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Carbon Monoxide Enhances Human Neutrophil Migration in a Cyclic GMP-dependent Way

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Carbon monoxide (CO) enhanced random migration of human neutrophils. An optimally stimulatory effect was observed with 10 μM CO. CO caused a rapid and transient increase in intracellular level of guanosine-3′,5′-cyclic monophosphate (cGMP). The enhancing effect of CO on random migration was reversed to a large extent by inhibitors of cGMP accumulation, and by antagonists of cGMP-dependent protein kinase (G-kinase). These results strongly suggest that the enhancement of random migration by CO is mediated by cGMP and G-kinase. Using hemoglobin, a scavenger of CO, we could show that stimulation of soluble guanylate cyclase over an extended period of time, rather than the observed fast and transient increase in intracellular cGMP levels, is responsable for CO-activated migration. We postulate that CO, like nitric oxide (NO), acts as a biological signal in the immune system.

Neutrophil migration in response to extracellular signals is considered to be of crucial importance for their rapid action during host defense. In a number of studies, guanosine-3′,5′-cyclic monophosphate (cGMP), which is synthesized by guanylate cyclase, was shown to be an important intracellular messenger involved in the modulation of neutrophil migration (reviewed in (1)).

Carbon monoxide (CO), which can be formed endogenously from heme catabolism by heme oxygenase, is able to activate soluble guanylate cyclase (2,3). It has been proposed that CO plays an important role in the regulation of cell functions and communication (4). Substantial evidence exists for CO as a neuronal messenger (5,6). Furthermore, CO has been shown to inhibit platelet aggregation, and to have vasodilator actions (reviewed in (7)). Up till now, only limited information on a role for CO in the immune system is available. It has been reported that the inducible form of heme oxygenase (HO-1) is present in macrophages (8). Furthermore, endogenously produced CO could decrease chemiluminescence (9), and enhance TNF-α production (10) by macrophages; both effects were mediated via activation of guanylate cyclase. It was also shown that CO inhibits ω-oxidation of leukotriene B_4 by human neutrophils (11).

We investigated the effect of CO on migration by human neutrophils to determine a possible role for CO in the immune system. In addition, we addressed the question of whether these effect was mediated by activation of soluble guanylate cyclase and subsequent elevations of intracellular cGMP levels.

MATERIALS AND METHODS

Materials. Carbon monoxide (99.9% purity) was obtained from Hoek Loos B.V. LY-83583 (6-anilino-5,8-quinolinedione) was from Calbiochem, and methylene blue from BDH Chemicals. Rp-8-pCPT-cGMPS (8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer) and Rp-8-Br-PET-cGMPS (8-bromo-β-phenyl-

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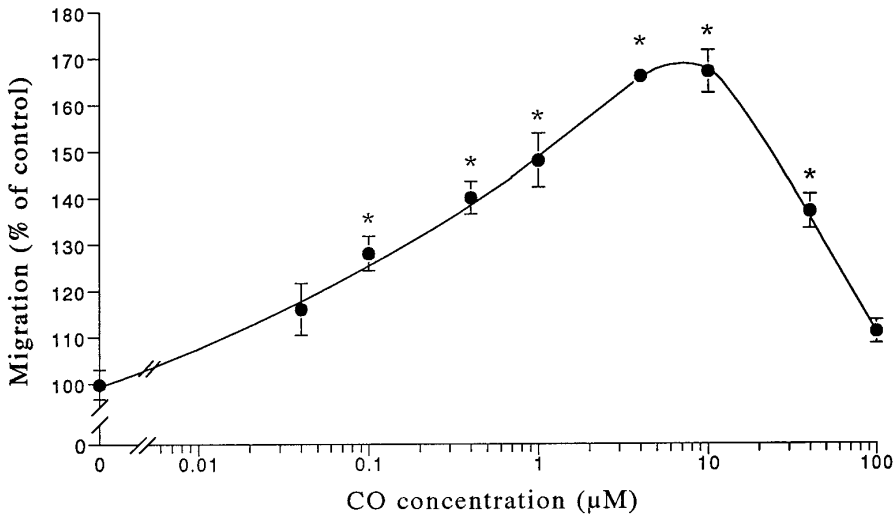

FIG. 1. The effect of increasing concentrations of carbon monoxide on neutrophil migration. CO was present in both compartments of the Boyden chamber. Values are expressed as % of control (i.e., random migration without CO present, which ranged from 49.6 to 51.0 μm, depending on the experiment). (*): $p < 0.05$ as compared with control.

1,N^2-ethenoguanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer) were obtained from BIOLOG Life Science Inst. All other chemicals were obtained from Sigma and were of the highest purity available.

Preparation of CO stock solutions. Saturated CO stock solutions were prepared in buffer containing 140 mM NaCl, 5 mM KCl and 20 mM Hepes pH 7.3. CO was bubbled through the medium for 20 minutes in a glass bottle (2 ml) with septum. CO stock-solutions were freshly prepared for every experiment. The CO-content of this stock solution (1.1 mM) was determined using oxyhemoglobin (12).

Neutrophil isolation. Neutrophils were isolated from the buffy coat of blood of healthy donors by starch-sedimentation and Ficoll-centrifugation. The neutrophils were suspended in medium consisting of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.5 % bovine serum albumin and 20 mM Hepes pH 7.3. The final suspension during the migration experiments contained 3×10^6 neutrophils per ml.

Migration measurements. Cell migration was measured with the Boyden chamber technique, as described before (13), using an incubation time of 35 minutes. Where indicated, neutrophils (3×10^6 cells/ml) were treated with different agents (inhibitors of cGMP metabolism) for the indicated time prior to incubation in the Boyden chamber. Cells were not washed: inhibitors were present both during pre-incubation and during incubation in the Boyden chamber. The assays were carried out in duplicate and the extent of migration was determined at five different filter sites.

cGMP assay. Intracellular cGMP levels were measured as described before (13).

Statistics. For the migration assay, measurements of five different filter sites were averaged to obtain a value representative for the distance migrated by cells into each filter. Data from three independent experiments (two filters each) were taken, recalculated as percentage of controls and expressed as mean ± SEM. Comparisons between means of multiple groups were analyzed by Scheffé's multiple comparison test. Other experiments were analyzed by Student's t-test for paired data. $p < 0.05$ was considered to be statistically significant.

RESULTS

When added to both compartments of the Boyden chamber, CO enhanced random migration of human neutrophils (Fig. 1). Maximal enhancement occurred with 10 μM CO, while at higher concentrations the enhancing effect decreased again. In order to determine whether CO is chemotactic, we studied the effect of CO on migration when it was present only in the lower compartment of the Boyden chamber. Under this condition, the enhancement was less when compared to the situation where CO was present in both compartments simultaneously (control/random migration: 100 ± 3%; CO present in both compartments: 168 ± 4%, $p < 0.05$ as compared with control; CO present in lower compartment only: 139 ± 9%, $p < 0.05$

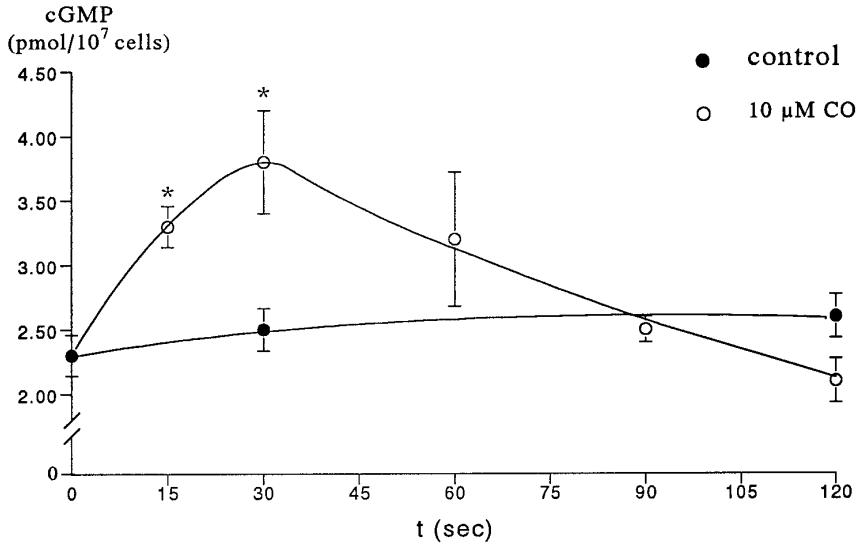

FIG. 2. The effect of CO on intracellular cGMP concentrations. Cells were stimulated with 10 μM CO for the indicated times, after which the reactions were terminated and the cGMP contents of the samples determined. (*): $p < 0.05$ as compared with basal levels (t=0 minutes).

as compared with both control and with CO present in both compartments). When CO was added to the cells in the upper compartment only, the enhancement was almost equal to the effect of CO present in both compartments (CO present in upper compartment only: 160 $\pm$ 7%, $p < 0.05$ as compared with control). We could not determine the contribution of chemokinesis *versus* chemotaxis by means of a checkerboard assay (14), because of the diffusion of CO between the two compartments.

CO, at the concentration which had a maximally stimulatory effect on random migration (10 μM), enhanced the intracellular level of cGMP (Fig. 2). Higher concentrations did not give a further increase in cGMP levels (data not shown). The effect of CO on cGMP levels was found to be rapid (maximal at 30 seconds after stimulation) and transient; intracellular cGMP levels decreased to basal levels within 2 minutes (Fig. 2).

Pre-incubation of neutrophils with LY-83583 (2.5 μM) and methylene blue (10 μM), inhibitors of cGMP accumulation (15,16), inhibited migration induced by 10 μM CO to a large extent (Table 1). One of the main effector enzymes of cGMP is cGMP-dependent protein kinase (G-kinase), which is present in neutrophils (17). Pre-incubation of the cells with two potent membrane-permeable antagonists of G-kinase, Rp-8-pCPT-cGMPS (18) and Rp-8-Br-PET-cGMPS (19), largely inhibited migration induced by 10 μM CO (Table 1).

To assess the contribution of the initial increase in intracellular cGMP levels (Fig. 2) to the enhancement of migration by CO, we incubated cells with CO (present in the upper compartment of the Boyden chamber only), and subsequently scavenged CO by adding excess hemoglobin. Hemoglobin was added at two different time points: at zero minutes (i.e. hemoglobin added together with CO) and at 2 minutes after addition of CO (Fig. 3). We chose for 2 minutes, because after this period of time, the CO-induced cGMP-elevation has decreased to basal levels again (Fig. 2). Immediate scavenging of CO (t=0 minutes) fully abolished the enhancement of migration (Fig. 3). Interestingly, scavenging of CO at 2 minutes after its addition to the cells, also markedly diminished the enhancing effect of CO on migration, although the enhancement was still significant (Fig. 3). Hemoglobin alone did not affect random migration (data not shown).

TABLE 1

The Effect of Inhibitors of cGMP-Accumulation and Antagonists of G-kinase on the Enhancement of Random Migration by CO

Inhibitor[a]	CO-activated migration[b]
(No inhibitor)	168 ± 3
LY-83583	93 ± 3*
Methylene blue	114 ± 8*
Rp-8-pCPT-cGMPS	125 ± 3*
Rp-8-Br-PET-cGMPS	111 ± 1*

[a] Cells were pre-incubated with LY-83583 (2.5 μM), methylene blue (10 μM), Rp-8-pCPT-cGMPS (4 nM), and Rp-8-Br-PET-cGMPS (50 nM), or without inhibitor for 30′, prior to stimulation with 10 μM CO.

[b] CO (10 μM) was present in both compartments of the Boyden chamber. Values are expressed as % of control (i.e., random migration without CO present, after incubation for 30′ without inhibitors or antagonists; this value ranged from 48.8 to 49.9 μm, depending on the experiment).

* $p < 0.05$ as compared to control (10 μM CO, after pre-incubation without inhibitors or antagonists).

DISCUSSION

In this study it is show that CO is able to enhance neutrophil migration. Based on the reversing effect of the inhibitors of cGMP accumulation, LY-83583 and methylene blue, and the antagonists of cGMP-dependent kinase, Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS, it can be concluded that the enhancing effect of CO on random migration is mediated by activation of soluble guanylate cyclase and subsequent cGMP-mediated stimulation of G-kinase. The involvement of G-kinase in CO-induced migration constitutes additional evidence for the hypothesis that this kinase promotes changes in morphology related to cellular movement (20).

At the concentration which gave a maximal stimulation of migration, CO caused a fast increase in intracellular cGMP levels. The enhancement of cGMP levels by CO was transient,

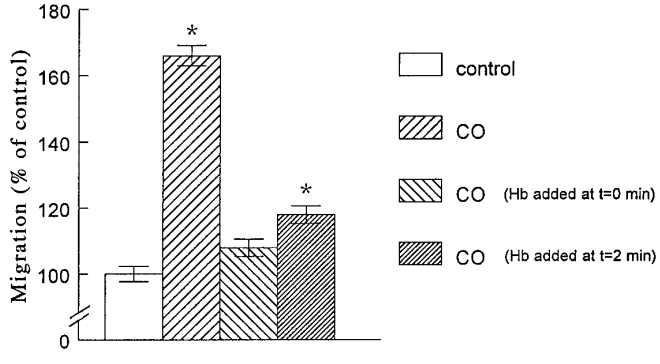

FIG. 3. The effect of hemoglobin on CO-activated migration. Hemoglobin (Hb, 50 μM) was added at the indicated times after addition of CO to the cells (set at t=0 minutes). CO was present in the upper compartment of the Boyden chamber only. Values are expressed as % of control (i.e., random migration without CO present, which ranged from 48.8 to 49.9 μm, depending on the experiment). (*): $p < 0.05$ as compared with control.

most likely because the intracellular concentration of cGMP is under rapid control of phosphodiesterases (21). However, the experiments where stimulation of neutrophils by CO (and thus activation of soluble guanylate cyclase) was abrogated by scavenging of CO with hemoglobin, suggest that the initial rise in cGMP cannot be responsible for the observed enhancement of migration by CO. Still, using inhibitors of cGMP accumulation and antagonists of G-kinase, it was shown that cGMP does play a crucial role in CO-activated migration. Clearly, the presence of CO as well as activation of soluble guanylate cyclase is required over a longer period of time. Therefore, it can be concluded that a sustained activation of guanylate cyclase is responsible for the enhancement of migration by CO, rather than the initial strong rise in intracellular cGMP concentration. The lack of a detectable elevation of cGMP above basal levels after 2 minutes of incubation can be explained by taking into account that synthesis and action of cGMP are likely to be compartmentalized. Over-all levels of cGMP may thus be of secondary importance in neutrophil functioning. The concept of functional compartments regulating cyclic nucleotide effectors has been described for neutrophils and other cellular systems ((22) and references therein).

Our observation that CO can enhance neutrophil migration supports the postulations that CO can act as a biological signal. It has been hypothesized that CO, nitric oxide (NO) and the hydroxyl radical ($OH^{\bullet}$), low molecular weight compounds formed under physiological conditions, all act on a wide-spread second messenger system (soluble guanylate cyclase/ cGMP), regulating physiological processes as diverse as smooth muscle relaxation, platelet aggregation, neurotransmission (23), and neutrophil migration (1). Although CO synthesis by heme oxygenase occurs in a large number of tissues, research into the role of CO in the immune system is a new and emerging field. The inducible form of heme oxygenase has been demonstrated in macrophages (8), but to our knowledge the presence of the enzyme in neutrophils has not been established yet. It is known that neutrophils are capable of synthesizing both NO and $OH^{\bullet}$, and that neutrophil functions can be affected by endogenously produced as well as exogenous NO (24,25). On the basis of the results presented in this paper we postulate that CO, like NO, may serve a biological role in the immune system. Therefore, measurement of CO production by neutrophils and other constituents of the immune system, and evaluation of its possible actions offer an interesting field for further research.

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