Intracellular but not extracellular conversion of nitroxyl anion into nitric oxide leads to stimulation of human neutrophil migration

Bart E. VANUFFELEN, Jolanda VAN DER ZEE, Ben M. DE KOSTER, John VANSTEVENINCK and Jan G. R. ELFERINK¹

Department of Molecular Cell Biology, Leiden University, POB 9503, 2300RA Leiden, The Netherlands

Considerable controversy exists in the literature with regard to the nature of the agent mediating the biological effects of nitroxyl (NO^-) donors. Here it is demonstrated that Angeli's salt (AS), a generator of NO^- , enhanced human neutrophil migration. Under aerobic conditions, AS was converted to peroxynitrite to a small extent. However, using methionine, a scavenger of peroxynitrite, it was shown that peroxynitrite was not involved in AS-induced migration. AS equally enhanced human neutrophil migration under aerobic and anaerobic conditions, which strongly suggests that extracellular conversion of NO^- to NO by oxygen was not required. Furthermore, metHb and L-cysteine, which react more readily with NO^- than with NO , inhibited AS-induced mi-

gration, whereas the response towards gaseous NO remained unaffected. AS induced an increase in the intracellular level of cGMP, although the curves for migration and cGMP level appeared to be slightly different in their concentration dependence. An inhibitor of soluble guanylate cyclase and antagonists of cGMP-dependent protein kinase had a more pronounced inhibitory effect on NO -induced migration than on AS-induced migration. This suggests that the cGMP signalling cascade is partially, but not solely, responsible for AS-induced migration. As it has been demonstrated that soluble guanylate cyclase can only be activated by NO , and not by NO^- , these data indicate that NO^- is at least partly converted intracellularly to NO .

INTRODUCTION

Considerable attention has been directed towards the chemistry and biology of NO and its redox-related forms nitrosonium cation (NO^+) and the nitroxyl anion (NO^-) [1]. Recently, it was shown that NO might not be the primary product of nitric oxide synthase [2], which catalyses the oxidation of a terminal guanidino nitrogen of L-arginine to form L-citrulline and (finally) NO . It was suggested that the direct product of the reaction catalysed by NO synthase is NO^- , the one-electron reduction product of NO , which can subsequently be oxidized to NO [2]. These results are in agreement with earlier observations that SOD enhances the generation of NO by NO synthase [3], and that *N*-hydroxyguanidines, intermediates of enzymatic conversion of L-arginine by NO synthase, can generate NO^- , depending on the oxidative conditions [4].

It has been proposed that NO^- , rather than NO , accounts for the biological activity of the elusive endothelium-derived relaxing factor (EDRF) [4]. The fact that EDRF does not pass an anion exchange column [5], and the lack of an ESR spectrum of NO-haem upon reaction of EDRF with deoxyhaemoglobin [6], match properties of NO^- . Therefore, NO^- has to be considered as a chemical species capable of similar, if not identical, behaviour to EDRF. However, it has been reported that NO^- can be converted to NO by a variety of ubiquitous biological oxidants such as superoxide dismutase, metHb and flavins [7,8]. It has also been reported that NO^- readily generates NO in aerobic solutions [8], but others were unable to reproduce these experiments [9]. Thus, controversy exists in the literature with regard to the nature of the agent mediating the biological effects of NO^- donors.

The studies mentioned above underline the potential physiological importance of NO^- and stress the need to investigate

whether NO^- itself is a biological activator or whether its actions require conversion to (an)other species. The objective of the present study, therefore, was to investigate whether NO^- can modulate cellular functions directly, or whether the extracellular conversion to NO or peroxynitrite (ONOO^-) is required [10,11]. As a model system human neutrophils were used, and it was studied how their migration was affected by exposure to a NO^- -donor. Furthermore, the involvement of the cGMP signalling cascade was investigated, as processes induced by nitrogen monoxides are often mediated by this pathway [12].

MATERIALS AND METHODS

Chemicals and solutions

Angeli's salt (AS) was a generous gift from Dr. Martin Feelisch (Schwarz Pharma AG, Monheim, Germany) or was purchased from Cayman Chemical Co., Ann Arbor, MI, U.S.A. and was stored under argon.

NO (98.5 % purity) was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. NO stock solutions were prepared in buffer gassed with argon, as described before [13]. Dilutions were made in the same deoxygenated buffer. Argon 6.0 (oxygen content < 0.1 ppm) was from Aga Gas, Amsterdam, The Netherlands.

Cellulose acetate filters (pore size 3 μm) were from Millipore BV, Etten-Leur, The Netherlands. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was from Tocris Cookson Ltd., Bristol, U.K. and Rp-8-pCPT-cGMPS, and Rp-8-Br-PET-cGMPS were obtained from Biolog Life Science Inst., Bremen, Germany. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and were of the highest purity available.

Abbreviations used: AS, Angeli's salt; EDRF, endothelium-derived relaxing factor; metHb, methaemoglobin; NO , nitric oxide; NO^- , nitroxyl anion; ONOO^- , peroxynitrite.

¹ To whom correspondence should be addressed.

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% BSA and 20 mM Hepes pH 7.3.

Migration measurements

Neutrophil migration was measured with the Boyden chamber technique, as described before [14], using an incubation time of 35 min. Where indicated, neutrophils (3×10^6 cells/ml) were treated with different agents for 30 min at 37 °C, 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. A number of experiments were performed under anaerobic conditions: all solutions and cell suspensions were gassed with argon, and the Boyden chambers were assembled under a continuous argon flow. The oxygen content of the buffers used in this set-up was monitored with a Clark-type electrode, and was found to be $< 0.1\%$, i.e. below the detection limit of the oxygen monitor used.

Serum albumin (containing a free sulphhydryl group) is routinely present during the migration assay, serving as a substrate over which the cells are able to move through the filter in the Boyden chamber. Reaction of NO^- with thiols gives formation of RSNOH, and leads to disulphide formation when a second thiol is available for reaction [1]. However, when a second thiol is not available, as is the case with the single sulphhydryl group of serum albumin, NO^- is released without being converted to NO . This phenomenon has been reported for metHb, where the β -93 cysteine sulphhydryl group is not oxidized by NO^- , but does play a role in determining the reactivity of the metal centre of the protein towards NO^- by competition and reversible addition [15].

Peroxynitrite assay

ONOO^- -mediated nitration of phenol was measured as described before [15a]. Briefly, 600 μM AS was added to 5 mM phenol in 50 mM sodium phosphate buffer pH 7.4 in a final volume of 2.0 ml. After incubation for 2 h at 37 °C, 15 μl 0.1 M NaOH was added, and the absorbance at 412 nm of the samples was immediately recorded. The yield of nitrophenol was calculated from $\epsilon = 4400 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

cGMP assay

Intracellular cGMP levels were measured as described before [14].

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 $\pm$ S.E.M. Comparisons between means of multiple groups were analysed by ANOVA and Scheffé's multiple comparison test. Other experiments were analysed by Student's *t* test for paired data. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

In the present study, AS ($\text{Na}_2\text{N}_2\text{O}_3$) was used as a source of NO^- [4,8,16]. At physiological pH, AS exists predominantly in the form of the monoanion HN_2O_3^- ($\text{p}K_1 = 2.5$, $\text{p}K_2 = 9.5$), which reversibly decomposes to form NO^- and nitrite [eqn. (1)] [17–19]. The protonated form of NO^- , HNO, is a weak acid ($\text{p}K_a = 4.7$) [20], which indicates that, at physiological pH, NO^- is the predominant form in aqueous solution.

Neutrophil migration was used as a model system for the biological effects of AS. Migration of neutrophils is crucial for their host, both for the protection against infection as well as for the induction of inflammation. AS strongly enhanced migration of human neutrophils, when added to both compartments of the Boyden chamber. The effect was significant at concentrations as low as 1 nM, while maximal enhancement of migration was obtained with 40 nM AS. At higher AS concentrations, the stimulatory effect decreased again (Figure 1A). In order to examine whether AS is chemotactic, we studied the effect of AS on migration when it was present only in the lower compartment

Figure 1 Migration and cGMP level of neutrophils stimulated with (A) AS and (B) NO

AS and NO were present in both compartments of the Boyden chamber. (●), Migration (values are expressed as % of control, i.e. random migration without AS or NO present, which ranged from 42.5 to 52.2 μm , depending on the experiment). (○), cGMP level (values are expressed as % of unstimulated control, which ranged from 0.67 to 0.92 $\text{pmol}/10^7$ cells, depending on the experiment). *, ** and ***: $P < 0.05$, 0.01, and 0.001 as compared with control, respectively.

Table 1 The effect of a scavenger of ONOO⁻, and of inhibitors of cGMP metabolism, on AS- and ¹NO-induced migration

Inhibitor†	Migration (% of control)‡	
	AS	¹ NO
No inhibitor	194 ± 9	152 ± 7
Methionine	178 ± 13 (17)	N.D.
ODQ	160 ± 12* (36)	110 ± 8** (81)
8-pCPT-cGMPS	149 ± 13* (48)	115 ± 5* (71)
8-Br-PET-cGMPS	151 ± 17* (46)	110 ± 5** (81)

* and **: $P < 0.05$ and 0.01 as compared with basal AS- or ¹NO-induced migration, respectively (after pre-incubation without inhibitors or antagonists). N.D., not determined.

† Cells were pre-incubated with ODQ (5 μ M), Rp-8-pCPT-cGMPS (4 nM), Rp-8-Br-PET-cGMPS (10 nM), methionine (10 mM) or without inhibitor for 30 min at 37 °C, prior to stimulation with 40 nM AS or 0.5 μ M ¹NO.

‡ AS and ¹NO were present in both compartments of the Boyden chamber. Values are expressed as % of control (i.e. random migration without AS or ¹NO present, after incubation for 30 min without inhibitors or antagonists; this value ranged from 43.2 to 50.4 μ m, depending on the experiment). The % inhibition by the respective inhibitors (of the activating effect by AS or ¹NO) has been indicated within parentheses after the migration values.

of the Boyden chamber. In that case the enhancing effect was also significant, but smaller than when AS was present in both compartments simultaneously (control/random migration: $100 \pm 3\%$; AS in both compartments: $198 \pm 17\%$; AS in the lower compartment only: $143 \pm 5\%$, $P < 0.05$ with respect to both control and AS present in both compartments). Thus, the effect of AS on neutrophil migration is mainly chemokinetic.

Thermal decomposition of AS in aqueous buffer generates singlet-state NO⁻ [21]. Upon relaxation of singlet-state NO⁻, or upon exposure of AS to UV radiation, triplet-state NO⁻ is formed. Triplet-state NO⁻, but not singlet-state NO⁻, rapidly reacts with oxygen in the buffer, yielding ONOO⁻ [eqns. (2) and (3)] [21].

ONOO⁻ is a potent and versatile oxidant that can attack a wide range of biological targets [10,11]. The reaction of phenol with ONOO⁻, resulting in nitrophenol formation, is a convenient assay to measure ONOO⁻ formation [15a,21]. Under aerobic conditions, AS nitrated phenol to a small but significant extent: $1.9 \pm 0.3\%$ of AS ($n = 3$) reacted with phenol, yielding nitrophenol. The reaction of AS with phenol thus reveals that under aerobic conditions a small amount of AS is converted to ONOO⁻. To discriminate between NO⁻ and ONOO⁻, methionine was used, a scavenger of the strong oxidant ONOO⁻. Whereas the conversion of phenol to nitrophenol by AS was completely blocked (results not shown), methionine did not significantly inhibit AS-induced migration (Table 1). It can therefore be concluded that AS-induced migration is not mediated by ONOO⁻.

It has been suggested that NO⁻ can be converted to ¹NO by reaction with oxygen according to eqns. (4) and (5) [8]:

Table 2 Modulation of AS-induced migration: the effect of metHb and L-cysteine (comparison with ¹NO-induced migration)

Inhibitor‡	Migration (% of control)†	
	AS	¹ NO
No inhibitor	218 ± 21	145 ± 7
metHb	107 ± 4*** (94)	133 ± 10* (27)
L-Cysteine	135 ± 11** (70)	137 ± 2* (18)

*, ** and ***: $P < 0.05$, 0.01 and 0.001 as compared with basal AS- or ¹NO-induced migration, respectively.

† AS (40 nM) and ¹NO (0.5 μ M) were present in both compartments of the Boyden chamber. Values are expressed as % of control (i.e. random migration without AS or ¹NO present, which ranged from 47.0 to 50.3 μ m, depending on the experiment). The % inhibition by the respective inhibitors (of the activating effect by AS or ¹NO) has been indicated within parentheses after the migration values.

‡ Where indicated, metHb (5 μ M) was present during incubation in the Boyden chamber, or cells were pre-incubated with L-cysteine (1 mM) for 30 min at 37 °C.

To investigate whether extracellular conversion of NO⁻ to ¹NO was required for AS-induced migration, we compared the effect of AS on migration under aerobic vs. anaerobic conditions. Anaerobically, the oxygen content of the buffer was less than 0.1 %, i.e. below the detection limit of the oxygen monitor used. This should result in at least a 2-log reduction in the rate of the reaction proposed by Fukuto et al. [eqns. (4) and (5)] [8], which is first-order in oxygen. The effect of AS on migration in the absence of oxygen was not significantly different from the effect in the presence of oxygen (AS/aerobic: $256 \pm 12\%$; AS/anaerobic: $257 \pm 12\%$ of control (i.e. random migration); random migration under anaerobic conditions did not significantly differ from random migration under aerobic conditions). This observation indicates that extracellular conversion of NO⁻ to ¹NO by oxygen is not required for the induction of migration by AS.

These results are in accordance with data in the chemical literature [21], that show that only triplet-state nitroxyl is able to react with oxygen [eqns. (2) and (3)]. Furthermore, the mechanism proposed to account for the conversion of NO⁻ to ¹NO by oxygen [eqns. (4) and (5)] [8] should lead to formation of ONOO⁻. The proposed electron transfer process between singlet-state nitroxyl and oxygen generates ¹NO and superoxide in close vicinity. As the reaction between these two species is diffusion-limited [22], their formation in close vicinity leads to ONOO⁻ formation and not to ¹NO [eqn. (6)]:

Indeed, as shown above, a small amount of ONOO⁻ is generated from AS under aerobic conditions, but this is most likely formed after relaxation of singlet NO⁻ [eqns. (2) and (3)], since reaction (4) has been reported not to occur with singlet-state NO⁻ [21].

In a number of studies, L-cysteine and metHb were used to discriminate between ¹NO and NO⁻, as NO⁻ seems to react more readily with these compounds than ¹NO [1,7,15,23,24]. Under aerobic conditions, both L-cysteine as well as metHb inhibited the effect of AS on migration. For comparison, neutrophil migration was induced by exposure of the cells to gaseous ¹NO [13]. In this case, L-cysteine and metHb exhibited only minor effects (Table 2). This confirms that AS acts as a NO⁻ donor, and does not mediate its effects via direct generation of ¹NO.

Cyclic GMP is an important intracellular messenger involved in the modulation of neutrophil migration [25]. AS induced an increase in the intracellular level of cGMP (Figure 1A); the

curves for migration and cGMP level appeared to be slightly different in their concentration dependence (Figure 1A). For comparison, the concentration dependence of 'NO on cGMP level and neutrophil migration was studied (Figure 1B). From Figure 1 it appears that cGMP level and migration are more closely correlated for 'NO than for AS. Therefore, the role of cGMP in AS- and 'NO -induced migration was investigated using a number of inhibitors of the cGMP signal-transduction pathway. An inhibitor of soluble guanylate cyclase, ODQ, and two antagonists of cGMP-dependent protein kinase, Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS, significantly inhibited migration induced by 40 nM AS, while almost completely blocking 'NO -induced migration (Table 1).

From these results it is clear that activation of soluble guanylate cyclase, and subsequent activation of cGMP-dependent protein kinase, are involved in the enhancement of migration by AS. However, the inhibitor of cGMP accumulation and antagonists of cGMP-dependent protein kinase had a more pronounced effect on 'NO -induced migration than on AS-induced migration (Table 1). This indicates that, whereas cGMP plays a predominant role in 'NO -induced migration, it is involved in, but not solely responsible for, AS-induced migration. This is underlined by the slight difference in concentration dependence of the curves of migration and cGMP level for AS (Figure 1A). It thus seems that other mechanisms than the cGMP signalling cascade participate in the enhancement of migration by AS.

It has been suggested that NO^- can bind to the Fe(III) state of soluble guanylate cyclase [7], or that activation of the enzyme may result from interaction of NO^- with regulatory thiol groups [1]. However, it was recently reported that 'NO is the only nitrogen monoxide redox form capable of directly activating soluble guanylate cyclase, whereas AS (NO^-) and NO^+ had no effect [26]. This means that NO^- has to be converted to 'NO in order to activate guanylate cyclase, and subsequently induce neutrophil chemotaxis. Our data clearly show that extracellular conversion of NO^- to 'NO is not involved in AS-induced migration, which indicates that NO^- is converted to 'NO intracellularly, for example by flavins or SOD [7,8].

The chemistry of NO^- has not received much attention [1], and more research is needed to clarify the interactions of NO^- with cellular components. Although it is, at present, not possible to measure directly the extent of intracellular conversion of NO^- to 'NO , our results indicate that NO^- is at least partly converted intracellularly to 'NO with subsequent activation of the cGMP-

signalling cascade. In addition, NO^- also exerts cGMP-independent effects. The latter may be through direct reaction of NO^- with protein thiols or metal centres, thus affecting cellular signalling and actions.

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