Developmental change in isoproterenol-mediated relaxation of pulmonary veins of fetal and newborn lambs

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Gao, Yuansheng, Jean-Francois Tolsa, Michael Botello, and J. Usha Raj. Developmental change in isoproterenol-mediated relaxation of pulmonary veins of fetal and newborn lambs. J. Appl. Physiol. 84(5): 1535–1539, 1998.—β-Adrenergic agonists are important regulators of perinatal pulmonary circulation. They cause vasodilation primarily via the adenyl cyclase-adenosine 3',5'-cyclic monophosphate (cAMP) pathway. We examined the responses of isolated fourth-generation pulmonary veins of term fetal (145 ± 2 days gestation) and newborn (10 ± 1 days) lambs to isoproterenol, a β-adrenergic agonist. In vessels preconstricted with U-46619 (a thromboxane A₂ analog), isoproterenol induced greater relaxation in pulmonary veins of newborn lambs than in those of fetal lambs. The relaxation was eliminated by propranolol, a β-adrenergic antagonist. Forskolin, an activator of adenyl cyclase, also caused greater relaxation of veins of newborn than those of fetal lambs. 8-Bromoadenosine 3',5'-cyclic monophosphate, a cell membrane-permeable analog of cAMP, induced a similar relaxation of all vessels. Biochemical studies show that isoproterenol and forskolin induced a 10-fold increase in the intracellular cAMP content, and adenyl cyclase activity, we determined whether there is a developmental change in β-adrenergic-agonist-mediated response of pulmonary veins of perinatal lambs.

MATERIALS AND METHODS

Fifteen term fetal lambs (143–149 days gestation, term being 150 days gestation; either sex) from 7 pregnant ewes and 13 newborn lambs (8–13 days old, either sex) from Nebek Ranch (Lancaster, CA) were used. After an overnight fast, the ewe was anesthetized with ketamine hydrochloride (30 mg/kg im). Then, the fetus was delivered by cesarean section and killed by a lethal dose of pentobarbital sodium (100 mg/kg iv) via the umbilical vein. The ewe was killed with an overdose of pentobarbital sodium. Newborn lambs were anesthetized with ketamine hydrochloride (30 mg/kg im) and then killed with an overdose of pentobarbital sodium.

The lungs were immediately removed, and fourth-generation pulmonary veins (OD: 1.5–2.5 mm) were dissected free of parenchyma and cut into rings (length: 3 mm).

Organ chamber study. Vessel rings were suspended in organ chambers filled with 10 ml of modified Krebs-Ringer bicarbonate solution [composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2; KH₂PO₄, 25.0 NaHCO₃, 11.1 glucose] maintained at 37 ± 0.5°C and aerated with 95% O₂, 5% CO₂ (pH 7.4). Each ring was suspended by two stirrups passed through the lumen. One stirrup was anchored to the bottom of the organ chamber; the other one was connected to a strain gauge (model FT03C, Grass Instruments, Quincy, MA) for the measurement of isometric force (10).

At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension. This was achieved by step-by-step stretching in 0.1-g increments until the active contraction of the vessel ring to 100 μM KCl reached a plateau. The optimal resting tension of pulmonary veins of fetal lambs (0.29 ± 0.08 g; n = 15) was not significantly different from that of newborn lambs (0.30 ± 0.06 g; n = 13; P > 0.05).

After the vessels were brought to their optimal resting tension, 1 h of equilibration was allowed. Then, indomethacin [10⁻⁵ M; an inhibitor of cyclooxygenase (31)] and nitro-L-arginine [10⁻⁴ M; an inhibitor of nitric oxide synthase (18)] were administrated to eliminate the possible involvement of endogenous prostanooids and endothelium-derived nitric oxide (5, 8–10). Indomethacin (10⁻⁵ M) plus nitro-L-arginine (10⁻⁴ M) caused an increase in resting tension by 0.78 ± 0.10 g (n = 15) and 0.85 ± 0.13 g (n = 13) for pulmonary veins of fetal and newborn lambs, respectively. These values are not significantly different (P > 0.05).

Effects of isoproterenol [a β-adrenergic agonist (3)], forskolin [a direct activator of adenyl cyclase (14)], and 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP) [a cell membrane-permeable analog of cAMP (15)] were determined in vessels preconstricted with U-46619 (3 x 10⁻⁸ M; a stable analog of thromboxane A₂, 6). To determine whether the effect of isoproterenol is mediated by β-adrenergic recep-
tors, the effect of the agonist was examined both in the presence and absence of propranolol (5 × 10⁻⁶ M; an antagonist of β-adrenergic receptors (3)). Experiments with isoproterenol were performed in the presence of hydrocortisone (3 × 10⁻⁵ M) and phentolamine (5 × 10⁻⁶ M) to block the extraneuronal uptake and α-adrenoceptor receptors, respectively (4, 30). All experiments mentioned above were performed in a parallel manner. For each vessel ring, only one vasodilator was tested.

Measurement of cAMP.Venous rings were incubated in modified Krebs-Ringer bicarbonate solution (37°C, 95% O₂-5% CO₂) in the presence of indomethacin (10⁻⁵ M) and nitro-arginine (10⁻⁴ M) to eliminate the involvement of endogenous prostanooids and endothelium-derived nitric oxide (5, 8-10). To prevent the degradation of cAMP by phosphodiesterases, isobutylmethylxanthine (3 × 10⁻⁴ M) was used (29). In experiments with isoproterenol, hydrocortisone (3 × 10⁻⁵ M) and phentolamine (5 × 10⁻⁶ M) were present to block the extraneuronal uptake and α-adrenoceptor receptors, respectively (4, 30).

After 45 min of equilibration, isoproterenol (3 × 10⁻⁸ M) or forskolin (10⁻⁶ M) was added. The vessel rings were rapidly frozen in liquid nitrogen at 2 and 10 min after the administration of isoproterenol and forskolin, respectively. Preliminary data showed that maximal accumulation of cAMP in the vessels in response to isoproterenol and forskolin occurred at 2 and 10 min, respectively. Then, vessels were thawed in trichloroacetic acid (6%), homogenized in glass with a motor-driven Teflon pestle, sonicated for 5 s, and centrifuged at 13,000 g for 15 min. The supernatant was extracted with four volumes of water-saturated diethyl ether and lyophilized; the pellets were weighed. The lyophilized samples were resuspended in 0.5 ml of sodium acetate buffer (0.05 M, pH 6.2), and the content of cAMP was determined by using a cAMP kit (Biomedical Technologies, Stoughton, MA). The content of cyclic nucleotide is expressed as picomoles per milligram protein.

Table 1. Tension levels in fetal and newborn ovine pulmonary veins induced by U-46619 (3 × 10⁻⁶ M) before determination of vasodilating effects of various agents

<table>
<thead>
<tr>
<th>Isoproterenol groups</th>
<th>Fetal PV</th>
<th>Newborn PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.89 ± 0.18</td>
<td>2.03 ± 0.20</td>
</tr>
<tr>
<td>With propranolol</td>
<td>1.88 ± 0.26</td>
<td>1.79 ± 0.16</td>
</tr>
<tr>
<td>Forskolin groups</td>
<td>1.77 ± 0.28</td>
<td>1.97 ± 0.30</td>
</tr>
<tr>
<td>8-Bromoadenosine 3',5'-cyclic monophosphate groups</td>
<td>1.90 ± 0.15</td>
<td>1.88 ± 0.29</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals for each group. PV, pulmonary veins.

the measurement of the CAMP formed by using a radioimmunoassay kit (Biomedical Technologies). Adenylyl cyclase activity is expressed as picomoles cAMP per minute per milligram protein.

Drugs. The following drugs were used (unless otherwise specified, all were obtained from Sigma Chemical, St. Louis, MO): L-ascorbic acid, 8-BrcAMP, forskolin, hydrocortisone 21-hemisuccinate, indomethacin, isobutylmethylxanthine, isoproterenol, nitro-arginine, phentolamine (Research Biochemicals International, Natick, MA), propranolol, and U-46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂α).

Isobutylmethylxanthine and forskolin were dissolved in ethanol (final concentration: 0.1%). Preliminary experiments showed that ethanol at the concentration used had no effect on contraction to U-46619 and relaxation induced by isoproterenol in fetal and newborn lambs. Indomethacin (10⁻⁵ M) was prepared in equal molar Na₂CO₃. This concentration of Na₂CO₃ did not significantly affect the pH of the solution in the organ chamber. Isoproterenol was prepared in 0.1% ascorbic acid stock solution to prevent oxidation of the agent. The other drugs were prepared by using distilled water. Vessels were exposed to all inhibitors and antagonists for at least 30 min before the effects of β-adrenoceptor agonists were tested.

Data analyses. Data are shown as means ± SE. When mean values of two groups were compared, Student’s t-test for unpaired observations was used. When the mean values of the same group before and after stimulation were compared,
Student's $t$-test for paired observations was used. Comparison of mean values of more than two groups from same vessel type was performed with one-way analysis of variance test with Student-Newman-Keuls test for post hoc testing of multiple comparison. All these analyses were performed by using a commercially available statistics package (SigmaStat, Jandel Scientific, San Rafael, CA). Statistical significance was accepted when the $P$ value (2 tailed) was $< 0.05$. In all experiments, $n$ represents the number of animals.

RESULTS

Organ chamber studies. Effects of isoproterenol, forskolin, and 8-BrcAMP were examined after vessel tension was raised with U-46619 ($3 \times 10^{-8}$ M). There were no significant differences in the increase in tension induced by U-46619 among different vessel groups (Table 1).

Isoproterenol induced greater relaxation of pulmonary veins of newborn lambs than those of fetal lambs. Relaxation in response to isoproterenol was eliminated by propranolol ($5 \times 10^{-6}$ M) (Fig. 1). Forskolin also induced greater relaxation of pulmonary veins of newborn lambs than those of fetal lambs (Fig. 2). 8-BrcAMP induced similar relaxation of all veins (Fig. 3).

cAMP content. The basal intracellular content of cAMP was 25.3 ± 5.1 and 30.6 ± 3.6 pmol/mg protein for pulmonary veins of fetal and newborn lambs, respectively ($n = 8$ for each group). These values are not significantly different from each other ($P > 0.05$).

Isoproterenol ($3 \times 10^{-8}$ M) and forskolin ($10^{-6}$ M) caused a greater increase in cAMP content of pulmonary veins of newborn lambs than that of fetal lambs (Fig. 4).

Adenyl cyclase activity. The basal activity of adenyl cyclase was 8.76 ± 0.75 and 11.22 ± 1.23 pmol·mg$^{-1}$·min$^{-1}$ for the crude membrane preparations of pulmonary veins of fetal and newborn lambs, respectively ($n = 7$ for each group). They are not significantly different from each other ($P > 0.05$).

Isoproterenol ($3 \times 10^{-8}$ M) and forskolin ($10^{-6}$ M) caused a greater increase in adenyl cyclase activity of pulmonary veins of newborn than those of fetal lambs (Fig. 5).

DISCUSSION

In the present study, isoproterenol induced greater relaxation of pulmonary veins in newborn than in fetal lambs. The relaxation was eliminated by propranolol. These results suggest that there is a developmental increase in $\beta$-adrenergic-agonist-mediated response in pulmonary veins of the perinatal lambs.

A primary mechanism by which $\beta$-adrenergic agonists cause vascular smooth muscle to relax is by activating adenyl cyclase and subsequently increasing production of cAMP (1, 19). In comparison to isoproterenol...

end, which activates adenyl cyclase by a receptor-mediated mechanism, forskolin directly stimulates the activity of adenyl cyclase (14). In our study, forskolin also caused greater relaxation of veins of newborn lambs than those of fetal lambs. Furthermore, both isoproterenol and forskolin caused a greater increase in the intracellular content of cAMP and a greater increase in adenyl cyclase activity of pulmonary venous preparations in newborn lambs than in fetal lambs. These results suggest that a developmental increase in adenyl cyclase activity may contribute to the differential responses of perinatal pulmonary veins to isoproterenol.

In the present study, isoproterenol, at a concentration inducing comparable relaxation of veins as that of forskolin, caused a smaller increase in cAMP content and adenyl cyclase activity than did forskolin. Such a phenomenon has been observed in both pulmonary and nonpulmonary smooth muscles (11, 20, 27). The underlying mechanism is not well understood. One possible explanation is that there are multiple subcellular compartments for adenyl cyclases and cAMP. Some of the cAMP elevated after stimulation of adenyl cyclase with forskolin may be in subcellular compartments that are not accessible to the protein kinases involved in vasodilation (16, 32). Alternatively, isoproterenol may cause smooth muscle to relax by a mechanism that does not depend on cAMP. For instance, in porcine coronary arterial smooth muscle cells, isoproterenol may cause vasodilation by the stimulation of Ca\(^{2+}\)-activated K\(^+\) channels, independent of cAMP (26).

cAMP induces vasodilation by multiple mechanisms, including inhibition of Ca\(^{2+}\) influx, stimulation of Ca\(^{2+}\) efflux, opening of Ca\(^{2+}\)-dependent K\(^+\) channels, stimulation of Ca\(^{2+}\) uptake by sarcoplasmic reticulum, and inhibition of myosin phosphorylation (17). These effects are believed to be primarily mediated by cAMP-dependent protein kinase (1, 17). However, cAMP may cause smooth muscle to relax by other mechanisms such as activation of guanosine 3’5’-cyclic monophosphate-dependent protein kinase (12, 13). Authentic cAMP is a poor cell penetrant. Therefore, we used 8-BrcAMP, a cell membrane-permeable analog of cAMP (15), to examine the response of perinatal ovine pulmonary veins to cAMP. We found that all veins responded to the cAMP analog similarly, suggesting that the difference in isoproterenol- and forskolin-induced relaxation between pulmonary veins of fetal lambs and those of newborn lambs is not due to a differential response to cAMP.

In comparison to our knowledge of pulmonary arteries, our knowledge of pulmonary veins is rather limited. In perinatal lungs, pulmonary veins actively participate in regulation of the pulmonary vascular resistance (9, 10, 22, 28). Thromboxane, platelet-activating factor, endothelin, and hypoxia cause pulmonary veins of newborn lambs to constrict equally or even more than do arteries (21, 23, 24). In fetal and newborn lambs, relaxation of pulmonary veins induced by endothelium-derived nitric oxide is developmentally regulated (9, 10, 28). Our present study demonstrates that a developmental increase in β-adrenoceptor-agonist-mediated relaxation of pulmonary veins occurs during the perinatal period. Furthermore, an increase in adenyl cyclase activity may contribute to such a change.

It is worthy to note that the response of isolated vessels obtained under in vitro conditions may not necessarily represent the response of vessels under in vivo conditions. In our study, isoproterenol induced a greater response of pulmonary veins of newborn lambs than that of fetal lambs. This phenomenon was observed not only from vessel tension studies but also from measurements of cAMP content and adenyl cyclase activity. It is likely that it reflects a genuine developmental change in β-adrenergic-agonist-mediated response. We only examined the response of the fourth generation of pulmonary veins of perinatal sheep. Whether this phenomenon also occurs in veins of other generations of the pulmonary venous tree and whether our results are applicable to human in vivo remains to be explored.

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