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Extract of *Rubus coreanus* Fruits Increases Expression and Activity of Endothelial Nitric Oxide Synthase in the Human Umbilical Vein Endothelial Cells

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This study aimed to investigate the effects of water extract of *Rubus coreanus* (RCE) on the expression and activity of endothelial nitric oxide synthase (eNOS), as well as its signal transduction pathways in human umbilical vein endothelial cells (HUVECs). The specific inhibitors of NOS show RCE treatment increases NO production in HUVECs due to the up-regulation of eNOS rather than iNOS. The real-time expression level of eNOS mRNA was also increased upon RCE treatment in HUVECs. While a PKC-specific inhibitor, RO-317549, did not alter RCE-induced NO production in HUVECs, tamoxifen (estrogen receptor-specific inhibitor), PD98059 (ERK-specific inhibitor) and LY-294002 (PI3K/Akt-specific inhibitor) did have suppressive effects. Increased NO production by RCE seems to result from a higher level of active eNOS (pSer1177). Specifically, inhibition of ERK not only decreased the level of active eNOS, but also increased the inactive form of the enzyme (pThr495) in HUVECs. This study suggests that RCE treatment increases NO production in HUVECs due to the increased expression and activity of eNOS. It is also shown that RCE-induced eNOS activation occurs partly through the binding of RCE to the estrogen receptor, along with ERK and PI3K/Akt-dependent signal transduction pathways. In addition, the regulatory binding proteins of eNOS including Hsp90 and caveolin-1 were related to these effects of RCE on eNOS activity in HUVECs.

Key words : Human umbilical vein endothelial cell, NO, eNOS, *Rubus coreanus*, estrogen receptor

Introduction

Nitric oxide (NO) plays various roles in regulation, mediating many physiological and pathophysiological functions such as vasodilation, neurotransmission/ neuromodulation and cytotoxic activity [2,24,42]. Vasodilation is primarily controlled by the endothelium-dependent production of NO, which acts to induce vascular smooth muscle relaxation in a cGMP-dependent manner [63].

In numerous mammalian cells and tissues, NO is synthesized by NO synthase (NOS), which oxidizes the terminal guanidinium residue of L-arginine (L-Arg) to NO and citrulline [53,54]. There are three NOS isozymes, Ca^{2+}-dependent endothelial and neuronal NOS (eNOS and nNOS, respectively) and Ca^{2+}-independent inducible NOS (iNOS).

The classic activity of estrogen is mediated by transcriptional activation of estrogen-responsive genes involving intracellular estrogen receptors (ER). Once estrogen binds to ER, the receptor-hormone complex then binds to a specific estrogen responsive element in the promoter region in the target genes, leading to transcriptional activation [28,34]. In addition to transcriptional regulation of estrogen-responsive genes, the rapid, membrane-initiated increase in NO release by estrogen, occurring within minutes and intact in the presence of transcriptional inhibitors, were observed [7,21]. Recent evidence implicates cell surface receptors in the rapid response to estrogen in a number of different cell types, including vascular endothelial cells [8,14,20,29,58].

eNOS exists in at least two distinct subcellular locations: within caveolae of the plasma membrane and in the Golgi/perinuclear region of the cell [41]. Caveolin-1, a major scaffolding protein in endothelial caveolae, significantly inhibits eNOS activity as it supplies anchorage and prevents calmodulin binding [16,41]. Conversely, the binding of Ca^{2+}/calmodulin to eNOS disrupts the eNOS/caveolin-1 complex, leading to enzyme activation [39]. The binding of Hsp90 (90 kDa heat shock protein) to eNOS triggers the transition from the Ca^{2+}-dependent activation to phosphorylation-mediated activation by recruiting Akt to the complex [4]. In fact, active eNOS can be co-immunoprecipitated with Hsp90 and kinases such as Akt [11,17].

Most adult-onset cardiovascular diseases are associated with age-related vascular dysfunction [10,37]. Age-related

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vascular dysfunction due mainly to reduced NO production by eNOS and reduced eNOS expression, which might make endothelial cells more vulnerable to apoptotic stimuli [3,12,23].

Phytoestrogens are biologically active phenolic compounds from plants that mimic mammalian estrogens, either structurally or functionally, and therefore play important roles in the prevention of cancer, heart disease, menopausal symptoms and osteoporosis [1,32,48,56,61]. Estrogens influence the growth and function of female and male reproductive tissues, maintain the skeletal and central nervous system, protect the cardiovascular system and prevent colon cancer and skin aging [19,51]. Considering the numerous effects estrogens have on the human body, it is not surprising to consider the potential of phytoestrogens for human health.

Phytoestrogens are naturally occurring molecules that include isoflavonoids, lignans, coumestanes, stilbens and the flavonoids quercetin and kaempherol. These phytochemicals are usually found in fruits, vegetables, legumes and tea and are generally concentrated in the fruit skin, bark and flowers of plants [43]. Resveratrol, daidzein, quercetin and genistein represent four of the most commonly ingested and most intensely studied phytoestrogens [38]. Moreover, they may act as "natural", selective ER modulators because their effects are mediated via the estrogen receptor (ER) in the cell membrane. Recently, the possible application of phytoestrogens in estrogen replacement therapy for postmenopausal women has been investigated. If successful, there is potential in preventing osteoporosis and cardiovascular diseases without adverse effects such as increased risk of breast and endometrial cancer, as well as irregular bleeding [5,13,38,64-66]. However, controversy over data requires further studies in order to confirm the health effects of phytoestrogens *in vivo* [44,61].

Rubus species have been cultivated as fruits for centuries and are used in various countries as natural remedies to several diseases such as diabetes, many types of infection, colic and burns [49]. *Rubus coreanus* Miq., a deciduous broadleaf shrub of the family Rosaceae, is popularly known as Bokbunja in South Korea and used in traditional folk medicines for treating spermatorrhea, enuresis, asthma and allergic diseases [57]. Bokbunja has been used to improve male reproductive function by treating symptoms like impotence, premature ejaculation, seminal emission, spermatorrhea and enuresis in combination with other herbal medicines in traditional Korean medicine [30].

Bokbunja has been reported to possess anti-oxidant, anti-nociceptive, anti-inflammatory, anti-cancer and anti-anaphylactic effects [9,33,57,67]. The fruits may also improve male fertility by enhancing spermatogenesis [46]. Many recent studies have investigated the scientific basis of these purported therapeutic effects on biological systems. However, the effect of Bokbunja on NO production in endothelial cells has not yet been reported. Therefore, in this study we investigated the effect the water extract of *Rubus coreanus* (RCE) on NO production, the expression and activity of eNOS and the intracellular signal pathway in cultured primary human endothelial cells.

Materials and Methods

Preparation of water extracts from *Rubus coreanus* fruits

Aqueous extract of *Rubus coreanus* Miq. fruits collected from Gochang county was prepared by the following methods. The fruits were frozen for overnight at -80°C and were lyophilized (Hanil Research & Development Co, Korea). The fruits once dried (5 g) were homogenized in distilled water (200 ml) for 5 min using a Polytron homogenizer (Model T-25B, IKA Co, Japan), followed by shaking for 20 min at 80°C in a water bath. Centrifugation was performed at 20,000 rpm for 20 min at 4°C. The resulting supernatant was frozen for overnight at -80°C and completely lyophilized thereafter. The weight of RCE was about 1.85 g, which was dissolved by distilled water into 10 mg/ml and stored at 4°C.

Quantification of phenol groups

The phenol content of RCE was measured with Folin-Ciocalteu Reagent (FCR, Sigma, St. Louis, MO, USA) [59]. An aliquot of diluted RCE (200 µl) was mixed with 10% FCR (1 ml) in test tube, which was reacted for 3 min at room temperature. The mixture was next mixed with 7.5% Na_2CO_3 (0.8 ml) and incubated for 2 hr in a shaking water bath. After incubation the absorbance was measured at 760 nm using UV-spectrophotometer (Model UV-1650PC, Shimadzu, Japan). The amount of phenol groups was quantified from the standard curve plotted with authentic catechin. The phenol content of RCE used in this study was $\leq$200 nM CE (catechin equivalent).

Cell culture

Human umbilical vein endothelial cells (HUVECs, PDL 21-23) were purchased from Modern Cell & Tissue Technologies (Seoul, Korea) and cultured in Cambrex microvascular endothelial cells medium-2 (EGM-2, Cambrex, MD, USA). The cells were incubated at 37°C in a humidified atmosphere under 5% CO_2.

Measurement of NO production

Production of NO was determined spectrophotometrically using Griess reagent (0.8% sulfanilamide, 0.75% N-(naphthyethylene)diamine in 0.5 N HCl, Sigma, MO, USA). An aliquot of the incubated supernatant (100 µl) was transferred to 96-well plates and mixed with 100 µl of Griess reagent. After 15 min incubation at room temperature, the nitrite concentration was measured at 540 nm using a microplate reader. Sodium nitrate (0.5 to 100 µM) was used as the nitrite standard and the absorbance was found to be linear over this concentrations range.

Western blot analysis

The cells were lysed in RIPA buffer (Santa Cruz Biotechnology, CA, USA, 50 mM Tris - HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1 mM PMSF, and 0.5 mM sodium orthovanadate) containing a cocktail of protease inhibitors (10-20 µl per 1 ml RIPA buffer) by incubating for 30 min at 4°C. The mixtures were transferred to microtubes and centrifuged at 12,000× *g* for 15 min at 4°C, and the supernatants were stored at -80°C before use. Proteins (40 µg) present in the cell lysates were separated by 8% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% skim milk in TBS-T (Tris buffered saline-Tween-20: 25 mM Tris-base, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20, pH 7.4) for 1 hr at room temperature and incubated with anti-human eNOS antibody (BD biosciences, Franklin Lakes, NJ, USA), anti-human pS1177 eNOS (BD biosciences) or anti-human pThr495 eNOS (BD biosciences) overnight at 4°C. After washing with TBS-T 3 times, the blot was incubated with secondary antibody (horseradish peroxidase-conjugated anti-goat antiserum) for 2 hr at room temperature. The antibody-specific proteins were detected using West-ZOL™ (plus) (iNtRON Biotechnology, Seongnam, Korea).

ELISA

Levels of eNOS protein were also measured using a commercially available ELISA kit (R&D System, MN, USA) following the manufacturer's instruction manual. Absorbances of the samples at 450 nm were converted to protein concentration (pg/ml) using standard curves generated with the recombinant human eNOS supplied with the kit.

RNA isolation

Total RNA was isolated from HUVECs using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). The cell lysate was then stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml chloroform per 1 ml of TRI reagent and shaken vigorously for 15 sec. The resulting mixture was stored at room temperature for 3 min and centrifuged at 12,000× *g* for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, the interphase and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, which was transfered to a fresh tube, whereas DNA and proteins are separate into the interphase and organic phase. The aqueous phase was transfered to a fresh tube. The supernatant was removed and the RNA pellet was washed by the addition of 75% ethanol and centrifugation at 7,500× *g* for 5 min at 4°C. After washing, at least 1 ml of 75% ethanol per 1 ml TRI reagent was added to the RNA pellet, which was briefly air-dried for 3～5 min. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC) water by passing the solution a few times through a pipette tip and incubating for 10～15 min at 55～60°C. Total RNA was quantified spectrophotometrically at 260 nm.

Real-time PCR

One microgram of total RNA was reverse transcribed using a maxime™ RT premix kit (iNtRON Biotechnology, Seongnam, Korea) at 42°C for 1 hr. Oligo $(dT)_{15}$ was used as primer. The cDNA was amplified by real-time PCR in the presence of AccuPower™ GreenStar qPCR premix (Bioneer, Daejeon, Korea). Specific primer pairs for eNOS were 5'-CCAGCTAGCCAAAGTCACCAT-3' and 5'-GTCT CGGAGCCATACAGGATT-3' for sense and antisense, respectively. For 28S, the sense primer was 5'-TTGAAAAT CCGGGGGAGAG-3' and antisense primer was 5'-ACATT GTTCCAACATGCCAG-3'. Real-time RT-PCR was conducted for 45 cycles under the following conditions: 95°C denaturation step for 15 sec, annealing at 54°C for 20 sec (eNOS) or 52°C (28S), and extension at 72°C for 20 sec in a real-time rotary analyzer (Rotor-Gene™ 6000, Corbett Life

Science, San Francisco, CA, USA). Final extension was performed at 72°C for variable times depending on product size (eNOS, 354 bp and 28S, 100 bp). The green fluorescent product was detected at the end of each cycle.

Measurement of protein content

The total protein concentrations of the conditioned media and cell extracts were determined using BCA™ protein assay kit (Pierce Biotechnology, Rockford, IL). Bovine serum albumin was used as a protein standard.

Immunoprecipitations

Immunoprecipitation (IP) was carried out using an Immunoprecipitation Starter Pack (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The IP procedure for detecting the protein level was performed according to the manufacturer's instructions. nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow are supplied preswollen in 20% ethanol. It was washed three times by RIPA buffer. It was centrifuged at 12,000× *g* for 20 sec and discarded the supernatant. And, it was mixed by equal volumes of media and RIPA buffer (50% slurry). It was stored at 4°C. Cell lysates (100 µl) were transferred to new tubes and added anti-human eNOS antibody (1 µg). These mixtures were gently mixed for 1 hr at 4 °C. These mixtures were added with the 50 µl nProtein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow suspension (50% slurry) and gently mixed for 1 hr at 4°C. After mixing, these mixtures were centrifuged at 12,000× *g* for 20 sec and save the pellet. These pellets were washed three times with 1 ml RIPA buffer and once with wash buffer (50 mM Tris, pH 8). The final pellets were suspended in 30 µl sample buffer (1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5) and heated to 95°C for 3 min. It was centrifuged at 12,000× *g* for 20 sec to remove the beads. The supernatants were carefully transferred in new tubes. These supernatants were analyzed by western blot. The following primary antibodies were used: anti-human eNOS from BD Biosciences (Franklin Lakes, USA), and anti-human Akt, anti-human Hsp90, and anti-caveolin-1 from Cell Signaling Technology (Danvers, USA).

Statistic analysis

For in vitro data analysis, the Students t-test was performed using the Sigma-plot computerized program and a P-value less than 0.05 was deemed significant. The comparison was carried out with control or reagent-treated data.

Results

NO production is increased by RCE in HUVECs

The effect of RCE on the bioavailability of NO in cultured HUVECs (from PDL 21 to 25) was analyzed spectrophotometrically by measuring nitrite concentration in culture media with Griess reagent. As shown in Fig. 1, NO production in HUVECs was significantly increased in a dose- (with increasing RCE concentrations from 0 to 100 nM) and a time-(with increasing time of incubation from 0 to 16 hr in 100 nM RCE) dependent manner at 37°C.

The relative contribution of NOS isozymes on RCE-induced NO production in HUVECs

Specific enzyme inhibitors (L-NIO, eNOS-specific inhibitor; 1400W, iNOS-specific inhibitor) were used to eval-

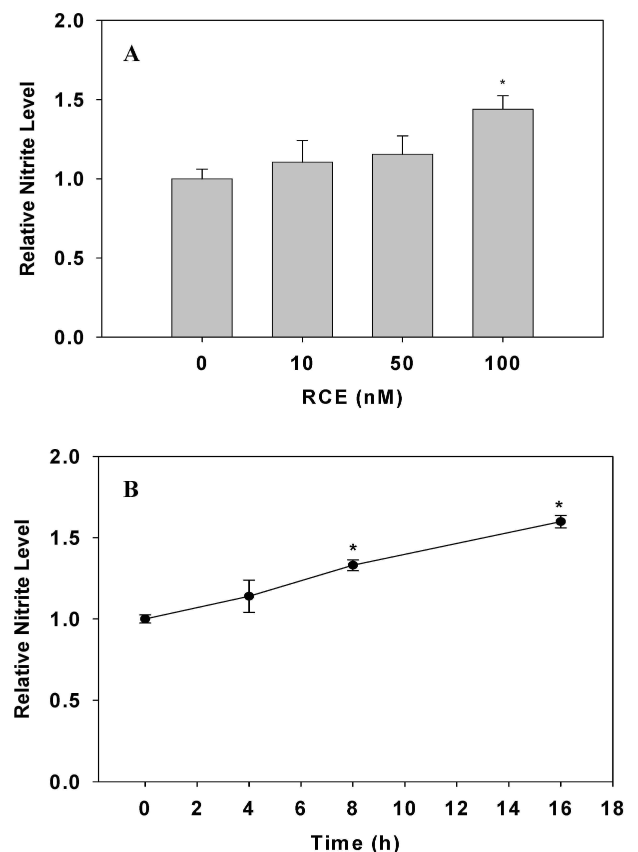

Fig. 1. Effect of RCE on NO production in HUVECs. HUVECs were treated with RCE in a dose-dependent manner for 16 hr (A) and with RCE (100 nM) in a time-dependent manner (B) at 37°C. Nitrite was measured by the Griess reagent method. Each result represents mean±SD (n=6~9). The significance was expressed as a comparison with NO production of the untreated group (*p<0.05).

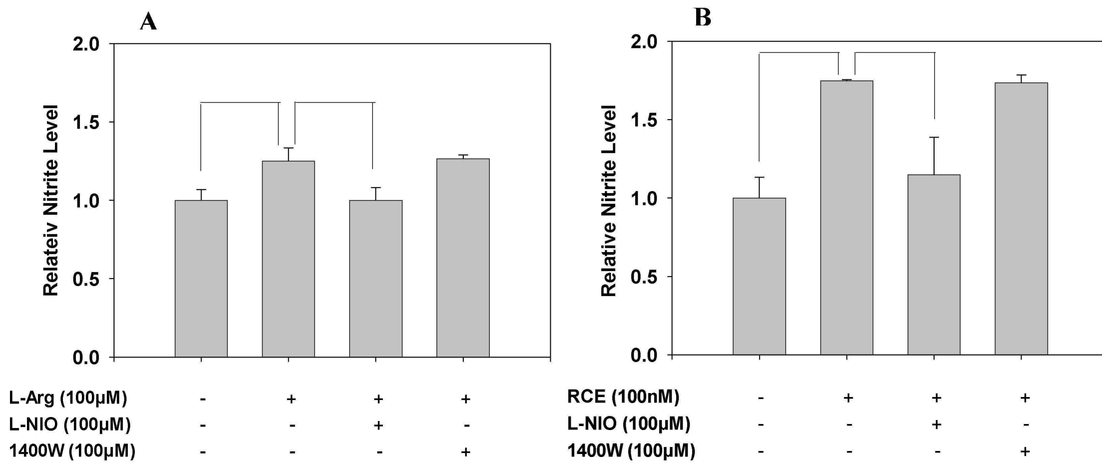

Fig. 2. Effect of NO synthase-specific inhibitors on RCE-induced NO production in HUVECs. HUVECs were treated with L-Arg (A) or RCE (B) in the presence or absence of NOS-specific inhibitors (L-NIO: eNOS inhibitor and 1400W: iNOS inhibitor) for 16 hr at 37°C. Nitrite was measured by the Griess reagent method. Each result represents mean±SD (n=6~9, $p<0.05$).

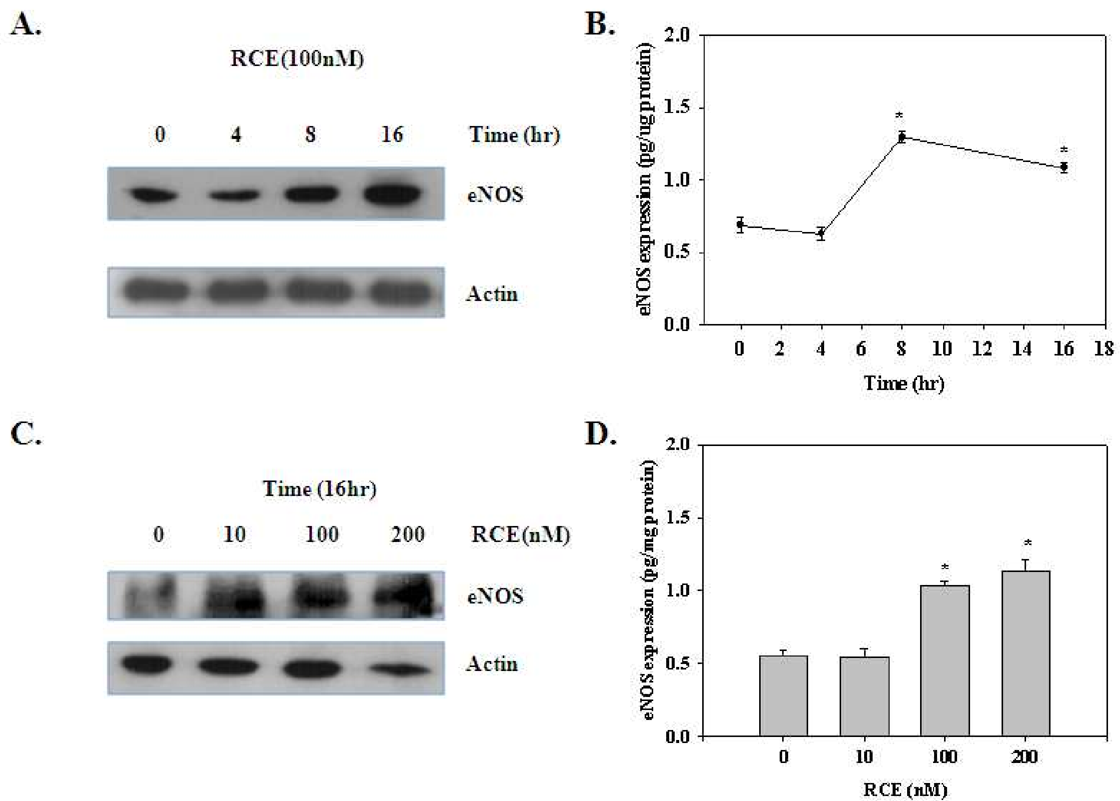

Fig. 3. Effect of RCE on content of endothelial NO synthase in HUVECs. HUVECs were treated with RCE in a time (0~16 hr, RCE 100 nM; A and B) and dose- (RCE 0~200 nM, 16 hr; C and D) dependent manner at 37°C under a humidified atmosphere of 5% CO_2. Western blot (A and C) and ELISA analysis (B and D) were performed as described in 'materials and methods'. Each ELISA result is represented as mean±SD (n=5). The significance was expressed as a comparison with eNOS expression of the untreated group (*$p<0.05$).

uate the relative contribution of NOS isozymes on RCE-induced NO production in human endothelial cells.

The increased NO production in HUVECs by both L-Arg- and RCE treatment was significantly suppressed by accompanying treatment of the eNOS-specific inhibitor, L-NIO. However, simultaneous treatment with the iNOS-specific inhibitor 1400W did not produce any effect on RCE-induced NO production in the cells (Fig. 2).

Expression of eNOS is increased by RCE in HUVECs

In this experiment, the effect of RCE on eNOS expression in HUVECs was evaluated by western blot and ELISA. HUVECs were treated with RCE in a time-(with increasing time of incubation from 0 to 16 hr in 100 nM RCE, Fig. 3A and 3B) and dose-(with increasing RCE concentrations from 0 to 200 nM for 16 hr, Fig. 3C and 3D) dependent manner at 37°C. Western blot analysis of HUVEC lysates was performed with anti-eNOS antibody. The content of eNOS protein is directly proportional to both RCE concentration and treatment time, as gradual increases in eNOS protein content were observed throughout. ELISA analysis again confirmed that eNOS expression is increased by RCE treatment.

To investigate real-time expression of eNOS, the expression of eNOS mRNA in RCE-treated HUVECs was analyzed using real-time PCR. HUVECs were treated with RCE in a time-(with increasing time from 0 to 24 hr in 100 nM RCE, Fig. 4A) and dose-(with increasing RCE from 0 to 200 nM for 4 hr, Fig. 4B) dependent manner at 37°C. As shown in Fig. 4B, any increase in eNOS mRNA expression caused by RCE treatment was not observed with a concentration of 10 nM. However, RCE at higher concentrations did effectively increase the expression of eNOS mRNA by 1.87-fold (100 nM) and 5.32-fold (200 nM). Treatment with 100 nM of RCE for 4~24 hr maintained a high level (1.87~2.02 fold) of eNOS mRNA expression (Fig. 4A). These results show RCE increases the expression of eNOS in a dose- and time-dependent manner in cultured HUVECs.

Effect of signal-specific inhibitors on RCE-induced NO production and eNOS activation in HUVECs

The inhibitory effect of inhibitors specific to the signal pathways of RCE-induced NO production was analyzed in cultured HUVECs (Fig. 5). Inhibitors used were Tamoxifen (ER-specific inhibitor, 10 μM), RO-317549 (PKC specific inhibitor, 10 μM), PD98059 (ERK specific inhibitor, 10 μM) and LY-294002 (PI3K/Akt specific inhibitor, 10 μM). HUVECs were treated with signal specific inhibitors in the absence or presence of RCE and western blots were performed with anti-pSer1177-specific eNOS antibody and anti-pThr495-specific eNOS antibody. As shown in Fig. 5, among the four inhibitors, Tamoxifen, PD98059 and LY-294002 suppressed RCE-induced NO production in HUVECs. However, RO-317549 did not have any effect.

The inhibitory effect of inhibitors specific to the signal pathways of RCE-induced eNOS activation was analyzed in

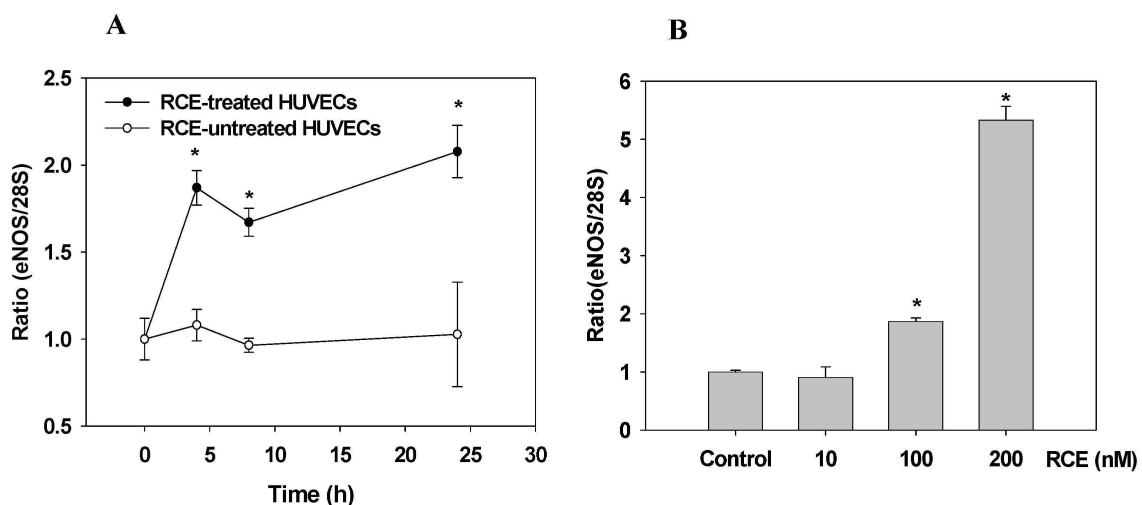

Fig. 4. Effect of RCE on mRNA expression of endothelial NO synthase in HUVECs. HUVECs were treated with RCE (100 nM) in a time-(A) and dose-(B) dependent manner for 4 hr at 37°C under a humidified atmosphere of 5% CO_2. Real-time PCR was performed as described in 'materials and methods'. Each result represents mean±SD (n=4). The significance was expressed as a comparison with mRNA expression of thhe untreated group (*p<0.05).

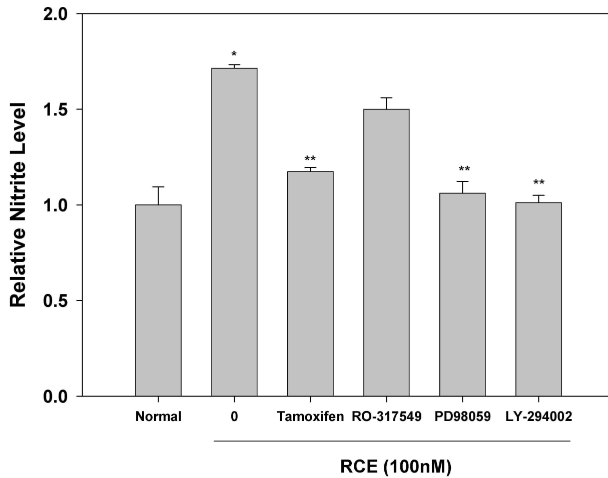

Fig. 5. Effect of signal transduction inhibitors on NO production induced by RCE in HUVECs. HUVECs were treated with 100 nM RCE and one signal transduction inhibitor (Tamoxifen, ER inhibitor; RO-317549, PKC inhibitor; PD98059, ERK inhibitor; LY-294002, PI3K inhibitor; each concentration, 10 μM) for 16 hr at 37°C. Nitrite was measured by the Griess reagent method. Each result represents mean±SD (n=4). The significance was expressed as a comparison with NO production of the control group (*p<0.05).

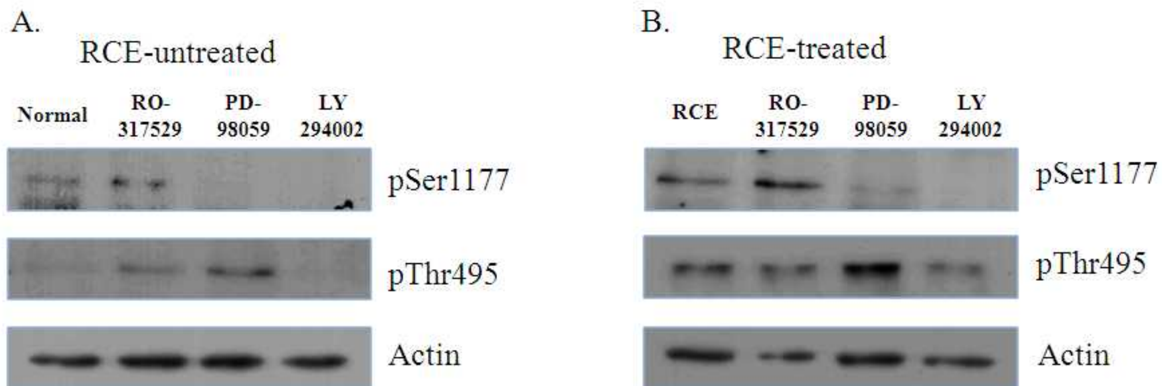

Fig. 6. Effect of signal transduction inhibitors on activation of endothelial NO synthase induced by RCE in HUVECs. HUVECs were treated with signal-specific inhibitors (RO-317549, PKC inhibitor; PD98059, ERK inhibitor; LY-294002, PI3K inhibitor; each concentration, 10 μM) in the absence (A) or presence (B) of RCE for 16 hr at 37°C. The phosphorylation of eNOS was detected by western blot using antibodies phospho-specific to the various eNOS forms (pThr495 and pSer1177). Western blot analysis was performed as described in 'materials and methods'.

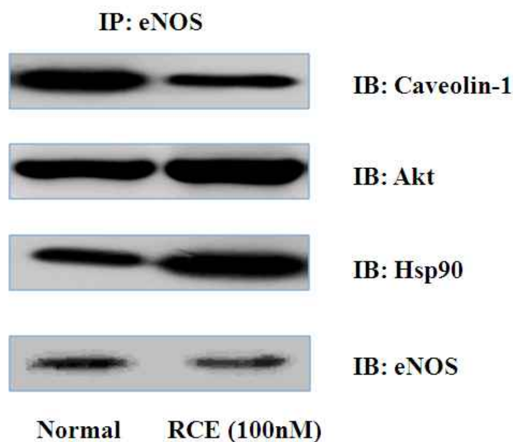

Fig. 7. Alterations in the directly coupling level of the regulatory binding proteins on eNOS in aged HUVECs. The young HUVECs were treated with RCE (100 nM) and incubated for 16 hr at 37°C in a humidified atmosphere of 5% CO_2. IP and Immunoblot analysis were performed as described in the materials and methods (IP: anti-human eNOS, IB: anti-human eNOS, anti-caveolin-1, anti-Akt, and anti-hsp90).

cultured HUVECs (Fig. 6). The amount of pSer1177 band indicating the active form of eNOS was decreased by PD98059 and LY-294002 treatment with or without RCE. In contrast, the amount of pThr495 band indicating the inactive form of eNOS was increased by PD98059 treatment with or without RCE. In addition, RO-317549

combined with RCE specifically increased the pSer1177 band and decreased pThr495 band.

Effects of RCE on the regulatory binding proteins associated with eNOS in HUVECs

To investigate the directly coupling change of caveolin-1, Akt and Hsp90 on eNOS in RCE-treated HUVECs, IP was carried out. Akt and Hsp90 directly coupled to eNOS were increased in RCE-treated HUVECs. In contrast, caveolin-1 bound to eNOS was decreased in RCE-treated HUVECs (Fig. 7). These results were shown that the regulatory binding proteins of eNOS including Hsp90 and caveolin-1 were related to these effects of RCE on eNOS activity in HUVECs.

Discussion

Both estrogen and NO play critical roles in blood vessel development, function and remodeling. Understanding the interplay between these two powerful, interdependent vascular modulators is possibly the first step in designing treatments for conditions such as ischemic, diabetic and postmenopausal vascular dysfunction [52].

It is well known that NO contributes to cardiovascular homeostasis by profoundly affecting blood pressure, vascular remodeling, platelet aggregation and angiogenesis [24]. Under normal conditions, the endothelial isoform of NO synthase expressed both in endothelial cells (EC) and cardiac myocytes is the major source of NO in the cardiovascular system [4]. This endothelium-dependent production of NO controls vasodilation in arteries and acts to induce vascular smooth muscle relaxation in a cGMP-dependent manner [63]. Furthermore, NO plays a variety of regulatory functions in vivo, mediating many physiological and pathophysiological functions including vasodilation, neurotransmission /neuromodulation and cytotoxic activity [2,24,42].

There exist two NOS isozymes, eNOS and iNOS, that generate NO within the vascular wall. It appears estrogen modulates a cell type- and tissue-specific NOS isozyme. Specifically, NO production by eNOS in cultured aortic endothelial cells was reported to be controlled by estrogen [22]. Estrogen increased iNOS mRNA expression through a classic receptor-mediated pathway in rat peritoneal macrophages [68]. In addition, phytoestrogen genistein and daidzein up-regulated LPS-induced iNOS activity through an estrogen-receptor pathway in RAW264.7 cells [45]. Long-term administration of a soy protein diet rich in genistein and daidzein increases expression of eNOS and key antioxidant defense genes such as MnSOD and cytochrome c oxidase [36].

In the present study, the NOS isozymes, eNOS and iNOS, were evaluated for their relative contribution to the RCE-induced increase of NO production in HUVECs (Fig. 2). Potentiation of NO production by RCE treatment was significantly suppressed by concomitant treatment of the eNOS-specific inhibitor L-NIO in HUVECs. However, an iNOS-specific inhibitor, 1400W, did not affect RCE-induced NO production. These results suggest NO production increased by RCE treatment occurs through the activation of eNOS instead of iNOS in HUVECs.

eNOS is constitutively expressed in endothelial cells and therefore its activity is most likely controlled by post-translational modifications such as protein-protein interactions, subcellular localization to specialized compartments and phosphorylation [25,50].

Caveolin-1 and eNOS are signaling partners that promote pulmonary vasodilation during the fetal-to-neonatal transition [26]. Importantly, the binding of eNOS to caveolin-1 through specific protein-protein interactions targets the enzyme into caveolae, thereby rendering it inactive. While eNOS in caveolae is held inactive by its association with caveolin-1, eNOS activity can be increased through association with Ca^{2+}/calmodulin and binding to Hsp90 and dynamin-2 [6,18,27,55]. PKC is highly localized to caveolae in lung (and other) endothelial cells and has been shown to interact with caveolin and affect the morphology and function of caveolae outside the cell [35,47,62]. However, it was shown the PKC-specific inhibitor RO-317549 did not affect RCE-induced NO production in HUVECs. Therefore, PKC seems not to be the major regulatory factor of eNOS in HUVECs.

Hsp90 facilitates the phosphorylation of eNOS by forming a ternary complex with eNOS and Akt [4]. Active eNOS located at the plasma membrane can be co-immunoprecipitated with Hsp90 and kinases, especially Akt [4,11,17]. Dynamin-2 regulates eNOS activity through the binding of its proline-rich domain to the FAD domain of eNOS, promoting electron transfer between the bound flavins of the reductase domain and increasing NO production [6]. Among the four inhibitors used in this study, tamoxifen (ER-specific inhibitor), PD98059 (ERK specific inhibitor) and LY-294002 (PI3K/Akt specific inhibitor) suppressed the RCE-induced NO production in HUVECs. However, RO-317549 (PKC inhibitor) did not have any effect on RCE-induced NO

production.

Phosphorylation of Ser116 and Thr495 negatively regulates eNOS activity, whereas phosphorylation of Ser635 and Ser1177 has the opposite effect of increasing eNOS activity [11,15,40]. eNOS located at the plasma membrane is usually phosphorylated at Ser1177 (human sequence) by Akt [17]. Conversely, inactive eNOS at the plasma membrane is phosphorylated at Thr495 (human sequence).

Although the active form of eNOS (pSer1177) is increased by RCE treatment, its activation by RCE is also decreased by PD98059 and LY-294002. Moreover, PD98059 effectively increases the inactive form of eNOS (pThr495) in HUVECs as detected by Western blot more than RCE alone.

Although the activation pathway of eNOS by 17β-estradiol and isoflavones varies in different cell types, the main transduction signal in equol-stimulated NO production occurs via PI3K/Akt, ERK1/2 and Hsp90 [60]. The stimulation of ERK1/2 and NO production in endothelial cells by trans-resveratrol was inhibited by the ER antagonists, ICI 182,780 and tamoxifen [31]. RCE also increases both the expression and activity of eNOS mainly via ER, ERK and the PI3K/Akt signal transduction pathway. And, RCE altered the eNOS coupling levels of the regulatory binding proteins including Hsp90, Akt, and caveolin-1.

This study suggests RCE treatment increases NO production in HUVECs, and this effect is due to the increase in eNOS mRNA expression and activity. It was also shown that RCE-induced eNOS activation occurs partly through binding to ER, and later through the ERK and PI3K/Akt-dependent signal transduction pathways. In addition, the regulatory binding proteins of eNOS including Hsp90 and caveolin-1 were related to these effects of RCE in HUVECs.

Interestingly, the band indicating the inactive form of eNOS (pThr495) was increased by RCE treatment. Among the three specific inhibitors, only PD98059 effectively increased the pThr495 band in HUVECs more than RCE alone (Fig. 6). These specific responses have to be studied thoroughly.

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초록 : 복분자 추출물에 의한 내피세포 NO 합성효소의 활성과 발현 증가

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본 연구는 복분자의 수용성 추출물(RCE) 이 배양된 제대정맥내피세포에서 내피세포 NO 합성 효소(eNOS) 의 발현과 활성에 미치는 효과를 연구하고, 이러한 RCE의 효과가 어떤 신호전달 과정을 거치는지를 밝히기 위한 것이다. 연구에 따르면 RCE가 제대정맥내피세포에서 NO의 생성을 증가시키는데 이는 iNOS 보다는 eNOS의 활성화에 의한 것임을 이들의 특이 억제제를 사용한 실험으로 확인할 수 있었다. 나아가 eNOS에 의한 NO 생성 증가는 이 효소의 활성 증가뿐만 아니라 mRNA 수준에서의 발현증가에도 기인함을 확인할 수 있었다. PKC-특이 억제제인 RO-317549는 RCE에 의한 NO 생성의 증가에 별다른 영향을 주지 않았으나, 에스트로젠 수용체-특이 억제제인 Tamoxifen, ERK-특이 억제제인 PD98059와 PI3K/Akt-특이 억제제인 LY-294002는 제대정맥내피세포에서 RCE에 의해 증가된 NO 생성을 억제하였으며, 이는 두 저해제가 eNOS의 활성형인 pSer1177의 양을 감소시키며, 특히 PD98059는 비활성형인 pThr495의 양도 증가시키기 때문임을 알 수 있었다. 이상의 결과로써, 복분자 수추출물은 사람의 제대정맥내피세포에서 NO 생성을 증가시키며, 이는 eNOS의 발현을 증가시킬 뿐만 아니라, ERK와 PI3K/Akt의 신호전달과정을 거쳐서 eNOS의 활성도 증가시키기 때문임을 확인할 수 있었다.