Genistein attenuates hypoxic pulmonary hypertension via enhanced nitric oxide signaling and the erythropoietin system

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Genistein attenuates hypoxic pulmonary hypertension via enhanced nitric oxide signaling and the erythropoietin system. Am J Physiol Lung Cell Mol Physiol 306: L996–L1005, 2014. First published April 4, 2014; doi:10.1152/ajplung.00276.2013.—Upregulation of the erythropoietin (EPO)/EPO receptor (EPOR) system plays a protective role against chronic hypoxia-induced pulmonary hypertension (hypoxic PH) through enhancement of endothelial nitric oxide (NO)-mediated signaling. Genistein (Gen), a phytoestrogen, is considered to ameliorate NO-mediated signaling. We hypothesized that Gen attenuates and prevents hypoxic PH. In vivo, Sprague-Dawley rats raised in a hypobaric chamber were treated with Gen (60 mg/kg) for 21 days. Pulmonary hemodynamics and vascular remodeling were ameliorated in Gen-treated hypoxic PH rats. Gen also restored cGMP levels and phosphorylated endothelial NO synthase (p-eNOS) at Ser1177 and p-Akt at Ser473 expression in the lungs. Additionally, Gen potentiated plasma EPO concentration and EPOR-positive endothelial cell counts. In experiments with hypoxic PH rats’ isolated perfused lungs, Gen caused NO- and phosphatidylinositol 3-kinase (PI3K)/Akt-dependent vasodilation that reversed abnormal vasoconstriction. In vitro, a combination of EPO and Gen increased the p-eNOS and the EPOR expression (3). In addition, a combined treatment with EPO and sildenafil acted synergistically to restore endothelial function after hypoxic exposure (12). These results indicate that the EPO/EPOR system has potential as a new therapeutic agent by virtue of its ability to activate NO-mediated signaling in victims of hypoxic PH.

Genistein (Gen), a phytoestrogen derived from soybeans and tested in numerous studies, has been shown to have vasodilative and cardioprotective effects. Gen enhanced eNOS activity and NO-mediated vasorelaxation, not only in the systemic circulation (40) but also in pulmonary arteries, independently of any estrogen-mediated mechanism (18). In addition, we previously found that a 21-day treatment with Gen significantly attenuated the development of monocrotaline (MCT)-induced PH in rats, another model of PH, by restoring eNOS expression (16). Therefore, this study was designed to investigate whether treatment with Gen would attenuate hypoxic PH through amelioration of the EPO/EPOR system and NO-mediated signaling.

MATERIALS AND METHODS

Animals and exposure to chronic hypoxia. All experimental and surgical procedures were approved by the Institutional Committee for “Use and Care of Laboratory Animals in Juntendo University” (Hongo, Tokyo, Japan), in accordance with the U.S. National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” Experiments were performed with adult male Sprague-Dawley rats (200–250 g) obtained from Charles River Laboratories (Yokohama, Japan).

The pulmonary normotensive rats (the control group; NL) were housed at the ambient barometric pressure (760 mmHg). Chronically hypoxic pulmonary hypertensive rats (the experimental group; HL) were housed in a hypobaric chamber (barometric pressure, ~380 mmHg; inspired O2 tension, ~76 mmHg), which was flushed continu-
ously with room air to prevent accumulation of CO₂, NH₃, and H₂O for a period of 21 days, as described previously (25).

Animal experimental protocols. For these studies, rats were randomly assigned to one of the following four groups (n = 6–8 animals in each group) to be administered either vehicle or Gen (60 mg/kg; Sigma Chemical, St. Louis, MO) (39): 1) control animals raised in normoxia; 2) Gen-treated animals raised in normoxia; 3) control animals raised in chronic hypoxia; 4) Gen-treated animals raised in chronic hypoxia. For treatment, Gen was dissolved in a mixture of DMSO (Sigma Chemical) and polyethylene glycol (PEG; Sigma Chemical). Rats were given subcutaneous injections of Gen or vehicle (100 µl of a mixture containing 1.25% DMSO and 98.75% PEG) daily throughout the experiments.

The efficacy of dietary Gen by gavage was also evaluated throughout the additional experiments. The Gen dosage of 60 mg/kg by gavage once/day was given to rats during hypoxic exposure for 3 wk. For comparison of the plasma concentrations of Gen between the control sample, subcutaneous and dietary delivery was examined by using the Gen TR-FIA (Labmaster, Aurora, Finland) at the end of hypoxic exposure.

Hemodynamic measurements. Animals were anesthetized by intraperitoneal injection with pentobarbital sodium (15 mg/kg) and implanted with catheters in the pulmonary and right carotid arteries and right jugular vein, as described previously (16). Right ventricular systolic pressure (RVSP) and systemic arterial pressure (SAP) were measured with a polygraph system (AT-600G cardiotachometer; Nikon Kohden, Tokyo, Japan).

Measurement of RV hypertrophy. Each heart was dissected to assess the severity of PH. An index of RV hypertrophy was calculated as the ratio of wet weight of the RV wall to wet weight of the left ventricular (LV) wall plus septum (RV/LV + S).

Morphological studies. At the end of each hemodynamic study, the rats were killed with an overdose of pentobarbital sodium, and the thorax was opened. After blood samples were drawn from the right ventricle, the heart and lungs were removed en bloc. The trachea was intubated, and the left lung was inflated with 10% formalin at 36 cm H₂O pressure and fixed in the inflated state for 3 days. The right lung was frozen in liquid nitrogen for further molecular analysis.

Sections of pulmonary arteries were treated with elastic van Gieson stain for morphometric analysis of the arteries’ medial-wall thickness to assess the degree of their muscularization, as described previously (16). In each tissue section, at least 50 consecutive arteries (>30 µm external diameter) were examined at ×400 magnification using an image analysis system (KS500; Carl Zeiss Imaging Solutions GmbH, Hallbergmoos, Germany). The medial-wall thickness was measured at two locations of each artery and calculated according to the following formula: (medial-wall thickness/external diameter) × 100 (in percent).

Pulmonary sections were also stained with EPOR antibody (1:50, anti-EPOR; Santa Cruz Biotechnology, Dallas, TX) to quantitate the EPOR immunoreactions. The paraffin-embedded, formalin-fixed lung specimens were washed and incubated with EPOR antibody overnight, followed by a 30-min incubation with the secondary antibody (1:300, biotinylated goat anti-rabbit IgG; Dako, Produktionsvej, Denmark). The color reaction was performed with 3,3-diaminobenzine.

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For quantitative analysis of the proportion of the EPOR immunoreactions. The paraffin-embedded, formalin-fixed lung specimens were washed and incubated with EPOR antibody (1:50, anti-EPOR; Santa Cruz Biotechnology, Dallas, TX) to quantitate the EPOR immunoreactions. The paraffin-embedded, formalin-fixed lung specimens were washed and incubated with EPOR antibody overnight, followed by a 30-min incubation with the secondary antibody (1:300, biotinylated goat anti-rabbit IgG; Dako, Produktionsvej, Denmark). The color reaction was performed with 3,3-diaminobenzine.

Samples were centrifuged for 15 min at 15,000 × g at 4°C, and the supernatant protein concentration was estimated using a Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein suspensions were separated electrophoretically on 5–10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membranes (GE Healthcare UK, Buckinghamshire, UK). The membranes were incubated with anti-EPOR antibody (BD Biosciences, Franklin Lakes, NJ) or anti-p-eNOS antibody (Ser1177; Cell Signaling Technology, Danvers, MA) (39): 1% Trition X-100, and 1 mM protease inhibitor cocktail (Sigma Chemical), according to the manufacturer’s instructions, as described previously (20). The sample cGMP concentration was determined (as fmol/mg tissue) using the equation obtained from a standard curve. Each sample was evaluated in duplicate, and the process was repeated three times.

Western blots. Frozen lung tissues and pulmonary arteries were homogenized in lysis buffer containing 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% Triton X-100, and 1 mM protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) using a tissue homogenizer. Samples were centrifuged for 15 min at 15,000 × g at 4°C, and the supernatant protein concentration was estimated using a Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein suspensions were separated electrophoretically on 5–10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membranes (GE Healthcare UK, Buckinghamshire, UK). The membranes were incubated with anti-EPOR antibody (BD Biosciences, Franklin Lakes, NJ) or anti-p-eNOS antibody (Ser1177; Cell Signaling Technology, Danvers, MA). Signals were visualized by the ECL Prime detection system (Amersham Pharmacia, Uppsala, Sweden) was included as a colloid. Before starting all experiments, 3 mL/l sodium meclofenamate (Sigma Chemical) was added to inhibit synthesis of vasodilator prostaglandins (27, 36).

Initial experiments compared the vasoconstriction in responses to KCl (Sigma Chemical) of lungs from NL and HL rats. After equilibration, 5–20 mmol/l KCl was added to the perfusate in a concentration-response fashion every 5th min. Higher concentrations of KCl were not used because they caused lung edema. We next tested whether Gen has acute vasodilative effects against KCl-induced vasoconstriction in NL and chronic hypoxia lungs. At the peak of the KCl pressure response, 30 µmol/l Gen or vehicle (DMSO) was added to the perfusate. We also tested whether NOS-nitro-l-arginine (NLA; Sigma Chemical), an inhibitor of NOS, blocks the vasodilative response to Gen. NLA (200 µmol/l) was added to the perfusate at 20 min after administration of Gen or vehicle. Vascular effects were analyzed by measuring baseline perfusion pressure, peak KCl pressor response, and the ratio of spontaneous (vehicle) or Gen-induced vasodilation to the KCl-induced vasoconstriction, and NLA-induced vasoconstriction. The ratio of vasodilation was calculated by dividing the decrease in pressure occurring from the addition of either vehicle or Gen to the perfusate at the peak of the KCl pressor response by the magnitude of the KCl pressor response.

To confirm further the mechanism in vascular responses to Gen, we examined whether the vasodilative effect of Gen was mediated by a PI3K/Akt-dependent pathway. LY294002, a specific PI3K inhibitor (10 µmol/l; Cayman Chemical, Ann Arbor, MI), was added to the perfusate at the peak of the KCl pressor response before the administration of Gen in separate experiments. Then, the alteration in vasodilative effect of Gen caused by the PI3K inhibitor was calculated as the ratio of vasodilation. To control for differences in vasoactivity over time of perfusion, the inhibitor, antagonist, or respective vehicle was administered identically with respective time.

cGMP measurement by enzyme immunoassay. cGMP levels in lung tissue were determined (as fmol/mg tissue) using the cGMP enzyme immunoassay (EIA; Cayman Chemical), according to the manufacturer’s instructions, as described previously (20). The sample cGMP concentration was determined (as fmol/mg tissue) using the equation obtained from a standard curve. Each sample was evaluated in duplicate, and the process was repeated three times.

Cells and cell culture. Human umbilical vein endothelial cells (HUVECs; Clonetics, Walkersville, MD) were cultured in 25-cm² flasks with a phenol red-free endothelial basal medium-2 (Lonza, Walkersville, MD) Bullet Kit, supplemented with endothelial growth medium 2 (Lonza, Basel, Switzerland) containing 5% FBS and cytokines. The human hepatoma cell line (HepG2; American Type Culture Collection, Manassas, VA) was cultured in DMEM containing 10% FBS. The medium was changed each day, and cells were
used within three to five passages. Cells were incubated under both 21% (normoxia) and 1% (hypoxia) oxygen for 48 h and were washed in HEPES buffer before exposure to hypoxia.

**Combined treatment with Gen and EPO and protein analysis of HUVECs.** HUVECs were cultured with the medium, which was replaced with or without 5 U/ml EPO and 10 μmol/l Gen for a 48-h exposure to the normoxia or hypoxia environment. Cells were washed and scraped from the plate, and the lysate was centrifuged for 15 min at 15,000 g at 4°C. The supernatant protein concentration was then estimated using a Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology).

**Measurement of EPO production in the culture supernatant of HepG2 cells under hypoxia.** HepG2 cells were plated at a density of 5 × 10⁵ cells/35 mm dish. At 24 h after the onset of culture, the medium was replaced with or without 10 μmol/l Gen and then incubated for 48 h of exposure to normoxia or hypoxia. EPO protein concentration in the HepG2 cell culture supernatant was determined by chemiluminescent EIA by using Access EPO (Beckman Coulter, Brea, CA) as described previously (5).

**RESULTS**

Treatment with Gen ameliorated hypoxic PH by restoring hemodynamics, preventing RV hypertrophy, and averting vascular remodeling. Exposure to conditions of chronic hypoxia caused severe PH; that is, HL rats had 50.6 ± 4.32 mmHg of peak RVSP compared with 9.6 ± 0.74 mmHg of peak RVSP in NL rats (P < 0.05, n = 11–12). Gen significantly attenuated the elevation of RVSP in HL rats but did not affect the peak RVSP in NL rats (Fig. 1A). SAP and heart rates (HR) were similar in all groups (Fig. 1, B and C). The severity of PH was confirmed further by RV hypertrophy (RV/LV+S); that

*Fig. 1. Genistein (Gen) attenuated the chronic hypoxia-induced increases in (A) right ventricular systolic pressure (RVSP) and (B) RV hypertrophy [RV/left ventricular (LV) + septum (S)] of chronic hypoxia vehicle (HL) rats. However, Gen altered neither RVSP nor RV/LV + S in the normoxia group (NL). Gen did not affect (C) heart rate (HR) or (D) systemic arterial pressure (SAP). Values are mean ± SE; n = 11–12 animals/group. *P < 0.001 vs. NL; †P < 0.001 vs. HL.*

E: dietary Gen by gavage (Gen G) attenuated the chronic hypoxia-induced increases in RV/LV + S but not (E) RVSP of HL rats. However, dietary Gen altered neither RVSP nor RV/LV + S in the NL group. Values are mean ± SE; n = 5–6 animals/group. *P < 0.001 vs. NL; †P < 0.001 vs. HL. G: the plasma concentration of Gen was lower in gavage than in subcutaneous delivery. Values are mean ± SE; n = 5–7 animals/group. *P < 0.05 vs. control; †P < 0.05 vs. subcutaneous.

**Data analysis.** Data are presented as means ± SE. Statistical analysis was done by unpaired t-test or one-way ANOVA, followed by Tukey’s multiple comparisons test, or two-way ANOVA, followed by the Bonferroni test (Prism 5; GraphPad Software, San Diego, CA). Differences were considered significant at P < 0.05.
is, the RV value of 0.52 ± 0.02 in HL rats was increased significantly over that of 0.25 ± 0.01 in NL rats \( (P < 0.05, n = 11-12) \). Gen diminished the increase in RV/LV + S in the HL bearers but did not affect their NL counterparts (Fig. 1D).

Dietary Gen by gavage also attenuated the magnitude of RV/LV + S but did not affect the elevation of RVSP in HL rats (Fig. 1, E and F). However, dietary Gen did not alter RVSP or RV/LV + S in the NL group. The plasma concentration was lower in gavage than in subcutaneous delivery \( (27.1 \pm 21.2 \text{ nmol/l}, 970.3 \pm 549.7 \text{ nmol/l}, \text{ and } 398.2 \pm 338.8 \text{ nmol/l}, \text{ respectively, for control, subcutaneous, and gavage delivery; } P < 0.05, n = 5-7/each group) \). The coefficient of variation of the concentrations were 77.9%, 56.7%, and 85.1%, respectively, for control, subcutaneous, and gavage delivery (Fig. 1G).

In the hypoxic condition, medial-wall thickness of muscular pulmonary arteries corresponding to terminal bronchioles was increased significantly when compared with that of NL animals. However, Gen treatment reduced the increase in medial-wall thickness of each vessel (vessel diameter of 30–60 \( \mu \text{m}, 60–120 \mu \text{m}, \text{ and } >120 \mu \text{m} \) ) in HL rats (Fig. 2, A–D).

Gen restores cGMP levels and preserves p-eNOS at Ser\(^{1177} \) in hypoxic PH. To determine the cellular and molecular mechanisms used by Gen to lessen experimental hypoxic PH, we quantitated cGMP levels in lungs of all four groups used here. The cGMP levels in the whole lung were clearly lower in HL compared with NL tissues; comparatively, Gen preserved cGMP levels in lungs from the HL group. However, Gen increased cGMP levels slightly but not significantly in lungs from the NL group \( (P < 0.05, n = 6-8; \text{ Fig. } 3) \).

To examine the effects of Gen on eNOS and Akt activity, we assessed the p-eNOS at Ser\(^{1177} \) and p-Akt at Ser\(^{473} \) in lungs and pulmonary arteries. The representative Western blot analyses of lung homogenates are shown in Fig. 3, B–E. Although protein expression of eNOS or Akt, respectively, was increased or unaltered, the p-eNOS at Ser\(^{1177} \) and p-Akt at Ser\(^{473} \) decreased significantly in not only lungs but also pulmonary arteries from HL compared with NL rats. Gen attenuated the decrease in expression of p-eNOS but not p-Akt in the lungs manifesting HL but increased the expression of both p-eNOS and p-Akt in pulmonary arteries from the HL group. These
results suggest that Gen restored the expression of both p-eNOS and p-Akt selectively in pulmonary arteries.

Gen causes rapid vasodilation in isolated perfused lungs from NL and HL rats. In preparation for analyses of vasoconstriction and dilation, baseline levels were established. In 12–16 animals/group, we noted that the perfusion pressure was higher in vessels from HL rats (7.93 ± 0.37 mmHg) than in NL rats (6.25 ± 0.28 mmHg; P < 0.05, n = 12–16). Subsequently, KCl caused a concentration-dependent vasoconstriction that was greater in the HL group compared with NL animals (Fig. 4A). These results suggested the presence of increased vascular tone and abnormal vasoconstriction in pulmonary vessels during the course of HL. However, in the presence of KCl-induced vasoconstriction, Gen caused a similar extent of vasodilation in NL and HL vessels (Fig. 4B).

Rapid vasodilative effect of Gen is abolished by either NLA or a specific PI3K/Akt kinase inhibitor. The effect of Gen-induced vasodilation on KCl-induced vasoconstriction was abolished completely after NLA administration into the lungs of NL and HL rats (Fig. 4C). Pretreatment with LY294002, a specific PI3K/Akt kinase inhibitor, blunted the vasodilative effect of Gen significantly in both the NL and HL (Fig. 4D).
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These results suggest that the mechanism of a Gen-induced vasodilative effect was dependent on eNOS but, at least in part, mediated through the PI3K/Akt pathway.

Gen increases hemoglobin concentration, hematocrit levels, EPO production, and EPOR-positive endothelial cell counts of pulmonary arteries in hypoxic PH. Prolonged exposure to a hypoxic condition caused a significant increase of the hemoglobin (Hb) concentration and hematocrit (Hct) levels (17.10 ± 1.50 g/dl and 51.30 ± 4.16%, respectively) in the HL group above that in NL subjects (13.24 ± 1.12 g/dl and 39.72 ± 3.81%, respectively; P < 0.0001, n = 5–6). Gen potentiated the hypoxic increase of Hb concentration and Hct levels (Fig. 5, A and B).

Similar to the rise in Hb concentrations and Hct levels, Gen potentiated the increases of serum EPO levels and percentages of EPOR-positive endothelial cells. That is, EPO was 18.98 ± 6.45 mU/ml for HL vs. 40.73 ± 14.82 mU/ml for HL Gen (P < 0.05, n = 8), and percentages of EPOR-positive endothelial cell counts were 20.84 ± 7.38% for HL vs. 33.32 ± 6.06% for HL Gen (P < 0.0001, n = 4–6; Fig. 5, C–E). However, Gen did not affect Hb concentrations, Hct levels, EPO levels, or EPOR-positive endothelial cell counts in the NL groups.

EPO combined with Gen increases the p-eNOS at Ser1177 and EPOR expression in HUVECs under hypoxic exposure. Since exogenous EPO (5 U/ml) and hypoxia (2% O2) for 48 h increased expression of eNOS and EPOR in HUVECs (3), we examined whether Gen (10 μmol/l) would potentiate the p-eNOS at Ser1177 and the expression of EPOR in cultured HUVECs for 48 h under 1% O2 exposure with EPO (5 U/ml). Whereas 10 μmol/l Gen but not 5 U/ml EPO increased the p-eNOS at Ser1177, the combined administration of EPO and Gen further potentiated the p-eNOS at Ser1177 under hypoxic exposure (Fig. 6A). In addition, 10 μmol/l Gen alone and the combination of 10 μmol/l Gen and 5 U/ml EPO but not EPO alone upregulated EPOR protein expression, as shown by Western blot analysis in HUVECs under hypoxic exposure (Fig. 6B).

Hypoxia and Gen further increase EPO production in HepG2 cells. The human hepatoma cell lines, HepG2 and Hep3B, are widely used models for studying the production of EPO under hypoxic conditions. The mechanism is dependent on normal glycosylation of EPO, accumulation of cAMP, and interaction of cGMP and NO (26, 42). In our experiments, 1% O2 exposure for 48 h induced a significant increase in EPO production in the culture medium from HepG2 cells compared with cells in a state of normoxia. Resulting values were 1.20 ± 0.00 mU/ml for normoxia vs. 5.57 ± 1.40 mU/ml for hypoxia (P < 0.05, n = 4). The addition of Gen (10 μmol/l) enhanced the production of EPO in HepG2 cells under hypoxic exposure (9.63 ± 2.37 mU/ml for hypoxia Gen vs. 5.57 ± 1.40 mU/ml for hypoxia; P < 0.05, n = 4; Fig. 6C). However, Gen did not affect EPOR expression in HUVECs or EPO production in HepG2 cells under normoxic exposure.

DISCUSSION

In the present study, we demonstrate clearly that Gen is capable of attenuating hypoxic PH by correcting its chronic structural remodeling component and also its abnormal vasoconstrictive component in the lungs. In addition, for the first time, we showed that the EPO/EPOR system and PI3K/Akt pathway are likely contributors to the ability of Gen to improve NO-mediated signaling.

In the pathogenesis of hypoxic PH, reduced NO-mediated vasodilation is a major factor in the impairment of endothelial-dependent vasodilation (15). This impaired vasodilation occurs
The defect in responsiveness to endothelium-dependent vasodilation is thought to be a consequence of reduced cGMP activity and of downstream NO production in hypoxic PH (8). In other words, the normalizing of NO-mediated signaling may contribute to attenuating hypoxic PH. Chronic hypoxia-induced EPO production is a critical factor in the proliferation of red blood cells, and this response is traditionally believed to raise pulmonary vascular resistance, leading to the development of PH. In that context, the vascular EPO/EPOR system was recently invoked as a novel therapeutic target for cardiovascular disease (37). p-EPOR initiates a variety of signaling pathways, including PI3K, which is involved in NO-mediated signaling (23). In addition, in vivo studies showed that the effect of EPO on eNOS may be a physiologically relevant mechanism to counterbalance hypoxia (3).

The beneficial effects of Gen on cardiovascular diseases (9, 41) have been established and featured less toxicity and drug interactions than other treatments. Studies have shown that Gen interacted with vascular endothelial cells directly and increased NO production independently via an estrogen-mediated mechanism (31a, 45). We found that Gen significantly downregulated the development of MCT-induced PH by restoring eNOS expression in the lungs (16). These results indicate the strong potential of Gen as a therapeutic option for patients with hypoxic PH by virtue of its ability to mediate NO signaling and the EPO/EPOR system.

Long-term treatment with Gen restored normalcy to pulmonary hemodynamics and vascular remodeling, whereas a single dose of Gen produced rapid vasodilation to counterbalance vasoconstriction in this model. In experiments with isolated lungs perfused with a physiological salt solution, HL rats underwent elevations of baseline pressure, presumably produced by the increase in pulmonary resistance, resulting from vasoconstriction and vascular remodeling. In addition, the greater KCl pressor response of these HL animals indicated augmentation of abnormal vasoconstriction, probably caused by...
by boosting vascular tone in subjects exposed long-term to a low-oxygen environment.

Gen administered directly into the isolated perfused lung yielded a virtually immediate vasodilative effect that offset the abnormal augmentation of vascular tone in HL lungs. Since Gen-induced vasodilation was reduced by the NOS and PI3K inhibitors, apparently such vasodilation is dependent on PI3K/Akt- and NO-mediated signaling.

The present study showed that Gen suppressed the elevation of RVSP without causing systemic hypotension, reduced the HR, and prevented vascular remodeling in whole pulmonary arteries. It is generally agreed that the exacerbation of right heart failure caused by the elevation of RVSP and the systemic hypotension occurring after a decrease in cardiac output predispose PH patients to a fatal outcome. Because Gen did not affect the systemic blood pressure or the HR, Gen is a potentially useful agent for the prevention and treatment of hypoxic PH.

Mechanisms of Gen in preventing PH. We found that Gen restored levels of cGMP and p-eNOS at Ser1177 and p-Akt at Ser473 in hypoxic lungs and pulmonary arteries. The protein kinase Akt has been proposed to phosphorylate eNOS at Ser1177 and to increase eNOS activity in response to various stimuli in vascular endothelial cells (7), whereas Gen activated eNOS through a PI3K/Akt-dependent mechanism in pulmonary arterial endothelial cells (45). For the first time, however, the present study of HL rats demonstrated that Gen treatment upregulated the PI3K/Akt pathway in the lungs and pulmonary arteries. Since the vasodilative effect of Gen was abolished by administration of a NOS or PI3K/Akt inhibitor in isolated perfused lungs, Gen treatment may be attributable to the improvement of NO-mediated signaling, mostly through an Akt-dependent mechanism. In fact, Gen restored the p-eNOS at Ser1177 and p-Akt at Ser473 in hypoxic lungs manifesting PH and pulmonary arteries from HL rats.

Here, Gen treatment enabled an increase in Hb concentration and Hct levels in association with upregulation of EPO in serum and EPOR expression in endothelial cells of pulmonary arteries. The mechanism of action of Gen in pulmonary vasculature may function through the EPO/EPOR system. In vitro, 10 μmol/l Gen increased the p-eNOS at Ser1177, and p-Akt at Ser473 in hypoxic lungs manifesting PH and pulmonary arteries from HL rats.

Although our results raise the possibility that eNOS and the EPO/EPOR system are needed to improve each other under Gen treatment, the precise mechanism of their interaction is still unclear. The interaction of NO and cGMP is one of the pathways of hypoxia-induced stimulation of EPO gene expression (26). Administration of NLA, a NOS inhibitor, inhibited EPO production, although pretreatment with L-arginine prevented the inhibition (17). Whereas EPO increased eNOS transcription and activity through phosphorylation at Ser1177.
(3), transgenic mice overexpressing EPO produced increased amounts of eNOS activity (34). Since that observation is compatible with ours, the present study suggests that Gen may be effective in promoting the EPO/EPOR system, thereby ameliorating the interaction between EPO and eNOS.

Limitations. There are limitations of this study. First, in isolated perfused lung experiments, we used a Hb-free physiological saline solution for perfusate. It has the possibility that the half-life of circulating NO is able to prolong and that the decreased clearance of NO may enhance the dilator response to Gen. Second, although the PH model demonstrated several main features of hypoxic PH, such as vascular remodeling and endothelial dysfunction, no plexiform lesions were seen, which is a hallmark feature of vasculature in pulmonary arterial hypertension and fatal PH patients, in the present study. Third, the cardiac output was not estimated directly as hemodynamic measurements. Although Gen demonstrated no obvious effects on systemic blood pressure and HR, we cannot exclude a possibility that decreased cardiac output may contribute to the reduction of RVSP. Fourth, in in vitro studies, HUVECs and the HepG2 cell line did not necessarily accord with pulmonary arterial endothelial cells because of some difference of property between them. Fifth, the present study did not delineate the exact relationship between the increase in p-Akt at Ser\(^{473}\) and p-eNOS at Ser\(^{1177}\). Finally, the efficacy of Gen by other delivery, for example, dietary or inhalation, should be examined, because subcutaneous delivery was not necessarily pertinent. There raises a possibility of Gen as a promising option for treatment of hypoxic PH, since dietary Gen also attenuated RV hypertrophy of HL animals. However, contrary to subcutaneous delivery, dietary Gen did not affect the elevation of RVSP of HL animals. Although the reason of discrepancy was unclear, the lower and more inconsistent plasma concentration of dietary Gen may account for the different effects between subcutaneous and dietary delivery. On the other hand, as gut absorption and metabolism influenced large individual variations of plasma concentrations of Gen (33), suitable delivery of dietary Gen could answer these concerns. Further studies are required to elucidate these questions.

Conclusions. In the pathogenesis of hypoxic PH, endothelial cell dysfunction, characterized by the impairment of NO-mediated signaling, has been suggested to provoke abnormal vasoconstriction and vascular remodeling. Enhancement of the EPO/EPOR system in hypoxic conditions is thought to be a purposeful alteration that ameliorates endothelial function through upregulation of NO-mediated signaling. Our present study demonstrated for the first time that Gen, a phytoestrogen derived from soybeans, attenuated hypoxic PH by preventing vasoconstriction and chronic structural remodeling through restoration of NO-mediated signaling. In addition, Gen treatment enhanced the EPO/EPOR system function in the lungs and endothelial cells of pulmonary arteries from subjects with hypoxic PH. From these cumulative results, we envision that Gen may orchestrate the restoration of PI3K/Akt-dependent, NO-mediated signaling and enhancement of EPO/EPOR function, thereby preventing hypoxic PH. Based on our results and previous studies, combined with further examination and clinical studies, we propose that Gen may be an effective and safe option for the treatment of patients with hypoxic PH.

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DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS


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