

Original article

Hemin, a heme oxygenase substrate analog, both inhibits and enhances neutrophil random migration and chemotaxis

Background: Carbon monoxide (CO), is an endogenously produced gas, generated by the rate-limiting enzyme heme oxygenase (HO), present in man throughout the respiratory tract. CO can elicit important physiological responses like bronchial relaxation and vasodilation. Both HO expression and CO levels in the airways increase in response to hypoxic challenge and to a wide variety of inflammatory stimuli, such as intermittent allergic rhinitis, asthma and upper respiratory tract infections. A role for CO in airway regulation and inflammation has therefore been suggested. However, information about CO-induced effects on cells involved in airway inflammation is scarce. The present study was designed to investigate if the HO substrate analog hemin could affect neutrophil random migration, and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) induced chemotaxis.

Methods: Hemin was added to and incubated with whole blood and the effects of the anticipated CO production were then evaluated on isolated neutrophils using a chemotaxis chamber.

Results: A biphasic dose–response curve emerged for both the neutrophil spontaneous random migration and the fMLP-induced chemotaxis. Low concentrations of hemin (10^{-11} M to 10^{-9} M) enhanced the migratory response, whereas higher concentrations (10^{-7} M and 10^{-5} M) inhibited migration. The inhibition induced by hemin on fMLP-induced migration was abolished after pre-treatment with Rp-8Br-cyclicGMPs, an inhibitor of cyclicGMP.

Conclusions: The present data indicate that endogenously produced CO can affect both spontaneous and stimulated neutrophil migration, partly via a cyclicGMP-related process, hence strengthening the idea of a role for CO in airway inflammation.

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Neutrophils originate in the bone marrow, circulate in the blood for a few days and can be viewed as “enzymatic storehouses” capable of destroying engulfed microorganisms and antigen–antibody complexes (1). They are signaled to sites of infection and inflammation by a number of chemotactic stimuli and, in order to reach the airways, they have to adhere to the endothelium and migrate through to the airway epithelium (2). We have previously demonstrated how nitric oxide (NO), an endogenously produced gaseous mediator, is involved in neutrophil adhesion and migration *in vivo* (3). A role for NO in neutrophil migration and activation is also supported by data derived from numerous *in vitro* experiments (4–8). Carbon monoxide (CO) is another endogenously produced gas with similar physiological properties as NO. CO is mainly generated by the conversion of heme to biliverdin, free iron and CO (9). The rate-limiting enzyme for this process, heme oxygenase (HO), exists in two major isoforms: HO-1, an

inducible form, and HO-2, a constitutively expressed form (10). The presence of both isoforms has been demonstrated throughout the respiratory tract and the relation between HO and CO production has been established within the airways (11–14). Hypoxic challenge is reported to induce HO-1 expression in guinea pig airways, followed by a cyclicGMP-mediated bronchial relaxation (15, 16). Furthermore, CO levels in exhaled air increase in response to a wide variety of inflammatory stimuli, such as intermittent allergic rhinitis, asthma and upper respiratory tract infections (17–20). Altogether this suggests a role for CO in airway regulation and inflammation. However, the information about CO-induced effects on cells involved in airway inflammation is scarce (12). Therefore, the present study was designed to investigate if CO, in analogy with NO, could influence neutrophil migration. To this end hemin, an HO substrate analog, was added to whole blood, and the effects of the anticipated CO production were then

evaluated on isolated neutrophils using a chemotaxis chamber.

Material and methods

Isolation of neutrophils

Thirty-five millilitres of blood from healthy donors was drawn, heparinized and divided into test tubes. The blood was incubated at 37°C with either hemin (10^{-13} , 10^{-11} , 10^{-9} , 10^{-7} or 10^{-5} M) or vehicle. Other controls were incubated with different concentrations of hemin with the addition of Rp-8Br-cGMPS (10^{-5} M), a cyclic GMP antagonist, which does not affect neutrophil migration *per se* (data not shown). Neither hemin, nor Rp-8Br-cGMPS in concentrations up to 10^{-5} M, affected cell viability, as examined by cell counting after staining with trypan blue. After 4 h, a mixture of two parts Macrodex® (Dextran 70, 60 g/1000 ml 9% NaCl; Pharmalink Basläkemedel AB, Spånga, Sweden) and one part sodium metrizoate 32,8% (Nycomed Pharma AS, Oslo, Norway) was added to an equal amount of the heparinized blood. The solutions were allowed to sediment for 30 min at room temperature. The leukocyte-rich supernatant was then carefully layered upon Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 450 *g* for 20 min at room temperature (21–23). The supernatant was discarded and the remaining cells were washed with sterile Hanks balanced salt solution (HBSS; Life Technologies AB, Täby, Sweden), centrifuged again at 340 *g* for 20 min at room temperature and re-suspended in 1 ml of the same medium. Cells were counted in a Bürker chamber and the cell suspension was diluted with HBSS to a final concentration of 3×10^6 cells/ml. Staining of cells with May–Grünwald–Giemsa revealed that more than 95% of cells in the suspension consisted of neutrophils; contaminating cells were mononuclear cells and eosinophils.

Chemotaxis and random migration

A standard 48 well chemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD, USA) was used to assess neutrophil random migration and chemotaxis, as previously described by Falk and colleagues (24). Briefly, each well in the chamber was separated into two compartments by a PVP-free polycarbonate membrane filter with 3 μ m pores (Neuro Probe Inc., Gaithersburg, MD, USA). In assaying neutrophil chemotaxis, a suspension with 10^{-7} M N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma-Aldrich Sweden AB, Stockholm, Sweden), was applied in the bottom part of each well, whereas only a suspension with the corresponding vehicle was used when neutrophil random migration was evaluated. Finally, 50 μ l of the neutrophil suspension was added to the upper part of each well and the chambers incubated at 37°C for 20 min. At the end of incubation the filter was collected, washed in phosphate-buffered saline (PBS; Life Technologies AB, Täby, Sweden) and fixed in methanol (> 99%) for 1 min before staining with xanthene and thiazine (Svenska Labex AB, Helsingborg, Sweden). The filters were then mounted and allowed to dry before microscopic evaluation. Each assay was performed in duplicate, and the filters were randomly coded and read in a blind fashion. The number of cells that had completed their migration through to the underside of the filter in the area of a high-power field (HPF; 1000 $\times$ magnification) was counted. Data were calculated as the mean of five HPFs per well and the hemin-derived data was expressed as a percentage of control (vehicle and fMLP in random migration and chemotaxis experiments, respectively).

Reagents

Hemin (Sigma-Aldrich Sweden AB, Stockholm, Sweden) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Sweden AB, Stockholm, Sweden) and further diluted in RPMI-1640 medium (Life Technologies AB, Täby, Sweden) with 1-% fetal calf serum (FCS) to a maximum concentration of 10^{-4} M. A solution of RPMI-1640 and FCS was present in all experiments including the controls. DMSO in the concentration used in this study does not exert anti-inflammatory cell responses (25, 26). Rp-8Br-cGMPS (Biolog, Bremen, Germany) was dissolved in NaCl to yield a final concentration of 10^{-5} M.

Nomenclature

The revised nomenclature for allergy according to recent EAACI recommendations is used throughout the manuscript (27).

Statistics

Results are expressed as mean $\pm$ SEM, and *n* equals the number of subjects. Statistical comparisons were made by Student's *t*-test and *P*-values less than 0.05 were considered statistically significant.

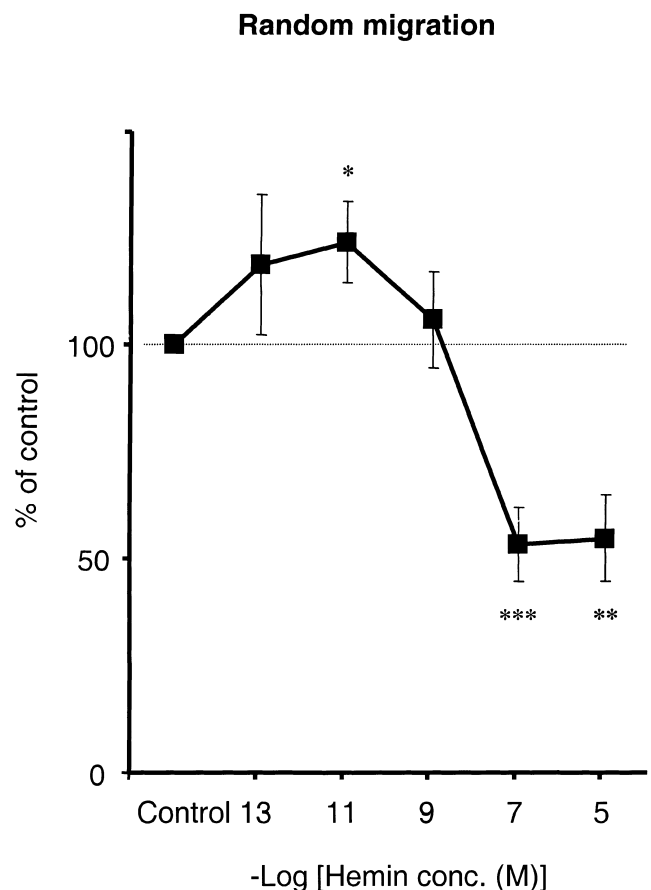


Figure 1. Random migration of human neutrophils. Neutrophils, incubated with increasing concentrations of hemin, migrating towards a control solution. The data points are derived from up to 12 subjects and presented as percentage of control $\pm$ SEM. ****P* < 0.001, **P* < 0.05.

Results

Human neutrophils were allowed to migrate in a multi-well chemotaxis chamber. During control conditions 13 ± 2 neutrophils/HPF ($n = 40$) randomly migrated through the chamber membrane. When the experiment was repeated, with fMLP present in the chamber, the number of neutrophil cells which migrated through the chamber membrane was markedly increased to 55 ± 5 neutrophils/HPF ($n = 37$, $P < 0.001$). After incubation with hemin, a biphasic dose-response curve emerged for both the spontaneous random migration (Fig. 1) and the fMLP-induced chemotaxis (Fig. 2). Low concentrations of hemin (10^{-11} M to 10^{-9} M) increased the migratory response, whereas higher concentrations (10^{-7} M and 10^{-5} M) inhibited the migration. Hemin, in a concentration of 10^{-11} M, enhanced the spontaneous neutrophil migration with $24 \pm 10\%$ ($n = 5$, $P < 0.05$), whereas 10^{-7} M hemin inhibited the same type of movement with $47 \pm 9\%$ ($n = 8$, $P < 0.001$) (Fig. 1). When chemotaxis was investigated, 10^{-11} M hemin increased the fMLP-

induced migration with $36 \pm 11\%$ ($n = 7$, $P < 0.01$) and 10^{-7} M hemin reduced the fMLP-induced migration with $31\% \pm 7\%$ ($n = 24$, $P < 0.001$) (Fig. 2). Further increases in hemin concentration did not increase inhibition of migration (Fig. 2). The inhibition induced by hemin on fMLP-induced migration was abolished after pre-treatment with Rp-8Br-cyclicGMPS (hemin: $58 \pm 11\%$ of control, $n = 8$ and hemin + Rp-8Br-cyclicGMPS: $108 \pm 31\%$ of control, $n = 5$, $P < 0.05$) (Fig. 3A). The stimulation of neutrophil chemotaxis at a hemin concentration of 10^{-11} M appeared to be further increased when Rp-8Br-cGMPS was used (hemin: $114 \pm 7\%$ of control, $n = 7$ and hemin + Rp-8Br-cyclicGMPS: $157 \pm 39\%$ of control, $n = 5$, $P < 0.05$) (Fig. 3B).

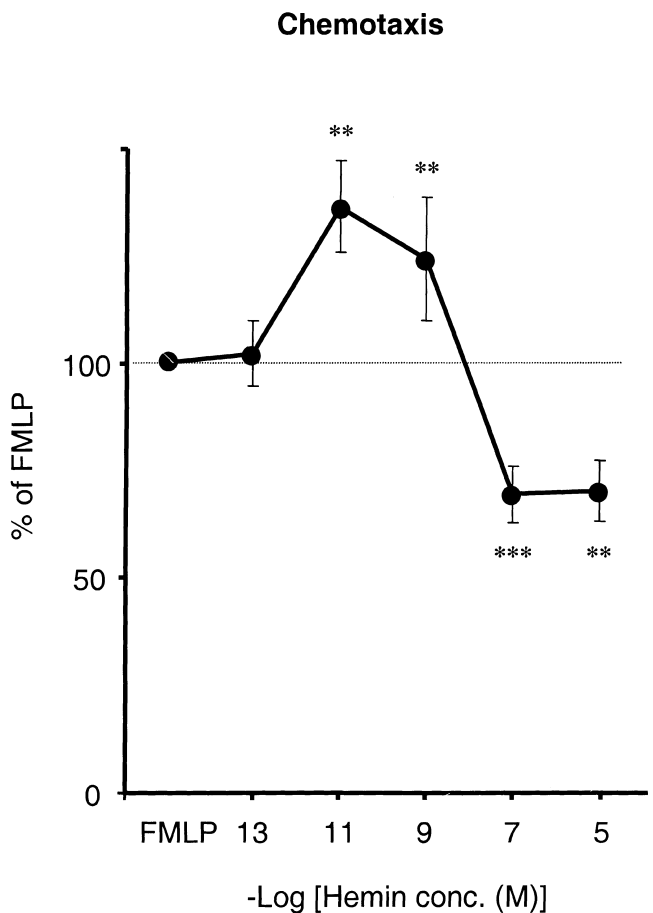


Figure 2. Chemotaxis of human neutrophils. Neutrophils, incubated with increasing concentrations of hemin, migrating towards a solution containing fMLP (10^{-7} M). The data points are derived from up to 24 subjects and presented as percentage of control $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$.

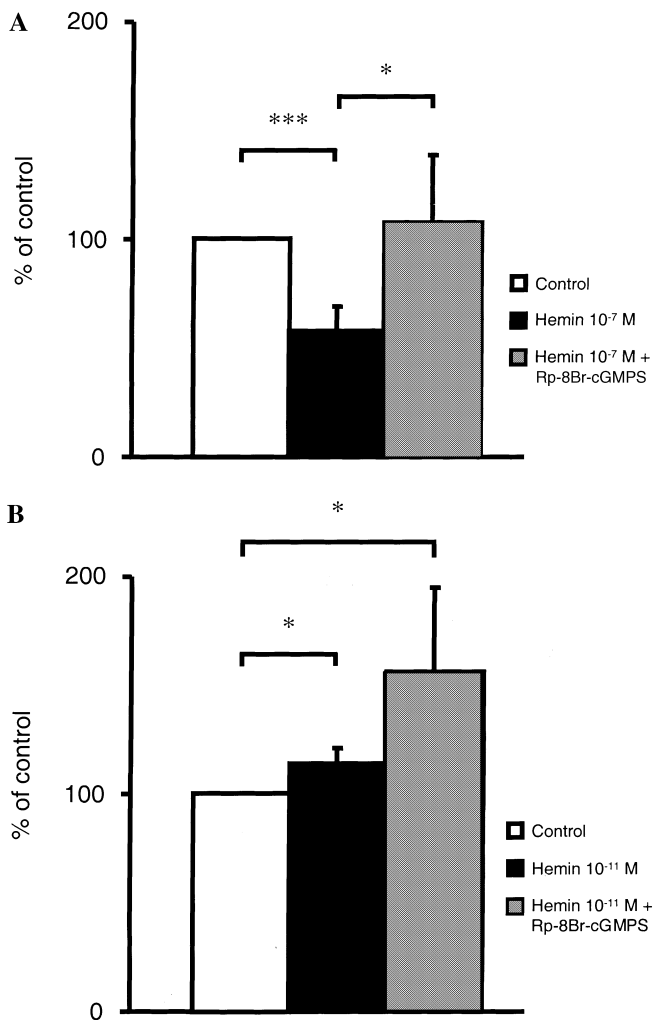


Figure 3. Chemotaxis of human neutrophils migrating towards a solution containing fMLP (10^{-7} M). A. Neutrophils, incubated with hemin (10^{-7} M) with or without addition of Rp-8Br-cGMPS (10^{-5} M) (gray or filled bar, respectively) B. Neutrophils, incubated with hemin (10^{-11} M) with or without addition of Rp-8Br-cGMPS (10^{-5} M) (gray or filled bar, respectively). The data points are derived from up to eight subjects and presented as percentage of control $\pm$ SEM. *** $P < 0.001$, * $P < 0.05$.

Discussion

The incubation of human neutrophils with low doses of hemin resulted in a stimulation of their spontaneous random migration, whereas higher concentrations inhibited their movements. A similar biphasic curve was seen when the effects of hemin on fMLP-induced neutrophil chemotaxis were analysed.

Hemin functions as an inducer of HO-1, as well as a substrate for endogenous CO production, a process facilitated by HO (27–30). Other products of hemin and HO activity include biliverdin and iron (9). CO has previously been reported to enhance random neutrophil migration (31), whereas biliverdin has been shown to attenuate monocyte chemotaxis (32). Neutrophil cell activation has also been shown to be affected after incubation with hemin (33). CyclicGMP has been described as exhibiting biphasic effects on neutrophil migration similar to the effects presently obtained by the use of hemin: low concentrations of cyclicGMP facilitates neutrophil chemotaxis, whereas higher concentrations inhibit chemotaxis (34). CyclicGMP is an important mediator of CO-induced physiological effects (15, 28, 30, 35), thus implying that the biphasic effects on neutrophil migration presently induced by hemin might be related to CO production and cyclicGMP. Further support for this assumption can be derived from the present experiments where Rp-8Br-cGMPS, an inhibitor of cyclicGMP, was shown to abolish hemin-induced inhibition of neutrophil chemotaxis. The potentiation induced by lower concentrations of hemin was increased following the addition of Rp-8Br-cGMPS. Altogether these findings suggest that there might be at least two different mediator pathways for the effects of hemin on neutrophil migration; one inhibitory related to cGMP and one stimulatory mediated by other mechanisms. The addition of Rp-8Br-cGMPS removed the inhibitory component, resulting in a net stimulatory effect. In accordance with the present data it has previously been reported that application of a medium, freshly bubbled

with CO, can enhance random neutrophil migration in a cyclicGMP-dependent way (31).

A number of studies have put forward CO as a new inflammatory marker in the airways (12, 18–20, 36), mainly since it shares a number of properties with NO, a well established inflammatory marker in both the upper and lower airways. In the aspect of chemotaxis and random migration, neutrophilic cells seem to respond much in the same way when exposed to CO as to NO; an initial stimulatory effect is replaced with an inhibitory effect at higher concentrations. This similarity might further increase the validity of the comparison of these two gaseous mediators (4–7, 37).

We have previously demonstrated that CO can be produced and released within the human airways and that CO might function as an intracellular messenger, mediating airway relaxation (13, 15, 16). Also, CO production is increased in patients suffering from intermittent allergic rhinitis, asthma and upper respiratory tract infections (17–20, 38). The present data indicate that hemin, probably by facilitating the endogenous production of CO, can affect both spontaneous and stimulated neutrophil migration. This strengthens the idea that CO might act as a biological signaling molecule within the immune system. However, further research will be needed in order to fully evaluate the role of hemin and CO in airway regulation and inflammation.

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