Nifedipine inhibits pulmonary hypertension but does not prevent decreased lung eNOS in hypoxic newborn pigs

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Fike, Candice D., and Mark R. Kaplowitz. Nifedipine inhibits pulmonary hypertension but does not prevent decreased lung eNOS in hypoxic newborn pigs. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L449–L456, 1999.—Therapies to prevent the onset or progression of pulmonary hypertension in newborns have received little study compared with those in adult models. We wanted to determine whether nifedipine treatment prevents the increased pulmonary vascular resistance, blunted pulmonary vascular responses to acetylcholine, and reduced lung endothelial nitric oxide synthase (eNOS) amounts that we have found in a newborn model of chronic hypoxia-induced pulmonary hypertension. Studies were performed with 1- to 3-day-old piglets raised in room air (control) or 10% O2 (hypoxia) for 10-12 days. Some piglets from each group were given nifedipine (3–5 mg/kg sublingually three times a day). Pulmonary arterial pressure, pulmonary wedge pressure, and cardiac output were measured in anesthetized animals. Pulmonary vascular responses to acetylcholine and eNOS amounts were assessed in excised lungs. The calculated value of the pulmonary vascular resistance for nifedipine-treated hypoxic piglets (0.09 ± 0.01 cmH2O·ml−1·min·kg−1) was almost one-half of the value for untreated hypoxic piglets (0.16 ± 0.01 cmH2O·ml−1·min·kg−1) and did not differ from the value for untreated control piglets (0.05 ± 0.01 cmH2O·ml−1·min·kg−1). Pulmonary arterial pressure responses to acetylcholine and whole lung homogenate eNOS amounts were less for both nifedipine-treated and untreated hypoxic piglets than for untreated control piglets. Nifedipine treatment attenuated pulmonary hypertension in chronically hypoxic newborn piglets despite the persistence of blunted responses to acetylcholine and reduced lung eNOS amounts.

METHODS

Animals. Newborn piglets (1–3 days old) were placed in chambers with either a room air environment (control; n = 13) or a hypoxic normobaric environment (chronic hypoxia; n = 24) for 10–12 days. Except for the case of one control piglet, two piglets were placed in each chamber. For the chronically hypoxic piglets, the normobaric hypoxic environment was produced by delivering compressed air and nitrogen to an incubator (Thermocare). The O2 content was regulated at 8–10% (P02, 60–72 Torr), and Pco2 was maintained at 3–6 Torr by absorption with soda lime. The chamber was opened two to three times a day for cleaning and to weigh the animals. The animals were fed ad libitum with an artificial sow milk replacer from a feeding device attached to the chamber. Piglets were randomly chosen from both groups (n = 5 control and 10 hypoxic) for nifedipine treatment (3–5 mg/kg sublingually three times a day). The dose of nifedipine was chosen because in pilot studies with chronically hypoxic piglets, we found that lower doses had no effect on pulmonary vascular resistance and that larger doses were associated with a high incidence of unexpected death. Some nifedipine-treated piglets were raised in the same chamber with an untreated animal from the same group (n = 3 control and 5 hypoxic).
Because Fike et al. (6) previously found no differences in endothelium-dependent responses or lung eNOS amounts between control piglets raised on the farm and control piglets raised in a normoxic chamber, 11 untreated control animals were studied on the day of arrival from the farm at 11–15 days of age.

Measurements in anesthetized animals. On the day of study, all piglets were weighed and anesthetized with ketamine (30 mg/kg im) and pentobarbital sodium (10 mg/kg iv). Additional intravenous pentobarbital sodium was given as needed via an ear vein to maintain anesthesia during placement of the catheters. First, the trachea of the piglet was cannulated so that the animal could be ventilated if necessary. Then, a catheter was placed into the right femoral artery for monitoring systemic blood pressure and arterial blood gases. Next, for most piglets (n = 16 untreated control, 5 nifedipine-treated control, 13 untreated hypoxic, and 11 nifedipine-treated hypoxic), another catheter was placed through the right external jugular vein into the pulmonary artery to monitor pulmonary arterial pressure. To obtain pulmonary wedge pressure, the pulmonary arterial catheter was advanced into a distal pulmonary vessel. The zero reference for the vascular pressures was the midthorax. To measure cardiac output by the thermodilution technique (model 9520 thermodilution cardiac output computer, Edwards Laboratory), a thermistor was placed into the aortic arch via the left femoral artery and a catheter that served as an injection port was placed into the left ventricle via the left carotid artery. Cardiac output was measured at end expiration as the mean of three injections of 3 ml of 0.9% saline (0°C). After blood gases were measured, all animals were given heparin (1,000 IU/kg iv) and additional anesthesia (3–5 mg/kg of pentobarbital sodium iv) and then exsanguinated. Most lungs were left in situ and used for perfusion as described in Measurements in isolated perfused lungs. A few lungs (n = 1 untreated control and 1 untreated hypoxic) were immediately frozen in liquid nitrogen and stored at −80°C for later analysis of eNOS as described in Immunoblot analysis.

Measurements in isolated perfused lungs. For lung perfusion, the tracheal cannula was attached to a large-animal piston-type ventilator, and the lungs were ventilated with a normoxic gas mixture (17% O2, 6% CO2, and balance N2), with a tidal volume of 15–20 ml/kg and a respiratory rate of 15–20 breaths/min (mean airway pressure of 4–6 cmH2O). A midline sternotomy was performed, and a clamp was placed across the ductus arteriosus. Saline-filled cannulas were placed into the pulmonary artery and left atrium through incisions in the right and left ventricles. The diaphragm and all abdominal contents were removed. The vascular cannulas were connected to the perfusion circuit as previously described (4, 5). The perfusion circuit was filled with 100–200 ml of the animal’s own blood collected during exsanguination (as described in Measurements in anesthetized animals) and mixed with 50–100 ml of 3% albumin-saline. The perfusion circuit included a rotary pump (model 7523-00, Cole Palmer Masterflex) that continuously circulated the perfusate from a reservoir through a bubble trap into the pulmonary arterial cannula, through the lungs to the left atrial cannula, and back to the reservoir. Pulmonary arterial, left atrial, and airway pressures were continuously monitored. The most dependent edge of the lung was used as the zero reference for vascular pressures.

After connection to the perfusion circuit, the lungs were perfused for 30–60 min until a stable pulmonary arterial pressure was achieved. The perfusate flow and left atrial pressure were adjusted to respective levels of 50

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room temperature with mouse anti-human eNOS (Transduction Laboratories) diluted 1:500 in PBS containing 0.1% Tween 20 and 1% nonfat dried milk (carrier buffer) followed by incubation for 30 min at room temperature with a biotinylated anti-mouse antibody (Vector Elite ABC kit, Vector Laboratories) diluted 1:5,000 in PBS containing 0.1% Tween 20 and 1% nonfat dried milk (carrier buffer) followed by incubation for 30 min at room temperature with streptavidin-horseradish peroxidase conjugate (Amersham) diluted 1:1,500 in PBS containing 0.1% Tween 20 after three times with PBS containing 0.1% Tween 20 and three times with the carrier buffer plus one time with PBS containing 0.1% Tween 20 after the final incubation. To visualize the biotinylated antibody, the membranes were developed with enhanced chemiluminescence reagents (Amersham), and the chemiluminescent signal was captured on X-Ray film (Bio-Max MR, Kodak) with laser densitometry. The absorbance of the eNOS band for the hematocrits in both groups of control piglets. The hematocrits in both groups of control piglets were slightly higher than those in the control piglets (Table 1).

Statistics. Data are presented as means ± SE. A one-way ANOVA with a multiple comparison test was used to compare the data between nifedipine-treated and untreated control and chronically hypoxic animals. P < 0.05 was indicative of significance.

RESULTS

After 10–12 days of hypoxia, nifedipine-treated and untreated chronically hypoxic piglets had higher hematocrits than both groups of control piglets (Table 1). The measured values of blood pH, PO2, and PCO2 obtained during hemodynamic measurements in anesthetized piglets breathing room air were similar between all groups of piglets except for a slightly higher PCO2 in the nifedipine-treated control group (Table 1). For the perfused lungs, the hematocrits in both groups of chronically hypoxic piglets were slightly higher than the hematocrits in both groups of control piglets. The measured values of pH, PO2, and PCO2 in the perfusate of isolated lungs from both the nifedipine-treated and untreated chronically hypoxic piglets were not different from those in the control piglets (Table 1).

Table 2. Hemodynamic measurements in anesthetized piglets

<table>
<thead>
<tr>
<th>Pulmonary Arterial Pressure, cmH2O</th>
<th>Pulmonary Wedge Pressure, cmH2O</th>
<th>Left Ventricular End-Diastolic Pressure, cmH2O</th>
<th>Aortic Pressure, cmH2O</th>
<th>Cardiac Output, ml·kg⁻¹·min⁻¹</th>
<th>Pulmonary Vascular Resistance, cmH2O·ml⁻¹·kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.0 ± 0.8 (16)</td>
<td>5.0 ± 0.4 (15)</td>
<td>4.4 ± 0.4 (14)</td>
<td>100 ± 8 (16)</td>
<td>242 ± 15 (16)</td>
<td>0.053 ± 0.004 (16)</td>
</tr>
<tr>
<td>Nifedipine-treated control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.1 ± 1.4 (5)</td>
<td>3.6 ± 0.6 (5)</td>
<td>3.6 ± 0.5 (5)</td>
<td>94 ± 7 (5)</td>
<td>372 ± 30*‡ (5)</td>
<td>0.034 ± 0.003 (5)</td>
</tr>
<tr>
<td>Untreated chronic hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41.0 ± 2.5*‡ (13)</td>
<td>9.6 ± 0.8*‡ (10)</td>
<td>8.6 ± 1.1*‡ (11)</td>
<td>84 ± 3 (13)</td>
<td>213 ± 23‡ (13)</td>
<td>0.16 ± 0.01*‡ (13)</td>
</tr>
<tr>
<td>Nifedipine-treated chronic hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.0 ± 7*‡ (11)</td>
<td>5.8 ± 0.5‡ (8)</td>
<td>5.2 ± 0.6‡ (11)</td>
<td>91 ± 6 (11)</td>
<td>319 ± 32‡ (11)</td>
<td>0.089 ± 0.01‡ (11)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses, no. of animals. Significantly different (P < 0.05) from: *untreated control group; † nifedipine-treated control group; ‡ all other groups (by 1-way ANOVA with multiple comparison test).
Measurements of pulmonary arterial pressure, pulmonary wedge pressure, left ventricular end-diastolic pressure, cardiac output, and aortic pressure in the anesthetized piglets are shown in Table 2. Pulmonary arterial pressure was greater in the untreated chronically hypoxic piglets than in the other three groups of piglets. The pulmonary arterial pressure in the nifedipine-treated group of chronically hypoxic piglets was intermediate in value between that in both groups of control piglets and that in untreated chronically hypoxic piglets. Pulmonary wedge pressure did not differ among nifedipine-treated chronically hypoxic, untreated control, and nifedipine-treated control piglets but was less in these three groups than in the untreated chronically hypoxic piglets (Table 2). Measurements of left ventricular end-diastolic pressure did not differ significantly from measurements of pulmonary wedge pressure (Table 2) and were used to calculate pulmonary vascular resistance if we were unable to obtain a pulmonary wedge pressure. Cardiac output did not differ between the untreated groups of control and hypoxic piglets but was greater in the nifedipine-treated piglets than in their corresponding group of untreated piglets (Table 2). The calculated value of pulmonary vascular resistance \([\text{pulmonary arterial pressure} - \text{pulmonary wedge pressure}]/\text{cardiac output}\) was greater for the untreated chronically hypoxic piglets than for all the other groups of piglets (Table 2, Fig. 1). Most importantly, the pulmonary vascular resistance was the same in the nifedipine-treated chronically hypoxic piglets as in the untreated group of control piglets (Table 2, Fig. 1).

In the perfused lungs, left atrial pressure and perfusate flow were the same in all groups and were maintained constant throughout the study so that changes and differences in pulmonary arterial pressure, respectively, represent changes and differences in pulmonary vascular resistance. Note that the baseline pulmonary arterial pressure did not differ between the nifedipine-treated and the corresponding group of untreated piglets (Table 3) and that the baseline pulmonary arterial pressure was greater in both groups of hypoxic piglets than in either group of control piglets (Table 3). After the addition of KCl, the pulmonary arterial pressure in the control groups was raised to a level comparable to that in the hypoxic groups (Table 3). Addition of all doses of acetylcholine resulted in similar decreases in pulmonary arterial pressure in the nifedipine-treated piglets as in the corresponding groups of untreated piglets (Table 3, Fig. 2). The response to all but the lowest dose of acetylcholine was less in both the nifedipine-treated and untreated groups of hypoxic lungs compared with either group of control lungs (Table 3, Fig. 2). The addition of papaverine and removal of vasomotor tone resulted in a pulmonary arterial pressure in the nifedipine-treated group of chronically hypoxic piglets.

Table 3. Pulmonary arterial responses to acetylcholine in perfused lungs of nifedipine-treated and untreated control and chronically hypoxic piglets

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline Pulmonary Arterial Pressure, cmH(_2)O</th>
<th>Elevated Pulmonary Arterial Pressure, cmH(_2)O</th>
<th>Absolute Change in Pulmonary Arterial Pressure, cmH(_2)O</th>
<th>%Change in Pulmonary Arterial Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>8</td>
<td>23.2 ± 0.2</td>
<td>30.5 ± 2.2</td>
<td>−6.2 ± 0.6</td>
<td>−20 ± 1</td>
</tr>
<tr>
<td>Nifedipine-treated control</td>
<td>5</td>
<td>19.4 ± 0.8</td>
<td>33.2 ± 1.0</td>
<td>−6.4 ± 0.5</td>
<td>−19 ± 1</td>
</tr>
<tr>
<td>Untreated chronic hypoxia</td>
<td>5</td>
<td>33.2 ± 3.6†‡</td>
<td>33.2 ± 1.0</td>
<td>−2.5 ± 0.8†‡</td>
<td>−6 ± 2†‡</td>
</tr>
<tr>
<td>Nifedipine-treated chronic hypoxia</td>
<td>10</td>
<td>31.0 ± 1.8†‡</td>
<td>33.2 ± 1.0</td>
<td>−2.7 ± 1.3†‡</td>
<td>−7 ± 4†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. Acetylcholine concentration was 10\(^{-6}\)M. Significantly different (P < 0.05) from: *untreated control group; †nifedipine-treated control group; ‡immediately preceding value (by 1-way ANOVA with multiple comparison test).
hypoxic piglets that was less than the pulmonary arterial pressure in the untreated group of chronically hypoxic piglets and that did not differ from the pulmonary arterial pressure in either the treated or untreated group of control piglets (Fig. 3).

One of the immunoblot analyses for eNOS in the whole lung homogenates is shown in Fig. 4. In both the nifedipine-treated and untreated groups of control and chronically hypoxic lungs, the antibody to eNOS detected eNOS at an apparent molecular mass of 135 kDa as determined from an exponential fit of molecular-mass standards. Figure 5 shows the mean data for the absorbance of the eNOS bands as determined by laser densitometry for the whole lung homogenates from the untreated and nifedipine-treated control and the untreated and nifedipine-treated chronically hypoxic lungs and shows that absorbance of the eNOS bands was less for the lungs from both the nifedipine-treated and untreated chronically hypoxic piglets compared with that for the lungs from the untreated control piglets.

**DISCUSSION**

In agreement with the findings of previous studies by Fike and colleagues (4–6), in this study we show that when exposed to 10–12 days of chronic hypoxia, newborn piglets develop pulmonary hypertension. Also in agreement with previous studies by Fike and colleagues (5, 6), the chronically hypoxic piglets exhibit blunted pulmonary vascular responses to the endothelium-dependent dilator acetylcholine and have reduced amounts of lung eNOS. A new finding is that the pulmonary vascular resistance in newborn piglets treated with nifedipine during chronic hypoxia did not differ from that in control piglets. This finding is consistent with the results of a study in adult models of pulmonary hypertension (17) and indicates that treatment with nifedipine ameliorates the development of pulmonary hypertension in our newborn model. Another new finding in this study is that the pulmonary arterial responses to acetylcholine were blunted and eNOS amounts were reduced in the lungs from chronically hypoxic piglets regardless of whether or not they were treated with nifedipine. This latter finding indicates that mechanisms other than an improvement in endothelial function or restoration of lung eNOS amounts underlie the ameliorative effect of nifedipine.

Although pulmonary vascular resistances in nifedipine-treated hypoxic and untreated control piglets did not differ in this study, the in vivo pulmonary arterial pressure was greater in the nifedipine-treated chronically hypoxic piglets than in the control piglets (Table 2). In addition, in the perfused lungs, the baseline pulmonary arterial pressure was greater in the nifedipine-treated hypoxic piglets than in the control piglets. This latter finding can be attributed to the influence of...
vasomotor tone in the isolated lung preparation because there was no difference between pulmonary arterial pressures in nifedipine-treated hypoxic and untreated control lungs once the vasomotor tone was removed (Fig. 3). In regard to the in vivo measurements, it is probable that the measurement of pulmonary arterial pressure was affected by differences in left atrial pressure and cardiac output. Moreover, even though not as low as in the control piglets, the pulmonary arterial pressure in the nifedipine-treated hypoxic piglets was less than that in the untreated hypoxic piglets. This latter finding clearly indicates that although pulmonary hypertension was not completely prevented, nifedipine treatment inhibited the development of the disorder.

Had we used higher doses of nifedipine as in a study with chronically hypoxic adult rats (17), we might have achieved even greater reductions in pulmonary vascular resistance in the hypoxic piglets. However, the dose that we used is high compared with common clinical usage in newborns (1, 7), and in pilot studies, we found that larger doses than these were not well tolerated by the newborn piglets. The mechanism by which nifedipine treatment inhibits the development of pulmonary hypertension in either our newborn model or adult models remains unknown. Of course, part of the difficulty in determining the mechanism underlying the ameliorative effect of nifedipine is the lack of understanding of the pathogenesis of pulmonary hypertension in either newborns or adults. Endothelial dysfunction is one potential cause of pulmonary hypertension (11, 20, 22). Another possibility is that either in association or separate from endothelial dysfunction, decreased production of endothelium-derived vasodilators such as nitric oxide could contribute to the development of pulmonary hypertension (11, 20, 22). Although the findings of Fike and colleagues (5, 6) and those of others (16, 19, 21) are supportive of the preceding mechanisms as a cause of neonatal pulmonary hypertension, our findings in the present study are also notable for providing evidence that mechanisms other than an improvement in endothelial function or restoration of lung eNOS amounts underlie the ameliorative influence of nifedipine. Indeed, one logical possibility is that nifedipine provided the reduction in smooth muscle tone, i.e., vasodilatory effect, that the dysfunctional pulmonary vascular endothelium and/or reduced lung eNOS could not.

The ability of nifedipine to reduce pulmonary arterial pressure and inhibit hypoxic pulmonary vasoconstriction from a smooth muscle cell vasorelaxant effect has been previously proposed as a mechanism for preventing pulmonary hypertension in chronically hypoxic adult animals (9, 17). That nifedipine can be an effective pulmonary vasodilator even in the nonhypoxic newborn pulmonary circulation is suggested by the slightly lower pulmonary vascular resistance in the nifedipine-treated than in the untreated control piglets (Fig. 1, Table 2). In addition to blockade of smooth muscle cell calcium channels, the vasodilatory effect of nifedipine could result from altered production of vasoactive agents. For example, nifedipine inhibited the
production of the vasoconstrictor thromboxane by cultured rabbit aortic endothelial cells (13). Yet, it is important to note that despite effectively lowering pulmonary arterial pressure, not all smooth muscle cell vasodilators have been shown to inhibit the development of chronic hypoxia-induced pulmonary hypertension (17). The ability of nifedipine to ameliorate pulmonary hypertension might be more complex than can be explained by smooth muscle cell dilation.

One possibility is that nifedipine has an inhibitory effect on smooth muscle cell proliferation (12). Another possibility is that nifedipine modulates metabolism of collagens within the extracellular matrix (15). Both of these effects would help maintain a lower pulmonary vascular resistance by preventing the pulmonary vascular remodeling associated with chronic hypoxia-induced pulmonary hypertension (17, 18). The mechanism for these effects could involve a decrease in intracellular calcium concentration due to the inhibition of voltage-gated L-type calcium channels. That is, nifedipine might ameliorate pulmonary vascular wall changes by inhibiting the calcium-triggered transcription of a myriad of genes (14). Additional pathways independent of calcium may also be involved (3).

Supportive of the potential for the above-noted protective effects of nifedipine, other investigators (17) found that nifedipine treatment inhibited pulmonary vascular remodeling in chronically hypoxic adult animals. Because we did not perform morphometry, we do not know with certainty that nifedipine prevented the pulmonary vascular remodeling that we (4) and others (8) have found in chronically hypoxic newborn piglets. However, our finding that with removal of vasomotor tone, the pulmonary vascular resistance in isolated lungs from nifedipine-treated hypoxic piglets did not differ from that in untreated control piglets provides evidence that the pulmonary vascular bed does not differ structurally in these two groups of piglets (Fig. 3). In addition, consistent with our previous findings (4), structural remodeling is the likely explanation for the persistently greater pulmonary arterial pressure in the untreated hypoxic lungs than in all other groups of lungs even with removal of vasomotor tone (Fig. 3).

Our finding that lung eNOS amounts were reduced in the lungs of hypoxic piglets regardless of nifedipine treatment merits some additional comments. Oxygen tension and shear stress are two stimuli that have been shown to regulate eNOS expression (2, 10). In the chronically hypoxic piglet model, oxygen tension changes but so do a number of determinants of shear stress including pulmonary arterial pressure, cardiac output, and hematocrit. Whether any or all of these stimuli are responsible for the reduced eNOS observed in the lungs of chronically hypoxic piglets remains unknown. Of these stimuli, it is of interest that findings in fetal lambs with pulmonary hypertension due to intrauterine closure of the ductus arteriosus support the notion that prolonged in vivo exposure to elevated pulmonary arterial pressure decreases lung eNOS in immature animals (16, 21). In this regard, the reason that lung eNOS amounts remained reduced despite nifedipine treatment could be due to the failure of nifedipine to completely reduce pulmonary arterial pressure in the hypoxic piglets to the level of control animals. Other possibilities are that the potential to restore lung eNOS by lowering pulmonary arterial pressure was offset by a direct effect of hypoxia or by some other effect of the nifedipine treatment such as increased cardiac output.

In summary, we found that nifedipine-treatment attenuated the development of pulmonary hypertension in a newborn model and that the effectiveness of the treatment was not due to an improvement in endothelial dysfunction nor to preventing a reduction in lung eNOS amounts. It seems logical that the presence of endothelial dysfunction and decreased lung eNOS amounts increase the likelihood that there will be worsening of the pulmonary hypertensive process so that counteracting, reversing, or preventing these alterations remains a reasonable therapeutic target that requires further exploration.

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