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Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide

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The toxic effects of carbon monoxide (CO) are attributed to severe tissue hypoxia resulting from a decrease in intracellular oxygen tension [1]. Carbon monoxide interacts reversibly with hemoglobin to form carboxyhemoglobin which in turn decreases the oxygen carrying-capacity of the blood and shifts the oxyhemoglobin saturation curve to the left. As a result, the limited amount of oxygen transported by the blood is more tightly bound to hemoglobin and results in functional anemia. There are, however, several studies which suggest that CO exerts toxic effects independent of those associated with carboxyhemoglobin formation [2–5]. Since significant partial pressures of CO are found in several tissues upon CO inhalation [6–8], the interaction of CO with intracellular constituents may account for the occurrence of direct (i.e. carboxyhemoglobin independent) toxic effects.

Duke and Killick [9] and Scharf *et al.* [10] have reported data which suggest that CO may inhibit the contractility of vascular smooth muscle. These reports are consistent with studies conducted in our laboratory which show that CO relaxes coronary and aortic vascular smooth muscle preparations [5, 11–13]. The ability of CO to decrease cellular calcium levels and relax vascular smooth muscle is not due to hypoxic or functional hypoxia nor mediated by adrenergic influences, adenosine or prostaglandins [5]. Since an elevation in cyclic nucleotide levels has been associated with a decrease in cellular calcium content and relaxation of vascular smooth muscle [14], the present study was conducted to determine if exposure of cultured aortic smooth muscle cells to CO is associated with alterations in cellular cyclic GMP levels.

Methods

Cell culture procedure. Male Sprague–Dawley rats (250–300 g) were obtained from Sasco, Inc. Animals were maintained in individual cages, and food and water were provided *ad lib*. Primary cultures of rat aortic smooth muscle cells were prepared as previously described [15]. Briefly, segments of thoracic aortae (18–24 mm) were excised from the animals and cleaned of clotted blood and connective tissue. The vessels were subjected to a series of enzymatic digestions to isolate medial smooth muscle cells. Cells were plated at a density of $1.8–2.0 \times 10^5$ cells in 35 mm petri dishes and grown in Medium 199 supplemented with fetal bovine serum, 10%; glutamine, 2 mM; penicillin, 10,000 units/ml; streptomycin, 10 mg/ml; and amphotericin, 50 μg/ml.

Treatments. Confluent cultures were exposed to a mixture containing air enriched with 5% CO_2:21% O_2:74% N_2 (control) or 5% CO_2:5% CO:21% O_2:69% N_2 (CO) for 30 or 60 min. Methylisobutylxanthine (50 mM) was added to the cultures to inhibit cyclic GMP phosphodiesterase [16]. At the end of the desired exposure periods, the culture medium was removed rapidly and 1 ml of ice-cold trichloroacetic acid (6%) was added. Samples were frozen and processed for cyclic GMP determination as described by Hirata *et al.* [17]. Cultures were subjected to three successive freeze–thaw periods. Cells were scraped off and centrifuged at 10,000 *g* for 15 min at 4°. The supernatant fraction was removed and combined with 10 ml of H_2O-saturated diethylether. The tubes were vortexed at room temperature for 60 sec and subjected to three successive ether extractions. Samples were incubated for 5 min at 80° to drive off all traces of ether. A 500-μl aliquot of the sample was lyophilized and resuspended in 500 μl of 0.05 M sodium acetate buffer (pH 6.2). Cellular cyclic GMP levels were assayed by radioimmunoassay (New England Nuclear).

Results and Discussion

Previous studies in our laboratory have shown that CO-induced relaxation of vascular smooth muscle is associated with a decrease in cellular calcium levels [13]. This vascular response is not secondary to hypoxia nor mediated by autonomic or humoral influences [5]. Furthermore, the ability of CO to relax vascular smooth muscle is not endothelium dependent since denuded aortic preparations respond to the same extent as intact vessels [12]. The results presented herein show that CO caused a time-dependent increase in the levels of cyclic GMP in cultured aortic smooth muscle cells (Fig. 1). Although no calcium measurements were conducted in the present study, the elevation of cGMP correlates temporally with a decrease in cellular calcium levels and a relaxation of aortic smooth muscle preparations under similar experimental conditions [12, 13]. Collectively, these results raise the possibility that carbon monoxide acts directly or through a second messenger system to alter vascular smooth muscle cell metabolism.

The concentration of CO used in this study was relatively high. However, our earlier studies demonstrating that CO relaxes vascular smooth muscle [5, 11–13] have all been conducted at 2.5 to 5% CO. Interestingly, these concentrations of CO are within the range used by both Ayres

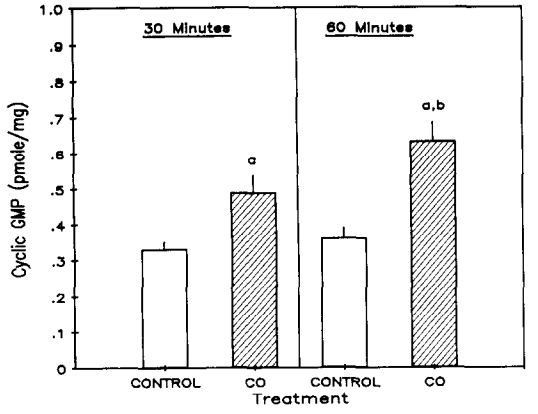

Fig. 1. Cyclic guanosine monophosphate levels in vascular smooth muscle cells upon exposure to 5% carbon monoxide. Key: (a) significantly different from respective control ($P < 0.05$); and (b) significantly different from 30-min CO ($P < 0.05$).

et al. [18] and Stewart *et al.* [19] in their studies with human subjects. A limited number of studies have been reported on the toxic effects of CO in cultured cardiac [20, 21] or vascular [22] cells. However, no direct correlations can be established with the present study since different durations of exposure and/or CO levels were used.

Carbon monoxide is known to react *in vivo* and *in vitro* with a variety of metal-containing proteins including hemoglobin [23], myoglobin [24], and cytochrome *c* oxidase [25]. These metalloproteins contain iron and/or copper centers that form metal–ligand complexes with CO in competition with molecular oxygen. The binding of CO to myoglobin in heart and skeletal muscle *in vivo* has been demonstrated at low levels of carboxyhemoglobin [24, 26]. Furthermore, CO binds to cytochrome oxidase *in vivo* in mammalian heart and brain tissues after severe intoxication in experimental animals [27, 28]. These reports support the concept that CO may interact directly with intracellular constituents to relax vascular smooth muscle.

The inhibition of cytochrome oxidase by carbon monoxide could result in vascular smooth muscle relaxation [29]. Bassett and Fisher [4] have shown that CO is more effective than N_2 in inhibiting oxidative metabolism and proposed that the enhanced effectiveness of CO may be due to its ability to bind with cytochrome oxidase competitively. However, this proposal is not consistent with the results of Coburn [30] which show that CO relaxes cyanide-treated aortic smooth muscle preparations. Since cyanide inhibits oxidative phosphorylation irreversibly, these findings raised the possibility that CO reacts with intracellular constituents other than cytochrome oxidase.

Guanylate cyclase is a ubiquitous enzyme which catalyzes the formation of cyclic GMP [31]. Recent studies have suggested that cyclic GMP relaxes vascular smooth muscle by inhibiting phosphatidylinositol hydrolysis [32]. The ability of carbon monoxide to elevate cyclic GMP levels may be due to direct stimulation of guanylate cyclase activity. This concept is supported by studies which show that the exogenous activation of guanylate cyclase is a heme-dependent process [33]. The ability of nitric oxide to stimulate guanylate cyclase activity requires the formation of an NO–heme complex. Carbon monoxide may interact with the heme group in guanylate cyclase in a manner analogous to its interaction with hemoglobin to stimulate enzymatic activity.

In summary, the present studies suggest that alterations in cellular cyclic GMP levels mediate the relaxation of vascular smooth muscle induced by CO. These data further support the concept that the toxic effects of CO differ from those of an equivalent degree of anemia. Future studies will be designed to determine if CO interacts directly with guanylate cyclase.

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