Ginsenoside-Rg1 Induces Vascular Endothelial Growth Factor Expression through the Glucocorticoid Receptor-related Phosphatidylinositol 3-Kinase/Akt and β-Catenin/T-cell Factor-dependent Pathway in Human Endothelial Cells

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Ginsenoside-Rg1, the most prevalent active constituent of ginseng, is a potent proangiogenic factor of vascular endothelial cells. This suggests that Rg1 may be a new modality for angiogenesis. Ginsenoside-Rg1 induced angiogenesis has not been elucidated. Here we showed for the first time that Rg1 was a potent stimulator of vascular endothelial growth factor (VEGF) expression in human umbilical vein endothelial cells, and importantly this induction was mediated through a phosphatidylinositol 3-kinase (PI3K)/Akt and β-catenin/T-cell factor-dependent pathway via the glucocorticoid receptor (GR). Rg1 stimulation resulted in an increase in the level of β-catenin, culminating in its nuclear accumulation, and subsequent activation of VEGF expression. Transfection of a stable form of β-catenin (S37A) or the use of a glycogen synthase kinase 3β inhibitor to stabilize β-catenin induced VEGF synthesis, whereas small interfering RNA-mediated down-regulation of β-catenin did not, confirming that the effect was β-catenin-specific. Using a luciferase reporter gene assay, we observed that Rg1 increased T-cell factor/lymphoid enhancer factor transcriptional activity. These events were mediated via a PI3K-dependent phosphorylation of the inhibitory Ser9 residue of glycogen synthase kinase 3β. In addition, the GR antagonist RU486 was able to inhibit Rg1-induced PI3K/Akt and β-catenin activation. These findings provide new insights into the mechanism responsible for Rg1 functions.

Ginseng, the root of Panax ginseng C.A. Meyer, has been a key component in Chinese traditional medicine for more than 1000 years. It is now one of the most extensively used alternative medicines throughout the world and appears in the pharmacopoeias of several countries, including the United States and Europe and employed for cancer, diabetes, and cardiovascular concerns. In the United States, it was the second largest selling herbal supplement in 2001, with gross retail sales of U.S. $62 million (1). The molecular components responsible for ginseng actions are ginsenosides, which are triterpene saponins that have a rigid steroidal skeleton with sugar moieties (2). Within more than 30 different ginsenosides, Rg1 is among the most abundant and active ingredients of Panax ginseng (3). Rg1 has been reported to trigger transcriptional activation of glucocorticoid-responsive element-containing reporter gene, suggesting that Rg1 can activate the glucocorticoid receptor (GR) (4, 5). In prior work, Rg1 has been demonstrated to promote functional neovascularization into a polymer scaffold in vivo and the proliferation, chemoinvasion, and tubulogenesis of endothelial cell in vitro (6, 7). Although these data indicate that Rg1 can be used as a novel therapeutic modality for inducing angiogenesis, for example in wound healing and tissue regeneration, the downstream targets that transmit these proangiogenic effects are not clearly understood.

Angiogenesis is the formation of capillaries as outgrowths from preexisting vasculature; it is a tightly regulated event integral to many physiological and pathological situations, including development, wound healing, and tumor growth (8). Vascular endothelial growth factor (VEGF) is probably the most important angiogenesis inducer because of its potency, selectivity for endothelial cells, and ability to regulate key steps in angiogenesis, including proliferation and migration of endothelial cells (9). Overexpression of VEGF and its receptors promote blood vessel formation, and VEGF inhibition blocks angiogenesis (8). Moreover, a number of other angiogenic cytokines and growth factors act, at least in part, by up-regulating VEGF expression (10).

Recent studies have implicated the Wnt/β-catenin signaling in vessel development in normal and pathological conditions (11). Stability of β-catenin is a critical point in Wnt signaling that is regulated by many cytoplasmic proteins, including glycogen synthase kinase 3β (GSK3β), axin, and adenomatous polyposis coli. In the absence of Wnt, cytoplasmic β-catenin is constitutively degraded by the ubiquitin–proteasome pathway.

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2 The abbreviations used are: GR, glucocorticoid receptor; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; TCF, T cell factor; LEF, lymphoid enhancer-binding factor; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; GSK3β, glycogen synthase kinase-3β; siRNA, small interfering RNA; DN, dominant negative.
Wnt signaling inhibits β-catenin degradation by inactivation of GSK3β as a result of phosphorylation at Ser9. As a result, β-catenin accumulates in the nucleus, where it interacts with T-cell factor (TCF)/lymphoid enhancer factor (LEF) family transcription factors and regulates Wnt target genes (12). Several studies have demonstrated the expression of Wnt ligands and frizzled (Fz) receptors in vascular cells (11). Mice lacking Wnt-2 or Fz-5 display severe vascular abnormalities, including defective placental vasculature, and are embryonic lethal (13, 14). Mutations in human Fz-4 have been linked to familial exudative vitreoretinopathy, a hereditary disorder in which retinal angiogenesis is severely impaired (15). β-Catenin is also frequently observed in the cytoplasm and nucleus of vascular cells during angiogenesis and vessel remodeling in disease states (11). The endothelial cell-specific, conditional inactivation of the β-catenin gene in mice leads to a defective vascular pattern, ultimately resulting in embryonic lethality (16, 17).

In the present study, we sought to characterize the molecular mechanism by which Rg1 mediates VEGF expression in human umbilical vein endothelial cells (HUVEC). We demonstrate a role for Rg1 in the regulation of VEGF expression, and this induction is through the activation of a PI3K/Akt → GSK3β → β-catenin–TCF pathway via the GR.

**EXPERIMENTAL PROCEDURES**

**Experimental Reagents**—Ginsenoside-Rg1 is a reference compound (purity >98%) purchased from the Division of Chinese Material Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Public Health, China. A stock solution of Rg1 (50 mM) was prepared in sterile double distilled H2O. Medium 199, endothelial cell growth supplement, heparin, lithium chloride (LiCl), staurosporine, and the polyclonal anti-endothelial cell growth supplement, heparin, lithium chloride (Lake Placid, NY), respectively. Biotinylated phosphatidylidylinositol 4,5-bisphosphate (PIP2) and the PhosphoSensor kit were purchased from PerkinElmer Life Sciences, whereas growth factor-reduced Matrigel was obtained from BD Biosciences. The polyclonal phospho-specific antibodies to Akt (Ser473) and GSK3β (Ser9) and polyclonal anti-Akt and GSK3β were purchased from Cell Signaling, Inc. (Austin, TX). Alexa Fluor 488-conjugated rabbit IgG was from Molecular Probes, Inc. (Eugene, OR), and peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA).

Human β-catenin was first amplified from a cDNA library by PCR, and site-directed mutagenesis was then used to generate the activated mutant form of β-catenin (S37A). The PCR product was confirmed by DNA sequencing and subcloned into KpnI and BamHI sites of pEGFP expression vector (Clontech, Palo Alto, CA). Small interfering RNA (siRNA) oligonucleotide targeting β-catenin (18) and a nonspecific RNA control were obtained from Dharmaco Research (Lafayette, CO). Dominant negative Akt, the TCF-binding site reporter plasmid (TOPFLASH), and a mutated control reporter (FOPFLASH) were purchased from Upstate Biotechnology. Constitutively active and dominant negative constructs of TCF were kindly provided by Dr. B. Gumbiner (University of Virginia, Charlottesville, VA).

**Cell Culture and Treatments**—HUVEC was obtained from Clonetics (San Diego, CA) and cultured in medium 199 supplemented with 20% fetal bovine serum, 20 μg/ml endothelial cell growth supplement, 90 units/ml heparin, and 1% penicillin/streptomycin/neomycin in a humidified incubator at 37°C with 95% air, 5% CO2. HUVEC between passages 2 and 8 were used in these studies to ensure the genetic stability of a culture. For inhibition assays, HUVEC was pretreated with inhibitors (10 μM LY294002, 10 μM SH-6, 10 mM LiCl, and 10 μM RU486) for 30 min before the addition of Rg1. Cell transfections were performed using Lipofectin reagent (Invitrogen) according to the manufacturer’s instructions. Cells transfected with siRNA duplexes were incubated for 24 h prior to protein level determination or TCF reporter gene assay. To express cDNA construct, 1.5 μg of plasmid DNA was used per 35-mm dish.

**Enzyme-linked Immunosorbent Assay**—VEGF protein in cell lysates and conditioned media were quantified using a commercially available enzyme-linked immunosorbent assay kit (Assay Designs) according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a microplate reader (BMG Labtech, Offenburg, Germany). VEGF protein levels were determined by serial dilutions of recombinant VEGF165 standards assayed at the same time. All experiments were carried out in triplicate.

**Cell Fractionation and Western Blot Analysis**—The purification of membrane, cytosolic, and nuclear fractions of β-catenin was performed using a commercially available kit (Calbiochem) following the manufacturer’s instructions. Calpain I and histone H1 antibodies were used to verify the separation of cytosolic and nuclear fractions, respectively. Protein concentration was determined using protein assay reagent from Bio-Rad. An equal quantity of proteins was separated by SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Biosciences). The membrane was then incubated with the indicated primary antibodies for 3 h, followed by horseradish peroxidase-conjugated secondary antibodies, and revealed by an ECL detection system (Bio-Rad). Where indicated, the membranes were stripped and reprobed with another antibody. The density of the bands was quantified by densitometric analysis using Metamorph software.

**Reverse Transcription-PCR**—Total RNAs from HUVEC were extracted with TRizol LS reagent (Sigma) and reverse transcribed using Superscript III First-strand Synthesis SuperMix (Invitrogen). The primers for human VEGF were 5'–CGA CAT GAA CTT TCT GC-3' (forward) and 5'–CCT CAG TGG GCA CAC ACT CC-3' (reverse), and primers for β-actin were 5'–GGG GGG CCC CTC TAG GCA C-3' (forward) and 5'–TTT GAT GTG TAC CAG CAC GAT TT-3' (reverse), purchased from Invitrogen. The number of amplification cycles during which PCR product formation was limited by template concentration was determined in pilot experiments. Amplification of human VEGF and β-actin cDNA were carried out for 33 and 30 cycles, respectively, with PCR SuperMix (Invitrogen).
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Confocal Microscopy—Cells were grown on coverslips and treated with Rg1 in the presence or absence of inhibitors as described above. Cells were then fixed in 4% paraformaldehyde for 15 min. Primary and secondary antibodies were diluted (1:100) in phosphate-buffered saline, 0.1% Triton X-100, and 2% normal goat serum. Coverslips were mounted on glass slides with fluorescence mounting medium (Vector Laboratories, Burlingame, CA) and viewed using a Zeiss LSM-510 multi-tracking laser-scanning confocal microscope (Frankfurt, Germany).

TOPFLASH Luciferase Reporter Assay—In 6-well plates, cells were transiently transfected with 1.5 μg of the TOPFLASH or FOPFLASH reporter plasmid using Lipofectamine (Invitrogen). As a control for transfection efficiency, 0.5 μg of the β-galactosidase construct was included in each transfection. Cells were harvested 24 h after transfection, and extracts were prepared in 200 μl of reporter lysis buffer (Promega). Luciferase and β-galactosidase activity were assayed according to the manufacturer’s protocol using the luciferase assay kit from Promega. Luciferase activity in each well was normalized to the β-galactosidase activity. All experiments were assayed in triplicates, and the assay was performed in three independent experiments.

Assay for PI3K Activity—PI3K activity was determined using the PhosphoSensor kit according to the manufacturer’s instructions. This kit measures PI3K activity by quantifying the phosphorylation of PIP2. Cellular extracts of HUVEC untreated or treated with Rg1 (150 nM) was incubated for 1 h at room temperature with biotinylated PIP2 (500 nM) and ATP (3 μM). Detection of phosphorylated PIP2 was performed by using fluorescent streptavidin conjugates and measuring the emission of fluorescence at 520 nm (iEMS analyzer; Labsystems). The addition of the generic protein kinase inhibitor staurosporine (10 μM) was used to control for nonspecific background signal.

Tube Formation Assay—HUVEC (1 × 10^5 cells/well) were seeded in a growth factor-reduced Matrigel-coated 24-well plate. Cells were untreated or treated with 150 nM Rg1 or with a combination of Rg1 and various dominant negative constructs or siRNAs for 16 h at 37 °C. Images were captured under phase-contrast microscopy (×10) using a CCD camera. Twelve microscopic fields were randomly selected for each well, and the number of tubelike structures per field was counted.

Statistical Analysis—Each experiment was repeated at least three times, with each experiment yielding essentially identical results. Data were expressed as means ± S.D. Statistical comparisons were carried out by one-way analysis of variance, with Tukey’s least significant difference t test for post hoc analysis (GraphPad software, San Diego). p < 0.05 was considered statistically significant.

**RESULTS**

Ginsenoside Rg1 Induces VEGF Production in HUVEC—Both in vivo and in vitro studies have shown that Rg1 increases neovascularization of endothelial cells (6, 7). Because VEGF is known to be a key activator of angiogenesis, we examined whether VEGF is up-regulated by Rg1. We found that VEGF production was significantly elevated in response to Rg1 stimulation as determined by an enzyme-linked immunosorbent assay (Fig. 1). Moreover, VEGF was mainly secreted outside of the cell, since the concentration of VEGF in tissue culture medium was much higher than that in the cell lysate (Fig. 1).

Treatment with increasing concentrations of Rg1 (50, 150, and 300 nM) increased the level of VEGF protein secretion by 38.0-, 41.2-, and 20.7-fold, respectively (Fig. 1A). In addition, the stimulatory effect of Rg1 on VEGF production in HUVEC was time-dependent. Elevated levels of VEGF were noted at 6 h, became more evident at 12 h, and peaked at 24 h following 150 nM Rg1. Cell lysates were subjected to immunoblotting with antibody against VEGF. cDNA was isolated, treated with 150 nM Rg1 for 6, 12, and 24 h. C (left), cell lysates and total RNA were prepared at 24 h after exposure to 150 nM Rg1. Cell lysates were subjected to RT-PCR for VEGF expression were also carried out using the antibodies and primers described under “Experimental Procedures.” The results confirmed the up-regulation of VEGF protein and mRNA after cells were exposed to 150 nM Rg1 for 24 h (Fig. 1C).

Rg1-mediated Up-regulation of VEGF Involves Accumulation of β-Catenin—To uncover the molecular mechanisms for increased VEGF expression, we focused on signaling events that converge on β-catenin, which has an essential role in the regulation of angiogenesis (11). First, we examined the subcellular distribution of β-catenin in Rg1-treated HUVEC by Western blot analysis on fractionated membrane, cytosolic, and nuclear extracts. Although changes in membrane or cytosolic pools of β-catenin were not detected by this method, a significant amount of β-catenin appeared in the nucleus of HUVEC...
after 1 h of incubation with Rg1 (Fig. 2A). Equal loading of the membrane, cytosolic, and nuclear fractions were confirmed by the presence of VE-cadherin, calpain I, and histone H1, respectively (Fig. 2A). To verify this highly intriguing result, we used immunofluorescence microscopy to directly visualize the localization of β-catenin. As shown in Fig. 2B, strong staining of β-catenin in the nucleus was detected in >90% of the cells following treatment with Rg1, confirming the data from the fractionation experiment (Fig. 2A). Together, these data suggest that Rg1 induced rapid nuclear accumulation of β-catenin in HUVEC.

Since we observed that Rg1-induced accumulation of β-catenin correlated with VEGF expression, we asked whether a reduction of the amount of β-catenin by siRNA would affect VEGF expression. Endogenous β-catenin protein levels were efficiently and specifically reduced in the presence of a specific siRNA for β-catenin (Fig. 3A). No inhibition was observed for nonspecific siRNA (Fig. 3A). Importantly, expression of β-catenin siRNA, but not nonspecific siRNA, reverted the effects of Rg1 on VEGF protein expression (Fig. 3B), indicating that β-catenin was required for Rg1 stimulation of VEGF. Similar results were obtained by measuring VEGF mRNA levels (Fig. 3C).

β-Catenin Stimulates VEGF Expression in a TCF-dependent Manner—In the nucleus, accumulation of β-catenin allows interaction with the TCF/LEF transcription factors and up-regulation of target genes. Hence we studied the effect of Rg1 on the transcriptional activity of β-catenin-TCF. The luciferase reporters, which have a minimal thymidine kinase promoter and either wild type (TOPFLASH) or mutated (FOPFLASH) binding sites for the β-catenin-TCF complex, have been widely used to characterize β-catenin-TCF-dependent gene expression (19). These reporter constructs were transfected into HUVEC, and luciferase activity was determined. The adenomatous polyposis coli mutant colorectal cancer cell line SW480 served as a positive control (data not shown). As shown in Fig. 4A, HUVEC had low basal TCF transcriptional activities, and Rg1 treatment increased TOPFLASH activation by ~2-fold.

To further test if β-catenin-TCF complex formation is necessary for mediating Rg1 effects, we utilized a dominant nega-
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FIGURE 4. Rg1 induces β-catenin-TCF transcriptional activation. A, HUVECs were transfected with 1.5 μg of either the TOPFLASH or FOPFLASH luciferase reporter constructs containing wild-type and mutated TCF binding sites, respectively, together with 0.5 μg of β-galactosidase for normalization of transfection efficiency. 24 h post-transfection, cells were treated with Rg1. In some cases, TOPFLASH or FOPFLASH reporter constructs were transiently transfected with empty vector or vector expressing dominant negative TCF (DN-TCF) or constitutively active TCF (VP16-TCF) into HUVECs. Luciferase and β-galactosidase activities were measured. Values are normalized luciferase activity (TOPFLASH activity minus the activity devoted to FOPFLASH and normalized to the β-galactosidase activity) and are shown as mean ± S.D. of three independent experiments performed in triplicate. B (left), in parallel experiments, the expression of VEGF was shown by immunoblotting of total cell lysates of transfected HUVECs using antibodies to VEGF. β-Actin served as a loading control. B (right), signal intensities were determined by densitometry. Data points shown represent the mean, with error bars representing S.D. (n = 3), *, significant differences from control, with p < 0.05.

Rg1 Treatment Induces Phosphorylation of GSK3β in a PI3K/Akt-dependent Manner—The results of Fig. 2 indicated that β-catenin accumulation in the nucleus was rapid (∼1 h) in response to Rg1 treatment, suggesting that the Rg1-induced β-catenin nuclear translocation in HUVECs is likely to be due to β-catenin stabilization rather than transcription induction. GSK3β is known to phosphorylate β-catenin on N-terminal serine residues, targeting β-catenin for rapid ubiquination and proteasome-mediated degradation (21). Conversely, inactivation of GSK3β by phosphorylation promotes β-catenin protein stability and transcription activity. We therefore investigated whether Rg1-mediated β-catenin up-regulation involves changes in GSK3β phosphorylation. As shown in Fig. 5A, the level of Ser9 phosphorylation of GSK3β was increased significantly after Rg1 treatment. Notably, Rg1 stimulation caused a parallel increase in the cellular level of β-catenin and subsequent VEGF expression (Fig. 5A). Treatment with LiCl, a pharmacological agent well known for its inhibitory effect on GSK3β activity (22, 23), enhanced the cellular level of β-catenin and VEGF expression (Fig. 5A). Next, we asked whether expression of a constitutively active mutant of β-catenin (S37A) that is refractory to protosomal degradation (24) could increase VEGF. Fig. 5B shows that the expression of S37A β-catenin increased β-catenin levels (2.7-fold), TOPFLASH activity (1.8-fold; data not shown), and VEGF expression (2.3-fold) to similar levels as Rg1 treatment, lending additional support for GSK3β-dependent action of Rg1.

Because GSK3β can be inactivated through phosphorylation at its Ser9 residue via a PI3K-dependent activation of Akt, such events have the potential of increasing the amount of free β-catenin (25). Fig. 6A shows that Rg1 stimulated a time-dependent increase in PI3K activity, with a maximal response by 15 min. The Rg1-induced increase of PI3K activity was specific, because it was abrogated by the PI3K inhibi-
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VEGF Is Involved in Rg1-induced Angiogenic Actions in HUVEC—To examine whether Rg1-mediated induction of VEGF plays a role in the angiogenic activity of HUVEC, we used a tube formation assay. This is the most simple and classic angiogenesis assay in vitro and has been described as reminiscent of the multistep process of angiogenesis involving cell adhesion, migration, differentiation, and growth (26). As shown in Fig. 7, endothelial cells aligned and formed tube-like structures on Matrigel, and Rg1 treatment (150 nM) caused a significant increase in tube formation. The angiogenic effect of Rg1 was completely reversed by the addition of VEGF-neutralizing antibody (p < 0.05) but not control IgG, confirming that Rg1 actions were VEGF-dependent (Fig. 7). Importantly, inhibition of PI3K/Akt by LY294002 or DN-Akt or inhibition of β-catenin by β-catenin siRNA markedly attenuated Rg1-stimulated tube formation (Fig. 7). No inhibitory effect on tube formation was observed for nonspecific siRNA. These data indicate a role for VEGF in the angiogenic activity of Rg1 in HUVEC, and the effect was mediated via PI3K/Akt and β-catenin.

Rg1 Effects on β-Catenin Require Glucocorticoid Receptor—Rg1 signals have been shown to be transduced via the GR (4, 5). To investigate the possible role of GR in the course of Rg1-induced angiogenic effect, we used the specific GR antagonist RU486 (10 μM) to block GR-related pathways. The addition of RU486 completely abolished Rg1-induced PI3K activity (Fig. 8A) as well as Akt and GSK3β phosphorylation (Fig. 8B), which is a critical step in Rg1-induced β-catenin activation and subsequent VEGF up-regulation. In support of this observation, RU486 also inhibited β-catenin nuclear accumulation, as revealed by Western blot analysis and immunofluorescent analysis (Fig. 8, C and D), and completely aborted the effect of Rg1 on VEGF expression (Fig. 8E). These studies suggest that the modulation of angiogenesis-related signaling by Rg1 is GR-mediated.

DISCUSSION

Previous study has revealed a proangiogenic role for Rg1 (6, 7). Here we outline a plausible mechanism whereby Rg1-induced angiogenesis of vascular endothelial cells can be explained. We provide the first evidence for a role of Rg1 in the...
induction of VEGF, a critical mediator of angiogenesis, in HUVEC. Furthermore, our data suggest that PI3K/Akt and GSK3β are signaling molecules necessary for the Rg1-mediated up-regulation of β-catenin, its translocation into the nucleus, and changes in VEGF expression in these cells. In support of our data, the PI3K/Akt signaling pathway has been shown to be required for the angiogenic phenotype of Rg1 (7), indicating the functional significance of angiogenic signaling downstream of PI3K/Akt. The concentration of Rg1 (150 nM) used in this study is the optimal stimulatory dose of VEGF in HUVEC. A lower dose is also effective in VEGF induction (Fig. 1). Pharmacokinetics of Rg1 have not been determined in humans. However, the dosage (150 nM; 0.12 g/liter) utilized in this study is similar to what is observed in consuming 0.65–3.65 g of Rg1, based on the oral bioavailability of 3.29–18.4% for Rg1 in animal studies (27, 28).

Increased levels of free β-catenin result in an increased translocation of this protein to the nucleus, where its main task is to activate the TCF/LEF family of transcription factors (29). The present study suggests that β-catenin activity is an important signaling pathway contributing to VEGF induction by Rg1 in assay, and tube-like structure formation. Moreover, mice lacking VEGF exhibit defective vascularity in most organs and are embryonic lethal (8). VEGF binds to a family of class III tyrosine kinase receptors, including Fms-like tyrosine kinase 1 (Flt-1 or VEGFR-1) and kinase insert domain-containing receptor (Flk-2 or VEGFR-2) (32). Recent works have demonstrated the co-expression of VEGF and its receptors in HUVEC, suggesting the presence of an autocrine mechanism of action to modulate angiogenesis (33). Therapeutic approaches that stimulate or inhibit VEGF have produced encouraging clinical results in treating disease resulting from insufficient (e.g. chronic wound) or excessive blood vessel formation (e.g. cancer) (8). Thus, it is rational to explore Rg1 as a source of a novel angiomodulator.

In this report, we also show that the transcriptional activator β-catenin is a novel target of Rg1 signaling. We showed that Rg1 increased β-catenin protein levels as well as β-catenin-dependent transcription. Moreover, this stimulation involves a signaling cascade consisting of PI3K/Akt/GSK3β, suggesting crosstalk between the PI3K/Akt and β-catenin signaling pathways, and GSK3β is the convergent point of these signaling path-
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ways. Studies in several cell types have shown that β-catenin is rapidly degraded by the proteasome in unstimulated cells via a GSK3β-dependent mechanism (21). Our finding that the proteasome inhibitor MG132 (data not shown) and inhibitor of GSK3β LiCl (Fig. 4C) both increased cellular β-catenin levels in HUVEC argues that proteasome-mediated degradation keeps β-catenin levels low in unstimulated cells. In addition, Rg1 caused an increase in the levels of β-catenin in the nuclei within 1 h, which is more consistent with the kinetics of inhibition of β-catenin degradation as opposed to an increase in transcription. It is noteworthy that Rg1, similar to the Wnt signal (12), can activate the β-catenin-TCF pathway. Unlike Wnt, Rg1 stimulates PI3K and Akt, which are not downstream effectors of the Wnt signal. However, the involvement of PI3K/Akt in the genomic but also rapid nongenomic effects resulting in interplay with signaling processes, including PI3K/Akt (37, 38).

Our recent studies (5) showing that Rg1 can exert its genomic effects by binding to GR ligand binding domain and that Rg1 is also able to trigger rapid nongenomic activation of PI3K/Akt through a GR-dependent, transcription-independent mechanism are highly suggestive of this view. It is noteworthy that unlike many other members of the nuclear receptors that are understood, but Rg1 has been shown to be a functional ligand of GR (4, 5). Experiments using the steroid antagonists and siRNA provided further evidence that the action of Rg1 is mediated through GR but not estrogen receptor or progesterone receptor (5). Our findings that Rg1 rapidly induced activation of PI3K/Akt (within minutes) suggest a nongenomic rather than genomic action. Although the rapid nongenomic effects of GR have attracted increasing attention in recent years, the underlying mechanisms of action are not yet clear. One possibility is through a membrane-bound GR. However, the existence of functional GR associated with the plasma membrane has been debated. Membrane-bound GR have so far been detected only in amphibian brain and on immune cells, although specific nongenomic effects are widely observed in humans (37). Another intriguing hypothesis to explain the rapid effects is through the cytosolic GR, which not only causes classical

FIGURE 8. Rg1 signals via GR in HUVEC. Cells were pretreated with the GR antagonist RU486 (RU) (10 μM) for 30 min before Rg1 treatment. A, PI3K activity was determined using a PhosphoSensor kit by quantifying the phosphorylation of PIP2, as described under “Experimental Procedures.” B, the Western blots were carried out using antibodies against phospho-Akt (p-Akt), Akt, phospho-GSK3β (p-GSK3β), or GSK3β. C, nuclear fractions from cells treated with Rg1 in the presence or absence of RU486 were blotted with the anti-β-catenin antibody. Histone H1 served as loading control. D, β-catenin localization was detected by immunofluorescence with the anti-β-catenin antibody. Micrographs were captured with a confocal microscope at ×63 magnification. N, nucleus. Scale bar, 20 μm. E, Western blot was performed with lysates from above using antibodies against VEGF. Equal loading was confirmed by the presence of β-actin. Right, signal intensities determined by densitometry. Data points shown represent the mean, with error bars representing S.D. (n = 3). *, significant differences from control, with p < 0.05.
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synthesis of VEGF and hence angiogenic activity. Whether Rg1 may play a role in other cellular functions via the PI3k/Akt pathway warrants further investigation.

In summary, the findings reported here provide novel information about the mechanisms utilized by Rg1 to control β-catenin signaling pathways that culminate in the production of VEGF. The results shed light on the mechanism of action of Rg1 and also provide a further insight into the possible therapeutic use of Rg1 for inducing angiogenesis, such as in wound healing and tissue regeneration.

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