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Endothelium-Dependent Relaxations of Piglet Pulmonary Arteries Augment with Maturation

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ABSTRACT. To determine whether maturation alters endothelium-dependent responses in porcine pulmonary arteries, rings, with and without endothelium, of small pulmonary arteries taken from piglets of 3, 10, and 30 d of age were suspended in organ chambers filled with buffered salt solution, bubbled with 95% O_2-5% CO_2, and maintained at 37°C. These studies were performed in the presence of indomethacin (10^{-5} M) to inhibit prostaglandin synthesis. In rings without endothelium, potassium chloride (10^{-2} to 8.5×10^{-2} M) and histamine (10^{-9} to 10^{-5} M) caused concentration-dependent contractions. When normalized to maximal contractions achieved to each agonist, the concentration-effect curves to potassium chloride and histamine in rings without endothelium were similar at each age. Rings with endothelium showed a progressive shift to the right of the concentration-effect curve to histamine, possibly secondary to an increase in the basal release of, or responsiveness to, the endothelium-derived relaxing factor with maturation. Relaxations to sodium nitroprusside (10^{-9} to 10^{-5} M) were unaffected by age. In precontracted rings, acetylcholine (10^{-9} to 10^{-6} M), bradykinin (10^{-10} to 10^{-6} M), and the calcium ionophore A23187 (10^{-9} to 10^{-6} M) caused relaxations in rings with endothelium, but not in those without endothelium, which were greater at 10 and 30 d compared to 3 d; further augmentation at 30 d compared to 10 d was not observed. In rings without endothelium, changes in the responsiveness to nitric oxide (10^{-9} to 10^{-5} M), one of the proposed endothelium-derived relaxing factors, with age were comparable to those observed with endothelium-dependent relaxing agents. These studies demonstrate that endothelium-dependent relaxations increase with age, possibly due to changes in sensitivity of the smooth muscle to the endothelium-derived relaxing factor. (*Pediatr Res* 30: 176–180, 1991)

Abbreviations

EDRF, endothelium-derived relaxing factor

Most mammals, including swine, exhibit elevated pulmonary arterial pressures and resistance *in utero* that decrease after birth in response to an increase in oxygen tension, vascular remodeling, and intrapulmonary production of prostaglandins (1–4). The exact mechanisms by which these stimulants induce the observed hemodynamic changes are unclear. However, endothelial cells from pulmonary vessels of most mammals (including humans) contain enzymes that activate or degrade potent vasoactive hormones such as angiotensin I and bradykinin, respectively (5). In addition, the endothelium synthesizes and releases prostacyclin and EDRF when exposed to acetylcholine, bradykinin, and other autocoids; these vasoactive substances contribute to the local regulation of vascular tone (6–13). Cherry and Gillis (14) have shown that the release of an EDRF, other than prostacyclin, can reduce pulmonary artery pressure and resistance *in situ*. The purpose of our study was to determine if a maturational change in the release and/or effect of EDRF from small porcine pulmonary arteries occurs during the neonatal period.

MATERIALS AND METHODS

Twenty-two white piglets of either sex, from four sows, were studied at 3 ($n = 6$), 10 ($n = 7$), and 30 ($n = 9$) d of age. The piglets were anesthetized with ketamine hydrochloride (Bristol Laboratories, Syracuse, NY), 10 to 15 mg/kg intramuscularly, and sodium pentobarbital (Fort Dodge Laboratories, Inc., Fort Dodge, IA), 12 to 15 mg/kg i.v., and exsanguinated in accordance with NIH guidelines for humane care. The heart and lungs were removed en bloc and placed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.7, H_2O 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, Ca EDTA 0.026, and glucose 11.1 (control solution).

The intralobar pulmonary arteries, taken just distal to the branch point in the lower lobes (5th order), were dissected free from the surrounding lung parenchyma, cut into rings (3–4 mm long), and cleaned of loose connective tissue, taking special care not to touch the luminal surface. The diameter of the rings varied with age, averaging (nonstretched, measured by microscopy) 0.7 ± 0.1 mm at 3 d, 0.8 ± 0.15 mm at 10 d, and 1.5 ± 0.4 mm at 30 d. In some rings, the endothelium was deliberately removed by gently rubbing the luminal surface with a thin (0.25-mm) stainless steel wire while rolling the vessel on thin tissue wetted with control solution. The rings were then suspended between two stainless steel stirrups; one was connected to a fixed stirrup within the organ chamber and the other to a force transducer (model no. UTC2; Gould Inc., Cleveland, OH) for recording isometric tension. The rings were then placed in organ chambers filled with 25 mL of control solution, bubbled with 95% O_2-5% CO_2, and maintained at 37°C. Immediately after mounting, the rings were stretched progressively until a maximal response to KCl (20 mM) was achieved (optimal length/tension relationship). The rings were incubated with indomethacin (10^{-5} M) and allowed to equilibrate for 40 min before further experimentation. Rings with and without endothelium were studied in parallel.

Histology. At the end of each experiment, rings with and without endothelium were fixed in 10% buffered formalin solu-

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tion (pH = 7.0) for at least 24 h, embedded in paraffin (tissue prep 2; Fair Lawn, NJ), cut into 5-μm sections, and stained with hematoxylin and eosin. The slides were studied under light microscopy. The presence or absence of endothelium and the integrity of the basement membrane, internal elastic lamina, and vascular smooth muscle were determined (Fig. 1).

Protocols. Contractions were studied in quiescent rings. Concentration-response curves to potassium chloride were determined in rings without endothelium that were previously incubated with phentolamine (10^{-5} M) plus propranolol (5×10^{-6} M; to prevent the effects of catecholamines released from adrenergic nerve terminals) and indomethacin (10^{-5} M; to prevent the formation of vasoactive prostaglandins) for 40 min. Contractions to histamine were studied in rings with and without endothelium, in the presence of indomethacin (10^{-5} M). To study relaxations, rings were first incubated with indomethacin for 40 min and then exposed to a concentration of prostaglandin $F_{2\alpha}$ (10^{-6} to 4×10^{-6} M) that would cause a contraction equal to 75% of the maximal response to histamine (10^{-5} M; determined in all rings). Concentration-response curves to the vasodilators were obtained after the prostaglandin contraction reached a steady state. Rings that did not develop at least 200 mg of tension were excluded from the analysis because these were considered to have damaged smooth muscle. Rings with endothelium were exposed to a single dose of bradykinin (10^{-7} M) during length-tension testing, and those rings that did not achieve at least 30% relaxation of the maximal histamine contraction were excluded from the experiment. Relaxations to acetylcholine, bradykinin, and the calcium ionophore A23187 were studied in rings with and without endothelium, and those to sodium nitroprusside and solutions of nitric oxide (one of the proposed EDRF) (15–18) were determined in rings without endothelium.

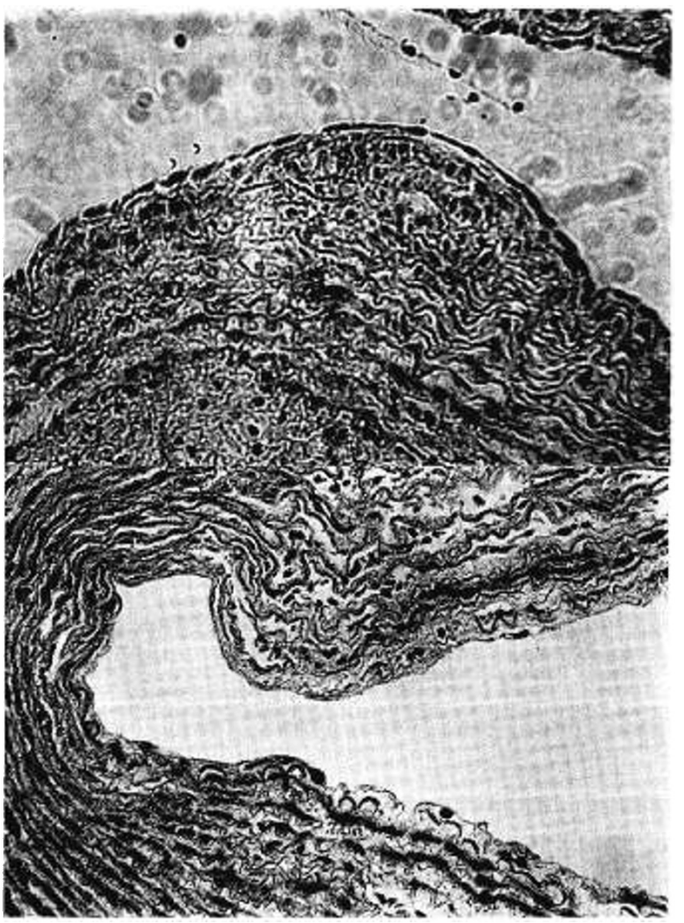

Fig. 1. Histologic sections of neonatal pulmonary artery with and without endothelium. The tissue has been stained with hematoxylin and eosin. The rings with endothelium (*top panel*) have a layer of endothelial cells present, whereas those without endothelium (*bottom panel*) are missing this intimal layer.

Drugs. The following drugs were used: acetylcholine, bradykinin, the calcium ionophore A23187, histamine hydrochloride, indomethacin, DL-propranolol hydrochloride, prostaglandin $F_{2\alpha}$, sodium nitroprusside (all from Sigma Chemical Co., St. Louis, MO), and phentolamine mesylate (Ciba-Geigy, Co., Summit, NJ). Indomethacin was first dissolved with Na_2CO_3 (10^{-5} M), and the calcium ionophore A23187 was dissolved in 100 μL of DMSO. All drugs were prepared daily with distilled water and kept on ice throughout the experiment. All drug concentrations are expressed as final molar (M) concentrations in the organ bath solution.

Nitric Oxide. A gas bulb fitted with a silicon rubber injection septum was filled with nitric oxide from a cylinder (Union Carbide, Chicago, IL). An appropriate volume (10–1000 μL) was removed with a syringe and injected into another gas bulb filled with 100 mL of distilled water (37°C), which has been gassed with helium for 3 h, giving stock solutions of nitric oxide of 4×10^{-6} M, 4×10^{-5} M, and 4×10^{-4} M as estimated by the solubility constant of 4.6 mL/100 mL for nitric oxide. Appropriate volumes of the stock solutions were then withdrawn and added to the organ baths in increasing concentrations in a noncumulative manner.

Statistical analysis. In all experiments, n equals the number of animals from which the rings were taken. Results are expressed as means ± SEM. Statistical analysis was performed on paired rings (with and without endothelium) using two-tailed t test for paired observations. To compare rings from animals of different age groups, an analysis of variance with Scheffe's test was used. Values were considered statistically significant when p was less than 0.05.

RESULTS

Histology. All vessels used were musculoelastic arteries. Rings with endothelium were found to have an intact endothelial layer with the exception of loss of endothelial cells where the stirrups were placed. Rings that were purposely denuded had nearly complete removal of the endothelial cell layer as determined by cross and longitudinal sections of the vascular lumen. Multiple sections of each vessel were examined to confirm the absence of the endothelial cell layer. The internal elastic lamina was intact in all rings examined.

Contractions. When normalized to the maximal contractions of the specific agonist, concentration-response curves to potassium chloride (10^{-2} to 8.5×10^{-2} M) or histamine (10^{-9} to 10^{-5} M) in rings without endothelium from 3-, 10-, and 30-d-old piglets were comparable (Figs. 2 and 3). Rings with endothelium also contracted in a concentration-dependent manner to histamine. When normalized to the maximal contraction, the concentration-response curves to histamine in rings with and without endothelium from 3-d-old piglets were comparable. Compared to rings (from animals of the same age) without endothelium, the concentration-response curves to histamine were shifted rightward significantly for rings with endothelium at both 10 and 30 d; concentration-response curves for rings from 30-d-old piglets were shifted rightward significantly more than those observed at 10 d (Fig. 3).

When expressed in absolute terms, as grams of tension developed, potassium chloride caused contractions that were statistically similar in rings without endothelium taken from 3- and 10-d-old piglets; contractions in rings from 30-d-old piglets were significantly greater than those observed at 3 or 10 d (Fig. 2).

Histamine-induced contractions, at 3 and 10 d, when expressed as grams of tension developed, exhibited a pattern in the concentration-response curves similar to that observed when these data were normalized to maximal contraction. At 30 d, the contractions to histamine were significantly larger than those

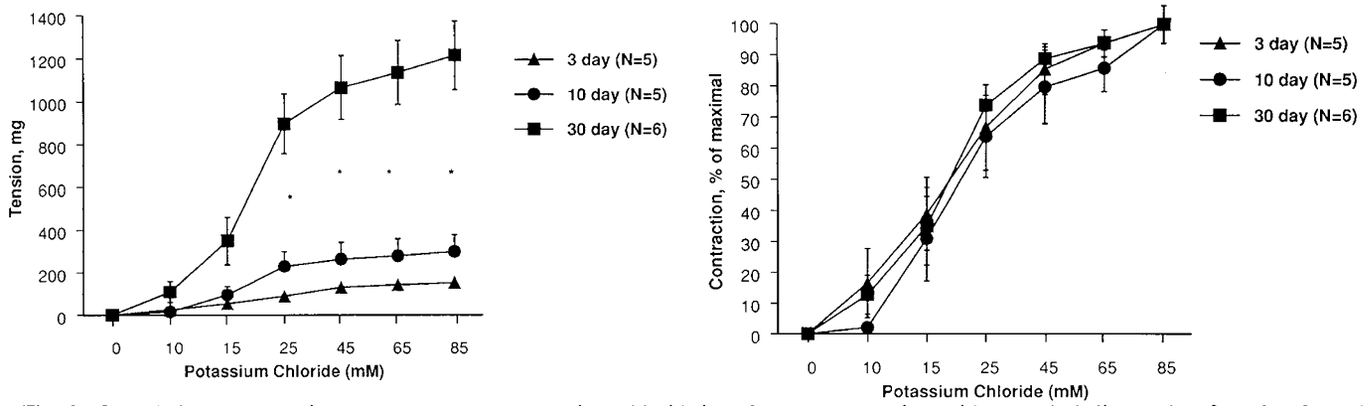

Fig. 2. Cumulative concentration-response curves to potassium chloride in pulmonary artery rings without endothelium, taken from 3-, 10-, and 30-d-old piglets. In the *left panel*, contractions are expressed in mg of tension developed to increasing cumulative concentrations of the agonist. In the *right panel*, these data are normalized to the maximal tension developed by the rings to the agonist and expressed as a percentage of change in tension to increasing cumulative concentrations of potassium chloride. Data are expressed as means ± SEM. Phentolamine (10^{-5} M) and propranolol (5×10^{-6} M) were present to prevent the effects of catecholamines released from nerve endings, and indomethacin (10^{-5} M) was used to prevent the synthesis of vasoactive prostaglandins.

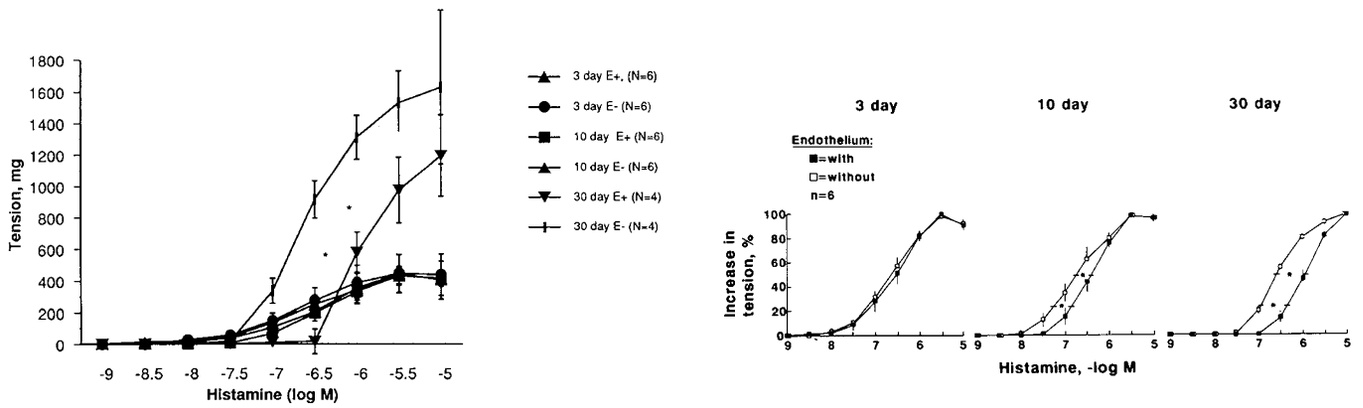

Fig. 3. Cumulative concentration-response curves to histamine in rings with and without endothelium taken from 3-, 10-, and 30-d-old piglets. In the *left panel*, the effects of increasing cumulative concentrations of histamine (10^{-9} to 10^{-5} M) are expressed as mg of tension developed to the agonist at each concentration. In the *right panel*, these data are normalized to the maximal amount tension developed to the highest concentration of the agonist in each individual ring. These data are expressed as the percentage of change (of the maximal contraction) in tension developed at each concentration. Data are expressed as the means ± SEM. Indomethacin (10^{-5} M) was present to prevent synthesis of vasoactive prostaglandins. *M*, molar concentration in the organ bath.

observed at either 3 or 10 d of age in rings with or without endothelium. At 30 d, the concentration-response curve was shifted rightward for rings with endothelium compared with rings without endothelium, but maximal contractions were not significantly different (Fig. 3).

Relaxations. Sodium nitroprusside (10^{-9} to 10^{-5} M) caused relaxations in rings without endothelium that were similar in all three age groups (Fig. 4). All rings relaxed 100% to the agonist.

Acetylcholine (10^{-9} to 10^{-6} M), bradykinin (10^{-10} to 10^{-6} M), and the calcium ionophore A23187 (10^{-9} to 10^{-6} M) caused concentration-dependent relaxations in rings with, but not without, endothelium that were significantly augmented at 10 and 30 d compared with 3 d of age (Fig. 5). However, the endothelium-dependent relaxations at 30 d were not significantly different from those observed at 10 d of age. The largest relaxations to the above three agonists were also significantly greater at 10 and 30 d compared with 3 d; the largest relaxations at 10 and 30 d were statistically similar.

Nitric oxide (10^{-9} to 10^{-5} M) caused concentration-dependent relaxations in rings without endothelium that were significantly augmented at 10 and 30 d compared with 3 d of age (Fig. 5). The direct relaxations evoked by nitric oxide at 30 d of age were not significantly different from those observed at 10 d of age. The greatest relaxations to nitric oxide were also significantly augmented at 10 and 30 d compared with 3 d; the relaxations at 10 and 30 d were statistically similar.

DISCUSSION

The endothelium most likely plays a role in the control of vascular tone through the release of EDRF (12, 14, 19–27). Alterations in the release or effect of EDRF have been demonstrated in animal models of systemic hypertension (28–32). The release of EDRF, other than prostacyclin, may be involved in the control of pulmonary arterial pressure and resistance (14, 33). Although the presence of EDRF from pulmonary and systemic vessels of the newborn guinea pig has been recently reported (34), the present experiments suggest a maturational change in the release from or effect of EDRF upon the pulmonary arterial vasculature during the neonatal period.

The contractile response of the vascular smooth muscle is qualitatively similar at 3, 10, and 30 d of age, as demonstrated by the concentration-response curves of rings without endothelium exposed to potassium chloride and histamine. This suggests that a difference in the contractile capabilities of the vascular smooth muscle or sensitivity to the agonist is not responsible for the augmented endothelium-dependent responses observed with maturation. In contrast, the rightward shift of the concentration-

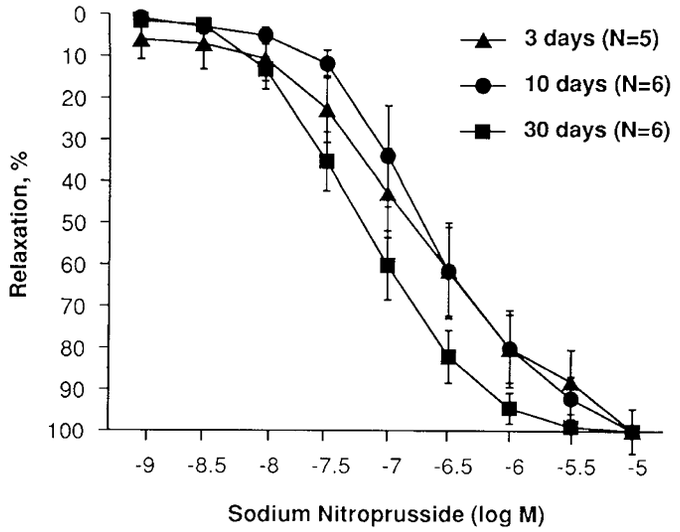

Fig. 4. Cumulative concentration-response curve to sodium nitroprusside in rings without endothelium taken from 3-, 10-, and 30-d-old piglets. The rings were contracted with prostaglandin $F_{2\alpha}$ to a tension approximating 75% of the maximal contraction to histamine before exposure to sodium nitroprusside. Relaxations are expressed as the percentage of inhibition of the prostaglandin contraction. Data are expressed as means ± SEM. Indomethacin (10^{-5} M) was present to prevent the synthesis of vasoactive prostaglandins. *M*, molar concentration in the organ bath.

response curve to histamine at 10 and 30 d of age in rings with, compared to rings without, endothelium suggests a possible maturational increase in basal release (7, 19) and/or effect of EDRF. An increased degradation or metabolism of histamine by the endothelial cell or a decreased production of endothelium-derived contracting factors such as superoxide anions (35, 36), metabolites of arachidonic acid (37, 38), or endothelin, (39) with maturation, are alternate explanations for these observations. A change in histamine metabolism by the endothelial cell is difficult to prove or refute and remains a possible explanation. Superoxide anion production is also possible, but treatment of piglet rings with superoxide dismutase (40) before stimulation with nitric oxide did not alter the responsiveness of these vessels (n = 2, unpublished observations), making this explanation unlikely. A cyclooxygenase product also is unlikely, inasmuch as all experiments were conducted in the presence of indomethacin. A decreased production of endothelin with time is also possible but untested.

These experiments, more importantly, demonstrate that relaxations to agonists known to stimulate the release of EDRF (6, 7) increase with maturation. The increased relaxations are not due to an altered ability of the vascular smooth muscle to relax, inasmuch as the responses to sodium nitroprusside were similar in vessels taken from piglets from the three age groups. Endogenous release of vasodilator prostaglandins cannot account for these differences because all experiments were performed in the presence of the inhibitor of cyclooxygenase, indomethacin. An increase in receptor number or receptor coupling with maturation also is unlikely because the response to the calcium ionophore A23187, a nonreceptor-mediated agonist, was similar to those observed with the receptor-mediated agonists, acetylcholine and bradykinin. An increased sensitivity of the smooth muscle to, rather than an increased release of, the endothelium-derived factor could explain the increased relaxations because the effect of nitric oxide, one of the proposed EDRF (16, 17), is similar to that observed with acetylcholine, bradykinin, and the calcium ionophore A23187 during the same time course. A maturational increase in the activation of or quantity of soluble guanylate cyclase and, subsequently, cyclic GMP to EDRF may be one of the molecular mechanisms involved (41). The data obtained with

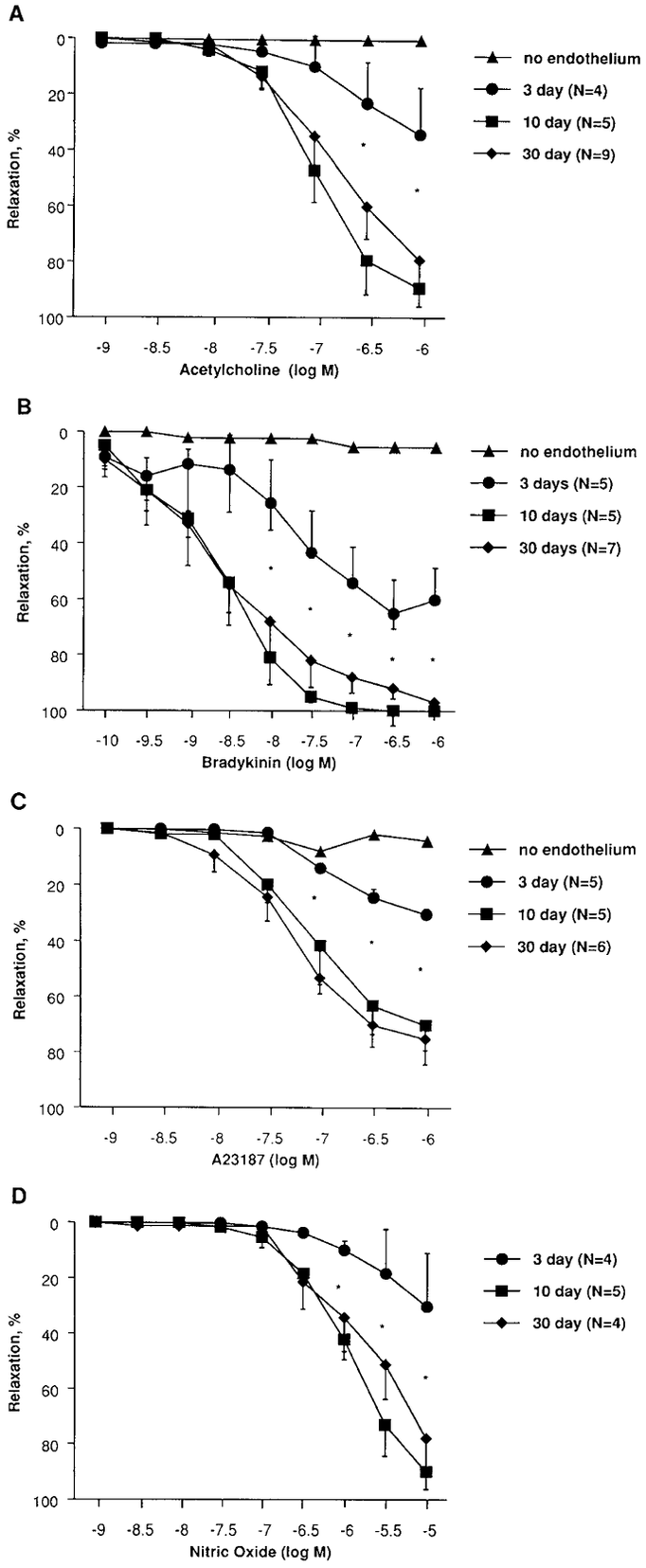

Fig. 5. Cumulative concentration-response curves to acetylcholine (*A*), bradykinin (*B*), the calcium ionophore A23187 (*C*), and nitric oxide (*D*) in pulmonary artery rings taken from 3-, 10-, and 30-d-old piglets. Rings were contracted with prostaglandin F2 alpha (contraction equal to 75% of the maximal contraction to histamine), and the relaxations at each age group are expressed as the percentage of inhibition of the prostaglandin contraction. Data are shown as means ± SEM. Indomethacin (10^{-5} M) was present to prevent synthesis of vasoactive prostaglandins. *M*, molar concentration in the organ bath.

sodium nitroprusside would suggest that this explanation is unlikely because both sodium nitroprusside and nitric oxide activate guanylate cyclase (16, 42). However, relaxations evoked by sodium nitroprusside can be mediated by the sodium-potassium ATPase pump (43, 44). This additional mechanism may explain the discrepancy between the results observed with nitric oxide and sodium nitroprusside. Alternatively, a concomitant increase in the synthesis and release of the EDRF could also, in part, explain the increase in relaxation to the various endothelium-dependent agonists observed with maturation, but the nitric oxide experiments strongly suggest a maturational change in the sensitivity of the vascular smooth muscle to EDRF.

Physiologically, an increase in the effect or release of EDRF, in both the basal and the stimulated state, may be important to the maintenance of pulmonary vascular tone during the neonatal period. The basal release of EDRF has been demonstrated in arteries from other species (19, 45) and is thought to be involved in the maintenance of the basal tone of the blood vessel. A decreased basal release of the endothelium-derived factors, as demonstrated in spontaneously hypertensive rats (28), may help explain the increase in the peripheral resistance characteristic of hypertension. This suggestion is supported by the finding that the antagonist of nitric oxide production, N^G-monomethyl-L-arginine, causes sustained increases in peripheral resistance and blood pressure (46). Similarly, the decreased effect of, and possible release of, the EDRF (in the stimulated and possibly in the basal state) at 3 d compared with 10 and 30 d observed in the present experiments may be related to the elevated pulmonary pressures and resistance observed in the immediate newborn period in the pig (3); an increased effect or release of EDRF with maturation may play an important role in the perinatal decrease in pulmonary arterial pressure and resistance. Although our results do not definitely demonstrate a causal relationship, they suggest a probable role for EDRF in the process.

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