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Resveratrol Inhibits AGEs-Induced Proliferation and Collagen Synthesis Activity in Vascular Smooth Muscle Cells from Stroke-Prone Spontaneously Hypertensive Rats

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Advanced glycation end-products (AGEs) of plasma proteins and/or matrix proteins are candidate mediators for various vascular complications such as atherosclerosis. We previously reported a significantly larger accumulation of AGEs of the aorta in stroke-prone spontaneously hypertensive rats (SHRSP) than in age-matched Wistar-Kyoto rats (WKY). In this study, we examined the effects of AGEs on vascular smooth muscle cells (VSMC) from SHRSP and WKY rats. We also studied the *in vitro* effects of resveratrol (3,4′,5-trihydroxystilbene), a natural phytestrogen, on VSMC proliferation, DNA synthesis, and collagen synthesis activity in SHRSP-VSMC. AGEs accelerated the proliferation of SHRSP- or WKY-VSMC in a time- and dose-dependent manner. VSMC from SHRSP were more sensitive to AGEs than VSMC from normotensive WKY. AGEs also significantly increased DNA synthesis and prolyl hydroxylase activity, a marker for collagen synthesis, in SHRSP-VSMC. AGEs-induced increases in TGF-β1 mRNA in SHRSP-VSMC were significantly greater than in WKY-VSMC. Resveratrol inhibited AGEs-stimulated proliferation, DNA synthesis, and prolyl hydroxylase activity in SHRSP-VSMC in a dose-dependent manner. ICI 182780, a specific estrogen receptor antagonist, partly blocked the inhibitory effects of resveratrol on AGEs-stimulated proliferation, DNA synthesis, and prolyl hydroxylase activity. Resveratrol significantly inhibited AGEs-induced TGF-β1 mRNA increases in a dose-dependent manner. Thus, resveratrol may confer protective effects on the cardiovascular system by attenuating vascular remodeling and may be clinically useful as a safer substitute for feminizing estrogens in preventing cardiovascular disease.

Key Words: **vascular smooth muscle cell; stroke-prone spontaneously hypertensive rats; advanced glycosylation end-products, phytestrogen.**

Vascular changes related to physiological aging and involving collagen and noncollagen components of vascular walls are augmented by hypertension [1, 2]. Molecular and cellular mechanisms of vascular smooth muscle cells (VSMC) hypertrophy, hyperplasia and remodeling in hypertension have been extensively studied [3–5]. Hypertension accelerates the thickening, stiffening, remodeling and sclerosis of arterial walls [2, 3], which thicken and stiffen gradually with advancing age. The thickening of arterial walls is induced even at the early or pre-hypertensive stage in spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP). Under tissue culture condition free from the influence of blood pressure, VSMC from SHR and SHRSP grow faster than VSMC from normotensive Wistar-Kyoto rats (WKY), and labeled thymidine or leucine incorporation is also significantly higher [4, 5], suggesting an unknown growth-accelerating factor other than hypertension in proliferation of VSMC from SHR and SHRSP.

Advanced glycation is a posttranslational modification process that results in the formation of glucose-derived heterocycles with diverse chemical structures [6–8]. This process occurs ubiquitously in extracellular matrix proteins, and advanced glycation end-products (AGEs) accumulate progressively in the vasculature and other tissues during aging [9–11]. Recently it has been suggested that the depletion of cellular antioxidant defense mechanisms and the generation of oxygen free radicals by AGEs may play a major role in the pathogenesis of various vascular complications [12–15]. We have previously reported a significantly larger accumulation of AGEs in the aorta in SHRSP than in age-matched WKY [16].

Some reports have shown that estrogens have various vascular protective effects [17–20]. In particularly, 17β-estradiol has a protective effect against various

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kinds of glycoxidative damage in the artery [20]. More recently, interest has focused on phytestrogens, such as resveratrol (3,4′,5-trihydroxystilbene), natural dietary plant compounds with estrogenic activity [21]. Several biological activities of resveratrol have been reported [22, 23]. These include (1) inhibition of the oxidation of low-density lipoprotein cholesterol [24] an effect which is expected to inhibit atherosclerotic changes, (2) attenuation of platelet aggregation by inhibition of the metabolism of arachidonic acid [25], and (3) inhibition of ovariectomy-induced hypertension in SHRSP [26]. We hypothesized that resveratrol may have a protective effect against AGEs-induced dysfunctions in VSMC. Accordingly, the aims of the present study were to determine whether in SHRSP-VSMC: (1) AGEs-induced proliferation and DNA synthesis are different to WKY-VSMC, (2) resveratrol inhibit AGEs-induced proliferation, DNA synthesis and collagen synthesis activity, and the inhibitory effects of resveratrol are (3) estrogen receptor-mediated, and (4) transforming growth factor-β1 (TGF-β1) gene expression-mediated.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), resveratrol, and [^{3}H]thymidine were obtained from Nissui (Tokyo, Japan), Gibco BRL Life Technologies (NY), Sigma Chemicals Co. (MO) and Amersham (Buckinghamshire, UK), respectively. Trizol reagent and Superscript II were purchased from Gibco BRL Life Technologies. ICI 182780 was a gift from Zeneca Pharmaceuticals (Cheshire, UK). Rat glycated albumin (Exocell Inc., PA) was used as the source of AGEs. Cell proliferation and prolyl hydroxylase activity were assayed using a CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega Co., U.S.A.) and a rat γ-prolyl hydroxylase activity ELISA kit (Fuji Chemicals, Tokyo, Japan), respectively.

Isolation and culture of rat VSMC. Cultured VSMC were obtained by an explant method (27) from the thoracic aortae of 12-week-old male WKY/Izm and age-matched male SHRSP/Izm. The medium was decanted, and the explants, cut into approximately 1 mm^2 sections, were transferred into tissue culture flasks (Falcon, U.S.A.) to which 5 ml of DMEM supplemented with 10% FBS was added carefully to avoid the detachment of explants from the flasks. The flasks were incubated at 37°C in a humidified incubator (95% air/5% CO_2 atmosphere). Cells migrating from these explants by 30 days after inoculation were trypsinized and subcultured by repeated passage. The medium was changed every 4 days. Confluent cultured VSMC (passages 6–8) were used all experiments.

Cell proliferation. VSMC were seeded in DMEM supplemented with 10% FBS at densities of 3×10^4 cells/cm^2 in 96-well tissue culture plates. After 48 h, serum-free medium containing various concentrations of AGEs in the presence or absence of resveratrol and ICI 182780 was added to the cells. At the indicated number of days after the addition of samples, cell numbers were determined using a cell counting kit.

DNA synthesis assay. VSMC were grown to semi-confluence in 96-well tissue culture plates, and serum-free medium containing various concentrations of AGEs in the presence or absence of resveratrol or ICI 182780 was added to the cells. The cells were treated for 24 h with [^{3}H]thymidine (46 kBq/ml). Ice-cold 10% trichloroacetic acid was then added to each well, and the plates were held at 4°C for 15 min. The extent of [^{3}H]thymidine incorporation was determined by scintillation counting.

Prolyl hydroxylase activity assay. VSMC were grown to semiconfluence in 6-well tissue culture plates, and serum-free medium containing various concentrations of AGEs in the presence or absence of resveratrol or ICI 182780 were then added to the cells. Prolyl hydroxylase activity was determined by a one-step sandwich enzyme immuno assay method as described below. Polystyrene beads conjugated with anti-γ-prolyl hydroxylase monoclonal antibody were added to the mixture of peroxidase-conjugated anti-γ-prolyl hydroxylase monoclonal antibody and each supernatant solution of culture medium, in 30 mM phosphate buffer containing 100 mM sodium chloride and 1% BSA. The mixture were incubated at 25°C for 60 min. After rinsed with a physiological salt solution, the beads were incubated with *o*-phenylenediamine, in 0.02% hydrogen peroxide at 25°C for 30 min. The enzyme reaction was stopped with 1.15 N sulfuric acid, and the optical density at 492 nm was determined.

Real-time quantitative PCR Total cellular RNA was isolated from VSMC essentially by the guanidine thiocyanate–phenol extraction method of Chomczynski and Sacchi [28], Trizol, total RNA isolation reagent as per the protocol provided with the reagent. Briefly, VSMC were quickly scraped in 2 ml Trizol reagent, and homogenized. The homogenate was vortexed vigorously, mixed with 0.2 ml chloroform and vortexed again for 15 s. The suspension was allowed to stand at room temperature for 5 min and then centrifuged in a microfuge for 15 min. The supernatant was mixed with 0.4 ml isopropanol and the RNA was allowed to precipitate for 10 min. Following centrifugation, the pellet was washed with 75% ethanol. The ethanol washed RNA pellet was air dried and dissolved in distilled water (DW). RNA content was determined by measuring $A_{260\ nm}$. RNA quality was evaluated on the basis of the 260/280 nm ratio. First-strand cDNA synthesis was performed by Superscript II.

Quantitative real-time PCR was performed for TGF-β1 using the ABI Prism 7700 Sequence Detector (TaqMan; PE Applied Biosystems, CA) which combines PCR technology with fluorescence detection [29–31]. The machine contains a 96-well PCR block and each well has a fluorescence detector which can monitor emissions within individual wells. We monitored the expression of a housekeeping gene, 18S ribosomal RNA (TaqMan ribosomal RNA Control Reagents). An 18S ribosomal RNA amplicon was used as an internal control for quantitation of the total amount of cDNA. Serial 1:5 dilutions (200, 40, 8, 1.6, 0.32, and 0.064 ng) of cDNA were used to create a standard curve for quantitation of target mRNA. We calculated the expressed mRNA using the standard curves of target mRNA and obtained the relative quantity of target mRNA.

A 50 μl total reaction volume consisted of 25 ng of the cDNA sample, 200 nM each of forward and reverse primer, 100 nM probe, 200 μM each dNTP, 5.5 mM $MgCl_2$, 1.25 units AmpliTaq polymerase, 0.4 unit/μl RNase inhibitor, 1× TaqMan buffer, and RNase-free water to volume. Amplification was performed using one cycle of 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The primer pair used in these experiments recognized the TGF-β1 gene: forward 5′-TGCTTCCGCATCAC-CGT-3′ and reverse 5′-TAGTAGACGATGGGCAGTGGC-3′. The probe sequence is 5′ (FAM)-CTGCGTGCC GCAGGCTTTGG-(TAMRA)-3′ . The T_m (forward; 58, reverse; 59, probe; 70) and GC% (forward; 59, reverse; 57, probe; 70) were developed using Primer Express 1.0 software (PE Applied Biosystems). To optimize the reaction we tested primer concentrations at 300/300 and 900/900 nM and each combination was tested with 100 and 200 nM of probe. Reaction kinetics were similar with all combinations and 100 nM probe with 200/200 nM of primers were chosen for subsequent reactions.

Statistics. Results are presented as the mean ± SD. Differences between the means were calculated using one-way analysis of variance (ANOVA) and Fisher's PLSD. Probability values less than 0.05 were considered significant.

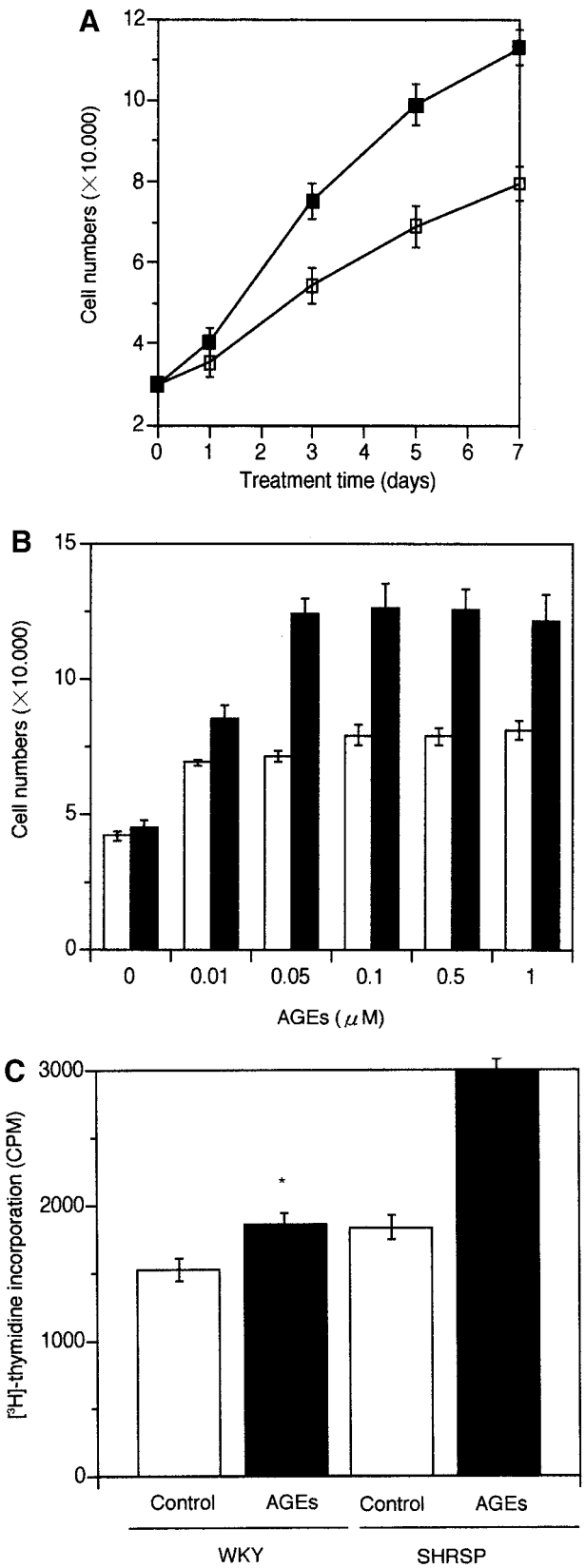

FIG. 1. (A) Time course of AGEs-induced cell proliferation in SHRSP- (■) and WKY-VSMC (□). Assays were performed by determination of cell numbers following stimulation with 0.1μg/ml AGEs for the indicated period. (B) Concentration–response curve of AGEs-induced cell proliferation in SHRSP- and WKY-VSMC. Assays were performed by determination of cell numbers following stimulation with the indicated concentrations of AGEs for 5 days. (C) Effects of AGEs on DNA synthesis in SHRSP- and WKY-VSMC. Assays were performed by incorporation of [^{3}H]thymidine over 24 h following stimulation with 0.1μg/ml AGEs. AGEs-induced proliferation and DNA synthesis were significantly greater in SHRSP-VSMC than in WKY-VSMC. The experiment was repeated 6 times with reproducible results. *$P < 0.05$ compared with the control.

RESULTS

Characteristics of SHRSP-VSMC Compared with WKY-VSMC

SHRSP-VSMC exhibited markedly faster proliferation in the presence of AGEs than did WKY-VSMC (Fig. 1A). VSMC cell proliferation was induced by AGEs in a time- and dose-dependent manner. AGEs-induced increases in DNA synthesis in SHRSP-VSMC were also significantly greater than in WKY-VSMC.

Effects of Resveratrol on AGEs-Induced Proliferation in SHRSP-VSMC

Resveratrol (10^{-6}–10^{-8} M) significantly inhibited AGEs-induced proliferation in SHRSP-VSMC (Fig. 2A). Data obtained for the incorporation of labeled thymidine (Fig. 2B) were similar to those obtained from cell counts at 3 days following resveratrol treatment. At the doses tested, resveratrol did not induce any significant changes in cell detachment. Resveratrol-induced inhibitory effects on AGEs-induced cell proliferation and DNA synthesis were partly reversed by the estrogen receptor antagonist ICI 182780.

Effects of resveratrol on AGEs-induced prolyl hydroxylase activity in SHRSP-VSMC.

At concentrations of 10^{-8} M or greater, resveratrol significantly inhibited the prolyl hydroxylase activity induced by AGEs (0.1 μ g/ml) (Fig. 3). The inhibitory effects of resveratrol on AGEs-induced prolyl hydroxylase activity were partly reversed by ICI 182780.

Effects of Resveratrol on AGEs-Induced TGF-β1 mRNA Expression in SHRSP-VSMC

AGEs-induced increases in TGF-β1 mRNA in SHRSP-VSMC were significantly greater than in WKY-VSMC (Fig. 4). Resveratrol inhibited AGEs-induced TGF-β1 mRNA expression in a dose-dependent manner.

DISCUSSIONS

AGEs have been identified as relevant mediators of vascular complications [12–15]. Recent epidemiological studies have revealed that the incidence of coronary

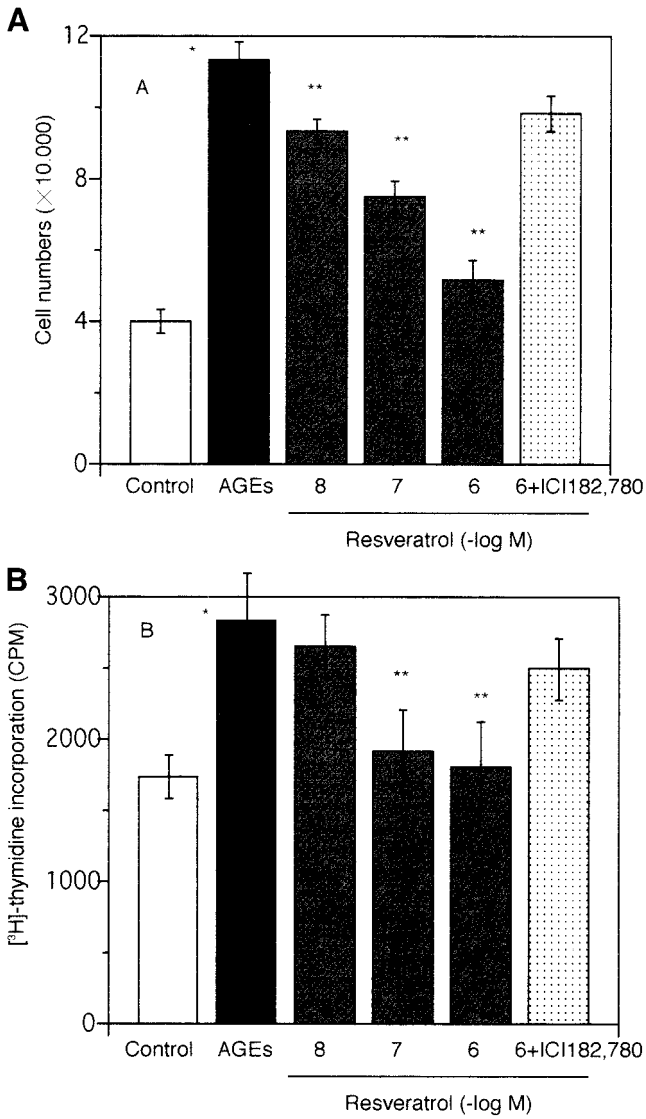

FIG. 2. Effects of resveratrol on AGEs-induced (A) proliferation and (B) DNA synthesis in SHRSP-VSMC. Assays were performed by determination of cell numbers or [^{3}H]thymidine incorporation over 24 h following stimulation with 0.1μg/ml AGEs in the presence or absence of resveratrol or ICI 182780. Resveratrol significantly inhibited AGEs-induced proliferation and DNA synthesis in SHRSP-VSMC. Resveratrol-induced inhibitory effects on AGEs-induced cell proliferation and DNA synthesis were partly reversed by ICI 182780. The experiment was repeated 6 times with reproducible results. *$P <$ 0.05 compared with the control. *$P < 0.05$ compared with the control.

heart disease is correlated with plasma concentrations of AGEs [12]. Bruel showed that age-related accumulation of AGEs was increased in both collagen and elastin of the aorta in SPF Wistar rats [9]. Furthermore, our previous study indicated that an increased content of AGEs was present in the aorta of 12-week-old SHRSP compared with age-matched WKY [16]. AGEs elicit their cellular effects by binding to specific cellular receptors, one of which, RAGE (receptor for AGEs), has been identified on endothelial cells, monocytes/macrophages, mesangial cells, neurons, and VSMC [32–34]. PDGF (platelet-derived growth factor), a potent chemoattractant and strong mitogen stimulates VSMC to migrate from the media to the intima and proliferate continuously [35, 36]. TGF-β1, which is released from platelets, VSMC, vascular endothelial cells and macrophages stimulates not only VSMC proliferation but also extracellular matrix synthesis [37, 38]. AGEs stimulate the production of PDGF and TGF-β1 by monocytes or VSMC, and some reports propose a role for PDGF and TGF-β1 in mediating abnormal cell proliferation and matrix synthesis by VSMC in response to AGEs [39, 40]. On the other hand, Satoh *et al.* [12] have confirmed that mitogen-activated protein kinase was activated by AGEs in VSMC.

The results of the present study demonstrate that AGEs accelerate proliferation, DNA synthesis, and collagen synthesis activity of VSMC. Proliferation and DNA synthesis in VSMC from SHRSP are more sensitive to AGEs than in the same cells from normotensive WKY. Some researches have demonstrated that VSMC from SHR or SHRSP proliferate and effect matrix production more rapidly and are more sensitive to TGF-β1 than those from WKY [41]. AGEs-induced increases in TGF-β1 mRNA in SHRSP-VSMC were significantly greater than in WKY-VSMC. Thus, it is possible that differences in cellular responses to AGEs between SHRSP-VSMC and WKY-VSMC are due to differences

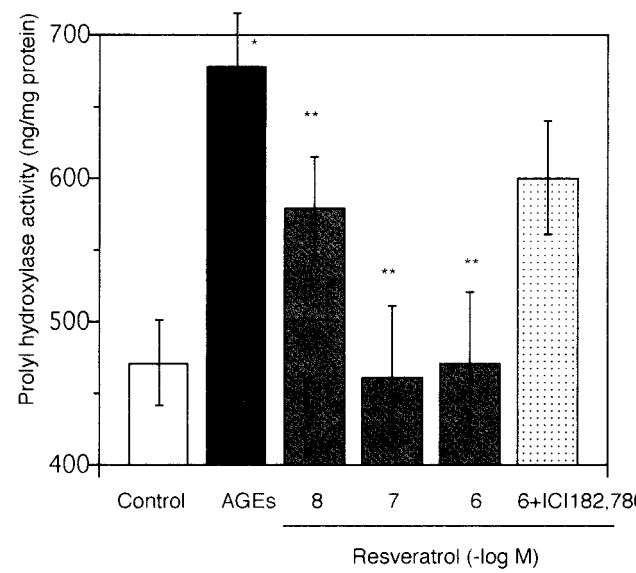

FIG. 3. Effect of resveratrol on AGEs-induced prolyl hydroxylase activity in SHRSP-VSMC. Assays were performed by determination of prolyl hydroxylase activity following stimulation with 0.1 μg/ml AGEs in the presence or absence of resveratrol or ICI 182780. Resveratrol significantly inhibited the prolyl hydroxylase activity induced by AGEs. The inhibitory effects of resveratrol on AGEs-induced prolyl hydroxylase activity were partly reversed by ICI 182780. The experiment was repeated 6 times with reproducible results. *$P < 0.05$ compared with the AGEs.

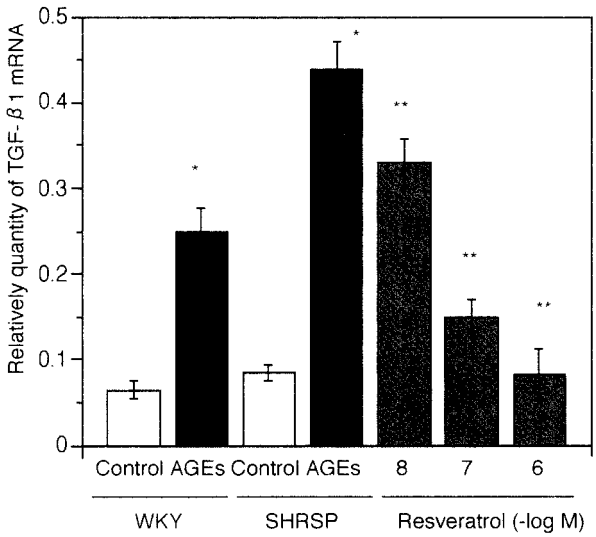

FIG. 4. Effects of AGEs and resveratrol on TGF-β1 mRNA in WKY- and SHRSP-VSMC. Assays were performed by determination of TGF-β1 mRNA expression following stimulation with 0.1 μg/ml AGEs in the presence or absence of resveratrol. AGEs-induced increases in TGF-β1 mRNA in SHRSP-VSMC were significantly greater than in WKY-VSMC. Resveratrol inhibited AGEs-induced TGF-β1 mRNA expression in a dose-dependent manner. The experiment was repeated 5 times with reproducible results. *$P < 0.05$ compared with the control. **$P < 0.05$ compared with the control.

in TGF-β1 mRNA increases or in response to TGF-β1 activated by AGEs.

Some reports have shown estrogens to have various vasculoprotective effects [17–19]. Recently, Anderson *et al.* found that postmenopausal women receiving oral estrogens for 3 months exhibited decreased AGEs-hemoglobin levels [42]. Beneficial effects of phytestrogens in the treatment of cardiovascular diseases including hypertension and atherosclerosis have been reported [24, 43–45], and much attention has been given to a possible influence on vascular remodeling. Resveratrol occurs naturally in grapes and a variety of medicinal plants. Because of its high concentration in grape skins, significant amounts of resveratrol are present in wine [46, 47]. The similarity in structure between resveratrol and the synthetic estrogen diethylstilbestrol prompted us to investigate whether resveratrol might exhibit estrogenic activity, a property that is known to have a protective benefit against cardiovascular diseases. In our results, resveratrol at a concentration of 10^{-8} M or greater significantly inhibited proliferation, DNA synthesis, and collagen synthesis activity of SHRSP-VSMC in culture. Resveratrol significantly inhibited AGEs-induced TGF-β1 mRNA increases.

Oxidative stress originates as the result of an imbalance between the generation of reactive oxygen species (ROS) and cellular antioxidants. ROS have been implicated as responsible for stimulating cell proliferation and matrix synthesis in VSMC [48, 49]. Although the mechanisms responsible for ROS-induced cell proliferation have not been definitively determined, possible explanations include protein oxidation, lipid peroxidation, enzyme-activity alterations and oxidative DNA damage. Our previous study has shown that resveratrol improve glutathione production deficiency induced by AGEs in VSMC, and it could lead to decrease in oxidative DNA damage (unpublished data). Therefore, the protective effect of resveratrol on AGEs-induced abnormal cell proliferation and matrix synthesis appeared to be also attributed to anti-oxidative action of resveratrol in VSMC.

Moreover, ICI 182780, a specific estrogen receptor antagonist, only partially blocked the inhibitory effects of resveratrol. It has recently been demonstrated that phytestrogens inhibit growth in both estrogen receptor-positive and estrogen receptor-negative cell lines [50]. This suggests that the inhibitory effects of resveratrol are only partly mediated by estrogen receptors. Earlier studies have shown that phytestrogens reduce cardiovascular risk factors and prevent neointima formation without affecting the reproductive system, thus reducing the risk of cancer [51]. Since hormone replacement therapy with estrogens is associated with side effects such as hot flushes, cancer, and bleeding, the use of dietary phytestrogens in women may be an attractive alternative. Moreover, unlike 17β-estradiol, phytestrogens do not have feminizing effects. Our previous study has demonstrated that resveratrol attenuates blood pressure in ovariectomized SHRSP [26]. Our observation that in SHRSP-VSMC, resveratrol inhibits AGEs-induced proliferation and DNA synthesis as well as collagen synthesis activity provides evidence that the cardioprotective effects of resveratrol may in part be mediated via inhibition of TGF-β1 mRNA gene expression-induced attenuation of vascular remodeling.

In summary, SHRSP-VSMC were more sensitive to AGEs than VSMC of normotensive WKY. Our data also give evidence that resveratrol, at concentrations of approximately 10^{-6}–10^{-8} M, which are attainable in plasma during therapeutic dosing, inhibits SHRSP-VSMC proliferation induced by AGEs, as well as having inhibitory effects on DNA synthesis and collagen synthesis activity. Thus resveratrol may provide a safe substitute for estrogen in protecting against cardiovascular disease.

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