Specific Activation of Insulin-like Growth Factor-1 Receptor by Ginsenoside Rg5 Promotes Angiogenesis and Vasorelaxation*

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Background: The mechanism by which ginsenoside Rg5 regulates vascular function remains unclear.

Results: Rg5 increases angiogenesis and vasorelaxation by activating multiple signal transduction pathways downstream of insulin-like growth factor-1 receptor (IGF-1R).

Conclusion: Rg5 promotes endothelial cell function through activation of IGF-1R.

Significance: These findings reveal a mechanism for the positive regulation of vascular function by Rg5-mediated IGF-1R activation.

Ginsenoside Rg5 is a compound newly synthesized during the steaming process of ginseng; however, its biological activity has not been elucidated with regard to endothelial function. We found that Rg5 stimulated in vitro angiogenesis of human endothelial cells, consistent with increased neovascularization and blood perfusion in a mouse hind limb ischemia model. Rg5 also evoked vasorelaxation in aortic rings isolated from wild type and high cholesterol-fed ApoE−/− mice but not from endothelial nitric-oxide synthase (eNOS) knock-out mice. Angiogenic activity of Rg5 was highly associated with a specific increase in insulin-like growth factor-1 receptor (IGF-1R) phosphorylation and subsequent activation of multiple angiogenic signals, including ERK, FAK, Akt/eNOS/NO, and Gα-mediated phospholipase C/Ca2+/eNOS dimerization pathways. The vasodilative activity of Rg5 was mediated by the eNOS/NO/cGMP axis. IGF-1R knockdown suppressed Rg5-induced angiogenesis and vasorelaxation by inhibiting key angiogenic signaling and NO/cGMP pathways. In silico docking analysis showed that Rg5 bound with high affinity to IGF-1R at the same binding site of Rg5. Rg5 blocked binding of IGF-1 to its receptor with an IC50 of ~90 nmol/liter. However, Rg5 did not induce vascular inflammation and permeability. These data suggest that Rg5 plays a novel role as an IGF-1R agonist, promoting therapeutic angiogenesis and improving hypertension without adverse effects in the vasculature.

Endothelial cells line blood vessels, which play an important role in vascular homeostasis and functions such as angiogenesis, vasorelaxation, and vascular inflammation and remodeling. Angiogenesis is a tightly controlled biological process characterized by changes in endothelial cell (EC) behavior that leads to increased growth, migration, and assembly into capillary structures (1). This process is accompanied by vasodilation and hyperemia of pre-existing capillaries (2). Angiogenesis is a pivotal process not only in embryonic development but also in the progression of a variety of pathologic conditions such as ischemic heart disease and wound healing (1). A large number of bioactive molecules, including vascular endothelial growth factor (VEGF), promote angiogenesis via stimulation of ECs to elicit multiple signal pathways (3, 4) and improve tissue and organ function in ischemia has been caused by defective blood circulation.

ECs constitutively express the endothelial isoform of endothelial nitric-oxide synthase (eNOS), in which catalytic activity is regulated by two distinct posttranslational modifications, specifically Ca2+-dependent dimerization and phosphorylation at Ser1177, leading to elevated endothelial NO production (5, 6). EC-derived NO plays a critical role in vasodilation via soluble guanylyl cyclase-mediated cGMP production and angiogenesis via promotion of EC survival (2). Impairments in endothelium-dependent vasodilation and angiogenesis are largely mediated

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by a reduction in the bioavailability of endothelium-derived NO. Indeed, mice lacking eNOS induce hypertension (7) and decrease VEGF-induced angiogenesis (2). Endogenous or exogenous molecules that regulate eNOS activity are suggested to improve hypertension (7).

Insulin-like growth factor-1 receptor (IGF-1R) signaling initiated by ligand binding mediates many crucial cell responses including angiogenesis (9). IGF-1R ligation has been shown to induce angiogenesis and endothelial proliferation in a retina model (9). Moreover, IGF-1R has also been shown to mediate neovascularization in human lung development and zebrafish cardiovascular development (10, 11) and promote vasculogenesis via differentiation of embryonic stem cells into endothelial cells (12). Moreover, a recent study determined that mouse overexpression of human IGF-1R in the endothelium enhances endothelium regeneration after denuding arterial injury and vasorelaxation (13), suggesting that IGF-1R activation is a useful target for treating vascular repair and hypertension disorders. Thus, IGF-1R agonists can be used as a beneficial means to treat human diseases with vascular dysfunction.

Although some ginsenosides present in ginseng are shown to regulate angiogenesis and vasodilation (14, 15), the beneficial effect of ginsenoside Rg5, a compound newly synthesized during the steaming process of Panax ginseng, on vascular function have not yet been studied. In the present study, we extensively investigated the pharmacological effect of Rg5 on angiogenesis and vasorelaxation as well as its cellular targets. We demonstrated that Rg5 regulates neovascularization and hypertension via action as a novel, nonbiological, IGF-1R agonist.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Rg5 was purified by a previously reported method (16), and its purity was >98%. Rg5 solutions (20 and 200 mM) were prepared in dimethyl sulfoxide as a stock solution. All cell culture media and reagents were purchased from Invitrogen. VEGF and basic fibroblast growth factor was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies used in this study were obtained from Cell Signaling Technology (Beverly, MA), R&D Systems (Minneapolis, MN), Santa Cruz Biotechnology (Santa Cruz, CA), and BD Transduction Laboratories (San Diego, CA). Recombinant human IGF-1 and [125I]IGF were obtained from R&D Systems and PerkinElmer Life Sciences, respectively. PD98059, LY294002, pertussis toxin (PTX), BAPTA-AM, U73122, PP2, N6-nitro-l-arginine methyl ester (l-NAME), and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) were from Calbiochem. Fluo-4-acetoxy-methyl (Fluo-4 AM) ester and 4-amino-5-methylaminonaphthalene-1,7'-difluorofluorescein (DAF-FM) diacetate were obtained from Molecular Probes (Eugene, OR). A human phospho-receptor tyrosine assay kit was purchased from R&D Systems.

The siRNAs for human and mouse IGF-1R, human phospholipase C-γ1 (PLC-γ1), and scrambled control were from Santa Cruz Biotechnology. The primers specific for human VEGF, 5′-GTGACATCTTCCAGGAGTA-3′ (sense) and 5′-GGACTCTGTGTGTGTTTGC-3′ (antisense), were purchased from Bioneer (Daejeon, Korea). All other chemicals were obtained from Sigma unless indicated otherwise.

Cell Culture—Human umbilical cords were obtained after full-term normal deliveries under protocols approved by the Institutional Review Board at Kangwon National University Hospital, and informed consent was obtained from all patients. The investigation conforms to the principles outlined in the Declaration of Helsinki. Human umbilical vein endothelial cells (HUVECs) were isolated and grown in M199 as described previously (17), and only passages 2–7 were used. Cells were grown in M199 media supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 ng/ml streptomycin, 3 ng/ml basic fibroblast growth factor, and 5 units/ml heparin at 37 °C under 5% CO2/95% air.

Animals—Seven-week-old male mice (C57BL/6) and ApoE−/−, and ApoE−/− and Sprague-Dawley rats were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained on a standard (normal) chow diet ad libitum in a laminar airflow cabinet under specific pathogen-free conditions. Some ApoE−/− mice were fed a high cholesterol diet (D12108C, Research Diet Inc., New Brunswick, NJ) for 8 weeks. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Ethics Committee of Kangwon National University. Moreover, this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (38).

In Vitro Angiogenesis Assay—Angiogenic activity was determined by measuring cell proliferation, migration, and tube formation as described previously (18). Cell proliferation was determined by a [3H]thymidine incorporation assay. HUVECs were pretreated with various inhibitors for 30 min and stimulated with the indicated concentrations, 20 μM Rg5 or 10 ng/ml VEGF, for 30 h followed by the addition of 0.5 μCi/ml of [3H]thymidine (Amersham Biosciences) for 6 h. H-Labeled high molecular DNAs were determined using a liquid scintillation counter. A chemotactic migration assay was performed using Transwell plates with 6.5-mm-diameter polycarbonate filters (8-μm pore size). The lower surface of the filter was coated with 10 μg of gelatin. The fresh M199 medium (1% FBS) containing the indicated concentrations, 20 μM Rg5 or 10 ng/ml VEGF was placed in the lower wells. HUVECs (1 × 104 cells/μl) were loaded into each of the upper wells. The chamber was incubated at 37 °C for 4 h. Migrated cells were stained with H&E and quantified using a phase-contrast microscope (×100). Tube formation was determined after culturing the HUVECs on a layer of growth factor-reduced Matrigel. Briefly, HUVECs treated with the indicated concentrations, 20 μM Rg5 or 10 ng/ml VEGF were plated onto the layer of Matrigel at a density of 2 × 103 cells/well. After 20 h, tube formation was observed by an inverted phase-contrast microscope (×40) and quantified using Image-Pro Plus, version 4.5 (Media Cybernetics, San Diego).

Ex Vivo and in Vivo Angiogenesis Assay—An aortic ring sprouting assay was performed using a modified method based on a previous report (19). Sprague-Dawley (7-week-old male) were deeply anesthetized with inhaled halothane (5%) and then humanely sacrificed. Dorsal aortas were isolated and carefully

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cut into 1-mm rings. The aortic rings were placed in the 48-well plates precoated with 120 μl of Matrigel, sealed in place with an overlay of 50 μl of Matrigel, and incubated with Rg5 (40 μM) or VEGF (20 ng/ml) in serum-free medium. On day 8, newly formed vessels were fixed and stained with FITC-labeled isoelectin B4. The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. A Matrigel plug assay was performed as described previously (20). C57BL/6J mice were injected subcutaneously with 400 μl of Matrigel containing 10 units of heparin combined with either 200 nmol of Rg5 or 100 ng of VEGF under anesthesia with pentobarbital (50 mg/kg, intraperitoneal injection). After 7 days, mice were sacrificed by cervical dislocation, and Matrigel plugs were carefully removed and photographed. Hemoglobin was measured using Drabkin’s reagent (Sigma-Aldrich) for quantification of blood vessel formation. Neovascularization was determined by intravital fluorescence microscopy as described previously (18). C57BL/6J mice were anesthetized by inhalation of 1.5% isoflurane and O2-N2O using a vaporizer (Surgivet, Waukesha, WI), and titanium-based imaging windows were surgically implanted between the skin and abdominal wall of the mice. Matrigel (100 μl) containing Rg5 or VEGF was applied to the inner space of the window, which was surgically implanted between the skin and abdominal wall of the mice. After 4 days, neovascularization was recorded by a Zeiss Axiowert 200M microscope (Carl Zeiss) after intravenous injection of 50 μl of 25 mg/ml FITC-labeled dextran (molecular mass, 250,000 Da) via the tail vein.

Mouse Model of Hind Limb Ischemia—Rg5 was delivered into the right gastrocnemius muscle of C57BL/6J mice by three injections (total, 300 μmol of Rg5/100 μl/mouse) following double ligation of the superficial femoral artery proximal to the deep femoral artery and distal femoral artery under anesthesia (100 mg/kg ketamine mixed with 2 mg/kg xylazine delivered by intraperitoneal injection). Sham-operated control animals were subjected to the same surgical protocol, but the femoral artery was not ligated. Pain caused by the surgical procedures was managed preoperatively and 1 and 2 days after surgery by intraperitoneal injection. Sham-operated control animals (100 mg/kg ketamine mixed with 2 mg/kg xylazine delivered by intraperitoneal injection). After 7 days, the gastrocnemius muscles were surgically removed and photographed. Hemoglobin was measured using Drabkin’s reagent (Sigma-Aldrich) for quantification of blood vessel formation. Neovascularization was determined by intravital fluorescence microscopy as described previously (18). C57BL/6J mice were anesthetized by inhalation of 1.5% isoflurane and O2-N2O using a vaporizer (Surgivet, Waukesha, WI), and titanium-based imaging windows were surgically implanted between the skin and abdominal wall of the mice. Matrigel (100 μl) containing Rg5 or VEGF was applied to the inner space of the window, which was surgically implanted between the skin and abdominal wall of the mice. After 4 days, neovascularization was recorded by a Zeiss Axiowert 200M microscope (Carl Zeiss) after intravenous injection of 50 μl of 25 mg/ml FITC-labeled dextran (molecular mass, 250,000 Da) via the tail vein.

Aortic Vascular Tension Assay—Male C57BL/6J wild type and eNOS−/− mice were anesthetized in a closed chamber ventilated with 2.5% isoflurane for 3–5 min, and the thoracic aorta was rapidly removed. The aortas were placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution and cleared of adherent connective tissues. The mouse aortas were cut into 1.5-mm rings and incubated for 30 min in DMEM containing Rg5 (20 μM) in the presence or absence of 1-NAME (1 mM) or ODQ (10 μM). Some aortic rings were transected with IGF-1R or scrambled siRNA using Lipofectamine 2000 reagent followed by incubation with Rg5. The aortic rings were suspended between two wire stirrups (150 μm) in a multiwire myograph system (DMT-USA Inc., Ann Arbor, MI) in 10 ml Krebs-Ringer buffer (95% O2, 5% CO2, pH 7.4, at 37 °C). One stirrup was connected to a three-dimensional micromanipulator, and the other was attached to a force transducer. The rings were passively stretched at 10-min intervals in increments of 200 mg to reach optimal tone (600 mg), and the response to a maximal dose of KCl was used to normalize the responses to agonist across vessel rings. Dose responses to the vasodilators acetylcholine (Ach) (10−9–10−5 M) were performed after preconstriction with U46619 (10−8 M) or phenylephrine (1 μM). At the end of the experiments, NO-dependent vasorelaxation activity was confirmed by adding the guanylyl cyclase inhibitor ODQ. Data were collected online using a MacLab system and analyzed using dose response software (AD Instruments).

Monocyte-Endothelial Cell Interaction—Monocytes (U937 cells) were labeled with 5 μM calcine-AM in RPMI 1640 containing 10% FBS at 37 °C for 1 h and washed twice with PBS by centrifugation. HUVECs were stimulated with Rg5 (20 μM) or VEGF (10 ng/ml) in 24-well plates for 8 h and then incubated with labeled monocytes (1 × 106 cells/ml) at 37 °C for 30 min. Non-adherent cells were removed by washing with RPMI 1640, and plates were photographed by fluorescence microscopy.

Endothelial Cell and Vascular Permeability Assays—[14C]Sucrose permeability in HUVECs was determined using Transwell plates (18). Confluent HUVECs in the upper compartment of Transwell plates were treated with Rg5 (40 μM) or VEGF (20 ng/ml) for 1 h. [14C]Sucrose (50 μl at 0.8 μCi/ml) was added to the upper compartment. The amount of radioactivity that diffused into the lower compartment after 30 min was determined using a liquid scintillation counter. For assaying Miles vascular permeability (18), Evans blue dye (100 μl of a 1% solution in 0.9% NaCl) was injected into the tail vein of C57BL/6J mice. After 10 min, 10 μl of Rg5 (100 μM) or VEGF (50 ng) was injected intradermally into the shaved back skin of mice. After 30 min, the animals were euthanized, and an area of skin (1 × 1 cm) that included the blue spot resulting from leakage of the dye was removed. Evans blue dye was extracted from the skin by incubation with formamide for 4 days at room temperature, and the absorbance of the extracted dye was measured at 620 nm with a spectrophotometer.

IGF-1 Binding Assay—HUVECs were cultured in 24-well plates overnight. The cells were changed to serum-free M199 and incubated for 1 h. The medium was removed, and the cells were incubated with fresh serum-free medium containing 1 × 10−7–5 × 10−2 M Rg5 at 37 °C for 20 min followed by the addition of 50 μl (1 μCi) of [125I]IGF-1 and then further incubated for 10 min. The medium was decanted, and cell plates were washed twice with serum-free medium. Cells were lysed in 300 μl of 0.1 N NaOH solution containing 0.1% SDS, transferred to scintillation vials, and mixed with 1 ml of Ultima Gold™ mixture solution (PerkinElmer Life Sciences). Cell-associated [125I]IGF-1 was analyzed in a scintillation counter. The nonspecific binding was determined by co-incubation with unlabeled IGF-1 (50 nm).

Measurement of [Ca2+]i, NO, and cGMP—The changes of [Ca2+]i were monitored by a confocal microscope (Olympus FV-300) according to a previously described method (21).
Briefly, cells grown on round cover slips were incubated with the Ca^{2+} probe Fluo-4 AM ester (2 μM) in M199 containing 1% FBS for 30 min and treated with 20 μM Rg5 in the presence or absence of several inhibitors. Then, [Ca^{2+}], was monitored using a confocal microscope. The levels of cellular NO production were measured in situ by using DAF-FM diacetate. Briefly, after treatment with or without 20 μM Rg5 and 1 mM L-NAME for 1 h, HUVECs were incubated with 5 μM DAF-FM diacetate for 30 min at 37 °C. After the excess probe was removed, the relative levels of intracellular NO were determined from the fluorescence intensity of DAF-FM by confocal microscopy. cGMP levels in the cell extract and vascular ring extracts were measured using a commercial ELISA kit.

Docking Simulations—Blind docking of Rg5 to IGF-1R (PDB code 1IGR) was performed using Autodock 4.2 with a Lamarckian genetic algorithm (22). A grid box of 0.2 Å spacing was set up to encompass the whole IGF-1R molecule. The number of energy evaluations was increased to 50 million to account for the large number (17) of rotatable bonds in Rg5. Chimera software was used for the graphic presentation (23).

Other Analytical Methods—Western blotting and immunoprecipitation were performed (17–19), and dimer and monomer of eNOS were separated using low temperature SDS-PAGE as described previously (24). All blots are representative of two to three independent experiments. The level of VEGF mRNA was determined by RT-PCR with primers specific for human VEGF.HUVECs were also transfected with 100 nM IGF-1R or scrambled siRNA using microporation (NanoEntek, Seoul, Korea) according to the manufacturer’s protocol. For the promoter assay, HUVECs were transiently transfected with a VEGF promoter (2.6 kb)/luciferase reporter construct by a Lipofectamine method. After 24 h, cells were treated with Rg5 (20 μM) or TNF-α (10 ng/ml) for 12 h. Promoter activity was measured in cell lysates by assaying the luciferase activity.

Statistical Analysis—Data are presented as means ± S.D. of at least three independent experiments. Statistical significance was determined using Student’s t test for unpaired observations between two groups or by analysis of variance with the Bonferroni correction for multiple group comparisons. p values of <0.05 were considered significant.

RESULTS

Rg5 Increases Angiogenesis in Vitro, ex Vivo, and in Vivo—When the angiogenic activities of 11 ginsenosides isolated from ginseng were examined in an EC culture system, Rg5 exhibited the strongest cell proliferation activity as compared with other ginsenosides (Fig. 1). Rg5 increased EC proliferation in a dose-dependent manner (Fig. 2A) as well as promoting chemotactic migration and tube formation (Fig. 2, B and C). The responses of ECs to 20 μM Rg5 were higher than the responses to 10 ng/ml VEGF (Fig. 2, A–C), suggesting that Rg5 stimulates the in vitro angiogenic behavior of ECs. We next examined whether Rg5 regulates angiogenesis ex vivo and in vivo. Rg5 significantly stimulated vessel sprouting in the cut edge of ex vivo explanted rat aortic rings, as compared with control (Fig. 2D). The Matrigel plug assay in mice was performed to evaluate the in vivo angiogenic activity of Rg5. Matrigel plugs containing Rg5 appeared dark red in color in the vessels, with increased hemo-globin inside the plugs, as compared with control plugs, indicating that Rg5 promotes extensive neovascularization (Fig. 2E).

Docking Simulations—Blind docking of Rg5 to IGF-1R (PDB code 1IGR) was performed using Autodock 4.2 with a Lamarckian genetic algorithm (22). A grid box of 0.2 Å spacing was set up to encompass the whole IGF-1R molecule. The number of energy evaluations was increased to 50 million to account for the large number (17) of rotatable bonds in Rg5. Chimera software was used for the graphic presentation (23).

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RESULTS

Rg5 Activates Multiple Angiogenic Signaling Pathways—Various angiogenic factors induce multiple signal pathways responsible for the survival, proliferation, migration, and tube formation of ECs (8, 25). We investigated whether Rg5 regulates the activation of angiogenic signal pathways. Treatment with Rg5 increased phosphorylation-dependent activation of...
ERK, Akt, eNOS, Src, FAK, and paxillin in a time-dependent manner (Fig. 4A). Activation of ERK, Akt, and Src was observed at 10 min; however, eNOS, FAK, and paxillin phosphorylation occurred 20 min after Rg5 stimulation. Because phosphorylated eNOS increases NO production, which is involved in angiogenesis (2, 3, 25), we examined the effect of Rg5 on eNOS-dependent NO production. Treatment with Rg5 promoted an increase in NO production, the effect of which was abrogated by co-treatment with the NOS inhibitor L-NAME (Fig. 4). Similarly, proliferation of ECs by Rg5 was inhibited by PD98059, LY294002, and L-NAME, and ERK, Akt, and FAK were suppressed by the inhibitors of MEK (PD98059), PI3K (LY294002), and Src (PP2), respectively (Fig. 4B). Activation of ERK, Akt, and FAK was suppressed by the inhibitors of MEK (PD98059), PI3K (LY294002), and Src (PP2), respectively (Fig. 4C). Similarly, proliferation of ECs by Rg5 was inhibited by PD98059, LY294002, and L-NAME, and the endothelial cell migration was suppressed by LY294002, PP2, and L-NAME. In addition, Rg5-mediated tube formation of ECs was significantly decreased by all of these inhibitor (Fig. 4D). These results suggest that Rg5 promotes angiogenesis by activating multiple signaling pathways, such as PI3K/Akt/eNOS, MEK/ERK, and Src/FAK/paxillin.

Rg5-mediated Angiogenesis Requires G i Protein, PLC, and Ca 2+ Mobilization—Some ginsenosides promote angiogenesis via activation of the glucocorticoid receptor or G i protein-mediated signaling (15, 26). We determined whether these mediators were involved in Rg5-induced angiogenesis. Rg5-induced activation of ERK and FAK (but not Akt and eNOS) was blocked by treatment with the G i protein inhibitor PTX but not by the glucocorticoid receptor inhibitor RU486 (Fig. 5A). Activation of the G i protein complex stimulates PLC activation and Ca 2+ mobilization, resulting in promotion of cell growth and migration (27, 28). We next examined whether Rg5 regulates the G i protein-mediated PLC/Ca 2+ pathway. Stimulation of ECs with Rg5 resulted in a significant increase in [Ca 2+] i, which was inhibited by PTX, siRNA-mediated PLC-γ1 knockdown, and the Ca 2+ chelator BAPTA-AM (Fig. 5B). In addition, Rg5-induced ERK activation was inhibited by the PLC inhibitor U73122, but not by PP2 and BAPTA-AM. However, all of these inhibitors suppressed Rg5-induced activation of FAK, but not Akt (Fig. 5C). Because [Ca 2+] i plays an important role in the active dimer formation of eNOS, we assessed the effects of Rg5 on eNOS dimerization and NO production. Rg5 increased the eNOS dimer/monomer ratio and intracellular NO level, which were suppressed by PTX, U73122, and BAPTA-AM (Fig. 5D). Furthermore, these inhibitors and PLC-γ1 knockdown significantly suppressed proliferation, migration, and tubule formation of ECs stimulated with Rg5, with the exception of the non-inhibitory effect of PP2 on Rg5-mediated EC proliferation (Fig. 5E). These results suggest that Rg5 activates the G i protein-dependent and -independent pathways, which are likely responsible for Rg5-induced angiogenesis.

Rg5 Promotes Angiogenesis via IGF-1R Activation—We investigated an angiogenesis-associated membrane receptor, which can be activated by Rg5 using a human phospho-receptor tyrosine kinase array kit. Treatment of HUVECs with Rg5 increased IGF-1R phosphorylation, which can be activated by Rg5 using a human phospho-receptor tyrosine kinase array kit.
resulted in a 4.3-fold increase in IGF-1R phosphorylation and non-significant increases in phosphorylation of insulin receptor and VEGF receptor-2 (Fig. 6A). However, Rg5 did not alter the phosphorylation of other receptors compared with untreated controls (Fig. 6A). We further confirmed that Rg5 elicited increases in IGF-1R and IRS-1 phosphorylation in HUVECs, which were not altered by a neutralizing human IGF-1 antibody (Fig. 6B). These data indicate that Rg5 stimulates IRS-1 phosphorylation via IGF-1R activation. To investigate the possible interaction of Rg5 with IGF-1R, a docking analysis was performed. Docking results showed that Rg5 binds strongly at two sites, A and B, with $K_d$ values of 20 and 27 nM, respectively, to the cysteine-rich domain of IGF-1R (Fig. 6C, left). The stronger binding site A, the hydrophobic moiety of Rg5 was anchored in a hydrophobic patch formed by Phe$^{241}$, Phe$^{251}$, Ile$^{255}$, and Phe$^{266}$, and the sugar moiety formed hydro-
Gen bonds with Lys180, Glu242, and Glu272 (Fig. 6C, top right). At the other binding site B, only Ile282 interacted hydrophobically with Rg5, and the hydrogen-bonding network between Rg5 and the IGF-1R residues, such as Gln321, Gly322, Glu345, Asn346, and Gly349, made a major contribution to the tight binding (Fig. 6C, bottom right). We next examined whether IGF-1R is critically involved in Rg5-mediated angiogenic signal cascades using a siRNA-mediated IGF-1R knockdown method. Specific knockdown of IGF-1R suppressed Rg5-mediated activation of angiogenic signal mediators ERK, Akt, eNOS, and FAK (Fig. 6D) as well as inhibiting Rg5-induced NO production and cGMP generation (Fig. 6E and F). In addition, IGF-1R knockdown significantly suppressed proliferation, migration, and proliferation of ECs stimulated with Rg5 (Fig. 6G). Pretreatment with Rg5 blocked the binding of radiolabeled IGF-1 to HUVECs with an IC50 value of ~90 μmol/liter (Fig. 6H), which was greater than an IC50 value of ~1.4 nmol/liter for unlabeled IGF-1 (data not shown). These results suggest that Rg5 promotes angiogenesis via activation of the angiogenic pathways, probably by directly binding to IGF-1R.

Rg5 Promotes Angiogenesis in Murine Model of Hindlimb Ischemia—We examined the angiogenic effect of Rg5 in a mouse model of experimental hind limb ischemia. Mice treated with Rg5 demonstrated significantly improved blood flow in the ischemic hind limb, compared with control animals (Fig. 7, A and B). Consistent with improved perfusion recovery, ischemic muscle from Rg5-treated mice showed higher capillary density than in control mice (Fig. 7C). These data suggest that Rg5 restores blood perfusion via neovascularization in ischemic tissues.

Rg5 Promotes NO-dependent Vasorelaxation via IGF-1R—Because eNOS-mediated NO production appears to play a critical role in vascular relaxation via cGMP production, we examined whether Rg5 regulates cGMP production in ex vivo cultured mouse aortic rings. Mouse aortic rings incubated with Rg5 increased cGMP production, which was abrogated by cotreatment with L-NAME and transfection with IGF-1R siRNA but not with scrambled siRNA (Fig. 8A). As expected, stimulation with Rg5 potentiated the vasorelaxant response to Ach as compared with control, and this response was significantly inhibited by L-NAME (Fig. 8B). Transfection of aortic rings with IGF-1R siRNA exerted a significant inhibitory effect on Rg5-mediated vasorelaxation as compared with scrambled siRNA (Fig. 8C). Interestingly, the aortic rings from ApoE−/− mice fed a high cholesterol diet showed reduced vasodilatory responses to Ach compared with those from normal diet-fed ApoE−/− mice, and this reduced vasorelaxation was significantly potentiated by treatment with Rg5 (Fig. 8D). In addition, aortic rings from wild type mice were more sensitive to the Rg5-induced vasodilatory response than those from eNOS−/− mice, and this response was significantly abrogated by L-NAME and ODQ (Fig. 8E). These data suggest that Rg5 reduces blood pressure via vasodilation.

DISCUSSION

Some angiogenic inducers, including VEGF, evoke angiogenesis mainly by activating multiple signal pathways, such as MEK/ERK, Akt/eNOS, and Src/FAK, through binding their receptors (20, 25). However, other factors indirectly activate angiogenic signaling by inducing angiogenic factor expression...
In the present study, Rg5 increased angiogenesis and blood perfusion in a mouse hind limb ischemia model by activating the angiogenic signal pathways without VEGF expression. These data suggest that Rg5 is a direct angiogenic inducer. In addition, this compound promoted vasodilation by activating the eNOS/NO/cGMP pathway. However, Rg5 did not increase vascular inflammation and permeability, which are known adverse effects induced during VEGF-based angiogenic therapy. These results suggest that Rg5 promotes therapeutic angiogenesis via activation of common angiogenic signal cascades and vasodilation via eNOS-derived increases in NO production without inducing harmful side effects on vascular function.
Rg5-mediated IGF-1R Activation Promotes Vascular Function

Although some angiogenic compounds elicit the common angiogenic signal pathways of MEK/ERK, Src/FAK, and Akt/eNOS, their apical targets or receptors have not been identified as yet (25, 29). We found here that Rg5 specifically activated IGF-1R, which is associated with regulation of the EC function. Silencing of IGF-1R inhibited Rg5-induced angiogenic events and signaling pathways, indicating that Rg5 promotes angiogenesis by directly binding to IGR-1R. This possibility was confirmed by in silico docking analysis that showed significant interactions of Rg5 with the cysteine-rich domain of IGF-1R. Indeed, we performed 1000 blind docking simulations from which 11 conformations were obtained with a $K_d$ value less than 100 nm and found that all of these were docked at two sites with $K_d$ values of 20 and 27 nm. The higher affinity binding site “A” is exactly the same as the interaction for the C-domain of IGF. This site of interaction occurs with the cysteine-rich domain of IGF-1R, which is associated with regulation of the EC function. These results also suggest that Rg5, rather than a contaminant, is the major natural product responsible for Rg5-induced angiogenesis. These data suggest that Rg5, which has been demonstrated using monoclonal antibodies and site-directed mutagenesis (30), indicating that this receptor evokes dual signaling mechanisms, such as receptor tyrosine kinase and Gi-mediated PLC pathways. Signal cascades induced by IGF-1 mediate many cellular responses, including proliferation, differentiation, and angiogenesis (8, 33), and are also involved in tumor angiogenesis and developmental neovascularization in human and zebrafish (12, 13, 34). A recent study demonstrated that IGF-1R activation by IGF-1 promotes differentiation of stem cells into mesoderm and endothelial progenitor cells into mature ECs, indicating IGF-1 as a potential drug for enabling vascular regeneration (12). Moreover, endothelium-targeted IGF-1R transgenic mice enhance endothelial remodeling and vasorelaxation (13), suggesting that an activator of IGF-1R may be a useful therapeutic strategy to treat vascular repair and hypertension disorders. In this study, we found that Rg5 activated common angiogenic signal pathways via IGF-1R. These signal mediators were independently blocked by their own upstream

Although IGF-1R is known as a receptor tyrosine kinase, its biological activity also requires PTX-sensitive Gi protein (28, 32), indicating that this receptor evokes dual signaling mechanisms, such as receptor tyrosine kinase and Gi-mediated PLC pathways. Signal cascades induced by IGF-1 mediate many cellular responses, including proliferation, differentiation, and angiogenesis (8, 33), and are also involved in tumor angiogenesis and developmental neovascularization in human and zebrafish (12, 13, 34). A recent study demonstrated that IGF-1R activation by IGF-1 promotes differentiation of stem cells into mesoderm and endothelial progenitor cells into mature ECs, indicating IGF-1 as a potential drug for enabling vascular regeneration (12). Moreover, endothelium-targeted IGF-1R transgenic mice enhance endothelial remodeling and vasorelaxation (13), suggesting that an activator of IGF-1R may be a useful therapeutic strategy to treat vascular repair and hypertension disorders. In this study, we found that Rg5 activated common angiogenic signal pathways via IGF-1R. These signal mediators were independently blocked by their own upstream

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inhibitors of PI3K, MEK, and Src, respectively, without cross-inhibition of each other. Such data indicate that these signal pathways are independently activated following the activation of IGF-1R by Rg5. Moreover, Rg5 promoted Ca\(^{2+}\)-dependent eNOS dimerization and NO production, which were mediated by the G\(_i\)-mediated PLC pathway. These data are consistent with previous results in which IGF-1R activated G\(_i\)-mediated PLC activation and Ca\(^{2+}\) mobilization (27, 28). Inhibitors of these signal mediators suppressed Rg5-mediated FAK activation and angiogenesis, suggesting that G\(_i\) protein-mediated elevation of [Ca\(^{2+}\)] is responsible for FAK phosphorylation (35). Thus, Rg5 directly promotes in vivo angiogenesis by activating both receptor tyrosine kinase and G\(_i\)-mediated PLC pathways via IGF-1R.

In the vasculature, NO is produced predominantly by constitutive, Ca\(^{2+}\)-dependent eNOS expressed in ECs and acts as a key determinant of vascular relaxation and angiogenesis (2, 7). The functional involvement of EC-derived NO in angiogenesis is strongly evidenced from results showing that eNOS mediates the angiogenic activity of VEGF (3) and from the observation of reduced angiogenesis in eNOS\(^{-/-}\) mice (2). On the other hand, NO released by the endothelium diffuses into vascular smooth muscle cells and causes vasodilatation by producing cGMP via activation of soluble guanylyl cyclase. In fact, eNOS\(^{-/-}\) mice are hypertensive and lack NO-mediated, endothelium-dependent vasorelaxation (7). Our previous studies show that natural compounds elevate eNOS-mediated NO production and promote angiogenesis and vasorelaxation (17, 25). In addition, several studies effectively demonstrate that the IFG-1R/IGF-1 axis activates the PI3K/Akt/eNOS pathway in endothelial cells and promotes angiogenesis in human and animal development and endothelium-dependent vasorelaxation (9–13, 34, 36, 37). Based on this evidence and our present data, we concluded that the responses of ECs to Rg5 are directly associated with IGR-1R activation. Additionally, L-NAME or eNOS deficiency suppressed the ability of Rg5 to increase NO production and cGMP synthesis or to induce angiogenesis and vasorelaxation, suggesting that eNOS-derived NO is an important axis for Rg5 for exertion of its vascular effects. We also found that the relaxation-response curves for Rg5 are significantly shifted to the left in the vascular rings from ApoE\(^{-/-}\) mice fed a high fat diet, which decreased the eNOS activity (31). This indicates that Rg5 improves vascular endothelial function in cardiovascular diseases associated with reduced eNOS-mediated NO production. Based on the current data, we concluded that Rg5 regulates vascular homeostasis by activating the eNOS/NO pathway via IGF-1R activation.

The regulation of eNOS activity involves a range of posttranscriptional mechanisms. Among them, [Ca\(^{2+}\)]\(^{-}\)-dependent eNOS dimerization and Akt-mediated eNOS phosphorylation at Ser\(^{1177}\) play a predominant role (4, 6). In line with these reports, our data show that Rg5 phosphorylates eNOS at Ser\(^{1177}\) via IGF-1R-dependent activation of the PI3K/Akt pathway, as well as enhancing eNOS dimerization via IGF-1R-mediated increases in Ca\(^{2+}\) mobilization, resulting in elevated NO production. Both mechanisms of action are directly linked to the promotion of angiogenesis and vasorelaxation. These findings suggest that Rg5 increases eNOS-derived NO production via two distinct posttranslational modifications following direct activation of IGF-1R, specifically Akt-dependent eNOS phosphorylation and Ca\(^{2+}\)-mediated eNOS dimerization.

Although IGF-1 was considered a pathogenic mediator of vascular disease in native arteries, increasing evidence indicates that the IGF-1/IGF-1R system protects against endothelial dysfunction, atherosclerotic development, and ischemic myocardial damage (31). Some of these effects are related to the induction of eNOS-derived NO production and the activation of multiple signal pathways. Because IGF-1 circulates almost entirely (>99%) bound to six IGF-1-binding proteins (IGFBP-1–6), the therapeutic effectiveness of exogenous IGF-1 is very limited. Thus, chemical IGF-1R agonists, which do not bind to IGFBPs, need to be developed for therapeutic propose. We found that Rg5 did not elicit any different angiogenic activity in serum-free versus 20% serum-supplemented media (data not shown). These data suggest that the angiogenic ability of Rg5 is not affected by serum components, including IGFBPs. Thus, this compound could be useful for treating vascular disease.

In summary, this work reports for the first time that Rg5 promotes neovascularization, local blood perfusion, and vasorelaxation via direct activation of IGF-1R, suggesting that Rg5 is a novel natural agonist IGF-1R. The present findings also offer a mechanistic explanation of the beneficial effect of Rg5 as a non-biological compound on neovascularization and endothelial function under pathological conditions, namely ischemic vascular diseases and hypertension. Our findings provide a rationale for novel therapeutic approaches, without affecting vascular inflammation and permeability, utilizing Rg5 for cardiovascular diseases caused by endothelial dysfunction such as ischemia and hypertension.

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