Soluble guanylyl cyclase during postnatal porcine pulmonary maturation

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NITRIC OXIDE (NO) is a key mediator in the regulation of pulmonary vascular tone (2, 6). Most of the physiological effects of NO occur through the activation of soluble guanylyl cyclase (sGC) in pulmonary arteries during early postnatal maturation. The expression of sGC in pulmonary arteries increased with postnatal age both at the level of mRNA and protein. The catalytic region of sGC β1 subunit in newborn; YC-1; vascular smooth muscle

Changes in the expression of sGC during rat postnatal development have been reported. In the rat heart and kidney, sGC increased postnatally (7, 9). In contrast, sGC expression in carotid and cerebral arteries was higher in 3- to 7-day-old lambs compared with adult sheep (37). In the pulmonary arterial system, an increase in the responsiveness to NO with postnatal age, which plays a key role in the reduction of pulmonary vascular resistance during early extruterine life, has been reported in most (3, 21, 25, 31, 38), but not all (27), studies on rabbits, lambs, and piglets. In rats, where functional changes in the NO/cGMP pathway in pulmonary arterial postnatal maturation have not been analyzed, lung sGC mRNA, protein, and activity were higher at 1 or 8 days than in adults (8, 12). Immunostaining in pulmonary vascular smooth muscle was estimated to be higher in newborns than in adult animals (8).

Impaired vasodilator responses to the NO/cGMP pathway have been observed in several pathological conditions. In fact, reduced NO synthesis, bioavailability, and/or activity have been involved in the pathogenesis of primary and secondary pulmonary hypertension, including the persistent pulmonary hypertension of the newborn (1, 17, 34). Desensitization of sGC describes a reduced response to a given NO challenge that may be caused by a change in enzyme reactivity or an alteration of the amount of the enzyme present (16). In fact, changes in the lung expression of sGC have been demonstrated in several animal models of lung injury and pulmonary hypertension (10, 11, 20).

Therefore, the aim of the present study was to analyze the changes in function and expression of sGC in pulmonary arteries during early postnatal maturation. We hypothesized that sGC expression and the responses to YC-1 could increase with age in parallel with the increased responsiveness to NO.

METHODS

All the procedures conform to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and are approved by our Institutional Review Board.

Tissue preparation. Male piglets of 3–18 h (newborn, n = 12) or 15–20 days (2 wk, n = 17) from a local farm were killed by exsanguination after being anesthetized with pentobarbital sodium (100 mg/kg). The lungs were rapidly immersed in cold (4°C) Krebs solution (composition in mM: 118 NaCl, 4.75 KCl, 25 NaHCO₃, 1.2 MgSO₄, 2.0 CaCl₂, 1.2 KH₂PO₄, and 11 glucose). Third-branch pulmonary arteries (external diameter of ~1–2 mm) were carefully dissected free of surrounding tissue and cut into rings under a microscope (28). The endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The endothelium removal procedure was verified by the inability of acetylcholine (1 μM) to relax arteries precontracted with norepinephrine (1 μM).

Contractile tension recording. Third-branch pulmonary arteries were mounted between two hooks in a 5-mI organ bath filled with Krebs solution and stretched to their optimal resting tension (0.5 and 0.7 g for newborn and 2-wk pulmonary arteries, respectively), at which the rings produced the maximal contractile response to KCIC in previous experiments. The contraction was measured by an isometric force transducer using data acquisition software and hardware (28). Krebs solution was maintained at 37°C and gassed with a 95% O₂-5% CO₂ gas mixture. In previous experiments, the relaxant response to NO was not modified by the concentration of oxygen in the bubbling mixture (35).

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Rings were stimulated with the thromboxane A<sub>2</sub> mimetic 9,11-dideoxy-11α,9-epoxymethano-prostaglandin F<sub>2α</sub> (U-46619, 0.1 μM), which induced a contractile response (628 ± 64 mg, n = 12 and 985 ± 89 mg, n = 12, in third-branch newborn and 2-wk-old piglets, respectively) of 70–75% of the maximal response to U-46619 in both age groups. In previous experiments, these contractions were equieffective in the two age groups when expressed as a percentage of the responses induced by 40 mM KCl (28). Then, concentration-response curves to YC-1, and to the cGMP analog 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP), were carried out by cumulative addition of the drugs. Because of the rapid disappearance of NO gas in the bath, the curves to NO were performed in a noncumulative fashion by addition of increasing volumes of Krebs solution saturated with NO.

Western blot analysis of sGC. Third-branch pulmonary arteries were isolated, frozen in liquid nitrogen, and stored at −80°C. Frozen tissues were homogenized and separated into cytosolic and particulate fractions as described (14). The protein concentration was determined using the Bradford assay (reagents from Bio-Rad). Western blotting was performed with 20 μg of protein from the cytosolic fraction per lane. SDS-PAGE (7.5% acrylamide) was performed using the method of Laemmli in a mini-gel system (Bio-Rad). Samples from newborns and 2-wk-old animals were run in parallel. The proteins were transferred to polyvinylidene difluoride membranes overnight and incubated with a rabbit anti-β-subunit of sGC polyclonal antibody (1:2,500, Alexis Biochemicals) and then with an anti-rabbit secondary horseradish peroxidase-conjugated antibody. The bands were visualized by chemiluminescence (ECL, Amersham). The membranes were then stripped and incubated with a mouse antibody against smooth muscle α-actin (1:10,000, Sigma) and then with an anti-mouse secondary horseradish peroxidase-conjugated antibody. Bands were quantified using image analysis software (TotalLab, Nonlinear Dynamics). The results were expressed as a percentage of the data of newborn animals.

RT-PCR. Pulmonary arteries were isolated and frozen in liquid nitrogen. Frozen arteries were homogenized in a glass potter with 1 ml of Tri Reagent (Sigma), and total RNA was extracted with the guanidine thiocyanate-phenol-chloroform method (13). The RNA was dissolved in RNA storage solution (Ambion) and stored at −70°C. The integrity of the purified RNA was determined by 1% agarose gel electrophoresis, and its concentration was determined spectrophotometrically at 260 nm. One microgram of total RNA was used in the PCR. The integrity of the purified RNA was determined by 1% agarose gel electrophoresis, and its concentration was determined spectrophotometrically at 260 nm. One microgram of total RNA was converted into complementary DNA by reverse transcription following the protocol of the manufacturer (AMV reverse transcriptase; Promega, Southampton, UK). The mRNA sequence of porcine sGC β1 has not been previously reported. Therefore, primers for the PCR were selected from the regions of the catalytic domain of the rat β1-subunit of sGC (NM012769, GenBank), which were highly conserved in human (BC047620) and mouse (NM017469). All sequences were compared with GenBank using the Blast software (National Center for Biotechnology Information website). The following primers, synthesized on request by Metabion ( Martinsried, Germany), were used for the amplification of sGC β1 sense: 5′-AGATAGCAACAATGTGACCATCCTC-3′ and antisense: 5′-GATAGAACACGGACCCCGGGCC3′ resulting in a product of 569 bp; and for β-actin, sense: 5′-GGACCTGGACCACTCCTA-3′ and antisense: 5′-CATGATCCGAGTGAAGGTCG-3′, which yielded a product of 301 bp. In preliminary experiments, we tested a number of cycle numbers ranging from 24 to 35, with cDNA diluted over a range of three magnitudes, to establish the linearity of the reaction (24). PCR of sGC β1 was performed using a MyCycler Thermal cycler (Bio-Rad) by using Taq DNA polymerase (Biotools, B&K Labs) for 27 cycles (92°C for 1 min, 59°C for 1.5 min, and 72°C for 3 min) followed by a final extension step at 72°C for 10 min. The same protocol was used for β-actin except for the annealing temperature, which was 56.5°C. The products of the PCR were separated in 1.5% agarose gels stained with ethidium bromide, and bands were quantified using image analysis software (Quantity One, Bio-Rad). Results were normalized to the bands of β-actin from the same samples and expressed as a percentage of the data of newborn animals. The nucleotide sequence of amplified products was determined in Unidad de Genómica y Proteómica (Universidad Complutense de Madrid) by automated sequencing using an ABI PRISM BigDye Terminator V3.0 Ready Reaction Cycle sequencing kit in a 3730 DNA analyzer (Applied Biosystems). The sequencing was performed using both upstream and downstream primers to check its accuracy.

Drugs. YC-1 was from Cayman Chemical, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was from Tocris Cookson (Bristol, UK), and all other drugs were from Sigma Chemical (Alcobendas, Spain). NO solutions were prepared by bubbling Krebs solution with NO (450 ppm) as described (23). Drugs were initially dissolved in distilled deionized water (except for YC-1 in DMSO) to prepare a 0.01 M stock solution, and further dilutions were made in Krebs solution. The vehicle DMSO at the maximal concentration used (0.1%) had no significant effect on U-46619-induced tone or NO-induced vasorelaxation.

Statistical analysis. Results are expressed as means ± SE, and n reflects the number of animals. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentrations producing 30% relaxation were calculated from the fitted concentration-response curves for each ring and expressed as negative log molar (−Log IC<sub>50</sub>). Statistically significant differences between groups were calculated by Student’s t-test for unpaired observations. P < 0.05 was considered statistically significant.

RESULTS

Maturational changes in NO-, YC-1-, and 8-BrcGMP-induced pulmonary vasorelaxation. To avoid the possible interference of endothelial-derived NO, the age-dependent changes on the relaxation induced by sGC activators and 8-BrcGMP were analyzed in endothe-lium-denuded arteries. The vasorelaxant responses induced by exogenously added NO in endothelium-denuded pulmonary arteries (third branch) increased significantly with postnatal age (Fig. 1A). As Fig. 1B shows, YC-1 also produced a significantly higher relaxant response in 2-wk-old animals compared with newborns. However, the relaxant responses of 8-BrcGMP, a membrane-permeable analog of cGMP, were not significantly different in the two age groups, even when a trend for increased response with age was also observed (Fig 1C). The age-dependent change in the potency of these drugs was calculated from the ratios of the IC<sub>30</sub> values in the two age groups, i.e., the distance between the two curves at the level of 30% relaxation. These ratios were 5.6, 4.0, and 1.9 for NO, YC-1, and 8-BrcGMP, respectively.

Expression of sGC protein and mRNA. In blots of the cytosolic fraction of homogenates from piglet pulmonary arteries (third branch) using an antibody recognizing the β1-subunit of sGC, a single band of ~70 kDa was detected (Fig. 2A). The expression of sGC increased significantly with postnatal age (Fig. 2, A and B). In spite of a trend for increased expression of smooth muscle α-actin in the cytosolic fraction with age, the change in sGC expression in 2-wk-old animals remained significantly elevated after normalization of the densitometric values with those of actin (212 ± 28%, P < 0.05). In contrast, the sGC protein content was negligible in the particulate fraction (not shown).

The expression of the β1-subunit of sGC was also analyzed at the level of mRNA. The amplified porcine cDNA corresponds with nucleotides 1328 to 1896 in the human BC047620 sequence (i.e., amino acids 416 to 604 located in the catalytic
region). This cDNA was sequenced and submitted to the GenBank database (accession no. AY661709) and showed a 92, 89, and 88% homology with sGCβ1 human, rat, and mouse sequences, respectively. However, the 47 differences found between the human and porcine nucleotide sequences (Fig. 3C) resulted in only three changes in the translated amino acid sequences (A432T, S574T, P575S), i.e., 98.5% homology at the protein level. Consistent with the increased protein sGCβ1-subunit levels, an increase in mRNA for the sGCβ1-subunit was also observed in the older animals (Fig. 3, A and B). This increase remained significant after normalization of the densitometric values with those of β-actin (155 ± 27%, *P < 0.05).

**DISCUSSION**

The aim of the present study was to analyze the changes in the function and expression of sGC in pulmonary arteries during early postnatal life. The main finding of this study is that the expression of sGC in homogenates from pulmonary arteries increased with postnatal age at both the level of mRNA and protein. This increase was associated with an increased responsiveness to the physiological sGC activator NO and to the exogenous sGC activator YC-1.
At birth, as the lung becomes responsible for blood oxygenation, there is an 8- to 10-fold increase in pulmonary blood flow, and pulmonary arterial pressure falls from suprasystemic levels in the fetus to about one-half of systemic values. NO is critically involved in these changes (30). A second gradual phase of reduction in pulmonary vascular resistance occurs over the first days and weeks of postnatal life to reach the low pulmonary arterial pressure characteristic of the adult (18). An increased responsiveness to the NO/cGMP pathway during this period is thought to be a key event in this maturational process and has been demonstrated in isolated pulmonary arteries from numerous species (3, 21, 28, 32, 35, 38).

The mechanisms responsible for the increased response to NO with postnatal maturation have been analyzed with pharmacological tools stimulating the NO/cGMP signaling pathway at different levels. The responses induced by acetylcholine increased with age (3, 21, 32, 35, 38). In these arteries, N\(^\text{G}\) -nitro-L-arginine methyl ester almost abolished the relaxant response to acetylcholine, indicating the prominent role of NO in this relaxation (35). Furthermore, exogenous NO gas also induced greater relaxant responses in pulmonary arteries from 2-wk-old animals than from newborn animals (28, 35, 38, present results). These relaxant responses were almost abolished by the sGC inhibitor ODQ in both age groups (28), ruling out possible sGC-independent mechanisms. In addition, we demonstrated that the responses to the sGC activator YC-1, analyzed in the absence of endothelium to avoid interferences with endogenous NO, increased with postnatal age. However, the responses to 8-Br-cGMP, the membrane-permeable analog of cGMP, were unchanged even when we found a nonsignificant trend for increased responses in piglets in the present and in a previous study (35). Finally, a trend for increased vasorelaxant responses to three different inhibitors of phosphodiesterase type 5 (PDE5), the major metabolic pathway for cGMP in pulmonary arteries, was also observed (26).

The expression and activity of key proteins involved in the NO/cGMP pathway during the first days of extrauterine life have also been analyzed. The endothelial NO synthase expression and activity did not change after birth (5, 19, unpublished observations), whereas the cytosolic superoxide dismutase (SOD-1) expression and activity decreased (28, 33) and PDE5 activity and expression increased with postnatal age (26). Herein, we found an age-dependent increase in the sGC \( \beta_1 \)-subunit protein expression that correlates with an increased vasorelaxant response to the sGC activators NO and YC-1. Therefore, we also analyzed by RT-PCR whether the age-
dependent changes were also observed at the level of the sGCβ1 mRNA in pulmonary arteries. The mRNA or protein sequences of porcine sGCβ1 have not been previously reported. Thus we amplified and sequenced the catalytic region of porcine sGCβ1. The amplified cDNA from porcine pulmonary arteries showed a 92, 89, and 88% homology with the human, rat, and mouse sequences, respectively. The sGCβ1 increase was also observed at the level of mRNA, indicating that the age-dependent change does not require posttranscriptional mechanisms. The mammalian α1- and β1-sGC genes are separated by 43 kb in human and 60 kb in mouse, and independent transcription of the two subunits in mammals is possible. However, the expression of the two subunits is synchronized due to the multiple shared putative binding sites for transcription factors (29). Thus even when we did not analyze the expression of the α1-sGC, we speculate that the changes in its expression in piglet pulmonary arteries parallel those of the β1-subunit. The data of increased sGC expression is also consistent with increased accumulation of cGMP stimulated by either acetylcholine or NO at 6 and 17 days of age compared with newborns (32). Changes in the expression of sGC can account for a reduced relaxant response of the NO/cGMP pathway (22). However, sGC can also be down-regulated as a counterregulatory mechanism when NO levels are increased (15). Our present results suggest that the lower neonatal sGC expression is not originated as a counterbalance mechanism but rather it seems to be causative of the reduced NO/cGMP activity in newborn arteries. Together, all these results suggest that the maturational changes in the efficacy of NO/cGMP to induce pulmonary relaxation are due to alterations in multiple steps in the signaling pathway. First, the bioavailability of NO during the first hours of life may be reduced due to an enhanced metabolism of NO involving SOD or cyclooxygenase (25, 28). Second, a reduced expression of sGC in newborns as described herein is consistent with a lower response to endogenous and exogenous NO and YC-1. Third, a reduced expression and activity of PDE5, and thus reduced cGMP degradation, in newborn piglets may partly counteract the above mechanisms (26). Finally, increased vascular smooth muscle cell responsiveness to cGMP cannot be excluded. In conclusion, an increased expression of sGC in pulmonary arteries from 2-wk-old compared with newborn piglets explains, at least partly, the age-dependent increase in the vasorelaxant response of NO and other activators of sGC. However, in the present study, we have used medium-sized pulmonary arteries, and thus the results may not exactly reflect the situation of resistance arteries and pulmonary vascular resistance.

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