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EPR SPECTROSCOPIC STUDIES OF DETECTION OF A CARBON-CENTERED FREE RADICAL DURING ACETYLCHOLINE-INDUCED AND ENDOTHELIUM-DEPENDENT RELAXATION OF GUINEA PIG PULMONARY ARTERY

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Vascular smooth muscle relaxation by several vasodilators, including acetylcholine (Ach) and ATP, depends on the presence of intact endothelium. Ach is thought to activate muscarinic receptors on endothelium to release an endothelium-derived relaxing factor (EDRF) which brings about relaxation of smooth muscle. In order to assess the role of free radicals in the endothelium-dependent relaxation of blood vessel, we have studied the effect of a spin-trapping agent, phenyl t-butyl nitrone (PBN), on Ach-, ATP-, and sodium nitroprusside-induced relaxation of guinea pig pulmonary artery. Arterial strips were mounted in a 5-ml organ bath containing Krebs solution equilibrated with 95% O_2 and 5% CO_2 at 37°C. After increasing vascular tone by a synthetic prostaglandin endoperoxide analog (50 ng/ml), the strips relaxed dose-dependently in response to Ach (5×10^{-8} M), ATP (1.5×10^{-6} M) or sodium nitroprusside (6×10^{-9} M). Removal of the endothelium abolished the relaxation by Ach or ATP, but did not affect the relaxation by sodium nitroprusside. PBN inhibited Ach-induced relaxation of pulmonary artery dose-dependently, but had no effect on relaxations by ATP or sodium nitroprusside. PBN did not block radioligand binding to muscarinic cholinergic membrane receptors on both chick embryonic heart and guinea pig pulmonary artery endothelial cells indicating that it does not block the muscarinic receptors. Spin trapping in combination with electron paramagnetic resonance (EPR) spectral analysis revealed a carbon-centered radical with hyperfine splitting constants of a_N = 16.0 G and a_β^H = 3.85 G in the lipid extracts of pulmonary artery (0.2–0.4 g) incubated with PBN (14 mM) and Ach (3×10^{-6} M) for 20 min. No signal was detected when endothelium was removed. Our data suggest that the endothelium-dependent relaxation of pulmonary artery by Ach is associated with the generation of a free-radical and can be prevented by a spin-trapping agent. ATP, however, relaxes the arterial smooth muscle by a different mechanism.

KEY WORDS: Free radical, acetylcholine, endothelium, EDRF, receptors, spin-trapping.

INTRODUCTION

The ability of acetylcholine (Ach) to relax isolated, precontracted arteries has recently been shown to be strictly dependent on the presence of an intact vascular lining.[1-5] Other vasodilators, such as adenosine di- and triphosphate (ADP and ATP),[3,4,6] serotonine,[7,8] histamine,[9] bradykinin,[4,10] arachidonic acid[11] and calcium ionophore A23187[12,13] can, in certain preparations, also cause relaxation by an endothelium-dependent mechanism. Intact endothelial cells, however, are not required for such vasodilators as isoproterenol,[10] nitrovasodilators,[1,12] atrial natriuretic peptide[14,15] and some prostaglandins.[10]

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Ach is thought to activate muscarinic receptors on endothelial cells to release an *Endothelium-Derived Relaxing Factor* (EDRF), which diffuses to and relaxes vascular smooth muscle.[1] Although the chemical identity of EDRF is still unknown, some experimental evidence has implicated an oxidation product of unsaturated fatty acids via the lipoxygenase pathway, possibly a free radical. Among this evidence is that the relaxing effect of Ach can be reversed by anoxia,[1] by inhibitors of phospholipase A2,[1,13,16] by several antioxidants,[1,2,17] and by inhibitors of lipoxygenase[1,3,5,13] but not of cyclo-oxygenase activity.[1,5]

In order to assess the role of free radicals in the relaxation of vascular smooth muscle, we have employed the techniques of spin trapping and electron paramagnetic resonance (EPR) spectroscopy. Spin-trapping agents react with certain transient free radicals to form a more persistent radical product, a spin adduct, which can be detected and examined directly by EPR spectroscopy.[18] Thus, the spin-trapping agent cannot only inhibit a free-radical mediated reaction by removing free radicals, but also provides the opportunity for identifying the nature of the primary free radical through EPR spectral analysis. In this report, we present evidence that a spin-trapping agent inhibits Ach-induced endothelium-dependent relaxation of guinea pig pulmonary artery by a mechanism other than muscarinic receptor blockade, and traps a free radical which can be detected and characterized by EPR spectroscopy.

MATERIALS AND METHODS

Preparation of pulmonary artery strips and relaxation experiments

Guinea pigs (Hartley strain, either sex, 350–650 g) were euthanized and exsanguinated. The main pulmonary artery was dissected, with care to avoid stretching or injury to the intima with instruments or rough handling. After dissection, the tissues were immersed in ice-cold Krebs Ringer Buffer solution (KRB) [mM concentration: NaCl 117.6, KCl 5.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, NaH_2PO_4 1.0, glucose 11.1, $CaCl_2$ 2.5] at 4°C. The arteries were carefully cleaned of any adherent fat and connective tissue and cut into 5 mm-wide ring segments. The rings were opened by a longitudinal cut along the length of the vessel with Wescott tenotomy scissors. Each preparation was attached to an isometric transducer (Harvard, model 363) by fine metal needles (micro-point spatula GS-9, Ethicon Inc.) placed through both cut edges. The change in tone was recorded graphically (Beckman type R Dynograph) throughout the experiment. The strips were suspended in a 5-ml organ bath filled with KRB equilibrated with 95% O_2 and 5% CO_2 at 37°C, and circulated continuously at 2 ml per min by an infusion pump (Cole-Parmer). An initial load of 1.0 g was applied to the strips, which were allowed 40–60 min to reach their basal tone. After the flow of KRB was stopped, vascular tone was increased (to facilitate the demonstration of relaxation) by the addition of 50 ng per ml of synthetic prostaglandin endoperoxide analog (PGH_2-A, 9,11-dideoxy-11, 9-epoxymethanol prostaglandin F2, Upjohn). After a new steady tension was reached, responses to Ach (10^{-9} to 10^{-6} M), ATP (10^{-8} to 10^{-4} M) and sodium nitroprusside (10^{-11} to 10^{-7} M) were obtained by cumulative additions (5–10 doses, 10 μl each) of the vasodilator to the organ bath at 5–10 min intervals. The strips were then washed with KRB (2 ml per min) for at least 40 min and re-exposed to PGH_2-A (50 ng per ml) before PBN (0.5 or 1.0 mM) was added. In one half of the experiments, concentration response curves to the vasodilators were obtained before addition of PBN; in the other half the order was reversed. Vascular

relaxation was expressed as percent decrease of the PGH_2-A induced tone. Only one vasodilator was tested on an individual strip. In order to investigate the role of endothelium in relaxation, the intimal surface of the strips was gently rubbed with a metal spatula to remove the endothelium. Removal of the endothelium was confirmed by the loss of vascular relaxation in response to Ach (10^{-6} M) despite its preservation in response to sodium nitroprusside (5×10^{-7} M).[1]

Binding to muscarinic receptor studies

In order to assess the possibility that PBN may inhibit Ach-induced relaxation at a muscarinic cholinergic receptor site, we tested the effect of PBN on the binding of [^{3}H]-methylscopolamine ([^{3}H]-NMS, New England Nuclear) to its receptors in both the chick heart membrane and guinea pig pulmonary artery endothelial cells. Hearts from ten-day old White Leghorn chick embryos or the endothelial cells were collected in 10 ml of ice-cold buffer (10 mM *Tris*-HCl, pH 7.4, 1 mM EDTA, 10% sucrose) and homogenized with 10 strokes in a glass-on-glass Dounce homogenizer. Homogenates were filtered through 4 layers of gauze and centrifuged at 20,000 × g for 20 min. The pellets were resuspended in 5 ml of ice-cold homogenization buffer. Membrane aliquots (50 μl; 143 μg protein/tube for heart membrane and 125 μg protein/tube for endothelial cells) were added to 50 μl of [^{3}H]-NMS (final radioligand concentration, 1.26 nM) in 50 mM *Tris*-HCl, pH 7.4, 0.5 mM EDTA, 15 mM $MgCl_2$ and various concentrations of PBN (10^{-2} to 10^{-7} M), and incubated for 60 min at 21°C. Following the incubation, the membrane samples were filtered over Whatman GF/C glass fiber filters, placed in 6-ml of liquid scintillation cocktail (Instagel, Packard instrument, IL), and counted in a liquid scintillation counter. Nonspecific binding, defined as radioactivity bound in the presence of 10 μM atropine, was less than 10% of total counts.

Electron paramagnetic resonance spectroscopy studies

Ring segments of pulmonary artery (0.2–0.4 g) and 14 mM PBN (phenyl t-butyl nitrone, Eastman Organic Chemicals) were incubated in 1 ml of KRB equilibrated with 95% O_2 and 5% CO_2 for 10 min at 37°C. Ach (final concentration 3 μM), ATP (20 μM) or sodium nitroprusside (1 μM) were added and the mixture was incubated for an additional 20 min. At the end of the incubation, the tissues were homogenized with a Polytron tissue disruptor (Brinkman) at a setting of 7 for two 10-sec periods, and total lipids were immediately extracted twice in chloroform-methanol (2:1) by the method of Folch *et al.*[19] The chloroform layers were combined and concentrated by evaporation under a stream of N_2 to approximately 200 μl. Each sample was examined in a Varian X-band EPR spectrometer having 9-inch magnet pole faces. The samples were contained in the heat-sealed tip of a Pasteur pipette and placed in the EPR cavity. The EPR spectrometer settings were: microwave power, 25 mW; modulation amplitude, 2 G; time constant, 3 sec; scan range, 100 G and sweep time, 8 min. The spectra were recorded at room temperature (24°C).

RESULTS

Effect of PBN on Ach-, ATP- and nitroprusside-dependent relaxation of pulmonary arterial strips

Ach, ATP and sodium nitroprusside induced concentration-dependent relaxatin of guinea pig pulmonary artery strips previously contracted with PGH_2-A. Removal of

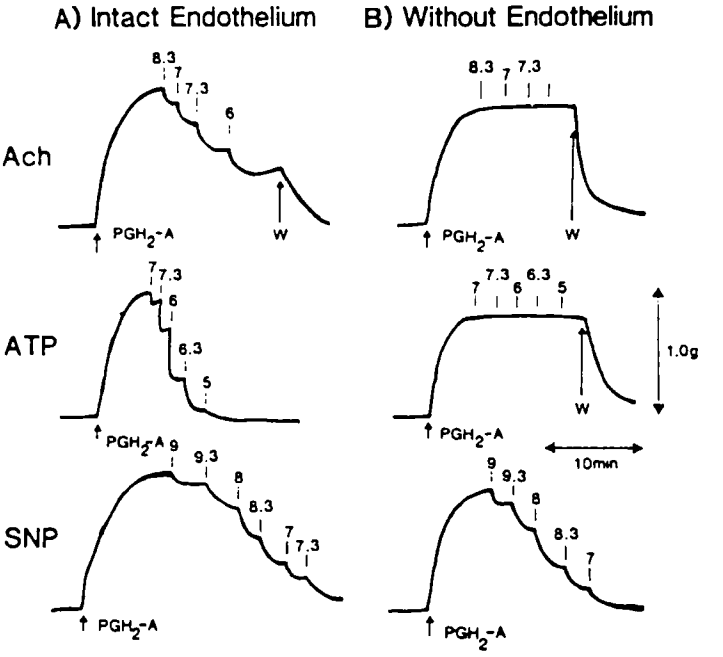

FIGURE 1 Comparative responses of guinea pig pulmonary artery precontracted with PGH_2-A (50 ng/ml) to acetylcholine (Ach), adenosine triphosphate (ATP), and Sodium nitroprusside (SNP) (A) in the presence of endothelium and (B) in the absence of endothelium. Selective removal of endothelium resulted in a complete loss of relaxant responses to Ach and ATP, whereas relaxant reponse to SNP was unaltered. Cumulative molar concentrations are expressed in -log units. W indicates washout of the organ bath.

endothelium abolished the relaxations by Ach and ATP, but did not affect the relaxation by nitroprusside (Figure 1). Half-maximal relaxations were obtained at approximately 5×19^{-8} M Ach, 1.5×10^{-6} M ATP and 6×10^{-9} M nitroprusside.

PBN alone elicited concentration-dependent relaxation of the arterial trip, with half-maximal relaxation achieved at 1 mM. This relaxation was reversed by washout, but was unaffected by removal of the endothelium. Unlike the relaxations by Ach, ATP and nitroprusside which lasted for at least 20–30 min at the higher concentrations, the relaxing effect of PBN was relative short lived (7.2 ± 1.3 min, mean ± SEM, n = 9), and the tension returned to 70–80% of its original value.

PBN also inhibited the relaxant effect of Ach in a dose-dependent manner. Thus, 0.5 mM PBN decreased the maximal relaxation (caused by 10^{-6} M Ach) by 42% (from 58% decrease of PGH_2-A induced tone to 34%) and 1 mM PBN decreased the Ach-dependent relaxation by 72% (from 58% to 16%) (Figure 2a). The ATP- and nitroprusside-induced relaxations, however, were unaffected by PBN (Figure 2b and 2c).

Effect of PBN on [^{3}H-N]methylscopolamine binding to muscarinic cholinergic receptors in membrane preparations

Under the conditions used in the study, 242 ± 11 and 200 ± 22 fmol/mg protein of [^{3}H]-NMS were specifically bound to the membrane of chick heart and pulmonary artery endothelial cells, respectively (Figure 3). PBN, in the concentration range of

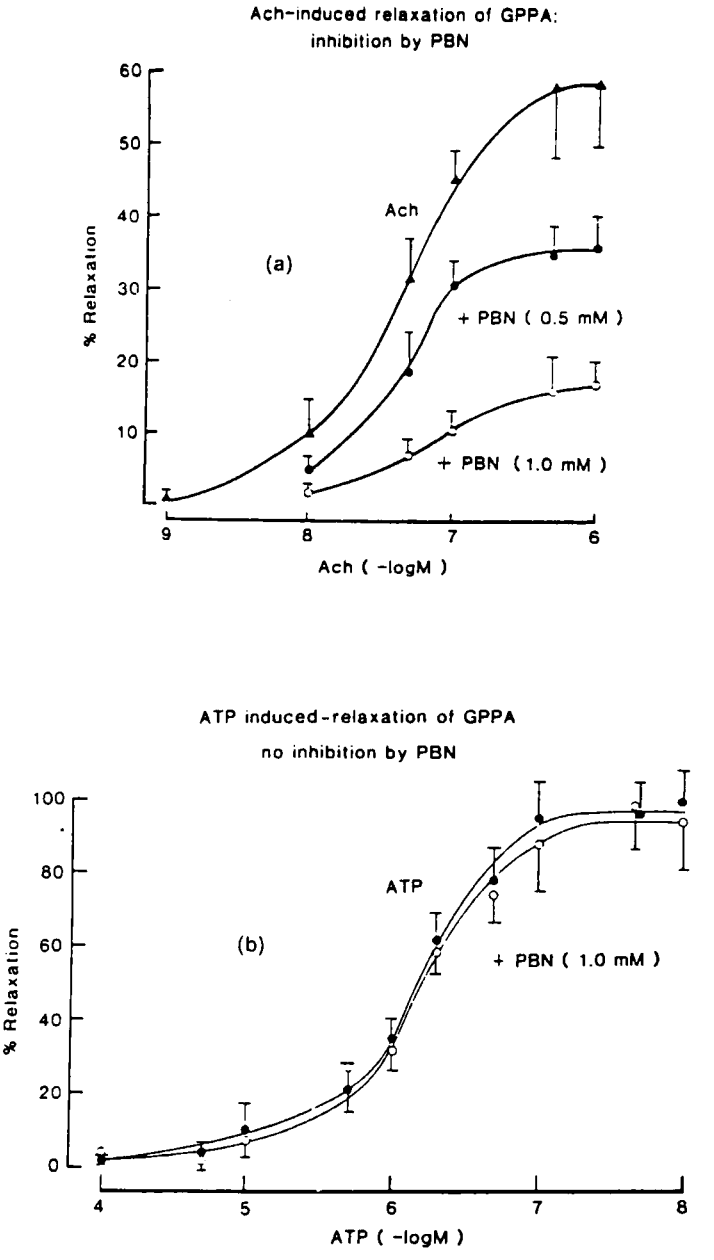

FIGURE 2 Effects of phenyl t-butyl nitrone (PBN) on Ach – (a) ATP (b) – and nitroprusside (c) — induced relaxation of guinea pig pulmonary artery strips. Strips were precontracted to a stable plateau tension by PGH_2-A (50 ng/ml) and then relaxed by the cumulative additions of a vasodilator. Values are mean ± SEM from 9 strips. The strips were preincubated with PBN 10 mins before the vasodilators were added in order to minimize the additive effects of PBN with vasodilators.

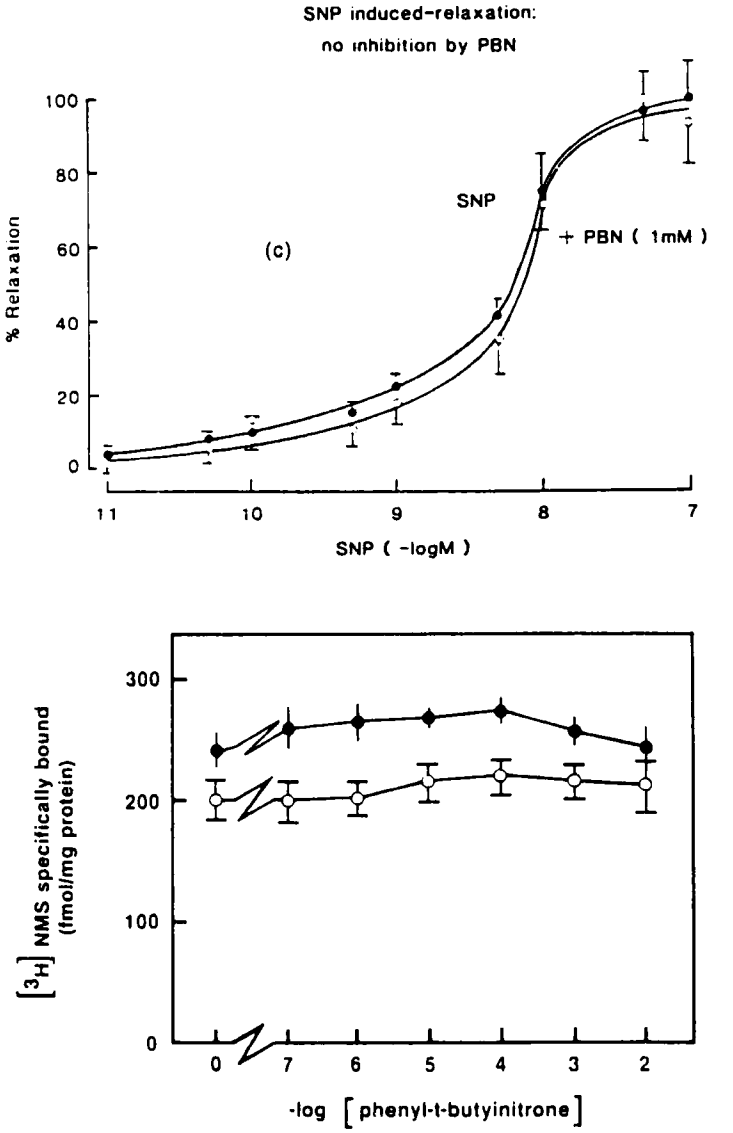

FIGURE 3 Effect of PBN on specific [^{3}]-N-methylscopolamine binding to muscarinic cholinergic receptors on chick heart membrane (●) and endothelial cell membrane (○). Assay mixtures containing appropriate amounts of membrane protein, [^{3}H]-NMS (1.26 nM), and various concentrations of PBN were incubated for 60 min at 21°C. Nonspecific binding, defined as radioactivity bound in the presence of 10 μM atropine, was less than 10% of total binding.

10^{-7} to 10^{-2} M, had no effect on the binding, indicating that PBN does not compete with [^{3}H]-NMS for the muscarinic cholinergic receptor sites.

Identification of a free radical

The incubation of arterial strips with Ach at 37°C for 20 min yielded free radicals that could be trapped by PBN and detected with electron paramagnetic resonance spec-

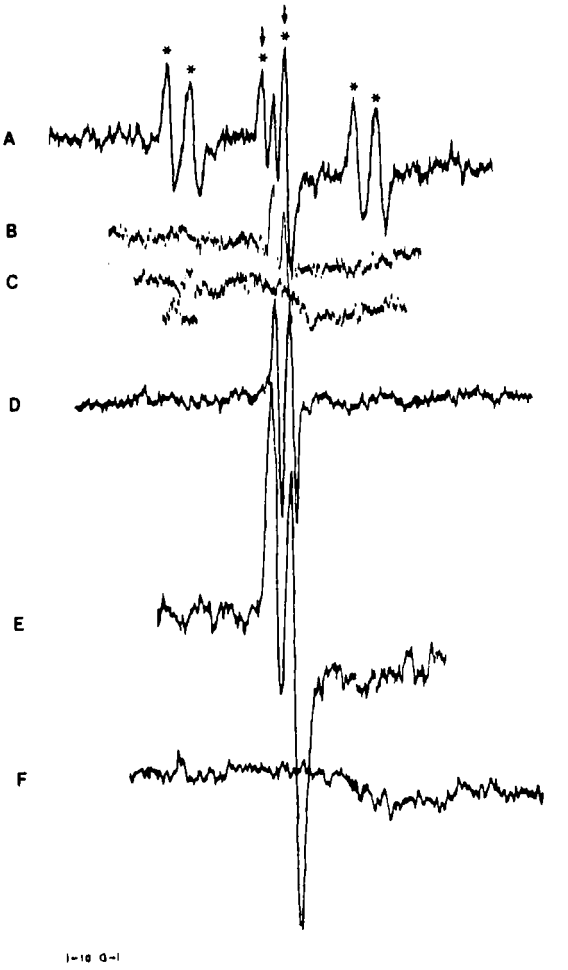

FIGURE 4 EPR spectrum: (A): PBN spin adducts obtained from lipid extract from enedothelium-intact guinea pig pulmonary artery incubated with Ach and PBN for 20 min at 37°C. The three doublets for a carbon centered free radical are indicated by **. The overlapping doublet of the ascorbic acid is marked by ↓↓. (B): Spectrum observed in the lipid extract of endothelium intact pulmonary artery incubated under similar conditions as in "A" but without Ach treatment. (C): Spectrum observed in the lipid extract of endothelium-denuded pulmonary artery incubated under identical conditions as in "A". (D): Spectrum of ascorbate at 1 mm concentration in KRB. (E): Spectrum of lipid extract from endothelium intact pulmonary artery incubated with Ach and without PBN. (F): Spectrum of PBN treated wtih Ach without tissue and extracted under similar conditions as in "A".

troscopy. The EPR spectrum of the spin adduct (Figure 4A) was complex and consisted of two components: 1) a spectrum having six equivalent lines with $a_N = 16.00$ G and $a_\beta^H = 3.85$ G, apparently representing a carbon centered free-radical;[20] and 2) a doublet signal in the middle due to an ascorbate signal which apparently overlaps with a portion of the spin-adduct signal. A doublet signal was obtained when arterial strips were incubated either with Ach but without PBN (Figure 4E), or with PBN but without Ach (Figure 4B), and the signal was identical with that of a standard

acorbate signal (Figure 4D). Although ascorbate radical is not lipid-extractable by the Folch procedure, presence of a persistent, relatively stable, radical by repeated extraction procedures may suggest that the extracts were free of aqueous medium and the ascorbate radical is co-extractable possibly by interacting with other organic materials. No signal was observed in lipid extracts from either endothelium-denuded pulmonary artery incubated with Ach and PBN (Figure 4C) or when Ach was incubated with PBN without the tissue (Figure 4F).

Although PBN was able to trap a radical generated by the interaction of arterial strips with Ach and inhibited the Ach-dependent smooth muscle relaxation, there is still reason to believe that the trapped radical may not be the primary EDRF species. Thus, it is possible that the Ach-dependent EDRF (RH) could diffuse to underlying smooth muscle cells for the direct action, but is unstable and can oxidize by the following reaction:

$$RH + O_2 \leftrightarrow R^{\cdot} + O_2^{-\cdot} + H^+$$

$$R. + O_2 \longrightarrow R^+ + O_2^{-\cdot}$$

When PBN was added it trapped R. and displaced the quasi equilibrium of the reaction to the right. PBN could thus have the effect of increasing the instability of the RH. By using another spin trapping agent, 5,5-dimethylpyrroline-N-oxide (DMPO), it was demonstrated that this was not the case.

DMPO is unknown to trap $O_2^{-\cdot}$ forming a spin adduct DMPO–OOH.[23] Additions of 2, 4 or 100 mM DMPO not only were ineffective in forming paramagnetic species when incubated with arterial strips and Ach, but also had no significant effect on arterial smooth muscle relaxation induced by Ach nor on PGH_2-induced tone of pulmonary artery.

There is, of course, a possibility that PBN might prevent relaxation by a mechanism unrelated to spin trapping (i.e. functional antagonism) or mechanism not related to free radicals. Alternately PBN-R spin adduct may be responsible for inhibiting the Ach-dependent relaxation of arterial smooth muscle cells. In order to lessen the likelihood of this subtle artifact, we investigated the time course of relaxation of pulmonary artery strips by PBN which had already trapped a radical, such as CH_3. If trapping of a carbon centered radical by PBN was responsible for the inhibition of Ach-dependent relaxation of arterial smooth muscle cells, PBN-CH_3 adduct should then no longer inhibit the relaxation. That this was the case is illustrated by the following studies:

Li–CH_3 is known to react with PBN to form a PBN–CH_3 spin adduct.[24] A well characterized PBN-CH_3 adduct was formed (a_N = 16.5 G and a_β^H = 3.5 G) when a molar excess of Li–CH_3 was incubated with PBN at room temperature.[24,25] The EPR signal of PBN–CH_3 adduct formed immediately and was stable for several minutes. Again, concentrations of 10^{-3}, 10^{-4} or 10^{-6} M PBN-CH_3 in the organ bath were found to have trivial effect on Ach-dependent relaxation of pulmonary arterial strips.

DISCUSSION

Our results confirm the observation that an intact endothelium is essential for the relaxation of guinea pig pulmonary artery by acetylcholine and ATP (but not by sodium nitroprusside), presumably because these two agents, stimulate the en-

dothelium to release a factor or factors (EDRF) that relaxes vascular smooth muscle. The identity of EDRF remains unknown. Free radicals have been suggested as possible EDRF for Ach-induced relaxation of rabbit thoracic aorta.[1,2] Our data provide evidence that a free radical is produced during the acetylcholine-induced relaxation of the pulmonary artery and a spin-trapping agent, PBN, abolished the relaxation. A carbon-centered free-radical was detected by spin-trapping combined with electron paramagnetic resonance spectroscopy. We have further shown that PBN does not inhibit acetylcholine-induced relaxation by blocking its binding to muscarinic cholinergic receptors on endothelium. In related experiments, Griffith *et al.*,[21] using superoxide dismutase, catalase, benzoate, and histidine and spin trapping agents (PBN and 2-methyl-nitrosopropane) with isolated superfused rabbit aorta, failed to fine evidence for a free-radical as EDRF. The apparent discrepancy between our results and theirs could be explained by the differences in species or tissues used, or, more likely, by the fact that Griffith *et al.* added the scavengers and spin-trapping agents to the effluent, rather than to the tissue itself, the presumed site of EDRF production. It is possible that a carbon-centered free radical is involved in a process to produce and/or release EDRF, and PBN can inhibit the synthesis or release of EDRF by trapping free radicals. This conclusion is consistent with the findings of Rubanyi and Vanhoutte[26,27] who postulated that EDRF is not likely to be an oxygen derived free radical. They, however, reported that superoxide radicals actually depress and hydraxyl radicals facilitate the effectiveness EDRF probably by activating the muscarinic receptors.

Our results also demonstrate that acetylcholine and ATP, both requiring intact endothelium, relax the pulmonary artery by different mechanisms. De Mey *et al.*[3] showed that ATP-induced relaxation of canine femoral artery, unlike that by Ach, was not inhibited by pretreatment with eicosatetraynoic acid or nordihydroguiaretic acid, and that the vascular relaxation produced by acetylcholine and ATP are mediated by different "signals" (EDRF).

Endothelium-dependent vascular relaxation has been found to be associated with an increase in the level of cyclic GMP in smooth muscle cells.[5,6,12,22] If in our experiments PBN prevented Ach-induced endothelium-dependent relaxation by inhibiting EDRF production or release, PBN would be expected also to prevent any increase in cGMP levels. Recently, we have found that PBN does, infact, inhibit cGMP accumulation as well as relaxation of rat thoracic aorta induced by acetylcholine. In the case of ATP, however, neither the relaxation and/or the cGMP accumulation was affected by PBN (unpublished observations).

In summary, we have found that acetylcholine-induced relaxation of guinea pig pulmonary artery was inhibited by PBN, a spin-trapping agent. The use of PBN allowed the detection of a carbon-centered free radical in lipid extracts of endothelium-intact pulmonary artery incubated with acetylcholine, but not with ATP. ATP-induced relaxation was not inhibited by PBN. We conclude: 1) that acetylcholine relaxes pulmonary vascular smooth muscle by an endothelium-dependent, free-radical mediated mechanism, and 2) that ATP induces vascular relaxation either by a different radical or by a non-radical mediated mechanism.

Acknowledgements

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