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Partial Inhibition of Hypoxia-Induced Erythropoietin Production by Cholinergic Blockade in the Dog[1] (39866)

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Introduction. While it is well known that several types of physiologic and pathophysiologic stimuli have the ability to trigger the release of erythropoietin (Ep) from the kidney, the mechanisms involved with this process are still unresolved (1). Recent investigations have demonstrated that the production of this hormone may be modulated by both humoral and neural factors (2). Evidence for a cholinergic mediation of Ep production has come from several sources. In 1967, Medado *et al.* (3) reported that the erythropoietic activity induced by electrical stimulation of the posterior hypothalamus of rats could be completely blocked by atropine. In addition, partial inhibition of Ep elaboration by atropine has been demonstrated in rabbits subjected to 8 and 18 hr of hypobaric hypoxia (4) and in rats treated with cobaltous chloride to induce histotoxic hypoxia (5). In the latter study (5), physostigmine, an inhibitor of acetylcholinesterase, was reported to potentiate Ep production. It has not been determined, however, whether the modulation of renal Ep elaboration caused by cholinergic drugs is mediated at central or peripheral nervous system sites.

The current studies were designed to evaluate the effects of atropine on Ep production in dogs subjected to hypoxic hypoxia and, further, to differentiate between central and peripheral sites of action with the use of the drug methylatropine. The site of action of this atropine analog is confined to the periphery, where it exerts cholinolytic effects similar to those of atropine.

Materials and methods. Female dogs weighing 11-26 kg were anesthetized with pentobarbital, 30 mg/kg, intubated, and ventilated with a Harvard respirator. Average tidal volume was 18 ml/kg body weight at a rate of 20 breaths/minute. End expiratory pressure was maintained at 5 cm H_2O. Femoral artery and vein were cannulated for continuous monitoring of systemic arterial blood pressure (Statham P23AC transducer), collection of arterial samples for blood gas analysis (PO_2 and PCO_2), pH and plasma erythropoietin determinations, and the administration of drugs. A right nephrectomy was performed through a midabdominal incision and a catheter was inserted into the left ovarian vein for collection of renal venous blood.

After a 30- to 60-min control period, hypoxia was induced by ventilating the animals with a gas mixture of 8% oxygen and 92% nitrogen. Control animals breathed room air. Blood samples for Ep assay and blood gas determinations were withdrawn at zero hour and at several time intervals during the 6-hr experimental period. One-half volume of 10% Dextran 40 was returned to the animals for each volume of blood removed.

A single dose of atropine sulfate, 0.5 mg/kg iv, was administered to seven dogs 30 min prior to the onset of hypoxia. This dose was sufficient to completely block the cardiovascular responses to 1.0 μg/kg of acetylcholine iv, while having no effect upon ganglionic stimulation by 10 μg/kg of 1,1-dimethyl-4-phenylpiperazinium (DMPP) or double carotid artery occlusion.

Methylatropine nitrate, 1.0 mg/kg iv, was administered by slow infusion to six dogs during a 45-min period prior to the onset of hypoxia. This dose completely blocked the cardiovascular responses to 1.0 μg/kg of acetylcholine, iv, but had no effect upon the hypertensive responses to DMPP or double carotid occlusion. Methylatropine was chosen for this study because, while its peripheral anticholinergic activity closely resembles that of atropine, this quarternary nitro-

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gen drug is effectively excluded from the central nervous system at the level of the blood-brain barrier (6).

Erythropoietic activity of arterial plasma samples was determined using a modification of the exhypoxic polycythemic mouse assay of Cotes and Bangham (7) as reported previously (8). It has been demonstrated that this assay is not affected by the presence of atropine in the assay samples in amounts which far exceed those used in the current study (4).

Comparisons between means and Dunnett's test for multiple comparison with a single control were performed according to standard statistical methods (9).

Results. Arterial PO_2 was decreased uniformly as early as 15 min after the induction of hypoxia in nontreated and in drug-treated dogs from a mean (±SEM) baseline value of 84.5 ± 2.8 to 29.0 ± 1.8 mm Hg (Fig. 1). This 66% decrease in PO_2 remained constant during the remaining 6-hr exposure to hypoxia. The magnitude of the hypoxic stimulus was therefore essentially identical in all three groups of hypoxic dogs: (i) hypoxia alone, (ii) hypoxia with atropine pretreatment, and (iii) hypoxia with methylatropine pretreatment (Fig. 1).

Arterial blood pH was not significantly different in hypoxic and control dogs during the 6-hr experimental period and did not change significantly from the zero-hour control values of 7.45 ± 0.04. Mean arterial PCO_2 was identical in all three hypoxic groups, with values of 22.7 ± 1.8, 22.0 ± 1.5, 21.5 ± 1.0, and 21.1 ± 1.7 at 0, 1, 3, and 6 hr of hypoxia, respectively. These values were significantly less ($P < 0.05$) than those for room air-ventilated control dogs at these same time intervals, which were 29.2 ± 4.0, 27.5 ± 2.8, 27.3 ± 3.1, and 26.7 ± 2.7, respectively.

Atropine and methylatropine pretreatment tended to increase heart rate in hypoxic dogs when compared to room air-ventilated control animals (Fig. 2). Hypoxia alone caused no significant change in heart rate from control animals. Figure 2 also shows that mean arterial blood pressure in the three hypoxic groups did not differ significantly from control dogs.

Figure 3 shows the effects of hypoxia,

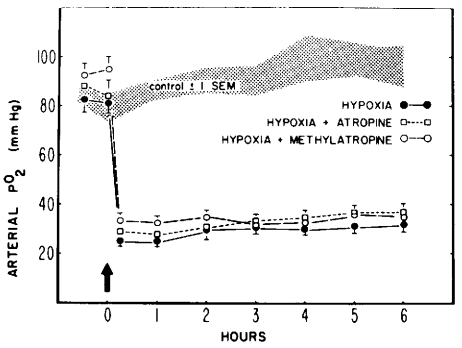

FIG. 1. Partial pressure of oxygen in arterial blood samples during the 6-hr experimental period. Values represent mean ± SEM. Number of animals for hypoxia and atropine = 7, all others = 6. Arrow represents onset of hypoxia.

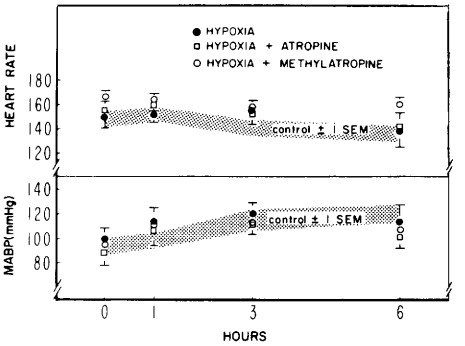

FIG. 2. Heart rate (beats per minute) and mean arterial blood pressure (MABP) during the hypoxic period. Number of animals same as in Fig. 1.

atropine, and methylatropine on 0-, 3-, 5-, and 6-hr plasma erythropoietic activity. Activity in plasma of control dogs was not significantly different from background activity elicited by saline in the assay system. Plasmas of nontreated hypoxic dogs showed a significant ($P < 0.05$) increase in erythropoietic activity after 5 and 6 hr of hypoxia, when compared to those of 0 hr or to 5- and 6-hr controls. This level of activity was not significantly different from that elicited by 0.1 unit of standard human urinary erythropoietin. Pretreatment with atropine or methylatropine decreased by 42% at 5 hr and by 56% at 6 hr, the ability of hypoxia to increase plasma erythropoietic activity (Fig. 3). These decreases at 6 hr were statistically significant ($P < 0.05$) when compared with the hypoxic control dogs. The activity in the

6-hr plasmas of drug-treated animals was not significantly elevated above that of the 0-hr sample; however, the 5- and 6-hr samples showed significantly more activity than did saline in the assay system.

Discussion. Experiments utilizing hypothalamic stimulation (3), hypobaric hypoxia (4), and histotoxic hypoxia (5) have provided convincing evidence for the involvement of the cholinergic nervous system in Ep production. In the present studies, atropine and methylatropine were found to cause more than 50% inhibition of the increase in plasma Ep activity induced by hypoxic hypoxia in dogs. Although atropine, in the doses employed here, affects primarily peripheral cholinolytic sites, the possibility existed that the drug was producing its effect by acting at a site somewhere in the central nervous system. Methylatropine has anticholinergic effects that are comparable to those of atropine, but, because of its quarternary structure, methylatropine is effectively confined to the peripheral circulation (6). The significant inhibition of Ep production induced by methylatropine, similar to that obtained with atropine, suggests therefore, that the effectiveness of the cholinergic blockade is not dependent upon access of either drug to the central nervous system.

It is not clear, at present, whether the peripheral actions of the two drugs involve effects at a ganglionic site. Atropine and methylatropine did not appear to decrease the carotid sinus pressor reflex produced by bilateral occlusion of the carotid arteries or the pressor response elicited by the ganglionic-stimulating effects of DMPP. This suggests that neither atropine nor methylatropine was interfering with acetylcholine-mediated nicotinic activity at the ganglionic level. However, these responses are less sensitive than others to the action of ganglionic blocking agents (10) and may not afford an accurate determination of the presence of low-level ganglionic blockade in the present study.

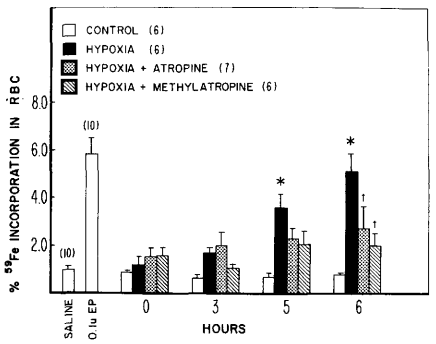

FIG. 3. Effects of hypoxia and drugs on plasma erythropoietic activity. Activity expressed as percentage total ^{59}Fe incorporation into red blood cells of exhypoxic, polycythemic mice. Saline and 0.1u Ep represent the activity elicited in the assay system by saline and 0.1 units of standard human urinary erythropoietin. Number of animals in each group is shown. *, Significantly different from 0 hr, $P < 0.05$; †, significantly different from hypoxic, $P < 0.05$.

The use of fluorescent antibody techniques has demonstrated the glomerular tuft of the renal cortex to be the probable site of Ep localization in the kidney (11, 12). Blood flow to the glomerulus arises from the arcuate artery by way of the interlobular artery and the afferent arteriole. All of these vessels have been demonstrated, in the dog, to have associated with them acetylcholinesterase-containing nerves suggested to be cholinergic (13). Since acetylcholine is known to be a renal vasodilator (14), it is possible that the hypoxic stimulation of Ep production is associated with vasodilation in these vessels which is at least partially inhibited by atropine or methylatropine. Atropine, in the dose employed in our experiments, was not found to effect total renal blood flow in two dogs studied in our laboratory. These results agree with those of Vander, who found renal hemodynamics to be unaffected by renal arterial administration of atropine in dogs (15). This does not preclude the possibility that atropine or methylatropine might produce their inhibition of erythropoiesis by interfering with cholinergically induced changes in the renal microcirculation. It is interesting to note that inhibition of the synthesis of prostaglandin E_2, a renal vasodilator, also results in decreased erythropoietin production following hypoxia in dogs (16).

A relationship has been demonstrated between cholinergic nervous input and cyclic GMP levels, suggesting that, in some tissues, this nucleotide may represent the intracellular "second messenger" of the cho-

linergic nervous system (17, 18). Rodgers *et al.* (5) have shown increases of cyclic GMP in renal homogenates from rats treated with cobaltous chloride that correlated with increased Ep production in these animals. These increases in renal cyclic GMP and Ep production were inhibited by atropine. It is possible that the results of the present studies are likewise related to alteration of local cyclic nucleotide levels in the kidney. Further work may demonstrate that atropine and methylatropine act to interfere with a similar rise in cyclic GMP during an early stage of the response to hypoxic hypoxia, thereby limiting Ep production in the pretreated animals.

It might alternately be suggested that the ability of atropine and methylatropine to interfere with Ep elaboration in the current study was associated with the modulation, by these drugs, of renal sympathetic nerve activity during hypoxia. A close anatomical association between cholinergic and adrenergic nerves in the dog kidney has been reported (13, 19). Demonstration of acetylcholinesterase in the renal adrenergic nerves of rats (20) has been presented as evidence in support of the theory that acetylcholine plays a role in adrenergic transmission (21). Recent studies by Fink *et al.* (22, 23) have convincingly demonstrated the involvement of adrenergic β-2 receptors in the erythropoietic response to 18 hr of hypoxia. However, further studies (24) showed that blockade of these receptors during short-term hypoxia (5 hr) did not decrease Ep production, while renal denervation was found to be effective in reducing the erythropoietic response to this 5-hr hypoxic stimulus. On the basis of these findings, one might speculate that the actions of atropine and methylatropine in the present study did not involve modulation of β-2 input, since our stimulus was also short-term (6 hr) and would presumably be independent of activity at these receptors (24). However, the studies by Fink *et al.* do not preclude the possibility that the short-term erythropoietic response to hypoxia involves a direct cholinergic mechanism within the kidney. The nonsympathetic, renal nerve-dependent response they describe (24) may represent such a cholinergically mediated event.

Summary. Anesthetized dogs exposed to hypoxic hypoxia showed elevated plasma erythropoietic activity by 6 hr. This activity was significantly decreased by atropine pretreatment. Methylatropine, a peripheral-acting analog of atropine, was equally effective in reducing the erythropoietic response. Neither atropine nor methylatropine, at the doses employed, appeared to exert their effect through blockade at the autonomic ganglia. These studies suggest that the erythropoietic response to short-term hypoxia in the dog involves a peripheral, cholinergically mediated event, perhaps within the kidney itself.

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