

Endothelium-Derived Relaxing Factor: Presence in Pulmonary and Systemic Arteries of the Newborn Guinea Pig

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ABSTRACT. Endothelium-derived relaxing factor (EDRF), believed to be nitric oxide or a compound that releases nitric oxide, is a potent vasodilator produced by some arteries in response to acetylcholine (ACh) and bradykinin (BK). ACh and BK are potent dilators of perinatal pulmonary and systemic arteries. The objectives of this study were to determine if EDRF is present in newborn vessels and if EDRF mediates the vasodilator actions of ACh and BK. Arterial rings from newborn guinea pigs, 1 to 3 d old, were obtained from a branch of the main pulmonary artery and the descending aorta for isometric force bioassays. At their optimal resting tension, the rings were precontracted with phenylephrine 10^{-5} M in Krebs-Henseleit solution before adding incremental doses of ACh or BK. If the endothelium was intact, ACh (10^{-5} M) relaxed pulmonary arteries and aortas ($64 \pm 7\%$, $72 \pm 9\%$ relaxation, respectively, mean \pm SE). ACh-induced relaxation (ACh 10^{-5} M) in the pulmonary artery and aorta, respectively, was significantly ($p < 0.05$) attenuated by 1) endothelial removal ($11 \pm 9\%$, $28 \pm 10\%$) by rubbing the ring lumen; 2) methylene blue, 10^{-6} M, ($6 \pm 8\%$, $7 \pm 3\%$) that inhibits EDRF-associated cGMP production in smooth muscle; and 3) methemoglobin, 10^{-5} M, ($13 \pm 9\%$, $17 \pm 7\%$) that binds EDRF. The results for BK were similar to ACh for the pulmonary artery but BK did not relax the aorta. Indomethacin diminished relaxation of the pulmonary artery and aorta to the submaximal dose (10^{-5} M) of ACh but indomethacin did not effect the relaxation to ACh 10^{-4} M or BK. We conclude that EDRF is produced in the guinea pig pulmonary artery and descending aorta at birth and that EDRF is a mediator of the vasodilator actions of ACh and BK. Vasodilation by ACh may also involve activation of the cyclooxygenase pathway. (*Pediatr Res* 27: 128-132, 1990)

Abbreviations

EDRF, endothelium-derived relaxing factor
ACh, acetylcholine
BK, bradykinin

EDRF, now believed to be nitric oxide or a compound that releases nitric oxide, was discovered in 1980 when acetylcholine

Received June 16, 1989; accepted September 22, 1989.

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Supported by an Edward Livingston Trudeau Scholar Award (D.D.) from the American Lung Association.

was found to relax precontracted rabbit artery strips *in vitro* only if the luminal endothelial cell lining was intact (1). Subsequently, EDRF has been identified by bioassay techniques in pulmonary and systemic arteries of many mammalian species and substances other than ACh (*e.g.* BK and calcium ionophore) have been shown to stimulate EDRF production. Based on a rapidly expanding body of work (2) that followed the discovery of EDRF, certain bioassay criteria have evolved for attributing vasodilation to EDRF. These include inhibition of vascular relaxation: 1) after removal of or injury to luminal endothelium of the blood vessel (1); 2) by pretreatment with methylene blue that inhibits the increase in cGMP in smooth muscle cells associated with EDRF production (3); and 3) by pretreatment with methemoglobin or Hb that binds EDRF (4).

EDRF has not previously been described in pulmonary and systemic blood vessels at birth. It is known that ACh (5) and BK are potent dilators of the perinatal pulmonary circulation and BK has been implicated as a mediator of the circulatory adjustments at birth (6). Therefore, our experiments were designed to determine if EDRF is present immediately after birth in the guinea pig pulmonary artery and abdominal aorta and to determine if EDRF mediates the vasodilator actions of BK and ACh in blood vessels at birth. A better understanding of the biochemical control of the perinatal pulmonary circulation is needed because an attenuation of the decrease in pulmonary vascular resistance at birth can lead to severe respiratory distress and persistent pulmonary hypertension of the newborn.

MATERIALS AND METHODS

Arterial ring bioassay. Newborn Duncan-Hartley guinea pigs, 1-3 d old (1.3 ± 0.3 d, mean \pm SE) were weighed (89 ± 3 g) and anesthetized with pentobarbital 100 mg/kg intraperitoneally. After a midline abdominal and thoracic incision, heparin (100 U) was injected into the right ventricle. The heart and lungs were rapidly removed together and placed in ice cold Krebs-Henseleit solution. The abdominal aorta distal to the renal arteries was then carefully dissected away from connecting tissues and placed in standard Krebs-Henseleit solution. One vascular ring, 3-4 mm in length, was taken from the abdominal aorta and the first branch of the pulmonary artery (distal to the ductus arteriosus). Care was taken, during dissection and hook insertion, to avoid injury to the endothelial lining. In a separate set of studies, the endothelium was intentionally rubbed off the lumen of some rings by pulling the ring by its adventitia along a stainless steel wire inserted through the ring lumen.

Each ring was mounted in a 10-mL, water-jacketed, organ chamber by stainless steel hooks inserted gently into the ring lumen. The Krebs-Henseleit bath (38°C) was bubbled with a gas mixture to maintain a pH of 7.42 ± 0.02 , PCO_2 of 30 ± 1 torr,

and PO_2 of 574 ± 10 torr (1). Blood vessel tension was recorded from a Grass FTO3C isometric force transducer and model 7 polygraph (Grass Instrument Co., Quincy, MA).

The rings were gradually stretched (3–4 h) using a micrometer to give an initial resting tension of 1 g. The selected resting force for optimal contraction was then chosen based on serial depolarizations using a modified Krebs-Henseleit solution in which NaCl was replaced by KCl, yielding a 9.4 mM KCl solution. Between drug administrations, the bioassay chamber was washed with standard Krebs-Henseleit without changing bath temperature and a steady baseline tension was recorded for 15 min. The rings were then exposed to cumulative doses of phenylephrine from 10^{-8} to 10^{-4} M in increments of 10-fold concentration. Based on preliminary experiments, we found that phenylephrine 10^{-5} M produced a near maximal response ($ED_{50} = 3 \times 10^{-7}$ M). Therefore, all rings tested for relaxation were evaluated during a contraction to phenylephrine 10^{-5} M. Any ring that could not increase tension by 0.25 g in response to phenylephrine 10^{-5} M was not studied.

Some rings with endothelium removed were treated with phenylephrine followed by incremental doses of papaverine, a direct smooth muscle relaxing agent, to determine if smooth muscle relaxation could still occur after rubbing. At the end of the experiment some rings (rubbed and unrubbed) were examined by light microscopy to assess the condition of the luminal surface. After cutting the rings longitudinally, the luminal surface was stained with 2% $AgNO_3$ for 5 min. $AgNO_3$ preferentially stains the endothelial cell membrane (1). Preliminary studies indicated that excessive rubbing to remove all endothelial cells from these neonatal arteries was one cause for a poor contractile response to phenylephrine. Therefore, limited rubbing that removed almost all the endothelial cells, was required.

Determination of EDRF production. After a steady increase in tension was achieved with phenylephrine, ring relaxation in response to both ACh or BK was studied at the following concentrations: 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M, in rings with intact and rubbed lumens. For rings with intact endothelial cells, ACh and BK experiments were repeated with methemoglobin 10^{-5} M added after phenylephrine constriction but before ACh or BK administration. Methemoglobin binds EDRF thereby inhibiting vascular relaxation (7). Some rings exposed to BK in the presence of methemoglobin were also exposed to papaverine, 10^{-8} to 10^{-5} M, to determine if smooth muscle relaxation could still occur. Methylene blue, 10^{-6} M, was administered to baths with rings having intact endothelium, after precontraction and before attempting relaxation with ACh. Methylene blue inhibits EDRF vasodilatation and production of cGMP in smooth muscle (3, 4). Inasmuch as baseline tension was variable after methylene blue was washed from the bath, methylene blue was only used at the end of the experimental protocol and for ACh studies only.

During a contraction to phenylephrine, pulmonary arterial and descending aortic rings with intact endothelium were used to study relaxation to cumulative incremental doses of ACh, BK, or arachidonic acid before and after an 0.5-h exposure to indomethacin $20 \mu M$ to block the cyclooxygenase pathway (3).

Materials. Phenylephrine hydrochloride, ACh chloride, BK, methemoglobin, methylene blue, papaverine, indomethacin-sodium, and arachidonic acid-sodium were obtained from Sigma Chemical Company (St. Louis, MO). All drugs were dissolved in deionized water. Indomethacin was made up fresh as a 10-mM solution with 100 mM $NaHCO_3$. Standard Krebs-Henseleit solution contains NaCl 118 mM, $NaHCO_3$ 25 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, MgCl 1.2 mM, NaH_2PO_4 1.1 mM, and dextrose 11.1 mM.

Statistical analysis. Results are expressed as mean \pm SE. The principal endpoints analyzed statistically were the relaxation responses to 10^{-5} M concentrations of ACh and BK. Comparisons were made by analysis of variance followed by unpaired *t* testing corrected by the Bonferonni method for multiple comparisons (8).

RESULTS

Baseline isometric tension was not different in intact compared to endothelium-removed pulmonary artery rings (1.1 ± 0.1 g) and intact compared to endothelium-removed descending aortic rings (1.3 ± 0.1 g). There was no difference between rings with intact endothelium or endothelium-removed regarding the percent increase in baseline tension after phenylephrine administration. Phenylephrine (10^{-5} M) raised pulmonary arterial ring tension by $48 \pm 7\%$ (to 1.63 ± 0.11 g) and abdominal aortic ring tension by $42 \pm 8\%$ (to 1.85 ± 0.15 g). Light microscopy of $AgNO_3$ -stained rings confirmed an intact endothelial lining (unrubbed vessels) and nearly complete removal of endothelium (rubbed vessels) as shown in Figure 1.

Effect of ACh on precontracted pulmonary artery and aortic rings. Figure 2 shows typical polygraph tracings depicting the effect of endothelial cell removal on ACh-induced relaxation in pulmonary arterial rings. ACh produced a concentration-dependent relaxation of both precontracted pulmonary artery and descending aorta (Fig. 3). Relaxation is defined as the percent decrease in tension caused by ACh or BK during a contraction to phenylephrine (10^{-5} M). For example, if phenylephrine (10^{-5}

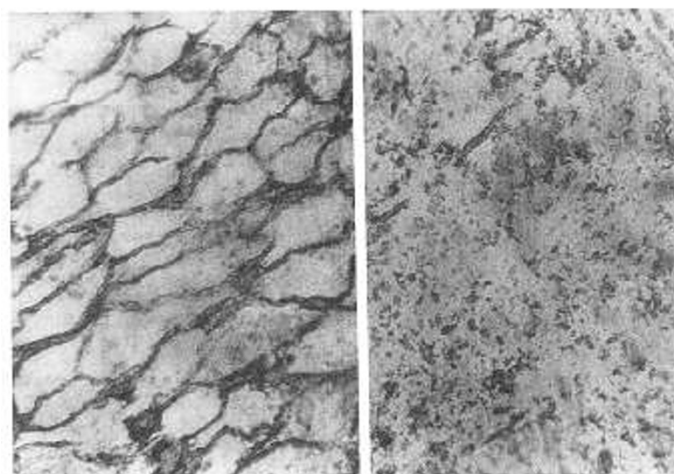


Fig. 1. Light microscopic views (x400, silver nitrate stain) of the luminal surface of a pulmonary artery from the newborn guinea pig. The panel on the left shows an intact endothelial surface, whereas the panel to the right shows the surface after removal of endothelial cells by rubbing.

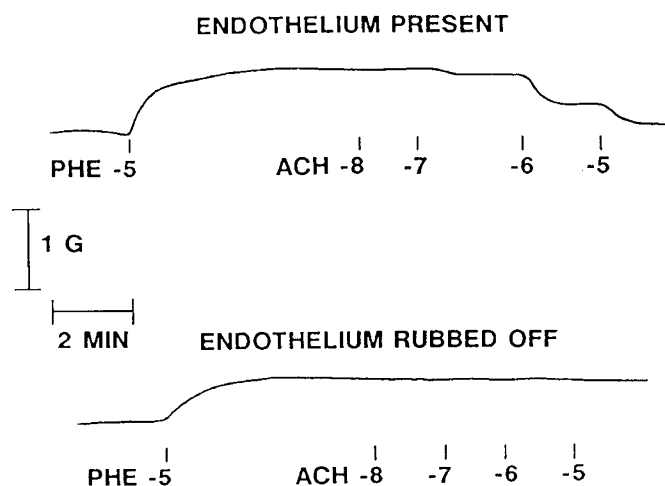


Fig. 2. Polygraph tracings demonstrating relaxation of a 1-d-old guinea pig pulmonary artery ring *in vitro* by ACh during a contraction by phenylephrine. Removal of the endothelial cell lining inhibited ACh-induced relaxation.

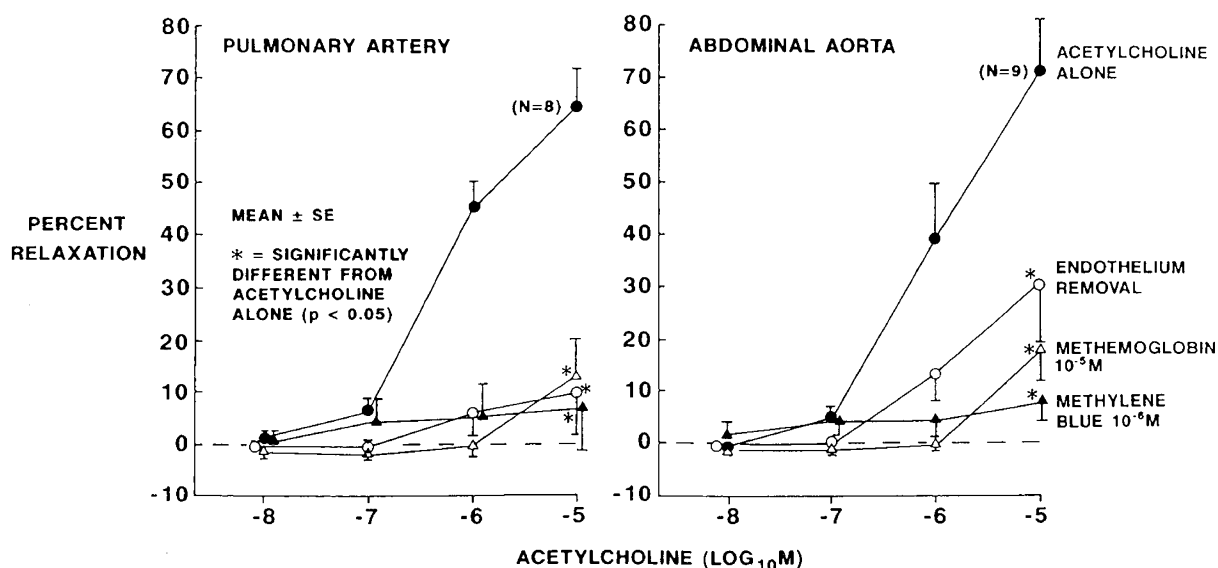


Fig. 3. ACh-induced relaxation of newborn guinea pig pulmonary arterial (*left panel*) and descending aortic (*right panel*) rings *in vitro* during a contraction by phenylephrine. Removal of the endothelial cell lining, methylene blue, and methemoglobin markedly attenuated ACh-induced relaxation.

M) increased ring tension by 1 g above baseline and ACh (with or without an EDRF inhibitor) reduced tension to 0.75 g above baseline, then a 25% relaxation has occurred. If an agent such as BK increases ring tension from 1 to 1.25 g then this is referred to as a -25% change in tension. ACh (10^{-5} M) relaxed precontracted pulmonary artery and aorta by $64 \pm 7\%$ ($n = 8$) and $72 \pm 9\%$ ($n = 9$), respectively.

Relaxation by ACh (10^{-5} M) was attenuated by: 1) endothelium removal (relaxation, $11 \pm 9\%$ pulmonary artery, $28 \pm 10\%$ aorta); 2) methemoglobin, 10^{-5} M ($13 \pm 9\%$ pulmonary artery, $17 \pm 7\%$ aorta), and 3) methylene blue, 10^{-6} M ($6 \pm 8\%$ pulmonary artery, $7 \pm 3\%$ aorta). When pulmonary arterial and aortic rings that had their endothelium removed were precontracted with phenylephrine, papaverine (10^{-5} M), a direct smooth muscle relaxing agent, totally relaxed the rings to baseline tension.

Effect of BK on precontracted pulmonary artery and aortic rings. BK produced a dose-related relaxation of the precontracted pulmonary arterial ring with intact endothelium (Fig. 4). Relaxation of the pulmonary arterial ring by BK 10^{-5} M was $53 \pm 6\%$ ($n = 6$) and was attenuated by 1) endothelium removal ($13 \pm 13\%$) and 2) methemoglobin, 10^{-5} M ($13 \pm 12\%$). BK did not relax the aorta ($n = 7$), the response was highly variable and the average response was one of increased tone (depicted by a negative percent change from base line in Fig. 4). The aortic response to BK was not different whether the endothelium was intact or removed, or whether the intact ring was exposed to methemoglobin.

Papaverine (10^{-5} M) could cause nearly total relaxation ($96 \pm 1\%$) of precontracted aortic rings after the BK experiments. Papaverine (10^{-5} M) led to total relaxation of two pulmonary arterial rings with intact endothelium that did not relax with BK (10^{-5} M) in the presence of methemoglobin (10^{-5} M).

Effect of indomethacin on arterial relaxation by arachidonic acid, BK, and ACh. Using rings contracted with phenylephrine, we examined the effect of indomethacin ($20 \mu\text{M}$, cyclooxygenase inhibitor) on pulmonary arterial ($n = 8$) and aortic ($n = 7$) ring tension during administration of arachidonic acid, ACh, or BK. Exogenous arachidonic acid (10^{-4} M) relaxed the pulmonary artery by $13 \pm 2\%$ but increased tension in the aorta by $47 \pm 7\%$. After indomethacin, arachidonic acid caused no relaxation in the pulmonary artery and changed the aortic response to $-9 \pm 9\%$, suggesting an appreciable inhibition of cyclooxygenase activity was present.

Indomethacin attenuated relaxation in the pulmonary artery and aorta as shown in Figure 5. For both vessels, the ACh concentration *versus* relaxation curve was shifted to the right and relaxation in response to ACh 10^{-5} was significantly reduced by indomethacin. Indomethacin had no significant effect on BK-induced relaxation in the pulmonary artery and no effect on the BK-induced variable change in tension of the aorta. BK-induced (10^{-5} M) relaxation of the pulmonary artery was 45 ± 6 and $38 \pm 8\%$ for the control and indomethacin groups, respectively. BK-induced (10^{-5} M) changes from baseline tension for the aorta were $-85 \pm 53\%$ (control) and $-32 \pm 17\%$ (indomethacin).

DISCUSSION

Our study demonstrates that EDRF can be produced in the guinea pig pulmonary artery and descending aorta at birth. ACh-induced relaxation of precontracted pulmonary arterial and descending aortic rings *in vitro* could be attributed largely to EDRF because its action was markedly attenuated by endothelial cell removal (1), methemoglobin (7), and methylene blue (3, 4). BK-induced relaxation of the pulmonary artery could also be attributed principally to EDRF, but BK had no consistent effect on aortic tone. To our knowledge, EDRF production has not been previously demonstrated in pulmonary and systemic blood vessels immediately after birth. An EDRF is produced in human umbilical vascular endothelial cells stimulated by histamine but not ACh (9).

After demonstrating ACh- and BK-induced relaxations of the newborn guinea pig blood vessels, we tested several methods known to inhibit EDRF production or action on vascular relaxation. First, the method of endothelial cell rubbing was effective in markedly diminishing relaxation but not impairing vasoconstrictive responses to phenylephrine and vasodilating responses to papaverine in comparison to the pulmonary arterial or aortic ring with intact endothelium. The effectiveness of rubbing the ring lumen on endothelial cell removal was confirmed by light microscopy. We found nearly complete endothelial cell removal; this may explain why we markedly attenuated but did not abolish relaxation in response to ACh and BK. Second, methemoglobin or Hb inhibition of EDRF is often used as a criteria that one must meet to demonstrate EDRF production by bioassay (2, 7). Methemoglobin and Hb, the latter used more commonly because it is more potent, appears to bind EDRF as it is produced by the endothelial cell. In our study we found that when pulmonary

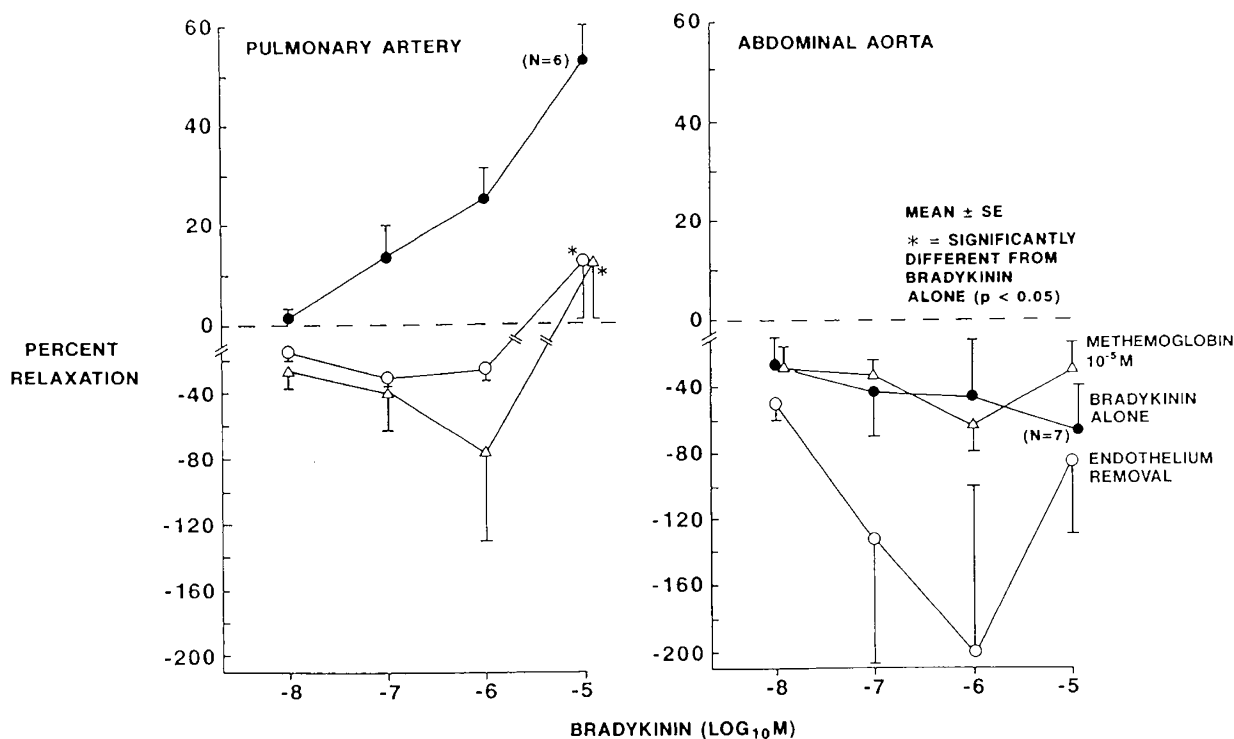


Fig. 4. BK-induced changes in vascular tone of newborn guinea pig pulmonary arterial (*left panel*) and descending aortic (*right panel*) rings *in vitro* during a contraction by phenylephrine. Removal of the endothelial cell lining and methemoglobin markedly attenuated BK-induced relaxation of the pulmonary artery. BK did not relax aortic rings.

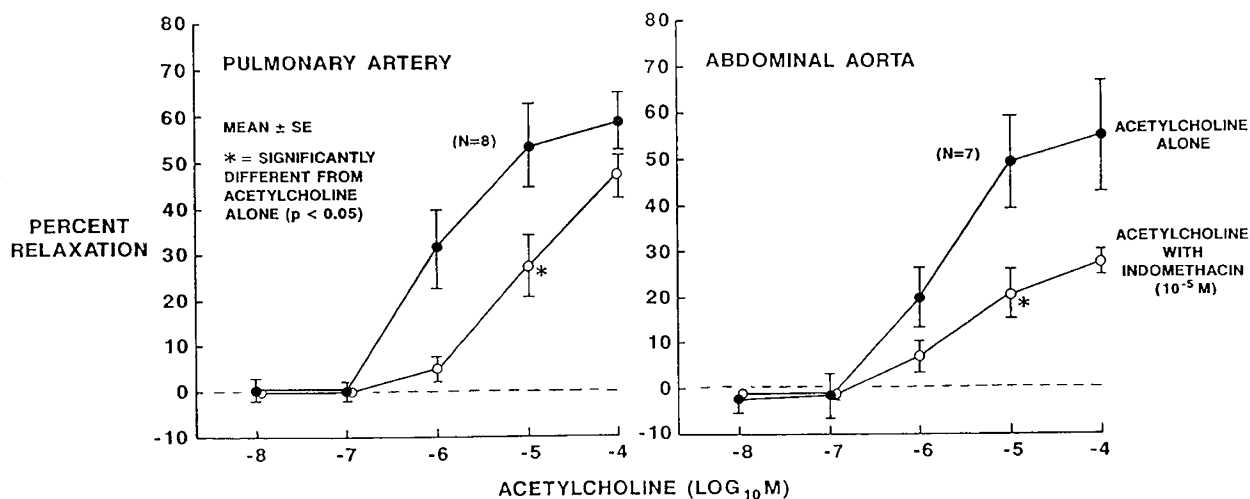


Fig. 5. ACh-induced relaxation of newborn guinea pig pulmonary arterial (*left panel*) and descending aortic (*right panel*) rings *in vitro* during a contraction by phenylephrine. Indomethacin attenuated acetylcholine-induced relaxation in both arteries.

vascular relaxation by BK was inhibited by methemoglobin, we could still achieve relaxation if papaverine (a direct smooth muscle relaxing agent) was then added to the bath. This indicates that methemoglobin was not having a direct inhibitory effect on smooth muscle relaxation and supports the proposal that methemoglobin combines with EDRF resulting in an inhibition of EDRF-induced relaxation (2). Third, methylene blue inhibited acetylcholine-induced relaxation in our study (2-4). Methylene blue inhibits the action of EDRF by inhibiting the associated increase in guanylate cyclase activity and thus cGMP production in smooth muscle.

The original report describing EDRF (1) and the extensive literature that has accumulated since (2), does not support the possibility that EDRF is a product of the cyclooxygenase pathway because indomethacin does not inhibit production of EDRF

(10). The identity of EDRF is now considered to be nitric oxide or a precursor compound that releases nitric oxide (2, 11). We did not expect to see an effect of indomethacin on ACh-induced relaxation because we had previously shown that this relaxation was almost completely inhibited by methylene blue and methemoglobin. These EDRF inhibitors do not theoretically block the production or action of a cyclooxygenase metabolite such as the vasodilator prostacyclin. Inasmuch as BK has been shown to stimulate prostacyclin production from endothelial cells (12), we expected that indomethacin might attenuate BK more than ACh-induced relaxation. In our study indomethacin did not attenuate the BK-induced relaxation of the pulmonary artery and this was compatible with the results that BK-induced relaxation was markedly inhibited by methemoglobin suggesting a principal role for EDRF. Our results with ACh could be explained by a

nonspecific action of indomethacin on ACh-induced EDRF production, *e.g.* lipoxygenase inhibitors can inhibit EDRF (13), or that products of the cyclooxygenase pathway may be coupled with EDRF production as demonstrated in coronary arteries of the adult pig (14) using indomethacin at 10^{-5} M. Despite our unexpected results with ACh, the fact that indomethacin had no effect on relaxation caused by the highest concentration of ACh (10^{-4} M) and BK, supports our conclusion that EDRF is present in guinea pig blood vessels at birth.

Whether EDRF plays an important role in the circulatory adjustments at birth will be an interesting area for future research. Vasodilator substances such as BK (6) and prostacyclin (15) have been implicated as mediators of the decrease in pulmonary vascular resistance and marked increase in pulmonary blood flow at birth. The results from our *in vitro* experiments indicate that the vasodilator effect of BK in the perinatal pulmonary circulation is mediated by EDRF. Prostacyclin production with the onset of breathing at birth appears to play a minor role in the pulmonary hemodynamic adjustments at birth based on experiments with acute cyclooxygenase blockade in chronically instrumented, spontaneously breathing lambs (16). Inasmuch as high circulating levels of vasoconstrictors such as catecholamines and angiotension II are elevated and contribute to the increase in systemic vascular resistance at birth (17, 18), EDRF could play a counterbalancing role in the pulmonary circulation. Attention is now turning to the relief of hypoxic pulmonary vasoconstriction as the principal process involved in the decrease in pulmonary vascular resistance at birth (19). The role of endothelial cell and EDRF production during hypoxia appears to be a new avenue for research (20).

Using the newborn guinea pig, our study demonstrates for the first time that EDRF is produced in pulmonary and systemic arteries immediately after birth. Further studies are needed in perinatal animals to determine whether there are differences in EDRF production or action between species and with development. Our study, taken together with recent work showing that ACh causes vasodilatation in pulmonary hypertensive calves (21), suggests that regional stimulation of lung EDRF production or administration of exogenous EDRF could play a therapeutic role in pulmonary hypertensive diseases of the newborn.

Acknowledgments. The authors thank Drs. P. Lipsitz, R. Bienkowski, and E. Scarpelli for their support and critical review of the manuscript. The multiple bioassay stand and controls were custom made by Fred Singer of Koehler Instrument Company, Bohemia, NY. The support of Diane Davidson is gratefully acknowledged.

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