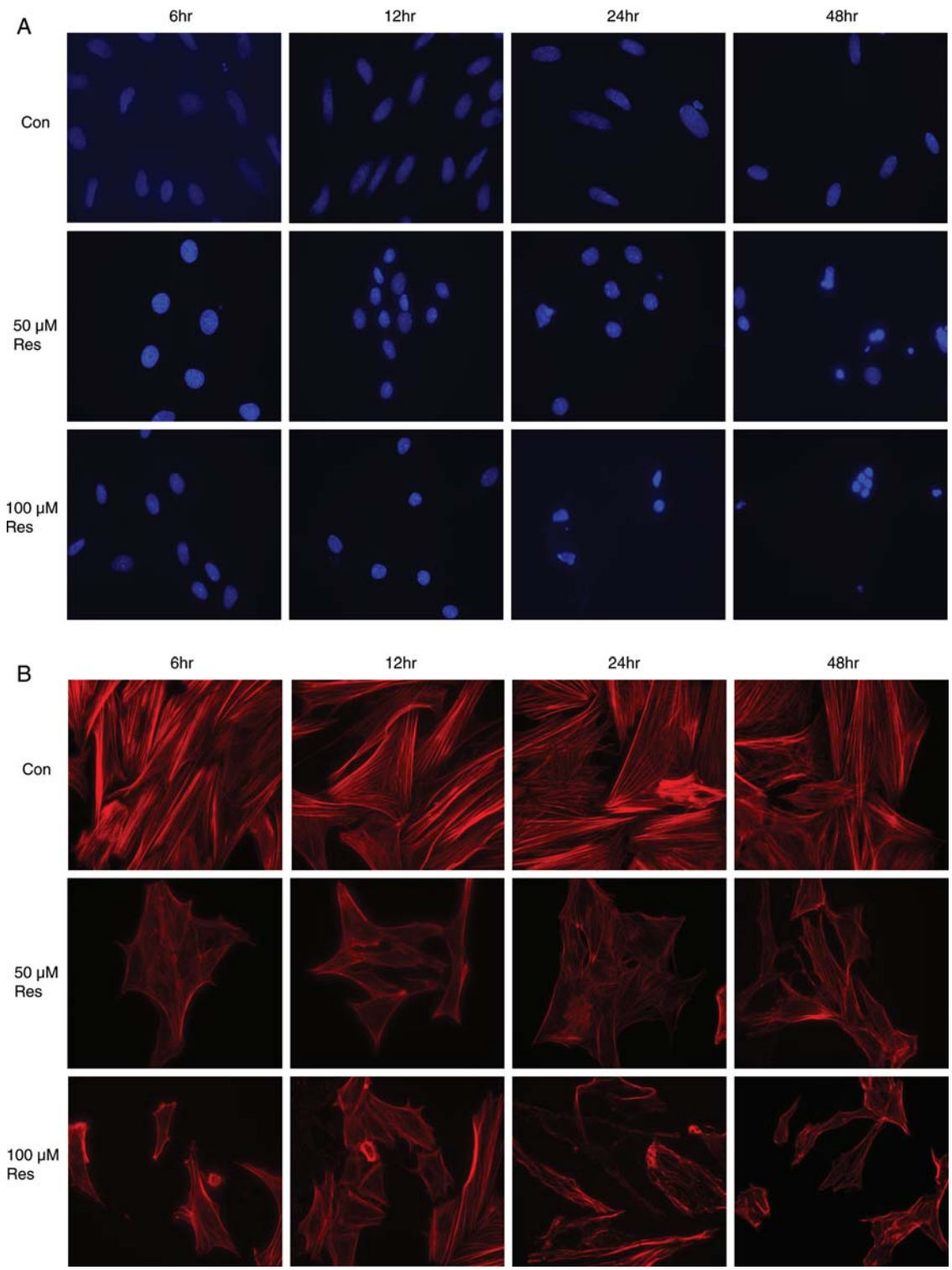

Figure 6 Effect of resveratrol (50 and 100 μM) on morphology.

Ovarian T-I cells were treated without (Con) or with 50 and 100 μM of resveratrol (Res) for 6, 12, 24 and 48 h, then fixed, stained and visualized under a fluorescent microscope (40× magnification) as described in Materials and Methods. Nuclear staining with DAPI (**A**) and F-actin (**B**) were used to observe morphological changes. Apoptotic cells were defined as chromatin condensation and nuclear fragmentation or formation of apoptotic bodies, membrane blebbing and cytoskeleton degradation.

resveratrol may arise from its ability to interfere with nuclear factor-κB (NF-κB), p38 MAPK and phosphatidylinositol 3-kinase/Akt survival pathways (Fulda and Debatin, 2006), resulting in the suppression of DNA synthesis and cell proliferation, the inhibition of cell cycle progression and the induction of apoptosis. It is also important to note that the effects of resveratrol on cellular growth are not universally inhibitory and, in several biological systems, resveratrol has been shown to protect cells from death (Brito *et al*., 2006; Gong *et al*., 2007; Anekonda and Adamus, 2008; Cao *et al*., 2008; Rubiolo *et al*., 2008; Shin *et al*., 2009).

Apoptosis is a key regulator of tissue homeostasis during normal development and disease states. One hallmark of apoptosis is caspase activation. Indeed, numerous studies have documented that resveratrol-mediated apoptosis involves the activation of caspase 3, the executer of apoptotic cell death, in several human cancer cell lines, including U251 glioma, malignant B cells and MDA231 cells (Jiang *et al*., 2005; Shimizu *et al*., 2006; Alkhalaf *et al*., 2008). Our data are in agreement with these observations and indicate that, in ovarian T-I cells, resveratrol induces the activation of executioner caspases 3/7. In addition, the apoptotic effects of resveratrol were further confirmed by increased DNA fragmentation using the TUNEL assay.

We also observed that resveratrol induced progressive dose- and time-dependent morphological changes that are consistent with apoptosis, such as cell and nuclei shrinkage and nuclear fragmentation. Under physiological conditions, the actin cytoskeleton contributes to the maintenance of plasma membrane integrity and cell shape. There is increasing evidence for an association between actin filament disruption and the initiation of apoptosis, as actin may act as an early modulator of apoptotic commitment (White *et al*., 2001; Bando *et al*., 2002). For example, in some cell types, cells with fragmented nuclei also displayed completely disorganized F-actin (Van de Water *et al*., 1996; Atencia *et al*., 2000). In another study, the cytoskeletal disruption in apoptotic cells potentiated mitochondrial membrane permeability, thereby enhancing cytochrome *c* release and the subsequent activation of the caspase cascade (Yamazaki *et al*., 2000). In the same study, it was also found that actin degradation accelerated caspase 3 activation (Yamazaki *et al*., 2000). Our findings on resveratrol-induced F-actin cytoskeletal changes are also in agreement with observations from other biological *in vitro* systems, indicating that resveratrol alters cell morphology (Bruder *et al*., 2001; Azios and Dharmawardhane, 2005; Azios *et al*., 2007).

In the present study, the effects of resveratrol were detected at concentrations ranging from 30 to 100 μM, which were comparable to those used in other studies, whereby resveratrol inhibited the proliferation of various cell types at concentrations in the range of 25–400 μM (Sun *et al*., 2006; Tang *et al*., 2006; Bhardwaj *et al*., 2007; Hwang *et al*., 2007; van Ginkel *et al*., 2007). These *in vitro* observations may be of clinical relevance as the bioavailability of resveratrol in human and in rodent models is in the micromolar range (Walle *et al*., 2004; van Ginkel *et al*., 2007). For example, in a phase I pharmacokinetic study of healthy volunteers, the peak plasma levels of resveratrol were above 2 μM and the peak levels of conjugated metabolites of resveratrol were up to 8-fold higher (Boocock *et al*., 2007). Interestingly, the effects of resveratrol *in vivo* may be detected at lower concentrations than those observed *in vitro*. For example, in the nude mouse model of neuroblastoma, resveratrol remained remarkably effective in inhibiting tumor growth, with serum levels in the 2–10 μM range within 30 min of oral gavage (van Ginkel *et al*., 2007). In the same study, resveratrol induced marked growth inhibition and subsequent loss of cultured human neuroblastoma cell viability, with IC_{50} values in the range of 70–120 μM (van Ginkel *et al*., 2007). Thus, the concentration of resveratrol (in the micromolar range) required to inhibit proliferation and to induce apoptosis in our model agrees with those used in other *in vitro* studies (Joe *et al*., 2002; Sun *et al*., 2006; van Ginkel *et al*., 2007), indicating that resveratrol, at pharmacological concentrations, may be effective in limiting excessive ovarian T-I cell growth.

Our present findings may have potential clinical applications. In PCOS, the typically enlarged ovaries are characterized by thecal and stromal hyperplasia (Hughesdon, 1982). This ovarian enlargement is associated with excessive ovarian androgen production and the disruption of menstrual cyclicity. Improvement of ovarian function, with restoration of ovulation and fertility, was observed with surgical reduction of ovarian size and/or partial destruction of ovarian tissues by procedures such as wedge resection and laparoscopic ovarian drilling (Donesky and Adashi, 1995; Duleba *et al*., 2003). It is tempting to speculate that the reduction in the growth of T-I cells as a result of resveratrol treatment may be of similar benefit. However, in view of the significant differences between the ovarian physiology of human and rodent, the present findings should be interpreted with caution and the present observations should be validated with human T-I cells.

In summary, our results suggest that resveratrol exerts its antiproliferative and pro-apoptotic actions by mediating caspase-3/7 activation and inducing morphologic changes in cultured ovarian T-I cells. These findings are of potential translational relevance to conditions associated with excessive growth of T-I cells, such as PCOS, whereby resveratrol may represent a novel therapeutic agent.

Authors' Roles

D.H.W. planned and ran the experiments, conducted statistical analysis and wrote the manuscript. J.A.V. assisted with experiments and reviewed the manuscript. A.B.C. assisted with experiments and reviewed the manuscript. A.J.D. planned the experiments, supervised and contributed to data interpretation and writing the manuscript.

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