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Basal and stimulated release of long-acting EDRF by bovine pulmonary arteries

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Endothelium-derived relaxing factor (EDRF) is supposed to be identical to nitric oxide (NO). A characteristic of NO is its half-life of 6–50 seconds. We report here bioassay experiments suggesting the presence of a long-acting EDRF. An endothelium intact segment of the main bovine pulmonary artery (generator) and an endothelium deprived artery strip (detector) were separately perfused with Krebs-Henseleit solution. The effluent of the generator was collected and permitted to stand in an open beaker at 37 °C for periods exceeding five minutes. Following this the collected effluent (CE) perfused the histamine precontracted detector, causing significant relaxation. The same degree of relaxation could be observed after storing CE for 80–120 minutes. After reaching steady state generator effluent (G) was directly superfused over the detector (time delay about two seconds) resulting in a more pronounced relaxation. Addition of pharmacological stimuli of EDRF mediated relaxation augmented, addition of haemoglobin inhibited relaxation as well as by CE and G. Our results show that perfusates from bovine pulmonary arteries contain a material with high degree of stability, being responsible for some part of endothelium mediated relaxation. *J Clin Basic Cardiol* 1999; 2: 117–9.

Key words: endothelium-derived relaxing factor, nitric oxide, superfusion, acetylcholine, lysophosphatidylcholine, bradykinin

Nitric oxide (NO) is an important bioregulatory molecule mediating intercellular signalling in many physiological processes by raising intracellular 3' 5' cyclic guanosine monophosphate (cyclic GMP) [1, 2]. It is partially responsible for endothelial regulation of vascular smooth muscle tone, accounting for action of endothelium-derived relaxing factor (EDRF) [1–4]. NO is synthesized from L-arginine by two NO synthases, a constitutive calcium NO synthase, active in vascular endothelium and in the central nervous system [5, 6] and, the inducible calcium-independent form, present in macrophages and heart muscle [7, 8].

Several publications have questioned the existence of a single EDRF [9–11]. By electron paramagnetic resonance spectroscopy Greenberg et al. [9] could not detect free nitric oxide. Using anion exchange resin, Long and Berkowitz [10] discriminated between NO and EDRF. NO is characterized by its short half-life of about 6–50 seconds due to rapid conversion to nitrate and nitrite. We report here on the presence of a long – up to 120 minutes – lasting EDRF.

Methods

Bovine lungs from animals of either sex were obtained from a local abattoir. Main bovine pulmonary arteries (12 cm length) and side branches were rapidly isolated, gently cleaned of adhering tissue and immediately put in Krebs-Henseleit solution (K-H). K-H consisted of 143 mM Na⁺/5.94 mM K⁺/2.54 mM Ca²⁺/1.19 mM Mg²⁺/1.19 mM H₂PO₄⁻/127.84 mM Cl⁻/25 mM HCO₃⁻/1.19 mM SO₄²⁻/10 mM glucose. The side-branches of the main pulmonary artery (referred to as generator) were ligated by means of titanium haemostatic clips. Polyethylene tubings of approximately 2 cm length were inserted into each end and tied into place. Dilute patent blue dye was installed into the generator to test for leaks. The artery was then mounted in plastic chambers, holding 60 ml K-H and perfused with oxygenated (95 % O₂ – 5 % CO₂) K-H at 3 ml/min by means of a perfusion pump at 37 °C [12]. Analysis of pH, pO₂ and pCO₂ gave values within physiological range. A side branch (5–10 mm diameter) of the main pul-

monary artery (referred to as detector) was cut into helical strips (length 20 mm, width 4 mm). The endothelium was removed by gently rubbing the intimal surface with moist filter paper. Two stitches were placed on each end of the strip using cotton thread (size 50). The lower threads were mounted on a stationary support, the upper one to an isometric pressure transducer. The initial tension was set at 4 gms and the detector was superfused with K-H at 6 ml/min. The strips were allowed to equilibrate for 1 hour. In the presence of 10⁻⁵ M indomethacin, to prevent formation of vasorelaxant prostaglandins, detector and generator were precontracted with histamine (10⁻⁵ M). Basal release of EDRF was tested by collecting the K-H effluent of the generator in an oxygenized open beaker and permitting it to stand at 37 °C for periods of 5–120 minutes to inactivate NO. Following this, the collected effluent (CE) superfused the precontracted detector, maintaining the previous flow rate of 6 ml/min. After reaching steady state a typical superfusion bioassay was performed. Immediately after disconnecting the line of CE to the strip, the effluent of the generator (G) was directly passed over the detector (time delay about 2 seconds). In control experiments, only superfusion bioassays were performed, ie, only generator effluent (G) superfused the precontracted detector. In another series of experiments, effluent of endothelium-deprived generators was also tested for relaxation in a superfusion bioassay.

By addition of acetylcholine (ACh) 10⁻⁶ M to the perfusion medium of endothelium intact generators, stimulated release of EDRF could be obtained [13]. The effluent (CE-ACh) was collected and superfused over the detector after reaching steady state by CE. Direct superfusion of the detector tissue with G-ACh was also performed in a manner similar to that described above. Beside ACh also lysophosphatidylcholine (LPC) and bradykinin (BKN) were tested for stimulated EDRF release [14].

Oxyhaemoglobin 10⁻⁵ M was added to the perfusion medium of the detector after having obtained relaxation due to CE and G. It was prepared according to the method of Martin et al. [15] by adding a 10-fold excess of sodium dithionite to a 1 mM solution of bovine haemoglobin in distilled water. So-

dium dithionite was removed by dialysis against 100 volumes of distilled water for two hours at 4 °C. Statistical analysis was carried out using Student's paired and unpaired t-test. A $p < 0.05$ was considered significant.

Results

Superfusion bioassay

Basal Release: Superfusion of the histamine precontracted detector with CE (Fig. 1) resulted in a relaxation of the detector by $14.9 \pm 1.9\%$ ($n = 26$, $p \leq 0.001$). The same degree of relaxation could be observed after storing CE for 80 to 120

minutes ($n = 4$). After relaxation and the attainment of a steady state, the detector was again superfused. This resulted in a further increase of relaxation to $36.6 \pm 5.1\%$ ($n = 7$, $p \leq 0.001$). The contribution of CE to total relaxation was 40.5%. In control experiments, relaxation induced by the perfusate of endothelium-intact generators was $31.1 \pm 7.4\%$ ($n = 10$, $p \leq 0.001$). Difference in total relaxation between these two experiments was not significant. Superfusates from endothelium-deprived generators caused no significant decline in tension ($0.4 \pm 1.7\%$; $n = 11$, n.s.). As compared to direct superfusion, which caused a steep decline in tension within a few seconds, onset of relaxation by CE had a time delay of about two minutes and the slope of relaxation was less steep. The addition of oxyhaemoglobin (10^{-5} M) significantly ($p \leq 0.001$) reduced relaxation due to CE and G ($84.9 \pm 20.4\%$, $n = 7$, versus 85.5 ± 43.9 , $n = 7$).

Stimulated Release: Addition of ACh (Fig. 2), LPC and BKN to the generator perfusate augmented relaxation as well as by CE and G (Table 1). Contribution of CE-ACh, CE-LPC and CE-BKN to total relaxation was 53.8 %, 53.9 % and 50.7 %, respectively.

Discussion

Perfusion of endothelium intact vessels allows the measurement of the generation of vasoactive substances, using endothelium denuded artery strips as detectors. Under these bioassay conditions bovine pulmonary arteries release EDRF(s) and prostacyclin upon mechanical (ie, shear stress) and pharmacological stimulation. Due to addition of indomethacin, which inhibits the formation of vasorelaxant prostacyclin, achieved relaxation is due to the liberation of EDRF. EDRF is supposed to be identical or related to nitric oxide, but also several studies exist postulating endothelium dependent vasodilating factors different from NO [5–7]. As techniques for detecting NO, chemiluminescence [4] and diazotization [16], require acidic conditions, thus converting all nitroso compounds into nitric oxide, a number of sources must be considered as potential donors of NO. The results of the present study demonstrate that endothelium intact pulmonary arteries (generator), in addition to releasing EDRF (NO), also produce a vasodilator substance with an extended half-life, resulting in a significant relaxation of the detector vessel even after periods of 80–120 minutes. After that period, which exceeds the half-life of NO, significant endothelium-mediated relaxation in bovine pulmonary arteries is still noticeable, inducing a relaxation which is about 50 % of total EDRF induced relaxation. This suggests the production of an endothelium-derived material with high degree of stability. The additional decrease in vascular tension due to direct generator superfusion with a time delay of only two

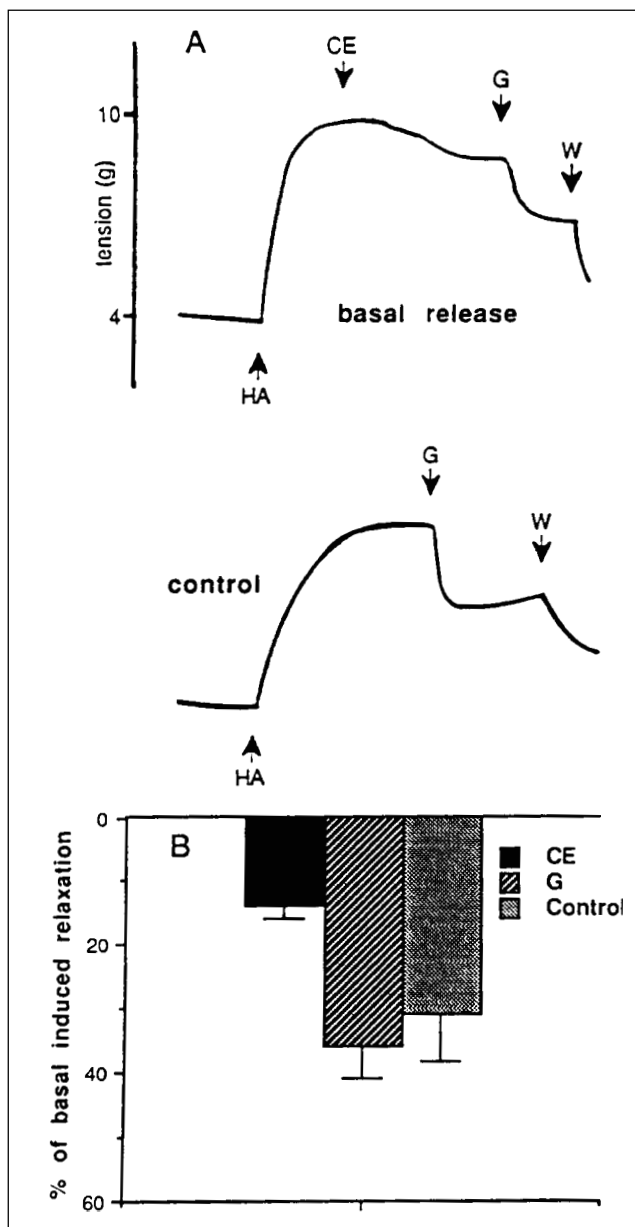


Figure 1. Relaxation of bovine pulmonary arteries under basal conditions. **A:** Representative tracing. Endothelium deprived artery strip (detector) was precontracted with 10^{-5} M histamine (HA). In the upper panel significant relaxation was induced by perfusing the detector with effluent of an endothelium intact pulmonary artery (generator) which was collected and stored prior to perfusion for five minutes (CE). Following direct superfusion (G), additional relaxation was induced. In control experiments (lower panel) only direct superfusion (G) was performed; W = wash. **B:** Average relaxation. Data shown as mean $\pm$ SEM.

Table 1. Stimulated relaxation by acetylcholine (ACh), bradykinin (BKN) and lysophosphatidylcholine (LPC) due to collected effluent (CE) and generator perfusate (G)

	% Relaxation		
	ACh 10^{-6} M (n = 7)	BKN 10^{-5} M (n = 5)	LPC 10^{-8} M (n = 9)
CE	9.2 ± 1.9	9.8 ± 1.3	16.2 ± 4.1
CE + Stimulans	22.7 ± 4.5	35.8 ± 4.4	37.3 ± 6.2
G + Stimulans	43.3 ± 8.4	62.2 ± 7.1	65.1 ± 7.2
Control	45.7 ± 7.2	51.8 ± 4.0	62.6 ± 7.8

Data are shown as mean $\pm$ SEM.

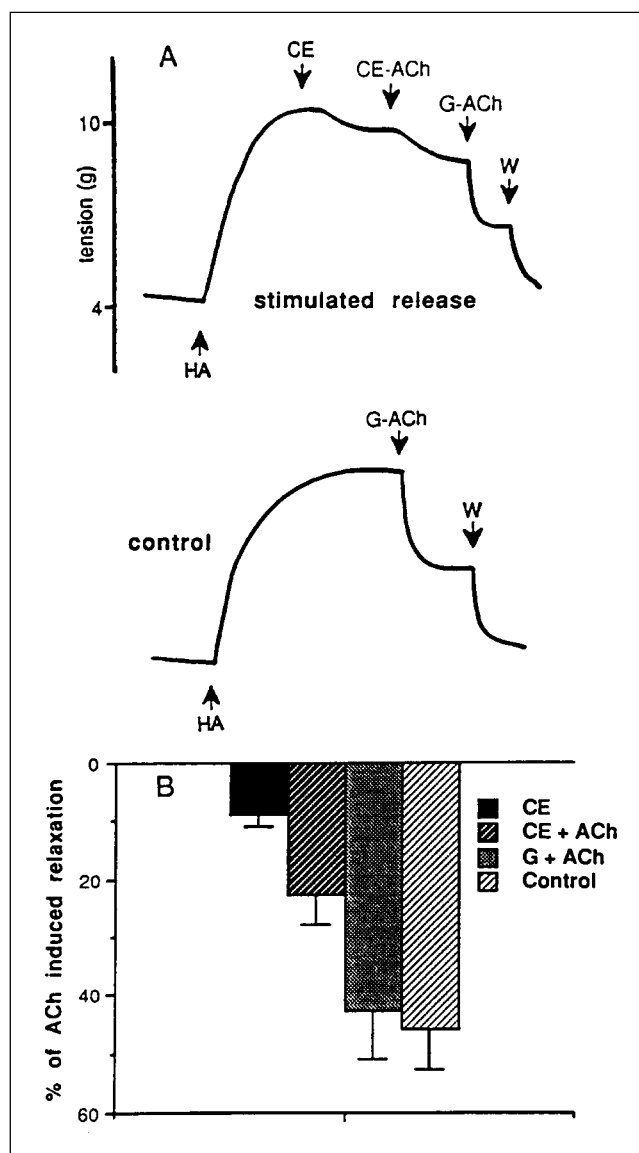


Figure 2. Relaxation of bovine pulmonary arteries under stimulated conditions. **A:** Representative tracing. In the upper panel significant relaxation was induced by perfusing the detector with effluent of an endothelium intact pulmonary artery (generator) which was collected and stored prior to perfusion for five minutes (CE). Addition of acetylcholine (ACh 10^{-6} M) to the perfusion medium of the generator (CE-ACh) augmented relaxation. Following direct superfusion (G-ACh), relaxation was more pronounced. In control experiments (lower panel) only direct superfusion was performed; W = wash. **B:** Average relaxation.

probably related to the presence of short acting substances, supposedly NO. Expectedly total relaxation was identical when this biphasic slope was compared to control experiments in which labile and more stable substances combined caused relaxation. As compared to direct superfusion, onset of relaxation by CE had a time delay of about two minutes and the slope of relaxation was less steep indicating a maybe different mode of action on vascular smooth muscle. The addition of pharmacological stimuli to the perfusion medium significantly

increased relaxation due to short and long lasting EDRFs. As perfusate of endothelium deprived generators failed to decrease vascular tension, released substances obviously derived from endothelial cells.

Oxyhaemoglobin is known to inhibit endothelium-dependent relaxation and to bind to nitric oxide. The addition of oxyhaemoglobin significantly reduced relaxation due to CE and G to the same extent, indicating that the substance inducing relaxation due to CE is bound, like NO, to haemoglobin.

Our results show that significant endothelium mediated relaxation by bovine pulmonary arteries still occurs after a time delay exceeding half-life of nitric oxide. The release of a substance with high degree of stability beside nitric oxide would provide a possible explanation. Another adequate hypothesis would be that only part of vasoactive nitric oxide gets inactivated by oxidation and remaining NO is capable of accomplishing relaxation. Physiologically, both theories could imply that EDRF, which is proposed to be rather a local than a circulating factor also could exhibit some systemic action. Further investigations will be necessary to elucidate the significance of these findings.

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