Diosgenin Induces Hypoxia-Inducible Factor-1 Activation and Angiogenesis through Estrogen Receptor-Related Phosphatidylinositol 3-kinase/Akt and p38 Mitogen-Activated Protein Kinase Pathways in Osteoblasts

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ABSTRACT

Diosgenin, extracted from the root of wild yam (Dioscorea villosa), has been reported to demonstrate an opportunity for medical application. Vascular endothelial growth factor-A (VEGF-A) plays an important role in bone-related angiogenesis, a critical process occurring during bone formation and fracture healing. In this study, we examine whether diosgenin is able to induce VEGF-A expression and to promote angiogenesis in osteoblasts. For murine MC3T3-E1 preosteoblast-like cells, VEGF-A mRNA and protein expression seemed to be significantly elevated in response to diosgenin in a concentration-dependent fashion. Conditioned media prepared from cells treated with diosgenin induced strong angiogenic activity in either in vitro or ex vivo angiogenesis assay. Furthermore, diosgenin treatment increased the stability and activity of HIF-1α protein. Inhibition of HIF-1α activity by transfection with DN-HIF-1α significantly diminished diosgenin-mediated VEGF-A up-regulation. The use of pharmacological inhibitors or genetic inhibition revealed that both the phosphatidylinositol 3-kinase (PI3K)/Akt and p38 signaling pathways were potentially required for diosgenin-induced HIF-1 activation and subsequent VEGF-A up-regulation. It is noteworthy that an estrogen receptor binding assay revealed that diosgenin has the strong ability to replace [3H]estradiol bound to estrogen receptor (IC50, 10 nM). In addition, the specific estrogen receptor antagonists ICI 182,780 (faslodex) and tamoxifen were noted to be able to strongly inhibit diosgenin-induced, src kinase-dependent Akt and p38 MAPK activation. Taken together, such results provide evidence that diosgenin up-regulates VEGF-A and promotes angiogenesis in preosteoblast-like cells by a hypoxia-inducible factor-1α-dependent mechanism involving the activation of src kinase, p38 MAPK, and Akt signaling pathways via estrogen receptor.

The importance of angiogenesis within bone is reflected by such processes as bone growth, repair, and remodelling (Brighton, 1978). Bone-fracture healing requires the restoration of blood supply (Glowacki, 1998), and defects in bone

ABBREVIATIONS: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor-1; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; ER, estrogen receptor; PCNA, peripheral cell nuclear antigen; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methyloxazol-5-yl)-5-(4-pyridyl)-1H-imidazole; LY294002, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one; ICI 182,780, faslodex; HUVEC, human umbilical vein endothelial cell; CM, conditioned medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; αMEM, α-minimal essential medium; PECAM-1, platelet-derived endothelial cell adhesion molecule-1; D-CM, conditioned media derived from diosgenin-stimulated MC3T3-E1 cells; CTL-CM, conditioned medium from control cells; DN, dominant-negative; HRE, hypoxia response element; kb, kilobase(s); OVX, ovariectomized.
liferate in situ and may subsequently differentiate into osteoblasts and migrate into the resorption lacunae (Erlebacher et al., 1995). Such a process provides an intriguing focus regarding the interaction between capillary endothelial cells and bone-derived cells (i.e., osteoblasts and osteoclasts) and such endothelial cells are actively involved in regulating bone metabolism (Erlebacher et al., 1995). These functional interactions between different cells are considered to influence one another via humoral factors as well as by direct cell-to-cell contact.

One of the factors involved in this cell-cell communication is vascular endothelial growth factor-A (VEGF-A) (Wang et al., 1997), an essential mediator of angiogenesis (Ferrara and Davis-Smyth, 1997). VEGF-A binds to its respective tyrosine kinase receptors, Flt-1 (VEGFR1) and Flk-1/KDR (VEGFR2), promoting the mitogenic and chemotactic actions of endothelial cells (Ferrara and Davis-Smyth, 1997). For bone metabolism, it has been reported recently that inactivation of VEGF-A by means of the systemic administration of a soluble-receptor chimeric protein causes complete suppression of blood vessel invasion to the newly forming bone, which is concomitant with impaired trabecular-bone formation and the expansion of the hypertrophic chondrocyte zone, as has been demonstrated at the mouse tibial epiphyseal growth plate (Gerber et al., 1999). VEGF-A is synthesized and secreted by several types of cells, including osteoblasts (Goad et al., 1996; Ferrara and Davis-Smyth, 1997); for example, prostaglandins E1 and E2 increase the level of VEGF-A mRNA and protein in primary cultured rat calvarial cells and RCT-3 osteoblast-like cells (Harada et al., 1994). Furthermore, 1,25-dihydroxy-vitamin D3, insulin-like growth factor-I, and parathyroid hormone also have been reported to induce the synthesis of VEGF-A in primary cultured human osteoblast-like cells and human osteosarcoma Saso-2 cells (Goad et al., 1996; Wang et al., 1997). VEGF has been shown to stimulate primary human osteoblast chemotaxis, differentiation, and antiapoptosis. A recent study suggests that VEGF stimulates bone repair in vivo and is an essential mediator in bone healing (Hiltunen et al., 2003). Based upon these findings, there would seem to be no doubt that VEGF secreted from osteoblasts plays a pivotal role in the regulation of bone metabolism and that VEGF-A can be significantly up-regulated by many kinds of agents and soluble factors in bone-derived cells.

Diosgenin is the primary furostanol saponin found in several plants, including Dioscorea species (yams), fenugreek, and Costus speciosus (Sautour et al., 2004). This compound has been shown to be useful for the maintenance of healthy blood cholesterol levels (Komesaroff et al., 2001), and it is also the starting material for the synthesis of a number of hormonal products, such as dehydroepiandrosterone (Scott et al., 2001). The estrogenic and anti-inflammatory effects of diosgenin have been previously hypothesized because of the molecule’s structural similarity to estrogen (Scott et al., 2001). It is interesting that sustained delivery of diosgenin has been shown, by means of bone histomorphometric analysis and three-point bending analysis, to be able to significantly prevent bone loss to the same extent as estrogen, although not contemporaneously altering the material properties of bone for ovariectomized mice (Higdon et al., 2001). These studies reveal important information as to the efficacy and safety of certain alternative treatment modalities for osteoporosis using diosgenin, and that diosgenin may possibly be used therapeutically for postmenopausal patients to attempt to reduce osteoporotic progression. The molecular mechanism of diosgenin’s activity in bone-derived cells, however, remains largely unknown.

In the present study, we examined the effects and mechanisms of action of diosgenin in the context of VEGF synthesis and the subsequent angiogenic effect. Our results reveal that diosgenin up-regulates VEGF-A and promotes angiogenic activity in MC3T3-E1 cells in a HIF-1α-dependent manner involving the activation of sar kinase, PI3K/Akt, and p38 MAPK through an estrogen receptor.

Materials and Methods

Antibodies and Reagents. Anti-human VEGF antibody and anti-β-actin antibody were purchased from R&D Systems (Minneapolis, MN). Anti-p-Akt1/2/3(Ser473) antibody, anti-Akt1 antibody, anti-p-ERK1/2 antibody, anti-ERK1/2 antibody, anti-p38 antibody, anti-HIF-1α antibody, anti-HIF-1β antibody, anti-estrogen receptor (ER)-α antibody, anti-PCNA antibody, anti-α-tubulin antibody, and goat anti-rabbit IgG rhodamine-conjugated antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-p38 MAPK (pT180/pY182) antibody, mouse VEGF-A ELISA kit, and recombinant VEGF were purchased from R&D Systems, Inc (Minneapolis, MN). Diosgenin (3β-hydroxy-5-spirostene, approximately 95%) and the kinase inhibitors PD98059 and SB203580 were obtained from Sigma (St. Louis, MO). Tamoxifen and LY294002 were purchased from Calbiochem-Novabiochem (Darmstadt, Germany). Matrigel was acquired from Collaborative Research (Bedford, MA). ER antagonist ICI 182,780 was purchased from Tocris Cookson, Inc (Ballwin, MO). The dominant-negative HIF-1α construct was obtained from The American Type Culture Collection (Manassas, VA).

Cell Culture. MC3T3-E1 mouse clonal osteogenic cells were cultured in an optimal minimal essential medium (Invitrogen) containing 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM penicillin, 100 µg/ml streptomycin (Invitrogen). Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. Human umbilical vein endothelial cells (HUVEC), endothelial cell growth medium, trypsin-EDTA, and trypsin-neutralizing solutions were purchased from Clonetics (San Diego, CA). All cell cultures were conducted according to the supplier’s recommendations. Cells were harvested between the third and fifth passages and were subcultured onto fibronectin-coated, 96-well tissue-culture plates 24 h before commencing the experiment. Cell viability was determined microscopically using a trypan-blue exclusion method. The cell numbers were determined by direct counting using a hemocytometer.

Collection of Conditioned Medium. MC3T3-E1 cells were grown in αMEM containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. At 90% confluence, cultured medium was changed to serum-free medium and cells were incubated for a further 24 h. Conditioned medium (CM) was collected, centrifuged to remove any cellular contaminants, and then stored at 80°C until use.

Mouse VEGF-A Immunoassay. Conditioned medium was concentrated with the use of Ultra Centrifugal Filter Devices (Millipore Corporation, Bedford, MA). VEGF-A levels in culture supernatants were assayed using a quantitative sandwich enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. In brief, 50 µl of cell supernatant was incubated with 50 µl of assay diluents for 2 h at room temperature in a 96-well tissue-culture plate coated with a monoclonal antibody against VEGF-A. After five consecutive washes, a conjugate consisting of a polyclonal VEGF-A antibody and horseradish peroxidase was added, and the mix was incubated for 2 h at room temperature. After the subsequent addition of a color reagent, absorbance was measured at 450 nm using a Thermo-Max microplate reader. For standardization pur-
poses, serial dilutions of recombinant murine VEGF-A were assayed at the same time. All experiments were carried out in triplicate.

**Determination of Human Umbilical Vein Endothelial Cell Proliferation.** HUVECs were plated onto six-well dishes (Falcon; BD Biosciences Discovery Labware, Bedford, MA) at a concentration of 2.5 x 10^4 cells/well in M199 medium supplemented with 15% fetal bovine serum. One day after seeding, HUVECs were stimulated with conditioned medium derived from cells that had undergone various treatments, and the conditioned medium had been mixed with M199 medium. Twenty-four hours later, the viable cells were counted by means of a trypsin blue exclusion method. For the trypsin blue exclusion assay, the cells were first washed with PBS, trypsinized, and then resuspended in 1 ml of PBS. The numbers of clear and trypsin blue-stained cells were then enumerated using a hemocytometer.

**RNA Isolation and Reverse Transcriptase-PCR.** Total RNA was isolated by using RNAzol B reagent according to the manufacturer’s instructions. Total RNA (3 μg) was reverse transcribed into single-stranded cDNA using a Mooney murine leukemia-virus reverse transcriptase and random hexamers (Promega, Madison, WI). The primer sequences are shown in Table 1. Primers were used at a final concentration of 0.5 μM. Reaction mixture was first denatured at 95°C for 10 min. The PCR conditions applied were 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Western Blot Analysis.** MC3T3-E1 cells were incubated in serum-free αMEM for 24 h before treatment with diosgenin (2 μM), and cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 100 mM NaCl) at a concentration of 0.5 x 10^6 cells/ml. The lysates were isolated by using RNAzol B reagent according to the manufacturer’s instructions. Total RNA (3 μg) was reverse transcribed into single-stranded cDNA using a Mooney murine leukemia-virus reverse transcriptase and random hexamers (Promega, Madison, WI). The primer sequences are shown in Table 1. Primers were used at a final concentration of 0.5 μM. Reaction mixture was first denatured at 95°C for 10 min. The PCR conditions applied were 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Promoter Activity Assay.** Two VEGF-A promoter reporters were kindly provided by Dr. Amit Maity (University of Pennsylvania) (Maity et al., 2000). For cell transfections, MC3T3-E1 cells were seeded in six-well plates. After reaching approximately 70% confluence, the cells were transfected with pGL3-1.5kbVEGFprom or pGL3-1.2kbVEGFprom promoter reporters using TransFast (Promega, Madison, WI); after transfection, the medium was replaced by fresh normal growth medium, and the cells were incubated for 24 h. Cells were then subjected to starvation in serum-free medium for a period of 16 h, after which the cells were treated with diosgenin (2 μM) for a period of 2 h, and the cells were harvested with passive buffer. After this, the luciferase activity was determined by using a dual-luciferase reporter assay system (Promega). **Immunofluorescence Staining.** Cells were cultured to 60 to 80% confluence on degreased glass coverslips in regular culture medium and then subjected to starvation for 16 h, after which cells were treated with diosgenin (2 μM) for a period of 90 min. Cells were then fixed in methanol/acetic acid [3:1 (v/v)] for 30 min at 4°C and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. After this, the cells were then rinsed and blocked for one h in 5% fetal bovine serum at room temperature. The cells were then incubated with anti-HIF-1α polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight, after which cells were washed in PBS and then incubated with a secondary rhodamine-conjugated antibody (1:100, Sigma) for 2 h at room temperature. After extensive washing, the coverslips were inverted onto glass slides using Mowiol (Calbiochem) as a mounting medium. The slides were examined with a fluorescence microscope; Hoechst 33258 was used to stain nuclei.

**Estrogen Receptor Binding Assay.** The whole-cell estrogen receptor binding assay was carried out as described previously (Gierthy et al., 1996). MC3T3-E1 cells were suspended in serum-free medium and seeded into 24-well plates at a density of 2 x 10^4 cells per well. Approximately 16 h later, the medium was changed to serum-free medium containing 1 nM [2,4,6,7,16,17-3H]E2 (118 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) and diosgenin or unlabeled PGE2, and the medium was incubated at 37°C for 3 h. The cultures were washed and solubilized using ethanol. Cellular radioactivity was determined using a Beckman LS 6000 IC scintillation counter (Beckman Coulter, Inc., Fullerton, CA). The amount of [3H]PGE2 bound to estrogen receptors was calculated.

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**Table 1**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Mouse VEGF-A</td>
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<tr>
<td>Forward</td>
<td>5′-CCTGGTTCTGGCGCCGGCTGA-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GTCGCGCGGCTTCCCATAC-3′</td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
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<tr>
<td>Forward</td>
<td>5′-CTGCTGGCTCTGGCGCCGGCTGA-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GTCGCGCGGCTTCCCATAC-3′</td>
</tr>
<tr>
<td>Human erythropoietin</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-ATGAGGAGGCCGCCGCCGG-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GATGAGGAGGCCGCCGCCGG-3′</td>
</tr>
<tr>
<td>Mouse HIF-1α</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-CCTGGTTCTGGCGCCGGCTGA-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GTCGCGCGGCTTCCCATAC-3′</td>
</tr>
</tbody>
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after correcting for nonspecific binding as measured by the amount of bound [3H]PGE2 in the presence of 200-fold excess unlabeled PGE2. Data are expressed as the ratio of bound [3H]PGE2 in the presence of a competitor to the bound [3H]PGE2 in the control cell medium (0.1% dimethyl sulfoxide) × 100. Triplicate probes were run at each dose level.

Fractionations of Cells and Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared by using a nonionic detergent method. In brief, nuclear extracts were prepared from MC3T3-E1 cells in extraction buffer (10 mM KCl, 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, and 0.5 mM dithiothreitol plus protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride). After centrifugation at 14,000 rpm in a microcentrifuge tube for 1 min, the supernatant was cytosol protein-extracted and then placed into extraction buffer (420 mM NaCl, 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, and 0.5 mM PMSF). After centrifugation at 14,000 rpm for 5 min, the supernatant fraction was harvested as the nuclear protein extract and stored at −70°C until use. Electrophoretic mobility shift assay for HIF-1 DNA binding in MC3T3-E1 cells was performed using the annealed and [32P]dCTP end-labeled HIF-1 consensus VEGF-A promoter (underlined) oligonucleotides probe (5'-CCA CAG CAT ACG TGG GCT CCA ACA-3', 3'-GGT GTC GTA CCA GGA GGT TGT-5') in a 20-µl reaction mixture containing 10–15 µg of protein of nuclear extract and 2 µg of poly(dI-dC) for 20 min at room temperature. For competition experiments, a 5-fold excess of unlabeled oligonucleotide was added to the binding reactions. The reaction products were analyzed by 5% nondenaturing PAGE using 12.5 mM Tris, 12.5 mM boric acid, and 0.25 mM EDTA, pH 8.3, for a period of 4 to 5 h at 280 to 300V/10 to 12 mA. The gels were then dried and exposed, for an appropriate time period, to Amersham blue autoradiography film (GE Healthcare) at −70°C whereas using an intensifying screen.

**Results**

**Diosgenin Induces VEGF-A Production in MC3T3-E1 Cells and Promotes Angiogenesis.** As described previously (Higdon et al., 2001), diosgenin has been shown to be capable of preventing ovariectomy-induced osteoporosis in rat. VEGF-A production and subsequent angiogenesis play a critical role in the process of bone growth, repair, and remodeling (Gerber et al., 1999). Thus, in an attempt to clarify whether diosgenin induces VEGF-A production and pro-
promotes subsequent angiogenic activity in osteoblastic cells, MC3T3-E1 cells were treated with diosgenin or not and were assessed for VEGF-A mRNA production and protein expression by, respectively, reverse transcriptase-PCR and Western blotting. Time-dependent studies pertaining to the incubation of MC3T3-E1 cells with diosgenin (2 μM) for various periods of time, revealed that VEGF-A mRNA induction was initially detected 3 h after diosgenin exposure, became more evident at 9 h, and peaked at 12 h after exposure (Fig. 1A). In comparison, the level of VEGF-A protein was noted to be elevated at 3 h, approached maximal levels at 12 h, and remained elevated until approximately 24 h after diosgenin treatment (Fig. 1A). In addition, the stimulatory effect of diosgenin on VEGF-A production in MC3T3-E1 cells seemed to be dose-dependent in the range between 0.5 and 2.5 μM (Fig. 1B). Furthermore, by the application of a mouse VEGF-A immunoassay, we were also able to detect the production of secreted VEGF-A in response to diosgenin stimulation. As shown in Fig. 1C, we further observed that the elevated levels of VEGF-A secretion that occurred as a consequence of diosgenin stimulation paralleled the increased expression of VEGF-A protein, indicating that a mature and functionally active VEGF-A protein was simultaneously being generated in diosgenin-treated cells. These data clearly demonstrate that diosgenin regulates VEGF-A gene expression in MC3T3-E1 cells in a time- and dose-dependent manner.

To examine whether diosgenin-treated MC3T3-E1 cells exhibited any significant angiogenic activity, we collected the CM from cells with or without diosgenin treatment to perform specific angiogenesis assays, such as proliferation and tube-like cell cord formation of HUVECs in vitro. As shown in Fig. 2A, conditioned media derived from diosgenin-stimulated MC3T3-E1 cells (D-CM) significantly increased the proliferation of HUVECs compared with the case for the control group (CTL-CM), and D-CM-induced HUVEC proliferation seemed to be greatly attenuated by the presence of VEGF-A neutralizing antibody (500 ng/ml) but not by control IgG. Although diosgenin seemed to induce HUVEC proliferation slightly (Fig. 2A, column 5), the extent of this induction did not seem to differ statistically compared with the corresponding control value (Student’s t test analysis; P > 0.05).

![Graph](https://example.com/graph.png)

Fig. 2. Effect of diosgenin-induced VEGF-A on HUVEC cells. A, HUVEC cell proliferation was measured after treatment with CTL-CM or D-CM in the presence or absence of VEGF-A (500 ng/ml) neutralizing antibody. Trypan blue exclusion method was performed to determine the level of HUVEC cells proliferation. Columns, means of at least three independent experiments performed in triplicate; bars, S.D. *, statistically significant increase compared with the corresponding control value (P < 0.05); #, statistically significant decrease compared with the corresponding D-CM value (P < 0.05).

B, the assay was performed in the presence of either CTL-CM or D-CM (which were collected as described under Materials and Methods), plus VEGF-A neutralizing antibody or mouse IgG for a period of 6 h. The experiment was conducted three times with similar results. C, quantitative results are derived from B. Columns, means of three independent experiments; bars, S.D. *, statistically significant increase compared with the corresponding value of CTL-CM (P < 0.05); #, statistically significant decrease compared with the corresponding value of D-CM (P < 0.05).
The effect of D-CM on the morphological differentiation of HUVECs was investigated by application of a tube-formation assay. HUVECs were placed onto growth factor-reduced Matrigel combined with D-CM or not for 6 h. D-CM stimulation led to the formation of elongated and robust tube-like structures (Fig. 2B, II), which were organized by a much larger number of cells than was the case for the control (Fig. 2B, I). In addition, we also found that D-CM–induced formation of tube-like cell cords was almost completely disrupted by VEGF-A–neutralizing antibody but not by mouse IgG (Fig. 2B, III-IV). Quantitation of these cell cords as described previously (Taraboletti and Giavazzi, 2004) revealed that D-CM induced enhanced tube-like structure formation (Fig. 2C, 5.4-fold induction compared with control) and that this effect was almost completely inhibited by VEGF-A–neutralizing antibody. The above data strongly suggested that diosgenin-induced angiogenic effects require the presence of VEGF-A in the in vitro assay.

Next, we used an ex vivo assay to confirm the angiogenic ability of diosgenin by means of the use of fetal bone explants, as reported by Deckers et al. (2001). To study the fate of the endothelial cells, dissected fetal metatarsals were cultured for 14 days and then stained with the endothelial cell marker PECAM-1. Treatment with D-CM resulted in a more densely branched network of PECAM-1–positive tube-like structure formation (Fig. 3B) compared with CM derived from untreated cells (Fig. 3A). The angiogenic response of cultures treated with D-CM was inhibited by treatment with VEGF-A–neutralizing antibody but not by mouse IgG (Fig. 3C, and D). These data indicate that diosgenin promotes angiogenic activity for MC3T3-E1 cells; such promotion is mediated by means of the stimulation of the production of mature and functionally active VEGF-A.

**Diosgenin Promotes HIF-1α Protein Stability and Induces HIF-1 Activation.** HIF-1α, a pivotal transcription factor, is a dominant regulator of VEGF-A gene expression for a number of cell types including osteoblasts (Kim et al., 2002). We therefore sought to determine whether HIF-1α was involved in diosgenin-induced VEGF expression in our cell model. To this end, we treated MC3T3-E1 cells with diosgenin for the times indicated in Fig. 4A and examined the expression level of HIF-1α mRNA and protein using RT-PCR and Western blot analysis, respectively. Results revealed that regulation of HIF-1α by diosgenin occurred in a time-dependent manner at protein level (Fig. 4A, top) but not at mRNA level (Fig. 4A, bottom). Regulation of HIF-1α seems to occur principally at the protein level by HIF-1α stabilization and activation (Jiang et al., 1996). We therefore attempted to determine HIF-1α protein stability and activity in response to diosgenin in MC3T3-E1 cells. To analyze the effect of diosgenin on HIF-1α protein stability, MC3T3-E1 cells were treated with diosgenin for 90 min, after which cycloheximide was added for a further 15 to 60 min to block ongoing protein synthesis. According to the Western blot analyses conducted thereafter and the resultant quantitative data elicited (Fig. 4B; quantification by Edit EZ-1D Software from EZLab Technology Co., Ltd., Taipei, Taiwan), the half-life of HIF-1α protein seemed to be more than 45 min for diosgenin-treated cells but less than 15 min for control cells (Fig. 4B, bottom). These results suggest that diosgenin increases HIF-1α protein, possibly by enhancing HIF-1α protein stability.

Nuclear translocation of HIF-1α is necessary for its transcriptional activation of a variety of HIF-1–regulated genes, including VEGF-A (Hofer et al., 2001). We therefore examined the nuclear translocation of HIF-1α protein in MC3T3-E1 cells after treatment with diosgenin by Western blot analysis and immunofluorescence staining. As shown in Fig. 4C, Western blot analysis revealed significant levels of HIF-1α in nuclear fractions of diosgenin-treated cells but not so for the control cells, whereas the level of nuclear proliferating cell nuclear antigen remained the same for diosgenin-treated and control cells. We did note a slight induction of HIF-1α in the cytosolic fraction of diosgenin-treated cells (Fig. 4C). Furthermore, we observed that immunofluorescent analysis also consistently revealed an evident nuclear staining for HIF-1α in diosgenin-treated cells, but this was not the case for control cells (Fig. 4D).

Thereafter, we performed an electrophoretic mobility shift assay to examine the DNA binding activity of HIF-1α in diosgenin-treated cells. Figure 4E shows that the DNA binding activity of HIF-1α increased significantly in nuclear extracts of diosgenin-treated MC3T3-E1 cells (Fig. 4E, lanes 5 and 6). We further noted that the increment in HIF-1α DNA binding activity thereby produced in diosgenin-treated cells was strongly attenuated when it was specifically competed against by the application of a nonradiolabeled probe (Fig. 4E, lane 7). In addition, the level of DNA binding activity of HIF-1α for diosgenin-treated cells was noted to reduce strongly after transfection with the dominant-negative mutant HIF-1α (DN-HIF-1α) carrying both of the deletions of the basic DNA binding domain (amino acids 4–27) and the carboxyl-terminal transactivation domain (amino acids 390–826), thus effectively inhibiting HIF-1α activity (Jiang et al., 1996). Such an observation was not made; however, by transfection with the control vector (Fig. 4E, lanes 1–4). Deletion of the basic region (amino acids 4–27) of HIF-1α protein has been shown to decrease its binding activity with DNA without affecting the complex formation between HIF-1α and HIF-1-β. Therefore, the excess amount of dominant-negative HIF-1α would strongly compete with wild-type HIF-1α for...
binding to HIF-1β and decrease the diosgenin-induced DNA binding activity of HIF-1α in our cell model. Based upon these findings, we suggest that diosgenin enhances the protein stability and DNA binding activity of HIF-1α.

**HIF-1 Is Critical for Diosgenin-Induced VEGF-A Expression.** We further explored whether HIF-1 activation is involved in diosgenin-induced VEGF-A expression in osteoblast MC3T3-E1 cells. To directly examine the effects of the HIF-1α on VEGF-A gene transcription, MC3T3-E1 cells were transfected with a luciferase reporter containing the 1.5 kb of VEGF promoter (pGL3–1.5kbVEGFprom; Maity et al., 2000) and a pGL3-Basic vector. Treatment with diosgenin resulted in a 2.6-fold increase in VEGF-A promoter activity compared with untreated cells, and this induction was weaker than CoCl₂, a standard agent for inducing VEGF-A transcriptional activity (4.1-fold induction compared with control; Fig. 5A). Furthermore, transfection with DN-HIF-1α significantly inhibited diosgenin-induced VEGF transcriptional activity in MC3T3-E1 cells (Fig. 5A). In addition, we transfected another VEGF promoter reporter, pGL3–1.2kbVEGFprom,
which lacks the promoter-binding element for HIF-1α (HRE) (Maity et al., 2000), into MC3T3-E1 cells and analyzed the VEGF-A promoter activity after treatment with diosgenin. It is interesting that when MC3T3-E1 cells were transfected with the pGL3–1.2kbVEGFprom reporter, their response to diosgenin seemed to have been lost (Fig. 5B). Transfection with DN-HIF-1α into diosgenin-treated cells strongly attenuated the induction of VEGF-A protein expression in response to diosgenin (Fig. 5C). In addition, diosgenin increased the expression of other HIF-1α–regulated genes, such as glucose transporter 1 and erythropoietin (Fig. 5C). These results suggest that the HIF-1/HRE axis is required for the transcriptional up-regulation of VEGF-A by diosgenin.

**Diosgenin-Induced HIF-1 Activation and Subsequent VEGF-A Expression via Estrogen Receptor-Mediated PI3K/Akt and p38 MAPK Pathways.** For certain cell types, the PI3K/Akt and MAP kinase pathways do seem to mediate growth factor- or cytokine-induced HIF-1α activation by enhancing HIF-1α transcriptional activity under non-hypoxic conditions (Semenza, 2002). Therefore, we investigated whether the activation of PI3K/Akt and MAP kinase pathways is involved in diosgenin-mediated HIF-1α and VEGF-A expression. Western blot analysis revealed that diosgenin treatment induced phosphorylation of p38 MAPK and Akt, the activation of which was not caused by the enhanced expression of the p38 and Akt proteins (Fig. 6A). Under the same treatment, however, we detected no significant difference in ERK1/2 activation between MC3T3-E1 cells with or without diosgenin treatment (Fig. 6A). To further examine whether p38 MAPK and Akt were involved in diosgenin-induced HIF-1α activation and subsequent VEGF-A expression, MC3T3-E1 cells were treated with SB203580 (p38 MAPK inhibitor), PD98059 (MEK kinase inhibitor), or LY294002 (PI3K inhibitor) for 1 h before treatment with diosgenin. Western blot analysis of nuclear fractions showed that the diosgenin-induced nuclear translocation of HIF-1α protein was abolished by SB203580 and LY294002 but not by PD98059 (Fig. 6B). The inability of PD98059 to reduce diosgenin-induced HIF-1α nuclear trans-

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Fig. 5. HIF-1 is required for diosgenin-induced VEGF-A expression. A, VEGF-A transcriptional activity was measured by the application of a luciferase assay using the pGL3–1.5kbVEGFprom. MC3T3-E1 cells transfects with various plasmids were treated with diosgenin or not (2 μM) for 90 min or CoCl2 for 12 h, after which samples were collected and analyzed for luciferase activity. The data were the average of three independent experiments. Columns, means of three independent experiments; bars, S.D. ∗, statistically significant increase compared with the corresponding value for column 1 (∗P < 0.05); #, statistically significant decrease compared with the corresponding value for column 2 (∗P < 0.05). B, MC3T3-E1 cells were transfected with the pGL3 control vector, pGL3–1.5kbVEGFprom or pGL3–1.2kbVEGFprom (lacks the HRE site). Forty-eight hours later, cells were treated with diosgenin or not (2 μM) and assayed for luciferase activity. Columns, means of three independent experiments; bars, S.D. ∗, statistically significant increase compared with the corresponding value of vehicle (∗P < 0.05). C, MC3T3-E1 cells (left) and HeLa cells (right) were transfected with control vector or DN-HIF-1α with diosgenin or not and determined the protein and mRNA expression by Western blot assay and RT-PCR, respectively. Results are representative of at least three independent experiments. Numbers below lanes indicate level of protein expression compared with the control.
location, however, was not due to the ineffectiveness of PD98059 at inhibiting MEK-1 activity, because the phosphorylated ERK1/2 was effectively reduced in PD98059-treated cells (Fig. 6A). Supporting such an observation was that diosgenin-induced VEGF-A expression and VEGF-A promoter activity were also reduced by SB203580 and LY294002 but this was not so for PD98059 (Fig. 6C).

Furthermore, we used another approach to block the PI3K/Akt and p38 MAPK signaling pathways by transfection of dominant-negative mutant Akt (DN-Akt) and dominant-negative mutant p38 (DN-p38) expression vector into MC3T3-E1 cells. We subsequently examined VEGF-A expression and HIF-1α translocation by Western blot analysis. As expected, the diosgenin-induced phosphorylation of p38 MAPK (Fig. 6D, top) and VEGF-A expression (Fig. 6D, middle) were both reduced in DN-p38–transfected cells, and this was not the case for vector control cells. We further observed that DN-p38 also decreased the nuclear translocation of HIF-1α in response to diosgenin (Fig. 6D, bottom). In addition, transfection of DN-Akt expression vector significantly inhibited diosgenin-induced VEGF expression and HIF-1α nuclear translocation (Fig. 6E). These data suggest the potential involvement of PI3K/Akt and p38 MAPK, but not ERK1/2, in diosgenin-induced HIF-1α activation and subsequent VEGF-A expression in MC3T3-E1 cells.

The structure of diosgenin is similar to that of estrogen, and estrogen deficiency seems to have a marked effect on bone metabolism, resulting in osteoporosis (Scott et al., 2001). To investigate the possible role of the ER in the course of a diosgenin-induced angiogenic effect, we used the highly specific ER antagonist ICI 182,780 and another ER antagonist, tamoxifen, to block ER-related pathways.
The addition of ICI 182,780 (10 μM) or tamoxifen (2.5 μM) almost completely abolished diosgenin-induced Akt and p38 phosphorylation (Fig. 7A), which is a critical step in diosgenin-induced HIF-1α activation and subsequent VEGF-A up-regulation. In support of this observation, the ER antagonists also reduced the diosgenin-induced activation of HIF-1α and VEGF-A (Fig. 7B). To investigate the effect of diosgenin estrogenic activity in MC3T3-E1 cells, estrogen receptor binding study was conducted. In the estrogen receptor binding assay, we found that treatment of MC3T3-E1 cells with 0.8 nM unlabeled E2 or 10 nM diosgenin for 3 h displaced 50% [3H]E2 from the receptor (Fig. 7C). These data indicated that diosgenin has the competitive ability with E2 to bind to ERα in MC3T3-E1 cells. As shown in Fig. 7D, Western blot analysis revealed that the expression levels of ERα in nuclear fraction were not changed after treatment with diosgenin or not. The above data suggest that diosgenin-induced the ER-mediated nongenomic effects rather than transcriptional induction. Src kinase has been reported to act as a critical signal transducer in nontranscriptional estrogenic action in response to estradiol (Kousteni et al., 2001). Thus, we examined whether src kinase was involved in diosgenin-induced ER-dependent Akt and p38 MAPK signaling pathways, which were required for HIF-1 activation and VEGF-A expression in response to diosgenin. We found that diosgenin-induced phosphorylation of src kinase could be diminished by treatment with either ICI 182,780 or tamoxifen (data not shown). Furthermore, transfection with dominant-negative mutant src (DN-src) expression vector significantly decreased diosgenin-induced phosphorylation of Akt and p38 MAPK and subsequent VEGF-A expression.

Fig. 7. Diosgenin-induced VEGF-A expression and related signaling pathways requiring the estrogen receptor and src kinase. A, MC3T3-E1 cells were treated with diosgenin (2 μM) and/or ICI 182,780 (10 μM) and tamoxifen (2.5 μM). The level of Akt and p38 MAPK phosphorylation was determined by Western blot analysis. Data are representative of three independent experiments. B, MC3T3-E1 cells were treated with diosgenin combined (or not) with ICI 182,780 (10 μM) or tamoxifen (2.5 μM) and analyzed for the nuclear translocation of HIF-1α (top) and VEGF-A expression (bottom) by Western blot assay. Data are representative of three independent experiments. C, displacement of [3H]17β-estradiol by diosgenin and unlabeled 17β-estradiol in MC3T3-E1 whole-cell estrogen receptor binding assay. Each data point represents the mean ± S.E.M. for four replicate determinations from a representative experiment. These data were a representative of three reproducible experiments. D, cytosolic and nuclear fractions were subjected to Western blot analysis by using the indicated antibodies for ERα. α-Tubulin and PCNA acted as the internal loading control for cytosolic and nuclear fractions, respectively. E, MC3T3-E1 cells were transfected with control vector or DN-src expression vector after treatment with diosgenin or not and analysis of protein expression by Western blot assay as described under Materials and Methods. F, MC3T3-E1 cells were pretreated with the indicated signaling inhibitors for a period of 1 h before being treated with diosgenin (2 μM) for a further 90 min. After the nuclear extracts were isolated and subjected to electrophoretic mobility shift assay, as described under Materials and Methods. To test for the specificity of binding samples were incubated with a 5-fold excess of unlabeled wild-type (URE) probe. The HIF-1α-specific complex is indicated by an arrow. Results are representative of at least three independent experiments.
in MC3T3-E1 cells (Fig. 7E). Taken together, our study reveals that diosgenin binds to ER and activates the angiogenesis-related signaling through the src kinase.

Applying an electrophoretic mobility shift assay, we further tested whether these signaling pathways were involved in the diosgenin-induced DNA binding activity of HIF-1α. As shown in Fig. 7F, the DNA binding activity of HIF-1α in diosgenin-treated cells was reduced by treatment with SB203580 or LY294002, but not so after treatment with PD98059 (Fig. 7F, lanes 1–5). Furthermore, the ER antagonist ICI 182,780 also consistently abolished the increment of HIF-1α DNA binding activity in diosgenin-treated MC3T3-E1 cells (Fig. 7F, lane 6). Furthermore, these chemical inhibitors, including SB203580, LY294002, and ICI 182,780, have also been demonstrated to be able to reduce diosgenin-induced HUVEC proliferation and tube-formation as well as HIF-1α activation (data not shown). Together, the data presented here strongly suggest that the signaling cascade from estrogen receptor via src kinase to PI3K/Akt and p38 MAPK and subsequent activation of HIF-1α is required for diosgenin-mediated up-regulation of VEGF-A in mouse osteoblasts.

**Discussion**

Invasion of capillaries into the metaphyseal end of the growth plate was known to be an important step in endochondral bone formation, resulting in bone growth (Brighton, 1978). On the one hand, diosgenin has been shown to decrease bone loss from ovariectomized mice (Higdon et al., 2001), although to the best of our knowledge, the detailed mechanism by which diosgenin thus protects against bone loss has not yet been characterized. In this study, we have shown that src kinase-dependent phosphorylation of p38 MAPK in the process of diosgenin-induced HIF-1α activation and VEGF-A expression requires the participation of an estrogen receptor. Our findings provide the first indication that osteoblasts respond to diosgenin by elevating the level of intracellular angiogenic signal transduction and angiogenesis through HIF-1α-mediated VEGF-A induction, providing a clear molecular explanation for diosgenin-promoted angiogenesis and neovascularization of bone tissue.

From the results of our current study, we suggest that VEGF-A plays a crucial role in diosgenin-stimulated angiogenesis (Figs. 2 and 3), although in this study, we did not examine the effect of diosgenin on the other angiogenic factors that have been reported to be involved in bone-derived cell-induced angiogenesis (e.g., fibroblast growth factor, platelet-derived growth factor, and bone morphogenic protein-2) (Barnes et al., 1999). Based upon our findings (Figs. 2 and 3), we suggest that osteoblast-synthesized VEGF-A might act as an important intercellular mediator between osteoblasts and vascular endothelial cells. In addition, the contemporaneous expansion of the capillary network, providing microvasculature, is believed to be essential for the process of bone repair/remodelling. Thus, it is possible that diosgenin may be a therapeutic candidate for bone repair, acting via the induction of VEGF-A synthesis in osteoblasts. Furthermore, we have also observed that diosgenin is able to induce connective tissue growth factor expression, which has been reported to increase angiogenesis within bone tissue (Nishida et al., 2000) (data not shown). The role of connective tissue growth factor in diosgenin-mediated effects for bone metabolism clearly requires further investigation. Our findings provide support for the notion that a novel function of diosgenin as regards stimulation of angiogenesis exists and that such activity continues via an increase in VEGF-A expression in osteoblasts.

In the present study, we used fetal mouse long bones as an ex vivo angiogenesis assay (Fig. 3). The tube-like structures exhibit an endothelium-like morphology and express the endothelial marker PECAM-1 (CD31) (Fig. 3 and Deckers et al., 2001). This ex vivo angiogenesis assay model combines both the flexibility and accessibility of in vitro models with the complexity of in vivo assays and, in particular, focuses on the contribution made by stromal cells that interact with endothelial cells. With the growing demand for easy and reproducible in vitro angiogenesis assays and the growing interest in the specific properties of endothelial cells, this assay provides a new tool for screening agents for potential clinical use and a comprehensive vehicle for undertaking further research in the field of angiogenesis.

HIF-1α has been reported to activate VEGF-A expression by binding to the HRE site within the VEGF-A promoter in response to hypoxia (Shima et al., 1996). Likewise, the activation of HIF-1α by diosgenin also resulted in an induction of VEGF-A transcription activity through the HRE site in MC3T3-E1 cells (Figs. 2 and 5). Although there are many other possible response elements, including activator protein-2, nuclear factor-κB, and simian virus 40 promoter factor 1, located within the pGL3–1.2kbVEGFprom (Shima et al., 1996), diosgenin treatment failed to display any significant induction of the VEGF-A promoter reporter activity in pGL3–1.2kbVEGFprom, which lacks the HRE. It is apparent that diosgenin-induced VEGF-A promoter reporter activity is substantially mediated by the HRE (Fig. 5). Because the level of induction of VEGF-A promoter activity was only approximately half the level of expression of VEGF mRNA by diosgenin, we are not able to exclude the possibility that other response elements, located beyond the 1.5-kb VEGF promoter region, might be involved in this induction. Several half-palindromic estrogen response elements were found to be located within the pGL3–1.2kbVEGFprom (Shima et al., 1996). Although diosgenin did demonstrate estrogen action in our recent study (Fig. 7), it did not seem to exhibit any significant effect upon the luciferase activity of pGL3–1.2kbVEGFprom. We thus suggest that estrogen-receptor activation by diosgenin might act as a type of signaling mediator instead of the transcription factor directly binding to the VEGF gene promoter. Taken together, such results suggest that it is probably that the presence of HRE is indispensable for diosgenin-induced VEGF-A gene expression.

A number of different signaling pathways, including PI3K/Akt or MAPK pathway, have been reported to be involved in VEGF-A gene induction by way of converging in activation of HIF-1α (Conrad et al., 1999; Jiang et al., 2001; Arsham et al., 2002; Fukuda et al., 2002; Gao et al., 2002). In general, in consensus with the results of some other studies, our data also revealed that the activation of both the PI3K/Akt and p38 MAPK pathways is required for the induction of HIF-1α and VEGF-A expression by diosgenin, whereas the ERK pathway would seem to not be involved (Fig. 6). Moreover, we also found that there was no direct cross-talk between these
two pathways in response to stimulation with diosgenin, because the blockage of either pathway did not affect the other pathway (data not shown). It would seem probable that the effect of MAPKs, such as p38 MAPK and ERK1/2, on the stimulation of HIF-1α transcriptional activity occurs via HIF-1α–interacting proteins rather than via the influence of HIF-1α itself (Huang and Bunn, 2003). Such results, in concert with other studies and ours, would seem to suggest that the diosgenin-induced phosphorylation of Akt and p38 MAPK might coordinate function at different aspects in regulating the complex process of HIF-1α activation and subsequent VEGF-A expression.

The estrogenic effects of diosgenin on the mammary epithelium of the ovariectomized (OVX) mouse have been reported previously (Aradhana et al., 1992). Decreased osteoclast progenitor development and/or decreased osteoclast recruitment and the promotion of apoptosis among mature osteoclasts leading to a slowing of the rate of bone remodeling are thought to be the main mechanisms of the so-called “anti-resorptive” agents such as estrogen. Our unpublished data, however, have revealed that diosgenin did not seem to exert any significant antiresorptive activity on osteoclasts. It has been reported that diosgenin is the more viable alternative form for treatment of osteoporosis in OVX rats than its derivative compound, dehydroepiandrosterone (Scott et al., 2001). Previous studies revealed important information as to the efficacy and safety of the alternative treatments diosgenin or estrogen for osteoporosis in OVX rats (Higdon et al., 2001). More importantly, the three-point bending test of the femora showed that OVX-induced decline in mechanical strength could be restored by treatment with diosgenin but not estrogen (Scott et al., 2001). In addition, in the present study, ER antagonists can not completely reduce the diosgenin-induced angiogenic response (Fig. 7). According to our study and that of Scott et al. (2001), diosgenin has an ER-independent mechanism in treatment of osteoporosis. Whether or not this favorable profile of diosgenin has clinical relevance is presently unknown.

Estrogens have been previously reported to regulate HIF-1α activation and VEGF-A expression in a biphasic manner (Gao et al., 2004; Mukundan et al., 2004). Furthermore, 17-β estradiol has been reported to attenuate the hypoxic induction of HIF-1α and erythropoietin in Hep3B cells, although 4-hydroxyestradiol has been reported to induce HIF-1α and VEGF-A activation in human ovarian carcinoma cells (Gao et al., 2004; Mukundan et al., 2004). Our present data have demonstrated that diosgenin-induced HIF-1 activation could possibly be mediated by estrogen receptor as is evident from the action of the specific ER antagonists ICI 182,780 and tamoxifen (Fig. 7, B and C). The connection between the estrogen receptor and PI3K/Akt and MAPKs has already been demonstrated, but the detailed mechanism underpinning the connection is not fully understood (Kousteni et al., 2001; Mori-Abe et al., 2003; Gao et al., 2004). Our study demonstrated for the first time that diosgenin-induced VEGF-A expression, unlike other estrogens-induced angiogenic signaling, required the involvement of src kinase. Consistent with such reported findings, we have found that the estrogen receptor operated as an upstream effector to activate the src kinase-mediated PI3K/Akt and p38 MAPK signaling pathways in diosgenin-treated cells (Fig. 7A), suggesting that activation of HIF-1α and enhanced HIF-1α protein stability may be the possible mechanism of VEGF-A induction by estrogen-like compounds such as diosgenin.

In conclusion, we not only demonstrated that diosgenin was able to generate angiogenic activity in mouse MC3T3-E1 osteoblasts by elevating VEGF-A levels but also dissected the molecular mechanism by which diosgenin induces VEGF-A up-regulation through activation of HIF-1 by the estrogen receptor-dependent PI3K/Akt and p38 MAPK signaling pathways (Fig. 8). Our study also provides a further insight into the possible therapeutic use of diosgenin for the treatment of certain bone-related diseases.

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