Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle

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In rat aortic rings, hydroxocobalamin (10–30 μ M) produced concentration-dependent reductions of the relaxant action of nitric oxide (NO) and the endothelium-dependent, NO-mediated, relaxant action of acetylcholine. In anococcygeus muscles, hydroxocobalamin (10–30 μ M) reduced but also prolonged, NO-induced relaxations, but had no effect on non-adrenergic, non-cholinergic-mediated relaxations. Hydroxocobalamin had no effect on the NO-independent relaxant action of papaverine in either tissue. It is suggested that hydroxocobalamin sequesters NO by forming nitrosocobalamin. Nitrosocobalamin did not relax aortic rings, but produced a slowly developing and prolonged relaxation of anococcygeus muscles.

Keywords: Aortic rings (rat); anococcygeus muscle; endothelium-dependent relaxation; hydroxocobalamin; nitrgic transmission; nitric oxide; nitrosocobalamin

Introduction Hydroxocobalamin (vitamin B_{12a}) can be readily converted to nitrosocobalamin (vitamin B_{12c}) in a mild nitrosylating reaction, by use of sodium nitrite and acetic acid (Kaczka *et al.*, 1951). It occurred to us that hydroxocobalamin might sequester nitric oxide (NO) in a biological milieu and thereby inhibit the smooth muscle relaxant action of NO. We tested this hypothesis by studying the effects of hydroxocobalamin on responses to NO and the NO-mediated relaxations produced by acetylcholine (ACh) in endothelium-intact rat aortic rings and non-adrenergic, non-cholinergic (NANC) nerve stimulation in rat anococcygeus muscles, which are thought to be NO-mediated (see Rand, 1992).

Methods Male Sprague-Dawley rats (250–400 g) were killed by decapitation and the thoracic aorta and anococcygeus muscles were removed. Aortic rings of approximately 5 mm in length were mounted under a tension of 2 g in a 10 ml organ bath for isometric recording. In some experiments the endothelium was removed by rubbing the intimal surface of the aortic ring. Relaxations were elicited, during sustained submaximal contractile responses to phenylephrine (1 μ M) by the addition of ACh (in rings with intact endothelium), NO or papaverine before or 15 min after exposure to hydroxocobalamin.

The preparation of anococcygeus muscles was as described previously (Li & Rand, 1989). Guanethidine (10–30 μ M) was used to raise the tone and relaxations were elicited by field stimulation (1 ms pulses at 1–5 Hz for 10 s), NO or papaverine before and after exposure to hydroxocobalamin. The physiological salt solution (PSS) used for both preparations had the following composition (mM): NaCl 118, KCl 4.7, NaHCO₃ 25, MgSO₄ 0.45, KH₂PO₄ 1.03, CaCl₂ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14. The PSS was gassed with 5% CO₂ in O₂ and maintained at 37°C.

Drugs acetylcholine perchloride (BDH, UK), guanethidine sulphate (Ciba, Australia), hydroxocobalamin acetate (Sigma), nitric oxide gas (Commonwealth Industrial Gases, Melbourne), phenylephrine hydrochloride (Sigma, St. Louis,

Figure 1 Log concentration-response curves for nitric oxide (NO) in rat aorta (a) and anococcygeus (b) before (●) and after (○) hydroxocobalamin (30 μ M). Vertical axis: decreases in tension expressed as a percentage of pre-existing tone. Horizontal axis: concentration of NO in μ M. Symbols are means $\pm$ s.e.mean (vertical bars).

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Mo.) and papaverine hydrochloride (Sigma) were used. NO solutions (2 mM) were made by passing NO gas through de-oxygenated distilled water (Di Iorio, 1981). Nitrosocobalamin was prepared as described by Kaczka *et al.* (1951).

Results Responses to NO In rat aortic rings with or without endothelium, and in anococcygeus muscles, hydroxocobalamin produced shifts to the right of the concentration-response curves for NO (Figure 1). In addition, in anococcygeus muscles, the rate of development of relaxation was decreased and the duration was prolonged (Figure 2b).

Responses to papaverine Hydroxocobalamin (30 μM) had no effect on sub-maximal relaxations elicited by 30 and 100 μM papaverine in aortic rings and 100 μM hydroxocobalamin had no effect on relaxations elicited by 0.3 and 1 μM papaverine in anococcygeus muscles (results not shown).

Effects of nitrosocobalamin The presence of a nitroso group in nitrosocobalamin suggests that it might act like a NO-donating nitrovasodilator. It was devoid of relaxant activity in aortic rings in concentrations up to 300 μM , but it produced a slowly developing and prolonged relaxation of anococcygeus muscles (Figure 2c).

Endothelium-dependent relaxant action of ACh Hydroxocobalamin (10 μM) produced a concentration-dependent enhancement of phenylephrine-induced contractions in endothelium-intact but not endothelium-denuded rings. The concentration-response curve for the relaxant action of ACh was shifted to the right. A concentration of 30 μM hydroxocobalamin abolished the responses to ACh (Figure 2a).

NANC nerve stimulation-induced relaxations Hydroxocobalamin (10–30 μM) had no effect on the tone or on the relaxations of the anococcygeus muscle elicited by NANC stimulation at 1–5 Hz (Figure 2b). However, in some experiments with 100 μM hydroxocobalamin, there was a

Figure 2 Tracings of responses in (a) endothelium-intact aorta rings: phenylephrine (Phe) and acetylcholine (ACh) before and after hydroxocobalamin (HC, 10 and 30 μM); (b) anococcygeus: non-adrenergic, non-cholinergic nerve stimulation and NO before and after hydroxocobalamin (HC, 30 μM) and (c) anococcygeus: nitrosocobalamin (NC).

slight reduction of the relaxations at the lower frequencies of stimulation.

Discussion Since hydroxocobalamin readily combines with NO to form nitrosocobalamin (Kaczka *et al.*, 1951), we suggest that hydroxocobalamin reduces responses to NO in rat aortic rings by sequestering NO so that the concentration of NO available for activating soluble guanylate cyclase and relaxing the smooth muscle is reduced. The postulated mechanisms of action of hydroxocobalamin is, therefore, like that of haemoglobin which binds NO and thereby reduces the effects of NO and nitrovasodilators in activating guanylate cyclase (Murad *et al.*, 1978) and relaxing vascular smooth muscle (Martin *et al.*, 1985). Since nitrosocobalamin did not produce relaxation of aortic rings, the NO bound in it is not available for activation of the soluble guanylate cyclase in the smooth muscle of this tissue.

Haemoglobin inhibits endothelium-dependent relaxations in vascular preparations (Martin *et al.*, 1985; Ignarro *et al.*, 1987). Likewise, hydroxocobalamin inhibited the endothelium-dependent relaxant action of ACh in aortic rings and, as was reported for haemoglobin (Martin *et al.*, 1985), hydroxocobalamin was more effective in reducing endothelium-dependent than NO-induced relaxations. A possible explanation for this difference is that the total amount of NO generated from ACh-activated endothelial cells is likely to be considerably less than the amount of exogenous NO producing an equivalent relaxation; hence, a given amount of hydroxocobalamin or haemoglobin in the organ bath would more completely mop up the endothelium-derived NO.

Phenylephrine-induced contractions were enhanced by hydroxocobalamin, and this effect has also been reported with haemoglobin (Martin *et al.*, 1985; Khan *et al.*, 1992).

In anococcygeus muscles, hydroxocobalamin reduced the relaxant action of NO but also changed the nature of the relaxation, in that it developed more slowly and was more prolonged. Nitrosocobalamin produced slowly developing and prolonged relaxations in anococcygeus muscles. Therefore it appears that nitrosocobalamin can donate NO to guanylate cyclase in the smooth muscle cells of the anococcygeus.

The concentration of hydroxocobalamin (30 μ M) that abolished the endothelium-dependent, NO-mediated, relaxant action of ACh in aortic rings had no effect on NANC nerve stimulation-induced relaxations of the anococcygeus muscle, although there is compelling evidence that the transmitter is NO or a closely related compound (see Rand, 1992). However, haemoglobin blocks NANC transmission in the anococcygeus muscle (Gillespie & Sheng, 1989); the reason for the difference from hydroxocobalamin in this respect is not entirely clear, but one possibility is that nitrosohaemoglobin does not donate NO to guanylate cyclase.

In conclusion, hydroxocobalamin may serve as an additional tool for investigating NO-mediated physiological processes. If it can be established as a general rule that it has a differential action in blocking responses to endothelium-derived but not neurogenic NO, it may help in distinguishing the source of NO in nerve stimulation-induced NO-mediated vasodilatation in such tissues as the salivary gland and corpus cavernosum.

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