Impairment of endothelium-dependent relaxation is thought to contribute to the pathogenesis of various forms of pulmonary hypertension (Higenbottam and Laude, 1998). Reduced nitric oxide (NO)-mediated relaxation accounts for at least a portion of this impairment as evidenced by the blunted response of pulmonary arteries to agonists that stimulate endogenous NO production such as acetylcholine and carbachol. This effect has been demonstrated in pulmonary arteries isolated from chronically hypoxic adult and neonatal animals (Eddahibi et al., 1991; Durmowicz et al., 1993; Maruyama et al., 1995; Karamsetty et al., 1996; Berkenbosch et al., 2000), and from patients with chronic obstructive lung disease (Dinh-Xuan et al., 1993) and primary pulmonary hypertension (Brett et al., 1996). Clinically, inhaled NO has been used to restore NO-mediated pulmonary vasodilation in some patients with pulmonary arterial hypertension (Channick et al., 1994, 1996), but this application is limited by the need for continuous inhalation. Other approaches to restoring endogenous NO-mediated relaxation are needed if more practical potential therapies for pulmonary hypertension are to be developed.

Genistein (4’,5,7-trihydroxyisoflavone) is a phytoestrogen derived from soybeans that binds to estrogen receptors (Kuiper et al., 1998) and has estrogen-like cardiovascular effects. Genistein enhances the dilator response to acetylcholine in atherosclerotic coronary arteries of female macaques (Honore et al., 1997). Additionally, supplementation with genistein enhances NOS activity in the lungs and NO-mediated relaxation in aortic rings isolated from ovariectomized rats (Squadrito et al., 2000). Based on these findings, we hypothesized that genistein would restore NO-mediated relaxation in pulmonary arteries isolated from chronically hypoxic rats. To test our hypothesis, we compared the effects of genistein on relaxation responses to carbachol in pulmonary arteries isolated from normoxic and chronically hypoxic rats (Squadrito et al., 2000). Based on these findings, we hypothesized that genistein would restore NO-mediated relaxation in pulmonary arteries isolated from chronically hypoxic rats. To test our hypothesis, we compared the effects of genistein on relaxation responses to carbachol in pulmonary arteries isolated from normoxic and chronically hypoxic rats. In addition, to gain insight into whether the effects of genistein on carbachol-induced relaxation are by its inhibitory effect on tyrosine kinases (Akiyama et al., 1987; Akiyama and Ogawara, 1991), we assessed the effects of another phytoestrogen derived from soybeans that binds to estrogen receptors. Akiyama et al., 1993) and primary pulmonary hypertension (Channick et al., 1994, 1996), but this application is limited by the need for continuous inhalation. Other approaches to restoring endogenous NO-mediated relaxation are needed if more practical potential therapies for pulmonary hypertension are to be developed.

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determine whether phytoestrogens are exerting estrogen-like effects, we compared actions of the phytoestrogens on carbachol-induced relaxation with that of the mammalian estrogen 17β-estradiol and evaluated the effect of estrogen receptor blockade on this response.

**Materials and Methods**

**Animals and Exposures to Hypoxia.** Adult male Sprague-Dawley rats (4–6 weeks old, 250–275 g) were exposed to 2 weeks of normoxia or hypobaric hypoxia (0.5 atmospheres). Hypoxic rats were removed from the hypobaric chamber for 30 min every other day to replenish food and water and to clean their cages. Normoxic rats were kept in adjacent cages exposed to room air.

**Hematocrit and Right Ventricular Hypertrophy.** After 2 weeks of exposure to normoxia or hypoxia, rats were anesthetized with pentobarbital (100 mg kg⁻¹ i.p.) and exsanguinated by cutting the abdominal aorta. Heart and lungs were removed en bloc and immersed in oxygenated Earle’s balanced salt solution containing 116.3 mM NaCl, 5.4 mM KCl, 0.83 mM MgSO₄, 19.0 mM NaHCO₃, 1.04 mM NaH₂PO₄, 1.8 mM CaCl₂, 2H₂O, 5.5 mM D-glucose, and 0.031 mM phenol red. Hematocrit of the aortic blood was measured by centrifugation. The right ventricle (RV) and left ventricle (LV) were isolated, cut into rings (2–3 mm long), and mounted in 10-ml organ baths filled with Earle’s balanced salt solution and bubbled with 95% O₂ and 5% CO₂ as described previously (Klinger et al., 1997). Fifteen minutes after mounting, the hearts were allowed to stabilize for 60 min at 1-g resting tension. The viability of the smooth muscle was determined from normoxic and hypoxic rats (Fig. 2A).

**Isolated Pulmonary Artery Preparation.** The main intralobar pulmonary artery of the left lung and the middle lobe of the right lung (i.d. ~1.5–2.0 mm) were isolated, cut into rings (2–3 mm long), and mounted in 10-ml organ baths filled with Earle’s balanced salt solution and bubbled with 95% O₂ and 5% CO₂ as described previously (Karamasety et al., 1996). Force was measured in grams using isometric force transducers (Grass FT03) and recorded on a Grass polygraph (model 790). Pulmonary arterial rings were allowed to stabilize for 60 min at 1-g resting tension. The viability of the smooth muscle and endothelium was confirmed by obtaining a contractile response to phenylephrine (10⁻⁶ M) and a subsequent relaxation response to the endothelial NOS activator carbachol (10⁻⁶ M). After washout of the drugs from the organ chamber and return to baseline tone (20–30 min), the rings were contracted again with phenylephrine (10⁻⁶ M). When the contractile response was stable, a concentration-response curve to carbachol (10⁻¹⁰–10⁻⁴ M) was obtained.

**Effects of Phytoestrogens and Mammalian Estrogen on Contractile and Relaxation Responses of Pulmonary Arteries from Normoxic and Hypoxic Rats.** To determine effects of genistein on carbachol-induced relaxation, we incubated pulmonary artery rings from normoxic and hypoxic rats with genistein (30 μM) (Uzun et al., 1998) for 30 min before contracting the rings with phenylephrine (10⁻⁶ M) and measuring relaxation to carbachol. To test whether an alternative phytoestrogen that lacks tyrosine kinase inhibitory activity would mimic the responses of genistein on carbachol-induced relaxation, we examined the effects of daidzein (30 μM), a structural analog of genistein with no inhibitory effects on tyrosine kinases (Sargeant et al., 1993). In addition, we tested the effects of 17β-estradiol (10 μM) on carbachol-induced relaxation to determine whether the effects of a mammalian estrogen would parallel those of the phytoestrogens. We also determined whether the estrogen-like effects were mediated via estrogen receptors by incubating rings with the estrogen receptor blocker ICI 182,780 (10 μM) (Wakeling and Bowler, 1992) before adding genistein, daidzein, and 17β-estradiol. Finally, to determine whether the restoration of carbachol-induced relaxation was mediated via endothelium-derived NO, we repeated the above-mentioned experiments in pulmonary artery rings pretreated with the NO synthase inhibitor L-NA (100 μM).

**Data Analysis.** Results are expressed as mean ± S.E.M. Carbachol-induced relaxation was expressed as percentage reversal of phenylephrine-induced contraction. The potency was calculated as the negative logarithm of the concentration causing a 50% relaxation response. Differences between mean values were evaluated using Student’s t-test. When more than two means were compared, one-way analysis of variance followed by the Fisher’s least-significant difference test was used. Differences were considered significant when P < 0.05.

**Results**

**Chronic Hypoxia-Induced Pulmonary Hypertension.** Rats exposed to hypobaric hypoxia (0.5 atmospheres) for 2 weeks manifested the characteristic features of hypoxia-induced pulmonary hypertension, including polycythemia and right ventricular hypertrophy as indicated by significant increases in the RV/(LV + S) and RV/body weight ratios (Table 1).

**Contractile and Relaxation Responses of Pulmonary Arteries Isolated from Normoxic and Hypoxic Rats.** The contractile response to phenylephrine (10⁻⁶ M) was not significantly different between pulmonary arteries from normoxic (0.32 ± 0.04 g) and chronically hypoxic (0.26 ± 0.05 g, P > 0.05) rats. In pulmonary arteries from normoxic rats, carbachol caused a concentration-dependent relaxation that almost completely reverses the phenylephrine-induced contraction. Carbachol also caused a concentration-dependent relaxation of phenylephrine-contraction pulmonary arteries isolated from chronically hypoxic rats, but the relaxation response was significantly attenuated (46.13 ± 6.7%, P < 0.001) compared with the response in pulmonary arteries from normoxic rats (90.37 ± 2.3%, Fig. 1).

**Effects of Genistein on Contractile and Relaxation Responses of Pulmonary Arteries Isolated from Normoxic and Hypoxic Rats.** In pulmonary arteries from normoxic rats, genistein (30 μM) had no effect on baseline tone, but significantly decreased contractile force generated by phenylephrine (Fig. 2A). In pulmonary arteries from chronically hypoxic rats, treatment with genistein decreased baseline tone (~0.11 ± 0.01 g, P < 0.01) and tended to reduce contractile force generated by phenylephrine, but the reduction did not reach statistical significance (Fig. 2A). The phenylephrine-induced contractions, both before and after genistein treatment, were similar in pulmonary arteries isolated from normoxic and hypoxic rats (Fig. 2A).

**Table 1**

<table>
<thead>
<tr>
<th>Effects of chronic hypoxia on body weight, hematocrit, and heart weights</th>
<th>Normoxic Rats (n = 14)</th>
<th>Chronically Hypoxic Rats (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>339 ± 11</td>
<td>271 ± 7***</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>41 ± 0.4</td>
<td>66 ± 1.0***</td>
</tr>
<tr>
<td>RV/(LV + S)</td>
<td>0.29 ± 0.01</td>
<td>0.55 ± 0.02***</td>
</tr>
<tr>
<td>RV (mg/body weight (g)</td>
<td>0.68 ± 0.05</td>
<td>1.4 ± 0.08***</td>
</tr>
</tbody>
</table>

*** P < 0.001 vs. normoxic rats.
In pulmonary arteries from normoxic rats, genistein (30 \( \mu \)M) caused a slight but significant decrease in the EC_{50} value for the relaxation response to carbachol (\(-6.29 \pm 0.05\) and \(-6.75 \pm 0.09\) M in the absence and presence of genistein, respectively, \( P < 0.05 \)) but had no significant effect on the magnitude of the maximum relaxation response (Fig. 2B). In hypoxic rat pulmonary arteries, genistein markedly increased the relaxation response to carbachol as shown by a marked decrease in the EC_{50} (\(-5.83 \pm 0.13\) and \(-6.28 \pm 0.19\) M in the absence and presence of genistein, respectively, \( P < 0.05 \)) and an increase in maximum relaxation response (from \(46.13 \pm 6.7\) to \(115.62 \pm 4.6\)%, \( P < 0.001 \)). Following genistein, the relaxation response to carbachol in pulmonary arteries from hypoxic rats was similar to that seen in pulmonary arteries from normoxic rats (Fig. 2C). Treatment with vehicle dimethyl sulfoxide alone had no significant effect on the relaxation response to carbachol (data not shown).

**Effects of Daidzein on Contractile and Relaxation Responses of Pulmonary Arteries Isolated from Normoxic and Hypoxic Rats.** Daidzein (30 \( \mu \)M), the structural analog of genistein that is inactive as a tyrosine kinase inhibitor (Sargeant et al., 1993), had no effect on either baseline tone or contractile force generated by phenylephrine in pulmonary arteries isolated from normoxic rats (Fig. 3A). In pulmonary arteries from chronically hypoxic rats, treatment with daidzein decreased baseline tone (\(-0.1 \pm 0.01\) g, \( P < 0.01 \)) but had no significant effect on contractile force generated by phenylephrine (Fig. 3B). Daidzein significantly increased the relaxation response to carbachol in pulmonary arteries isolated from hypoxic rats (Fig. 3C) and this was comparable with that observed with genistein (Fig. 2C).

**Effects of 17\( \beta \)-Estradiol on Contractile and Relaxation Responses of Pulmonary Arteries Isolated from Normoxic and Hypoxic Rats.** The mammalian estrogen, 17\( \beta \)-estradiol had no effect on either baseline tone or contractile force generated by phenylephrine in normoxic pulmonary arteries (Fig. 4A). In hypoxic rat pulmonary arteries, 17\( \beta \)-estradiol decreased baseline tone (\(-0.1 \pm 0.01\) g, \( P < 0.01 \)) but had no significant effect on phenylephrine-induced contraction (Fig. 4A). Similar to genistein and daidzein, 17\( \beta \)-estradiol (10 \( \mu \)M) also significantly increased the relaxation response to carbachol in hypoxic pulmonary arteries (Fig. 4C) with no significant effect on normoxic pulmonary arteries (Fig. 4B).
Effects of ICI 182,780 on Genistein, Daidzein, and 17β-Estradiol-Induced Restoration of the Relaxation Response to Carbachol. To determine whether estrogen receptors are involved in genistein, daidzein, and 17β-estradiol-induced restoration of the vasorelaxant response to carbachol, additional experiments were done in the presence of the estrogen receptor antagonist ICI 182,780 (10 μM). The estrogen receptor antagonist did not attenuate the effects of genistein, daidzein, and 17β-estradiol on baseline tone and carbachol-induced relaxation in hypoxic (Fig. 5, A–C, respectively) and normoxic rat pulmonary arteries (data not shown).

Fig. 3. Effects of daidzein (30 μM) on phenylephrine-induced tone (A) and carbachol-induced reversal of phenylephrine (10⁻⁶ M)-induced contraction in pulmonary artery rings isolated from normoxic (B) and chronically hypoxic rats (C). Panel A: □, −daidzein; ■, +daidzein; panel B: □, −daidzein; ■, +daidzein; panel C: △, −daidzein; ▲, +daidzein. Values are means ± S.E.M. n = 4–6 in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with rings not exposed to daidzein.

Fig. 4. Effects of 17β-estradiol (10 μM) on phenylephrine-induced tone (A) and carbachol-induced reversal of phenylephrine (10⁻⁶ M)-induced contraction in pulmonary artery rings isolated from normoxic (B) and chronically hypoxic (C) rats. Panel A: □, −estradiol; ■, +estradiol; panel B: □, −estradiol; ■, +estradiol; panel C: △, −estradiol; ▲, +estradiol. Values are means ± S.E.M. n = 4–6 in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with rings not exposed to 17β-estradiol.
Effects of L-NA on Genistein, Daidzein, and 17β-Estradiol-Induced Restoration of the Relaxation Response to Carbachol.

To determine whether endothelium-derived NO is involved in genistein, daidzein, and 17β-estradiol-induced restoration of the vasorelaxant response to carbachol, additional experiments were done in the presence of the NO synthase inhibitor L-NA (100 μM). L-NA completely abolished the relaxation response to carbachol in normoxic and hypoxic rat pulmonary arteries (Fig. 6A), including the relaxation restored by genistein. Similarly, L-NA abolished the relaxation restored by daidzein and 17β-estradiol (Fig. 6, B and C, respectively).

Discussion

We found that the soybean-derived phytoestrogens genistein and daidzein, like the mammalian estrogen 17β-estradiol, restore the impaired relaxation response to agonist-stimulated NO release in pulmonary arteries isolated from chronically hypoxic rats. This observation parallels those made in previous studies on the systemic circulation, where treatment with genistein restores the relaxation re-

Fig. 5. Effects of ICI 182,780 (10 μM) on genistein-enhanced (30 μM) (A, ■ + genistein; ▲ + genistein + ICI), daidzein-enhanced (30 μM) (B, ■ + daidzein; ▲ + daidzein + ICI), and 17β-estradiol-enhanced (10 μM) (C, ■ + estradiol; ▲ + estradiol + ICI) relaxation response to carbachol in pulmonary artery rings isolated from chronically hypoxic rats. Values are means ± S.E.M. n = 4–5 in each group.

Fig. 6. Effects of L-NA (100 μM) on carbachol-induced reversal of phenylephrine (10⁻⁶ M)-induced contraction in pulmonary artery rings isolated from normoxic (■) and chronically hypoxic (▲) rats and treated with genistein (30 μM) (A), daidzein (30 μM) (B), and 17β-estradiol (10 μM) (C). Values are means ± S.E.M. n = 4–5 in each group.
response to acetylcholine in atherosclerotic coronary arteries from female macaques (Honore et al., 1997) and aortic rings isolated from ovariectomized rats (Squadrito et al., 2000).

NO is released from the endothelium under basal conditions, in response to shear stress and after stimulation with agonists such as acetylcholine and carbachol, and plays a pivotal role in regulating pulmonary arterial tone. Many studies have reported that endothelial dysfunction, characterized by diminished relaxation in response to NOS stimulators, contributes to the development of hypoxic pulmonary hypertension (Adnot et al., 1991; Dinh-Xuan et al., 1993; Durmovicz et al., 1993; Maruyama et al., 1995; Brett et al., 1996; Karamsetty et al., 1996; Berkenbosch et al., 2000). In line with these studies, we found an impairment of carbachol-induced NO-mediated relaxation in pulmonary arteries isolated from chronically hypoxic rats. We also found that the phytoestrogens genistein and daidzein, as well as the mammalian estrogen 17β-estradiol, restore this impaired relaxation response to normal.

Our findings indicate that increased production and/or release of NO from the vascular endothelium induced by the phytoestrogens is involved in the mechanism by which phytoestrogens restore the relaxation response to carbachol in pulmonary arteries from chronically hypoxic rats. This is supported by our finding that the NOS inhibitor L-NA inhibits the effects of genistein on basal tone and carbachol-induced relaxation. This observation is consistent with previous reports showing that in vivo treatment with genistein reverses the endothelial dysfunction and increases enzymatic activity of endothelial NOS in ovariectomized rat lungs (Squadrito et al., 2000). In addition to increased synthesis and/or release of NO, it is also possible that genistein and daidzein act as antioxidants by virtue of their polyphenolic chemical structure (Wei et al., 1995; Cai and Wei, 1996; Ruiz-Larrea et al., 1997; Kerry and Abbey, 1998; Mitchell et al., 1998; Trieu et al., 1999). This action could potentiate the biological actions of NO by protecting it from scavenging by reactive oxygen species that abound in hypoxic rat pulmonary arteries (Wanstall et al., 1997). Alternatively, genistein could also potentiate the effect of NO by increasing the activity of soluble guanylate cyclase or decreasing the catabolism of cGMP by phosphodiesterases (Satake et al., 1999).

Genistein could also act by inhibiting tyrosine kinases (Akiyama et al., 1987; Akiyama and Ogawara, 1991), an action that has been shown to attenuate agonist-induced NO-mediated relaxation in the in situ rat basilar artery (Kitazono et al., 1998). However, daidzein also reduced basal tension and restored the relaxation response to carbachol in hypoxic rat pulmonary arteries. Considering that daidzein lacks tyrosine kinase inhibitory activity, this suggests that other mechanisms are responsible for the effects of phytoestrogens observed in this study. Some in vivo studies suggest that phytoestrogens may increase the activity of endothelial NOS gene (Squadrito et al., 2000). However, it is unlikely for any genomic changes to take place within the 30 min of pretreatment with phytoestrogens used in the present study.

Genistein could also act via binding to estrogen receptors (Miksicek, 1994) and exerting estrogen-like effects on the cardiovascular system, including stimulation of the NO pathway (Squadrito et al., 2000). Consistent with this possibility, 17β-estradiol restored the relaxation response to carbachol in hypoxic rat pulmonary arteries paralleling the effects of the phytoestrogens in restoring the relaxation response in hypoxic rat pulmonary arteries. This finding is also consistent with prior investigations showing that in vivo treatment with estrogen improves pulmonary hemodynamics and attenuates structural remodeling of small pulmonary arteries in rats with monocrotaline-induced pulmonary hypertension (Farhat et al., 1993) and fetal lambs with pulmonary hypertension induced by ductus arteriosus ligation (Parker et al., 2000).

However, despite the similar responses of the phytoestrogens and 17β-estradiol, we could not confirm that traditional estrogen receptors mediate these effects. The high-affinity estrogen receptor antagonist ICI 182,780 (Wakeling and Bowler, 1992) that blocks both estrogen receptor α and β did not inhibit the effects of genistein, daidzein, or 17β-estradiol on carbachol-induced relaxation. Thus, the known estrogen receptors do not appear to be involved in the effects of phytoestrogens observed in this study.

Although our results raise the possibility that phytoestrogens could have therapeutic potential in treating pulmonary hypertension, these observations are very preliminary and a number of limitations should be borne in mind. Our study was performed in vitro on isolated tissue and may not be reproducible in vivo. In addition we examined only conduit pulmonary arteries; more distal vessels may respond differently. Furthermore, the possibility of species-related differences should be considered.

In conclusion, the results of our study demonstrate that genistein and daidzein restore impaired NO-mediated vasodilatation in response to the endothelium-dependent agonist carbachol. Although, the mechanism(s) for this response is unclear, it does not appear to involve estrogen receptors or inhibition of tyrosine kinases. We suggest that genistein and daidzein, because of their ability to increase NO-mediated relaxation in hypoxic pulmonary arteries, and lack of the feminizing and oncogenic effects of the estrogens, deserve further investigation as potential therapeutic agents for the treatment of pulmonary hypertension.

References


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