*Am J Physiol Lung Cell Mol Physiol* 283: L839–L848, 2002;
10.1152/ajplung.00293.2001.

# Postnatal maturation in nitric oxide-induced pulmonary artery relaxation involving cyclooxygenase-1 activity

FRANCISCO PÉREZ-VIZCAÍNO,[1] JOSÉ G. LÓPEZ-LÓPEZ,[1] ROCÍO SANTIAGO,[1] ANGEL COGOLLUDO,[1] FRANCISCO ZARAGOZÁ-ARNÁEZ,[1] LAURA MORENO,[1] MARÍA J. ALONSO,[2] MERCEDES SALAICES,[2] AND JUAN TAMARGO[1]
[1]*Department of Pharmacology, Institute of Pharmacology and Toxicology, School of Medicine, Universidad Complutense, 28040 Madrid; and* [2]*Department of Pharmacology, School of Medicine, Universidad Autónoma, 28029 Madrid, Spain*

Received 30 July 2001; accepted in final form 14 May 2002

**Pérez-Vizcaíno, Francisco, José G. López-López, Rocío Santiago, Angel Cogolludo, Francisco Zaragozá-Arnáez, Laura Moreno, María J. Alonso, Mercedes Salaices, and Juan Tamargo.** Postnatal maturation in nitric oxide-induced pulmonary artery relaxation involving cyclooxygenase-1 activity. *Am J Physiol Lung Cell Mol Physiol* 283: L839–L848, 2002; 10.1152/ajplung.00293.2001.—The maturation in the vasodilator response to nitric oxide (NO) in isolated intrapulmonary arteries was analyzed in newborns and 15- to 20-day-old piglets. The vasodilator responses to NO gas but not to the NO donor sodium nitroprusside increased with age. The inhibitory effects of the superoxide dismutase inhibitor diethyldithiocarbamate and xanthine oxidase plus hypoxanthine and the potentiation induced by superoxide dismutase and $MnCl_2$ of NO-induced vasodilatation were similar in the two age groups. Diphenyleneiodonium (NADPH oxidase inhibitor) potentiated the response to NO, and this effect was more pronounced in the older animals. The nonselective cyclooxygenase inhibitors indomethacin and meclofenamate and the preferential cyclooxygenase-1 inhibitor aspirin augmented NO-induced relaxation specifically in newborns, whereas the selective cycloxygenase-2 inhibitor NS-398 had no effect. The expressions of α-actin, cycloxygenase-1, and cycloxygenase-2 proteins were similar, whereas Cu,Zn-superoxide dismutase decreased with age. Therefore, the present data suggest that the maturational increase in the vasodilatation of NO in the pulmonary arteries during the first days of extrauterine life involves a cycloxygenase-dependent inhibition of neonatal NO activity.

piglet; superoxide; newborn

AT BIRTH AND ALONG THE FIRST days and weeks of extrauterine life, several important functional and structural changes occur in the pulmonary circulation to replace the placenta for gas exchange (12). During the first minutes of extrauterine life, 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 half of systemic values (10). This acute decline in pulmonary vascular resistance is triggered by lung ventilation, oxygenation, and increased shear stress (12). The endothelium-derived vasodilators nitric oxide (NO) and cyclooxygenase (Cox)-derived metabolites, mainly prostacyclin ($PGI_2$), are critically involved in these changes (30). In fact, inhibition of Cox or NO synthases attenuates the birth-related decline in pulmonary vascular resistances in lambs (1, 9, 29).

A second postnatal maturational phase of pulmonary vascular resistance reduction takes place over the first 2–3 wk of extrauterine life in pigs and humans to reach the pulmonary pressure values characteristic of the adult life (14). During this period, the pulmonary circulation is highly vulnerable to develop pulmonary hypertension in response to exogenous insults. Several groups have consistently reported an increase in endothelium-dependent vasodilation to ACh or exogenous NO during the first days of extrauterine life in rabbit (21), lamb (2, 15, 32), and piglet (4, 18, 32, 37) pulmonary arteries, which seems to be a primary factor for the postnatal adaptative maturation. This maturational process is specific to the pulmonary circulation (20, 25, 33, 34), but the mechanisms involved in the age-dependent changes are unclear. Although the responses to exogenous NO gas increase with age (34, 37), the vasodilator responses to the NO donor sodium nitroprusside (18) or the cGMP analog 8-bromo-cGMP (34) remain unchanged. These results indicate that changes in the activity of soluble guanylate cyclase and cGMP-dependent phosphodiesterase are not essential to explain the maturation of NO-dependent relaxation. In fact, the activity and expression of soluble guanylate cyclase decrease postnatally in rat lung (3). Because NO and nitroprusside exhibit a different susceptibility to be inactivated by superoxide (19), an attractive hypothesis to explain the age-dependent difference in NO-induced relaxation involves an elevated metabolism of NO resulting from excess tissue levels of superoxide. Therefore, the aim of the present study was to further analyze the maturational changes in the re-

Address for reprint requests and other correspondence: F. Pérez-Vizcaíno, Dept. Pharmacology, School of Medicine, Universidad Complutense, 28040 Madrid, Spain (E-mail: fperez@ucmail.ucm.es).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

  

sponse to exogenous NO and to determine the possible role of superoxide-generating and -metabolizing enzymatic pathways.

## METHODS

All of the procedures conform with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996).

*Tissue preparation.* Male piglets of 3–18 h ($n = 25$) or 15–20 days of age ($n = 29$) were used in this study. The lungs were rapidly immersed in cold (4°C) Krebs solution (composition in mM: 118 NaCl, 4.75 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 2.0 $CaCl_2$, 1.2 $KH_2PO_4$, and 11 glucose). The pulmonary arteries (third branch with an internal diameter of ~0.5–1.5 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm length (26–28). Except where otherwise stated, 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 ACh ($10^{-6}$ M) to relax arteries precontracted with norepinephrine ($10^{-6}$ M). Rings were mounted between two hooks under 0.5 g of tension in a 5-ml organ bath filled with Krebs solution at 37°C gassed with a 95% $O_2$-5% $CO_2$ gas mixture as previously described (26–28). The contraction was measured by an isometric force transducer (model PRE 206–4 from Cibertec, Madrid, Spain; or Grass model FT03) using data acquisition software and hardware (REGXPC computer program from Cibertec or Powerlab hardware and Chart version 3.4 software from AD Instruments, Castle Hill, Australia).

Concentration-response curves to NO and sodium nitroprusside were carried out in rings precontracted with KCl (40 mM), endothelin-1 ($3 \times 10^{-9}$ M), or thromboxane $A_2$ mimetic 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U-46619, $10^{-7}$ M). These vasoconstrictors induced significantly lower contractile responses in the 3- to 18-h-old animals (320 ± 66, 664 ± 34, and 400 ± 48 mg, respectively) than in the 15- to 20-day-old animals (521 ± 52, 1,081 ± 76, and 694 ± 49 mg, respectively). However, when expressed as a percentage of the response induced by 40 mM KCl, the concentrations used were equieffective in the two age groups (206 ± 10 and 208 ± 18% for endothelin-1 in the 3- to 18-h- and 15- to 20-day-old animals, respectively, and 167 ± 13 and 179 ± 28% for U-46619, respectively, $n = 6–9$). U-46619 at $10^{-7}$ M produced a submaximal contraction (75–90% of the maximal response to $10^{-6}$ M U-46619). After the contraction reached steady state, rings were treated for 15–25 min with vehicle (Krebs solution or dimethyl sulfoxide) or one of the following drugs before constructing a concentration-response curve to NO: the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, $10^{-6}$ M), the inhibitor of the sarcoplasmic reticulum $Ca^{2+}$-ATPase thapsigargin ($2 \times 10^{-6}$ M), superoxide dismutase (SOD, 100 U/ml), the SOD mimetic $MnCl_2$ ($10^{-4}$ M), xanthine oxidase (XO, 5 mU/ml) plus hypoxanthine (HX, $10^{-4}$ M added just before initiating the concentration-response curves to NO), the SOD inhibitor diethyldithiocarbamate (DETCA, $10^{-3}$ M), the inhibitor of the mitochondrial electron transport chain rotenone ($5 \times 10^{-5}$ m), the NADPH oxidase inhibitor diphenyleneiodonium (DPI, $10^{-5}$ M), the NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ M), the nonselective Cox inhibitors indomethacin ($10^{-5}$ M) or meclofenamate ($10^{-5}$ M), the relatively selective Cox-1 inhibitor aspirin ($5 \times 10^{-5}$ M), the selective Cox-2 inhibitor NS-398 ($10^{-5}$ M), arachidonic acid ($10^{-5}$ M), the cytochrome *P*-450 inhibitor SKF-525A ($10^{-5}$ M), the lipooxygenase inhibitor AA-861 ($10^{-5}$ M), or the XO inhibitor oxypurinol ($10^{-4}$ M). Some of these treatments produced significant changes in tone either above or below the initial response, but the magnitude of these changes was always <20% of the initial tone so that the concentration of U-46619 was kept constant at $10^{-7}$ M. The concentration-response curves to nitroprusside were performed by cumulative addition of the drugs, whereas, because of the rapid disappearance of NO in the bath, the curves to NO were performed in a noncumulative fashion by addition of increasing volumes (up to 1,000 μl) of Krebs solution saturated with NO. To prepare the NO-saturated solutions, a 20-ml vial of Krebs solution containing the appropriate concentration of the vasoconstrictor used was initially bubbled with $N_2$ for 15 min and then continuously bubbled with NO (450 parts/million) as described (20, 37). The concentration of NO in the saturated solution was mea-

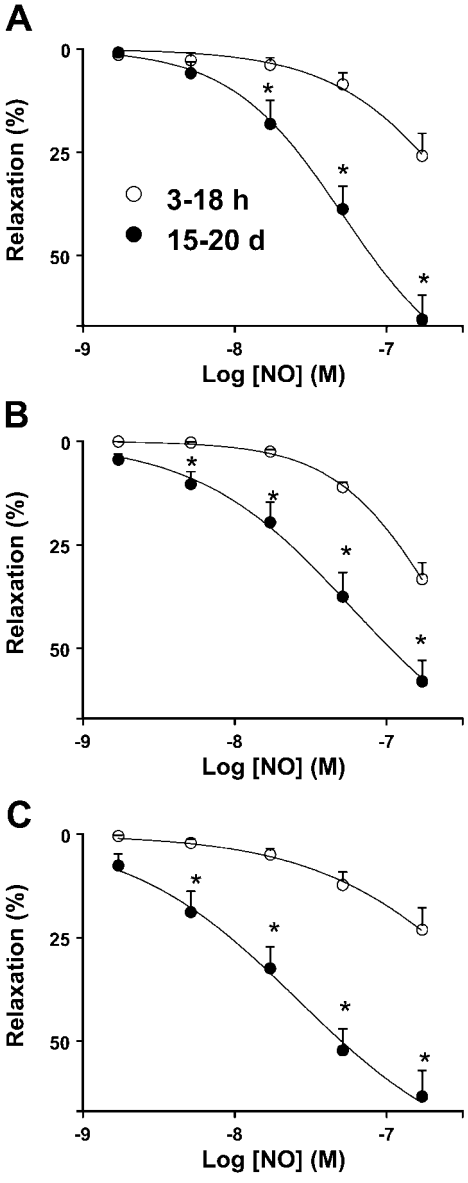


Fig. 1. Effects of nitric oxide (NO) in endothelium-denuded pulmonary arteries stimulated with 40 mM KCl (*A*), $10^{-7}$ M U-46619 (*B*), or $3 \times 10^{-9}$ M endothelin-1 (*C*) in animals of 3–18 h and 15–20 days (d) of extrauterine life. Results are expressed as means ± SE ($n = 8–14$). *$P < 0.05$ vs. 3–18 h. Brackets denote concentration.

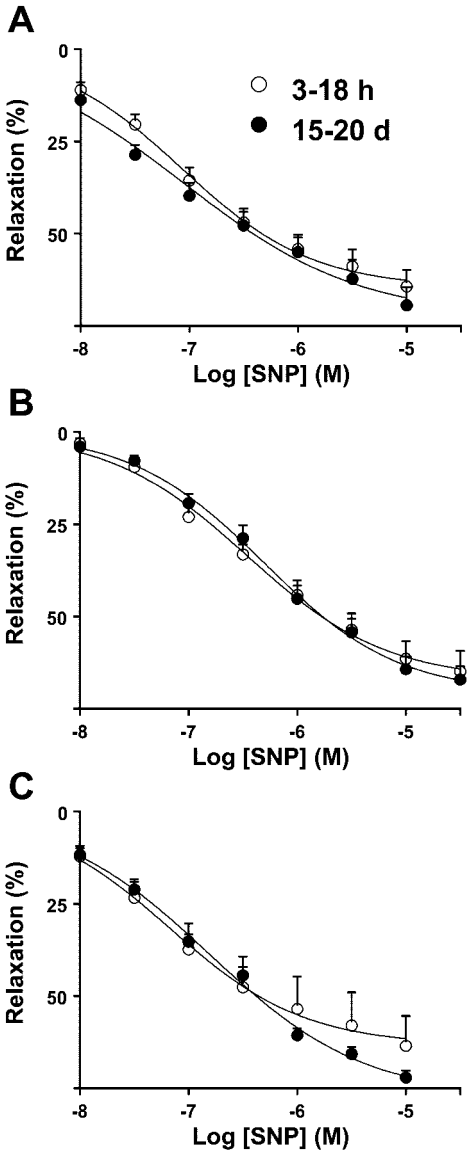


Fig. 2. Effects of sodium nitroprusside (SNP) in endothelium-denuded pulmonary arteries stimulated with 40 mM KCl (*A*), $10^{-7}$ M U-46619 (*B*), or $3 \times 10^{-9}$ M endothelin-1 (*C*) in animals of 3–18 h and 15–20 days of extrauterine life. Results are expressed as means ± SE ($n$ = 8–14).

sured by an amperometric NO-sensitive electrode (ISO-NO; WPI). The vehicles at the concentrations used (dimethyl sulfoxide, ethanol, or NaOH) had no significant effect on U-46619-induced tone or NO-induced vasodilatation; therefore, the control results shown indicate experiments in which Krebs solution was used as vehicle.

*Western blot analysis.* Pulmonary arteries were frozen in liquid nitrogen and stored at −70°C. Frozen arteries were homogenized in a glass potter in a buffer of the following composition: 1 mM sodium vanadate, 1% SDS, and 10 mM Tris. The homogenate was centrifuged at 10,000 revolutions/min for 1 min. The protein content in the supernatant was determined using the Bradford assay (reagents from Bio-Rad). Western blotting was performed with 20 μg protein from the supernatant/lane. SDS-PAGE (12% acrylamide) was performed using the method of Laemmli (16) in a minigel system (Bio-Rad). The proteins were transferred to polyvinylidene difluoride membranes overnight and incubated with rabbit anti-Cu,Zn-SOD polyclonal (1:1,400; StressGen Biotechnologies), goat anti-Cox-1 monoclonal (1:1,000 dilution; SantaCruz Biotechnology), rabbit anti-Cox-2 polyclonal (1:1,000; Cayman Chemical), or mouse anti-α-smooth muscle-actin monoclonal (1:400; Sigma) antibodies and then with the respective secondary horseradish peroxidase-conjugated antibodies. The bands were visualized by enhanced chemiluminiscence (Amersham) and quantified using image analysis software (NIH Image). The bands of actin in pulmonary arteries were similar in the two experimental groups (15- to 20-day-old animals were 103 ± 10% of those of newborns, $P > 0.05$) and were used as a reference for the expression of other proteins. Thus the results of SOD, Cox-1, and Cox-2 protein

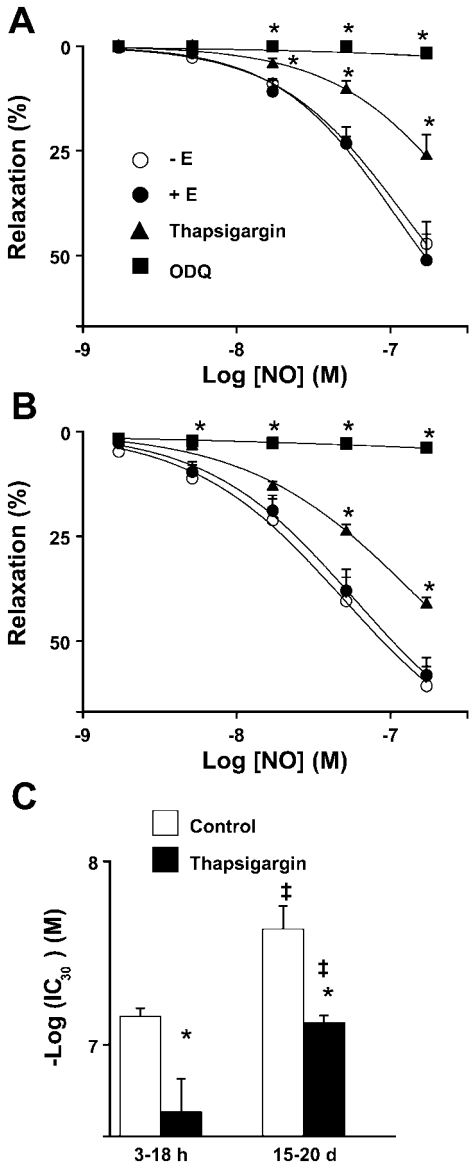


Fig. 3. Effects of the soluble guanylate cyclase inhibitor ODQ ($10^{-6}$ M) and the sacoplasmic $Ca^{2+}$-ATPase inhibitor thapsigargin ($2 \times 10^{-6}$ M) on NO-induced relaxation in endothelium-denuded pulmonary arteries (−E) and in endothelium-intact arteries (+E) stimulated with $10^{-7}$ M U-46619 in animals of 3–18 h (*A*) and 15–20 days (*B*) of extrauterine life. *C*: −log [$IC_{30}$] values calculated from data in *A* and *B*. Results are expressed as means ± SE ($n$ = 6–13). *$P < 0.05$ vs. control (−E) and ‡$P < 0.05$ vs. 3–18 h.

expression were normalized with respect to the bands of actin and expressed as a percentage of the data of 3- to 18-h-old animals.

*Drugs.* U-46619 was from Alexis Biochemicals (Läufelfingen, Switzerland), ODQ was from Tocris Cookson (Bristol, UK), SKF-525A was from RBI, AA-861 was from Takeda, meclofenamate was from Warner Lambert, and all other drugs were from Sigma Chemical (Alcobendas, Spain). Drugs were dissolved initially in distilled deionized water (except for thapsigargin, AA-861, and ODQ, which were dissolved in dimethyl sulfoxide, indomethacin and meclofenamate in ethanol, and HX in 0.1% NaOH) to prepare a $10^{-2}$ or $10^{-3}$ M stock solution, and further dilutions were made in Krebs solution.

*Statistical analysis.* Results are expressed as means ± SE, and *n* reflects the number of animals from which the arterial rings were obtained. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 30% relaxation was calculated from the fitted concentration-response curves for each ring and expressed as negative log molar (-log [$IC_{30}$]). The magnitude of the effect of agents modulating the response to NO agonists was quantified by the log dose ratios, which represents the distance between the two curves, i.e., log (dose ratio) = log [$IC_{30}$ (control)/$IC_{30}$ (drug)]. Statistically significant differences between groups were calculated by an ANOVA followed by a Newman Keuls test. $P < 0.05$ was considered statistically significant.

## RESULTS

*Developmental changes in NO- and NO donor-induced vasodilatation.* The vasodilator response to exogenously added NO increased significantly with postnatal development, and this effect was similarly observed in arteries preconstricted with KCl, U-46619, and endothelin-1 (Fig. 1). However, in contrast to exogenous NO gas, the NO donor nitroprusside produced a relaxant response in arteries stimulated with KCl, U-46619, or endothelin-1 that was similar in the two age groups (Fig. 2).

The mechanisms of the relaxant response to exogenous NO were analyzed using the inhibitor of guanylate cyclase ODQ and the inhibitor of the sarcoplasmic reticulum $Ca^{2+}$-ATPase thapsigargin. The vasodilator effects of NO were abolished by ODQ and partly inhibited by thapsigargin (Fig. 3) in the two age groups. The log dose ratios for the effects of thapsigargin were −0.52 ± 0.18 and −0.44 ± 0.05 at 3–18 h and 15–20 days, respectively ($P > 0.05$ when comparing the two groups).

The possible modulatory role of the endothelium was analyzed comparing the responses to NO in intact and endothelium-denuded arteries. Figure 3 shows that endothelium removal was without effect on NO-induced vasodilatation in the two experimental groups.

*Role of SOD and increased and decreased superoxide anion.* The inhibition of endogenous SOD by DETCA ($10^{-3}$ M) produced a strong inhibitory effect on NO-induced relaxation, whereas addition of exogenous SOD increased it (Fig. 4). A trend for an increased effect of exogenous SOD in 15- to 20-day old animals (log dose ratio = 0.91 ± 0.15) compared with that in 3- to 18-h animals (0.49 ± 0.15) was observed, but these

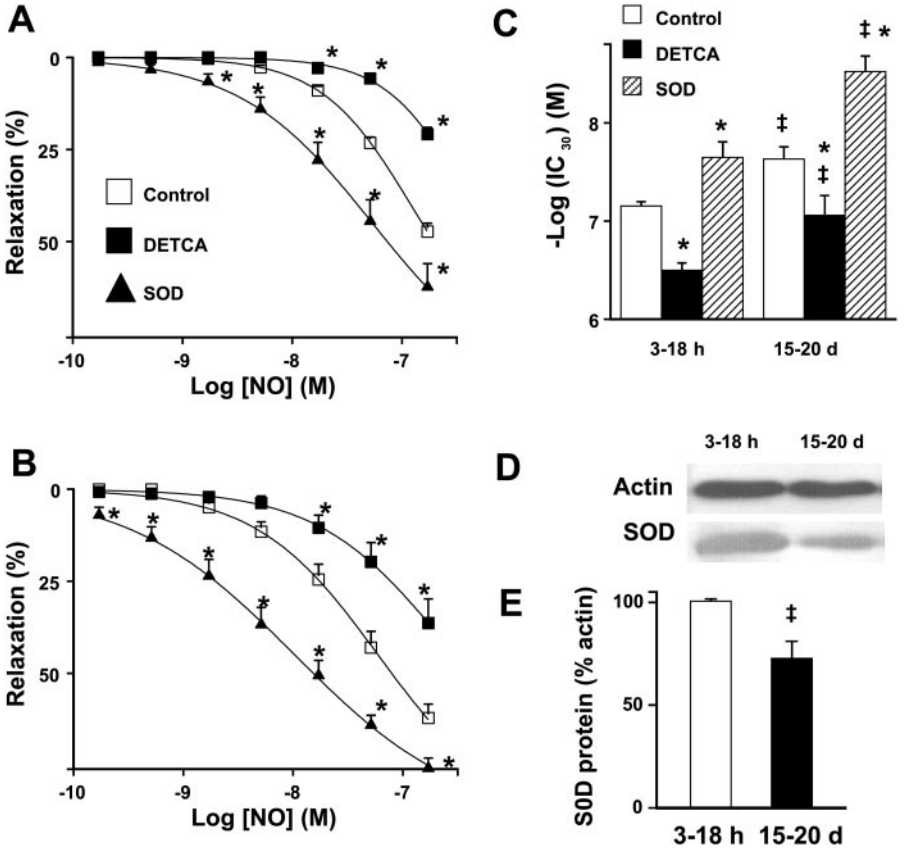


Fig. 4. Effects of superoxide dismutase (SOD, 100 U/ml) and the SOD inhibitor diethyldithiocarbamate (DETCA, $10^{-3}$ M) on NO-induced relaxation in endothelium-denuded pulmonary arteries stimulated with $10^{-7}$ M U-46619 in animals of 3–18 h (*A*) and 15–20 days (*B*) of extrauterine life. *C*: −log [$IC_{30}$] values calculated from data in *A* and *B*. Results are expressed as means ± SE ($n$ = 7–8). *$P < 0.05$ vs. control and ‡$P < 0.05$ vs. 3–18 h. *D*: representative Western blot of Cu,Zn-SOD and α-actin protein expression. *E*: averaged SOD protein expression (mean ± SE) at the two developmental stages ($n$ = 6 and 4, respectively) normalized with respect to the expression of α-actin and expressed as a percentage of 3–18 h.

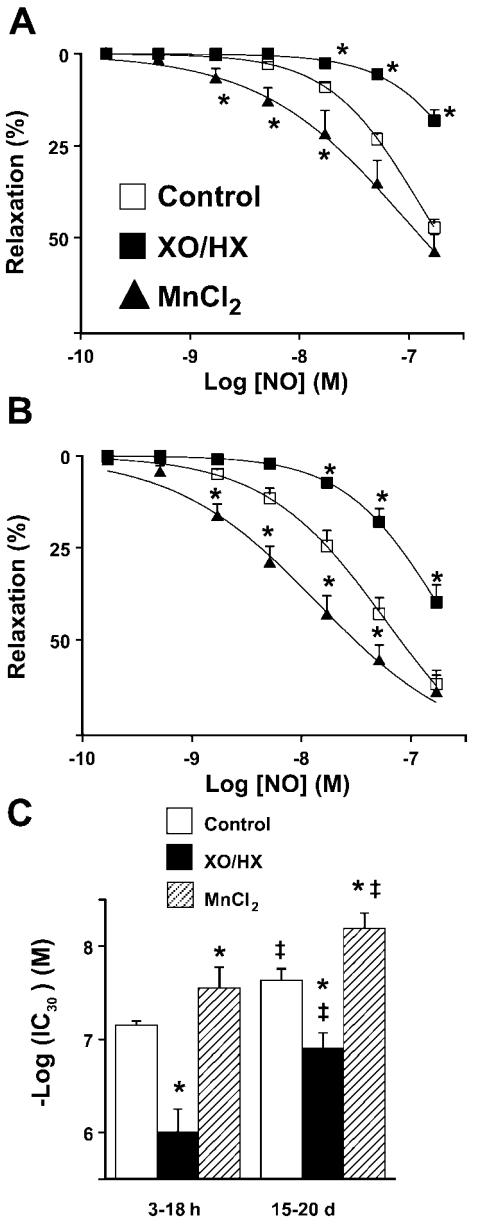


Fig. 5. Effects of the SOD mimetic ($MnCl_2$) and xanthine oxidase (XO, 5 mU/ml) plus hypoxanthine (HX, $10^{-4}$ M) on NO-induced relaxation in endothelium-denuded pulmonary arteries stimulated with $10^{-7}$ M U-46619 in animals of 3–18 h (*A*) and 15–20 days (*B*) of extrauterine life. *C*: $-\log [IC_{30}]$ values calculated from data in *A* and *B*. Results are expressed as means ± SE ($n = 6$). *$P < 0.05$ vs. control and ‡$P < 0.05$ vs. 3–18 h.

differences did not reach statistical significance. The effects of DETCA were similar at the two ages (log dose ratios = $-0.65 \pm 0.07$ and $-0.55 \pm 0.2$ in arteries from 3- to 18-h- and 15- to 20-day-old animals, respectively, $P > 0.05$). Therefore, in the presence of DETCA or SOD, the age-dependent increase in NO-induced relaxation was maintained (Fig. 4*C*). Furthermore, a weak but significant reduction in SOD protein expression was observed at 15–20 days compared with 3–18 h (Fig. 4, *D* and *E*). The effects of $MnCl_2$, which is a membrane-permeable SOD-like compound, on NO-induced relaxation were very similar to those observed for SOD (Fig. 5). A nonsignificant trend for an increased effect of $MnCl_2$ in 15- to 20-day-old animals (log dose ratio = $0.76 \pm 0.13$) compared with 3- to 18-h animals ($0.40 \pm 0.22$, $P > 0.05$) was also observed. Figure 5 also shows that the exogenous superoxide anion-generating system XO plus HX also produced a similar inhibitory effect on NO-induced relaxation at the two developmental stages.

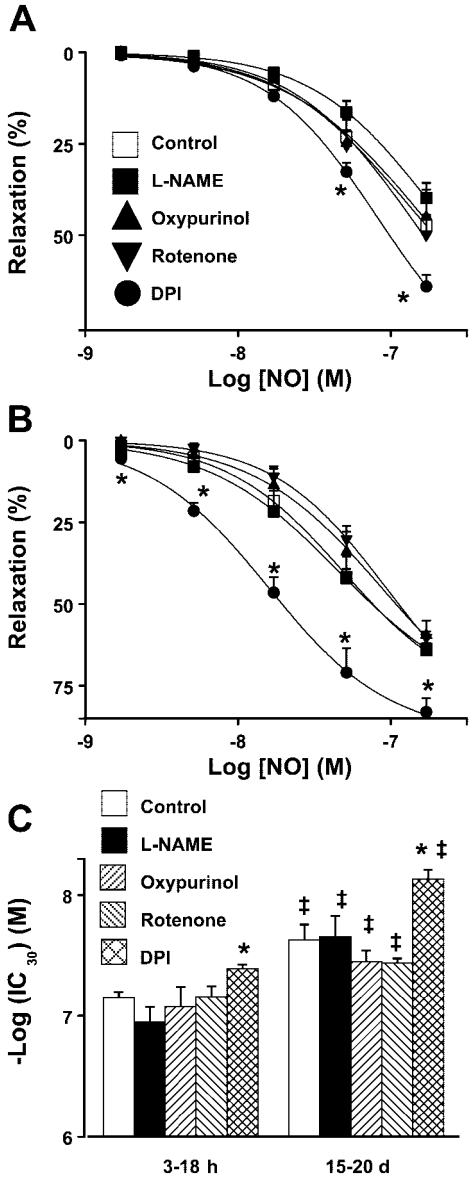


Fig. 6. Effects of inhibitors of potential enzymatic sources of superoxide anion: nitro-L-arginine methyl ester (L-NAME, $10^{-4}$ M, NO synthase inhibitor), oxypurinol ($10^{-5}$ M, inhibitor of xanthine oxidase), rotenone ($10^{-5}$ M, inhibitor of *complex I* of the mitochondrial electron transport chain), and diphenyleneiodonium (DPI, $10^{-5}$ M, inhibitor of the membrane NADPH oxidase) on NO-induced relaxation in endothelium-denuded pulmonary arteries stimulated with $10^{-7}$ M U-46619 in animals of 3–18 h (*A*) and 15–20 days (*B*) of extrauterine life. *C*: $-\log [IC_{30}]$ values calculated from data in *A* and *B*. Results are expressed as means ± SE ($n = 5$–6). *$P < 0.05$ vs. control and ‡$P < 0.05$ vs. 3–18 h.

*Role of endogenous superoxide-generating systems.* The source of superoxide that could be responsible for the destruction of NO was analyzed using pharmacological tools to inhibit different potential enzymatic superoxide-generating systems (7). We used DPI [inhibitor of membrane NAD(P)H oxidase], L-NAME (inhibitor of NO synthase), oxypurinol (inhibitor of XO), and rotenone (inhibitor of complex I of the mitochondrial electron transport chain). DPI significantly potentiated the relaxant effect of NO at the two ages, whereas the other inhibitors had no effect on these responses (Fig. 6). However, DPI-induced potentiation was significantly higher in the 15- to 20-day-old animals (log dose ratio = 0.58 ± 0.07) compared with the 3- to 18-h animals (0.24 ± 0.03, $P < 0.05$).

Other important potential enzymatic sources of superoxide are those involved in the metabolism of arachidonic acid. We used indomethacin as an inhibitor of Cox, AA-861 as an inhibitor of 5-lipooxygenase, SKF-525A as an inhibitor of cytochrome *P*-450, and arachidonic acid as a substrate for all these enzymes. None of these drugs produced any change in the effects of NO in the older animals (Fig. 7*B*). In contrast, indomethacin induced a significant increase in the vasodilator effects of NO in the 3- to 18-h animals (log dose ratios = 0.43 ± 0.04 vs. −0.15 ± 0.19 in the 15- to 20-day-old animals, $P < 0.05$, Fig. 7*A*). In fact, the age-dependent differences were no longer observed in the presence of indomethacin (Fig. 7*C*). On the other hand, AA-861 and arachidonic acid significantly inhibited the effects of NO specifically in the newborn, whereas SKF-525A was without effect. In contrast, the vasodilator effects of the NO donor nitroprusside were unaffected by indomethacin in both groups of animals (Fig. 7*D*).

Figure 8 shows that, when the arteries were stimulated with endothelin-1 ($3 \times 10^{-9}$ M), the effects of indomethacin were similar to those observed in U-46619-stimulated arteries, i.e., indomethacin potentiated the relaxant responses to NO in arteries only from newborns but not from older piglets, and it had no effect on the relaxations to nitroprusside.

*Isoforms of Cox involved.* To further determine the role of Cox isoforms (Cox-1 and Cox-2) involved in the reduced effect of NO, we studied the effects of meclofenamate, another nonselective Cox inhibitor chemically unrelated to indomethacin, aspirin, which at $5 \times 10^{-5}$ M is a fairly selective Cox-1 inhibitor, and NS-398, a selective Cox-2 inhibitor. None of these drugs produced any effect on NO-induced effects in the older animals (Fig. 9*B*). Meclofenamate and aspirin, however, significantly potentiated NO responses in neonates (log dose ratio 0.34 ± 0.06 and 0.35 ± 0.14, respectively, Fig. 9*A*) to a similar extent to that observed with indomethacin so that both drugs abolished the maturational differ-

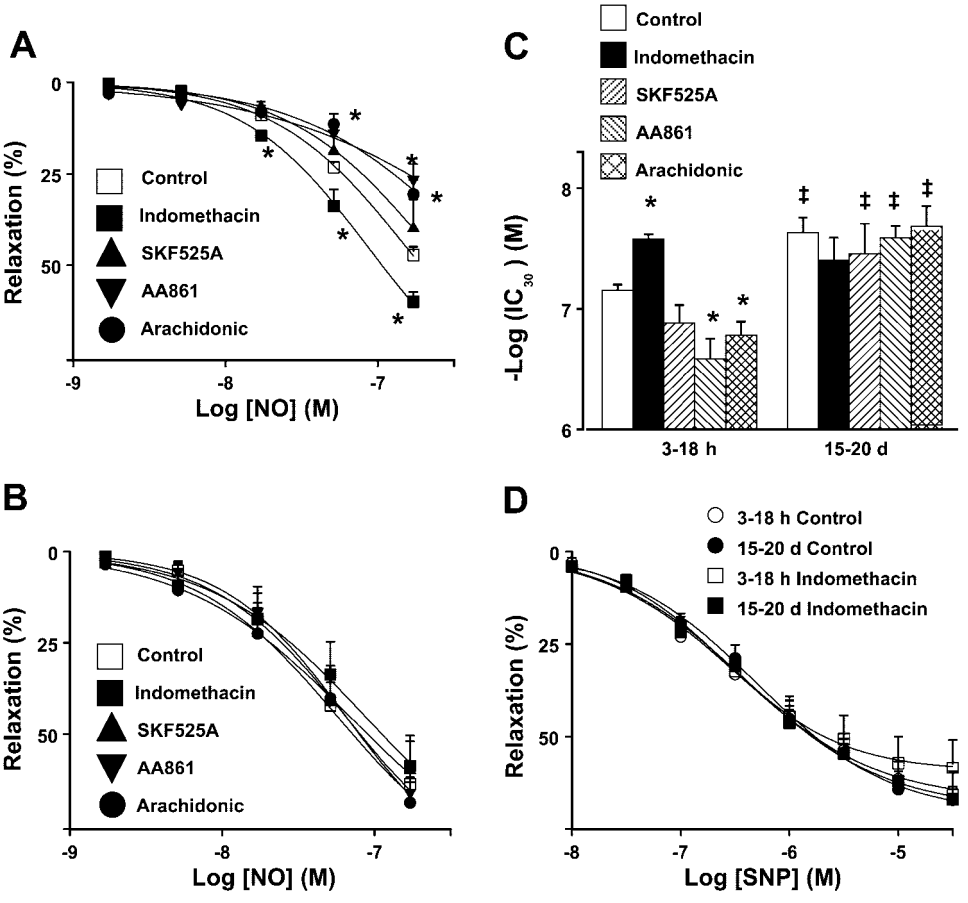


Fig. 7. Effects of arachidonic acid ($10^{-5}$ M) and inhibitors of arachidonic acid metabolism [indomethacin ($10^{-5}$ M, cyclooxygenase inhibitor), AA-861 ($10^{-5}$ M, inhibitor of 5-lipooxygenase), and SKF-525A ($10^{-5}$ M, inhibitor of cytochrome *P*-450)] on NO-induced relaxation in endothelium-denuded pulmonary arteries stimulated with $10^{-7}$ M U-46619 in animals of 3–18 h (*A*) and 15–20 days (*B*) of extrauterine life. *C*: −log [$IC_{30}$] values calculated from data in *A* and *B*. *D*: lack of effect of indomethacin on nitroprusside-induced relaxation in arteries from both age groups stimulated with U-46619. Results are expressed as means ± SE ($n$ = 5–7). *$P < 0.05$ vs. control and ‡$P < 0.05$ vs. 3–18 h.

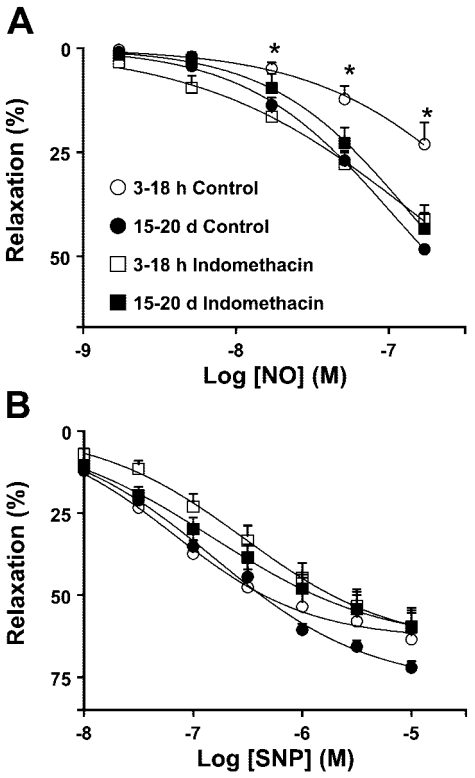


Fig. 8. Effects of indomethacin ($10^{-5}$ M) on NO (*A*)- and nitroprusside (*B*)-induced relaxation in endothelium-denuded pulmonary arteries stimulated with $3 \times 10^{-9}$ M endothelin-1 from animals of 3–18 h and 15–20 days of extrauterine life. Results are expressed as means ± SE ($n$ = 5–6). *$P < 0.05$ vs. control.

ences (Fig. 9*C*). In contrast, NS-398 had no significant effect.

To characterize if this Cox-dependent inhibition of NO-induced vasodilatation was related to increased Cox protein in the newborns, the expression of its isoforms was analyzed by Western blot. Both Cox-1 and Cox-2 proteins were expressed in pulmonary arteries in the two age groups. Cox-1 protein expression was remarkably similar in the two groups (Fig. 9, *D* and *F*). Cox-2 protein was also found to be expressed in neonatal and older animals, and a nonsignificant trend for increased expression of Cox-2 was observed in the older animals (Fig. 9, *E* and *F*).

## DISCUSSION

In the present paper, we found that, in isolated pulmonary arteries, the relaxant responses to exogenous NO increased during the first days of extrauterine life, whereas the responses to the NO donor sodium nitroprusside remained unchanged. All of these results are consistent with data previously published in piglet, rabbit, and lamb pulmonary arteries (2, 4, 15, 18, 21, 32, 34, 37). The most interesting finding of this study is that the inhibitors of Cox-1 specifically potentiated the responses to NO in the newborn animals so that, in their presence, the age-dependent changes were no longer observed. Other attempts to modulate the response to exogenous NO produced similar effects in newborns than in older animals. In addition, the maturation of the response to NO was not related to changes in the patterns of expression of SOD, Cox-1, and Cox-2 proteins.

NO- and nitroprusside-induced vasodilatation in piglet pulmonary arteries has been related to a cGMP-dependent activation of the sarcoplasmic reticulum $Ca^{2+}$-ATPase (8). The vasodilator effects of NO in the two age groups were similarly inhibited by ODQ and thapsigargin (inhibitors of guanylate cyclase and the sarcoplasmic reticulum $Ca^{2+}$-ATPase, respectively). Therefore, these results indicate that no differences in the NO/cGMP pathway beyond the activation of guanylate cyclase are responsible for the maturation of the NO response. Early maturational changes in phosphodiesterase 5 (PDE5) expression in mouse and sheep lung tissue have been proposed to account for the postnatal decrease in pulmonary vascular resistance (13). However, unchanged age-dependent responses to nitroprusside as described herein and by other authors (18) are not consistent with NO-dependent increases being the result of changes in PDE5 activity.

In contrast to NO, the responses to nitroprusside are unaffected by basal or exogenously stimulated superoxide production (19). Therefore, we hypothesized that age-dependent differences in NO- but not in nitroprusside-induced relaxation could be related to changes in the reactive oxidant species. Likewise, Morecroft and McLean (21) reported that the ACh-induced vasodilatation was preferentially increased by SOD in neonatal rabbits compared with older animals and suggested that the age-dependent differences could be the result of reduced SOD activity at birth. However, in piglets, the potentiation of the effects of exogenous SOD was, if any, higher in older animals (present results and Ref. 34). In the present study, the membrane-permeable SOD mimetic $MnCl_2$ also potentiated the vasodilator effects of NO, but these effects were similar to those of SOD. It is likely that the concentration of $MnCl_2$ used was not maximally effective, but the use of higher concentrations was limited by its solubility in Krebs solution. Conversely, Cu,Zn-SOD inhibition by DETCA produced similar inhibitory effects in both groups. Moreover, the inhibitory effects of the superoxide-generating system XO plus HX were also similar in the two groups. In addition, the expression of Cu,Zn-SOD protein was lower in older animals. Thus there is a maturation in the expression of Cu,Zn-SOD protein in piglet pulmonary arteries. However, these age-dependent changes in SOD expression and the effects of SOD mimetics and inhibitors cannot explain those of the responses to NO. Nevertheless, in the present study, we did not address a possible role of other isoforms of SOD (e.g., the mitochondrial Mn-SOD isoenzyme) or nonenzymatic scavengers of superoxide. Therefore, these results clearly indicate that a reduced Cu,Zn-SOD activity does not account for the reduced NO activity at birth but do not fully exclude an increased tissue superoxide in newborn animals.

In the search for a maturational change in the source of superoxide, we investigated the effects of inhibitors

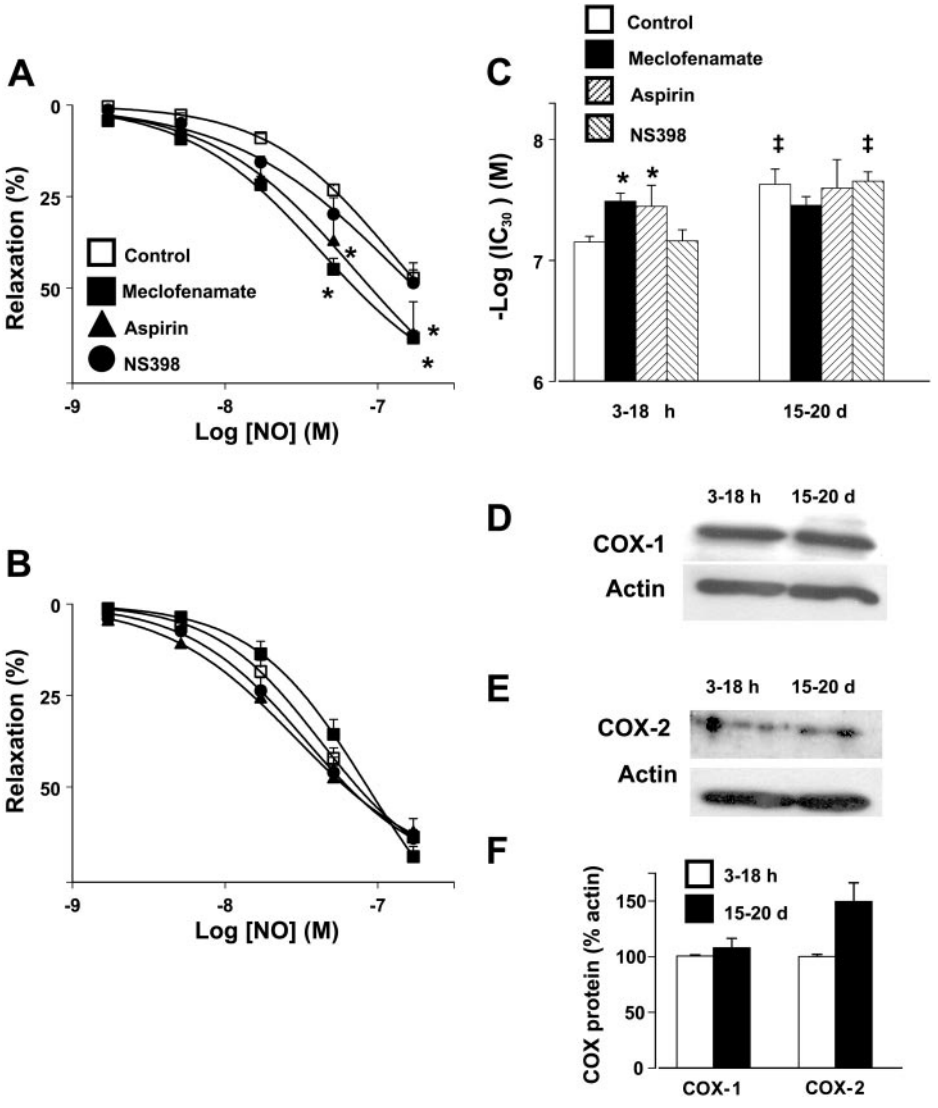


Fig. 9. Effects of the cyclooxygenase (Cox) inhibitors meclofenamate ($10^{-5}$ M, nonselective), aspirin ($5 \times 10^{-5}$ M, fairly selective Cox-1 inhibitor), and NS-398 ($10^{-5}$ M, selective Cox-2 inhibitor) on NO-induced relaxation in endothelium-denuded pulmonary arteries stimulated with $10^{-7}$ M U-46619 in animals of 3–18 h (*A*) and 15–20 days (*B*) of extrauterine life. *C*: $-\log [IC_{30}]$ values calculated from data in *A* and *B*. Results are expressed as means ± SE ($n$ = 5–8). *$P < 0.05$ vs. control and ‡$P < 0.05$ vs. 3–18 h. *D* and *E*: representative Western blots of Cox-1, Cox-2, and α-actin. *F*: Cox-1 and Cox-2 protein expression (mean ± SE) at the two developmental stages ($n$ = 6 and 4, respectively) normalized with respect to the expression of α-actin and expressed as a percentage of 3–18 h.

of several potential enzymatic sources of superoxide. In pulmonary arteries, XO, NO synthases, *complex I* of the respiratory electron transport chain, 5-lypoxygenase, and cytochrome *P*-450 are not important sources of superoxide, as indicated by the lack of inhibitory effect of oxypurinol, L-NAME, rotenone, AA-861, and SKF-525A, respectively. In contrast, DPI potentiated NO-induced relaxation. DPI is a membrane NADPH oxidase inhibitor but also inhibits *complex I* of the mitochodrial electron transport chain. Because rotenone (which does not affect NADPH oxidase) failed to modify NO-induced relaxation, the effects of DPI should be attributed to its inhibitory effect on NADPH oxidase. This is consistent with previous findings that indicate that NADPH oxidase is a major source of oxidative stress in pulmonary and systemic vessels under physiological and pathological conditions (11, 19, 36). However, the potentiation induced by DPI was greater in the older animals. Therefore, none of the above-mentioned enzymatic systems is involved in the maturation of the responses to NO. In contrast, the nonselective Cox inhibitor indomethacin specifically potentiated the responses to NO in newborns so that in its presence NO-induced relaxation was similar in neonates and in older animals. We then tested the effects of meclofenamate, another nonselective Cox inhibitor structurally unrelated to indomethacin; NS-398, a selective Cox-2 inhibitor; and aspirin, which at the concentration used ($5 \times 10^{-5}$ M) is a fairly selective Cox-1 inhibitor (35). Both meclofenamate and aspirin enhanced NO relaxation specifically in the newborn, whereas NS-398 was without effect. These results suggested that Cox-1 is responsible for a reduced vasodilator activity of NO during the first hours of extrauterine life. This suggestion was also supported by the further inhibitory effect of arachidonic acid (the substrate of Cox), specifically in neonates. The fact that the AA-861, an inhibitor of 5-lipooxygenase (which also uses arachidonic acid as its main substrate), also inhibited NO-induced relaxation specifically in neonates might be explained also through an increased substrate for Cox-1. The Cox-1 responsible for the reduced response of NO is not located in the endothelium because endothelium removal did not affect the response

to NO. Cox-1 produces reactive oxygen species as by-products of the synthesis of endoperoxide prostaglandins (31), which may account for the reduced response to NO in newborns reported herein. A role for Cox-1-derived superoxide has been recently suggested to play a major role in the regulation of cerebral circulation (22). In addition, indomethacin-sensitive Cox-1-dependent NO consumption during prostaglandin synthesis has been reported to play a physiological role in platelet function (24). However, prostaglandins or thromboxane synthesized by Cox could also inhibit NO-induced relaxation, even when thromboxane is unlikely to be involved, since the preparations were contracted by a thromboxane $A_2$ analog at a concentration that produced 70–90% of the maximal response.

We then tested whether the differences in the effects of Cox inhibitors were associated with changes in the expression of Cox proteins. In the late-gestation ovine fetus, pulmonary $PGI_2$ increases acutely with ventilation and enhanced oxygenation (17) resulting from rapid changes in the expression of Cox-1 (22). The lung mRNA and protein expression of the constitutive isoform of Cox (Cox-1) increases six- and twofold, respectively, from fetal to 1-wk-old newborn lambs (6). However, in piglets, we did not find any change in Cox-1 protein expression in the pulmonary arteries. The ovine lung Cox-2 mRNA also increased during the early postnatal period, even when Cox-2 protein was not detected (5). In contrast, Cox-2 was expressed constitutively in piglet cerebral arteries (25) and pulmonary arteries (present results) in both neonates and older animals. A nonsignificant trend for increased expression in the pulmonary arteries from older animals was observed. All these results indicate that the expression of Cox isoforms in the ovine and porcine pulmonary circulations does not change or increases postnatally. Therefore, the Cox-dependent maturation of the responses to NO in newborns reported herein appears to be related to changes in Cox activity, which is not associated with differences in the expression of Cox protein isoforms.

Cox are fundamental enzymes in vascular biology, playing a key role in the pulmonary adaptation to the postnatal circulation (23, 30, 31). Cox-1-dependent inhibition of NO-induced relaxation as described herein is likely to be only part of the role of Cox in the maturation of the pulmonary circulation. In fact, inhibition of Cox in the prenatal period in healthy subjects results in unpaired circulatory adaptation at birth (30) rather than in accelerated maturation. The physiological maturation of endothelial NO is probably more complex that the maturation of the responses to exogenously added NO. However, increased Cox activity may play a role in the development of persistent pulmonary hypertension of the newborn, and it is involved in several experimental models of newborn and adult pulmonary hypertension (30). In addition, Cox-dependent inhibition of NO might participate in the therapeutic failure of inhaled NO.

In conclusion, the present results indicate that, in the pulmonary arteries from newborn piglets, the activity of Cox-1 reduces the vasodilator response to NO, but this mechanism does not operate later in life. Therefore, the present data suggest that the maturational increase in the vasodilator response to NO in the pulmonary arteries during the first days of extrauterine life involves Cox activity.

We are grateful to Marta Miguel for excellent technical assistance.

This work was supported by SAF 99-069 from the Comisión Interministerial de Ciencia Y Tecnología, BXX 2000-0153 from the Comisión Interministerial de Ciencia Y Tecnología, PR48/01-9893 from the Universidad Complutense de Madrid, and 08.04.36.2001 from the Comunidad Autónoma de Madrid.

## REFERENCES

1. **Abman SH, Chatefield BA, Hall SL, and McMurtry IF.** Role of endothelium-derived relaxing factor during transition of pulmonary circulation at birth. *Am J Physiol Heart Circ Physiol* 259: H1921–H1927, 1990.
2. **Abman SH, Chatefield BA, Rodman DM, Hall SL, and McMurtry IF.** Maturational changes in endothelium-derived relaxing factor activity in ovine pulmonary arteries in vitro. *Am J Physiol Lung Cell Mol Physiol* 260: L280–L285, 1991.
3. **Behrends S, Kempfert J, Mietens A, Koglin M, Scholz H, and Middendorff R.** Developmental changes of nitric oxide-sensitive guanylyl cyclase expression in pulmonary arteries. *Biochem Biophys Res Commun* 283: 883–887, 2001.
4. **Boels PJ, Deutsch J, Gao B, and Haworth SG.** Maturation of the response in resistance and conduit pulmonary arteries. *Cardiovasc Res* 44: 416–428, 1999.
5. **Brannon TS, MacRitchie AN, Jaramillo MA, Sherman TS, Yuhama IS, Margraf LR, and Shaul PW.** Ontogeny of cyclooxygenase-1 and cyclooxygenase-2 gene expression in the ovine lung. *Am J Physiol Lung Cell Mol Physiol* 275: L66–L71, 1998.
6. **Brannon TS, North AJ, Wells LB, and Shaul PW.** Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. *J Clin Invest* 93: 2230–2235, 1994.
7. **Cai H and Harrison DG.** Endothelial dysfunction in cardiovascular diseases. The role of oxidant stress. *Circ Res* 87: 840–844, 2000.
8. **Cogolludo AL, Pérez-Vizcaíno F, Zaragozá-Arnáez F, Ibarra M, López-López JG, López-Miranda V, and Tamargo J.** Mechanisms involved in SNP-induced relaxation and $[Ca^{2+}]$i reduction in piglet pulmonary and systemic arteries. *Br J Pharmacol* 132: 959–967, 2001.
9. **Davidson D.** Pulmonary hemodynamics at birth: effect of acute cyclooxygenase inhibition in lambs. *J Appl Physiol* 64: 1676–1682, 1989.
10. **Dawes GS, Mott JC, and Widdecombe JG.** Changes in the lungs of the newborn lamb. *J Physiol (Paris)* 121: 141–162, 1953.
11. **Di Wang H, Pagano PJ, Du Y, Cayatte AJ, Quinn MT, Brecher P, and Cohen RA.** Superoxide anion from the adventitia of the rat thoracic aorta inactivates nitric oxide. *Circ Res* 82: 810–818, 1998.
12. **Fineman J, Soifer S, and Heymann M.** Regulation of pulmonary vascular tone in the perinatal period. *Annu Rev Pharmacol Toxicol* 57: 115–134, 1995.
13. **Hanson KA, Burns F, Rybalkin SD, Miller JW, Beavo J, and Clarke WR.** Developmental changes in lung cGMP phosphodiesterase-5 activity, protein, and message. *Am J Respir Crit Care Med* 158: 279–288, 1998.
14. **Haworth S and Hislop A.** Adaptation of the pulmonary circulation to extra-uterine life in the pig and its relevance to the human infant. *Cardiovasc Res* 15: 108–119, 1981.
15. **Kolber KA, Gao Y, and Raj JU.** Maturational changes in endothelium-derived nitric oxide-relaxation of ovine pulmonary arteries. *Biol Neonate* 77: 123–130, 2000.
16. **Laemmli UK.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685, 1970.

17. **Leffler CW, Hessler JR, and Terragno NA.** Ventilation-induced release of prostaglandin-like material from fetal lungs. *Am J Physiol Heart Circ Physiol* 238: H282–H286, 1980.
18. **Liu S, Hislop A, Haworth S, and Barnes P.** Developmental changes in endothelium-dependent pulmonary vasodilataion in pigs. *Br J Pharmacol* 106: 324–330, 1992.
19. **López-López JG, Pérez-Vizcaíno F, Cogolludo AL, Ibarra M, Zaragozá-Arnáez F, and Tamargo J.** Nitric oxide- and nitric oxide donors-induced relaxation and its modulation by oxidative stress in piglet pulmonary arteries. *Br J Pharmacol* 133: 615–624, 2001.
20. **Martínez-Orgado J, Salaices M, Rodríguez-Martínez MA, Sánchez Ferrer CF, and Marín J.** Role of nitric oxide on the endothelium-dependent vasodilation in newborn piglet cerebral arteries. *Gen Pharmacol* 25: 899–902, 1994.
21. **Morecroft I and MacLean MR.** Developmental changes in endothelium-dependent vasodilation and the influence of superoxide anions in perinatal rabbit pulmonary arteries. *Br J Pharmacol* 125: 1585–1593, 1998.
22. **Niwa K, Haensel C, Ross ME, and Iadecola C.** Cyclooxygenase-1 participates in selected vasodilator responses of the cerebral circulation. *Circ Res* 88: 600–608, 2001.
23. **North AJ, Brannon TS, Wells LB, Campbell WB, and Shaul PW.** Hypoxia stimulates prostacyclin synthesis by increasing cyclooxygenase-1 protein. *Circ Res* 75: 33–40, 1994.
24. **O'Donnell VB, Coles B, Lewis MJ, Crews BC, Marnett LJ, and Freeman BA.** Catalytic consumption of nitric oxide by prostaglandin H synthase-1 regulates platelet function. *J Biol Chem* 275: 38239–38244, 2000.
25. **Parfenova H, Massie V, and Leffler CW.** Developmental changes in endothelium-derived vasorelaxant factors in cerebral circulation. *Am J Physiol Heart Circ Physiol* 278: H780–H788, 2000.
26. **Pérez-Vizcaíno F, Cogolludo AL, Villamor E, and Tamargo J.** Role of $K^+$ channel opening and stimulation of cyclic GMP in the vasorelaxant effects of nicorandil in isolated piglet pulmonary and mesenteric arteries: relative efficacy and interactions between both pathways. *Br J Pharmacol* 123: 847–854, 1998.
27. **Pérez-Vizcaíno F, Villamor E, Duarte J, and Tamargo J.** Involvement of protein kinase C in reduced relaxant responses to the NO/cGMP pathway in piglet pulmonary arteries contracted by the thromboxane $A_2$ mimetic U46619. *Br J Pharmacol* 121: 1323–1333, 1997.
28. **Pérez-Vizcaíno F, Villamor E, Moro M, and Tamargo J.** Lack of endotoxin-induced hyporesponsiveness to U46619 in isolated neonatal porcine pulmonary but not mesenteric arteries. *J Vasc Res* 33: 249–257, 1996.
29. **Rairigh RL, Parker TA, Ivy DD, Kinsella JP, Fan ID, and Abman SH.** Role of inducible nitric oxide synthase in the pulmonary vascular response to birth-related stimuli in the ovine fetus. *Circ Res* 88: 721–726, 2001.
30. **Shaul PW.** Regulation of vasodilator synthesis during lung development. *Early Hum Dev* 54: 271–294, 1999.
31. **Smith WL, DeWitt DL, and Garavito RM.** Cyclooxygenases: structural, cellular and molecular biology. *Annu Rev Biochem* 69: 145–182, 2000.
32. **Steinhorn RH, Morin FC III, Gugino SF, Giese E, and Russell JA.** Developmental differences in endothelium-dependent responses in isolated ovine pulmonary arteries and veins. *Am J Physiol Heart Circ Physiol* 264: H2162–H2167, 1993.
33. **Stoen R, Brubakk A, Vik T, Lossius K, Jynge P, and Karlsson JOG.** Postnatal changes in mechanisms mediating acetylcholine-induced relaxation in piglet femoral arteries. *Pediatr Res* 41: 702–707, 1997.
34. **Villamor E, Pérez-Vizcaíno F, Cogolludo AL, Conde-Oviedo J, Zaragozá-Arnáez F, López-López JG, and Tamargo J.** Relaxant effects of carbon monoxide compared with nitric oxide in pulmonary and systemic vessels of newborn piglets. *Pediatr Res* 48: 546–553, 2000.
35. **Warner TD, Giuliano F, Voinovic I, Bukasa A, Mitchell JA, and Vane JR.** Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxigenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc Natl Acad Sci USA* 96: 7563–7568, 1999.
36. **Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Etavo JC, and Diez J.** Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension* 35: 1055–1061, 2000.
37. **Zellers T and Vanhoutte P.** Endothelium-dependent relaxations in piglet pulmonary arteries augment with maturation. *Pediatr Res* 30: 176–180, 1991.