N-Hydroxylamine is not an intermediate in the conversion of L-arginine to an activator of soluble guanylate cyclase in neuroblastoma N1E-115 cells

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This study evaluates the role of *N*-hydroxylamine (NH₂OH) in activating soluble guanylate cyclase in the mouse neuroblastoma clone N1E-115. It has been proposed that NH₂OH is a putative intermediate in the biochemical pathway for the generation of nitric oxide (NO)/endothelium-derived relaxing factor (EDRF) from L-arginine. NH₂OH caused a time- and concentration-dependent increase in cyclic GMP formation in intact cells. This response was not dependent on Ca²⁺. In cytosol preparations the activation of guanylate cyclase by L-arginine was dose-dependent and required Ca²⁺ and NADPH. In contrast, NH₂OH itself did not activate cytosolic guanylate cyclase but it inhibited the basal activity of this enzyme in a concentration-dependent manner. The formation of cyclic GMP in the cytosolic fractions in response to NH₂OH required the addition of catalase and H₂O₂. On the other hand, catalase and/or H₂O₂ lead to a decrease in L-arginine-induced cyclic GMP formation. Furthermore, NH₂OH inhibited L-arginine- and sodium nitroprusside-induced cyclic GMP formation in the cytosol. The inhibition of L-arginine-induced cyclic GMP formation in the cytosol by NH₂OH was not reversed by the addition of superoxide dismutase. These data strongly suggest that NH₂OH is not a putative intermediate in the metabolism of L-arginine to an activator of guanylate cyclase.

INTRODUCTION

Endothelium-derived relaxing factor (EDRF), first described by Furchgott & Zawadzki (1980), is a substance which is released by endothelial cells and causes relaxation of precontracted smooth muscle (see Ignarro, 1987*a,b* for reviews). It has been postulated that EDRF induces relaxation by activating guanylate cyclase located in the smooth muscle cells (Busse *et al.*, 1985; Ignarro & Kadowitz, 1985; Murad *et al.*, 1987). More recently, several investigators have demonstrated that nitric oxide (NO) possesses similar properties to those of EDRF (Rapoport & Murad, 1983; Ignarro *et al.*, 1987*a,b*; Palmer *et al.*, 1987; Radomski *et al.*, 1987). However, others have presented data suggesting that EDRF and NO are distinct chemical entities (Shikano *et al.*, 1988; Long & Berkowitz, 1989; Thomas & Ramwell, 1989). Thus the exact nature of EDRF and whether there are multiple forms of this mediator are not known at present.

Besides endothelial cells (Schmidt *et al.*, 1989*a*), activated murine macrophages (Iyengar *et al.*, 1987; Stuehr & Nathan, 1989; Tayeh & Marletta, 1989), rat and human neutrophils (McCall *et al.*, 1989; Rimele *et al.*, 1989; Schmidt *et al.*, 1989*b*; Salvemini *et al.*, 1989), differentiated HL-60 cells (Schmidt *et al.*, 1989*b*), cerebellar cells (Garthwaite *et al.*, 1988) and mouse neuroblastoma clone N1E-115 cells (Gorsky *et al.*, 1990) are known to generate EDRF/NO. In the case of activated macrophages, it has been proposed that NO may be one of the means by which these phagocytic cells kill invading organisms (Hibbs *et al.*, 1987*a,b*; Marletta, 1988). The mechanisms by which NO mediates its action are not well defined. There is evidence to support the concept that NO binds covalently to the haem group of soluble guanylate cyclase, and in so doing activates the formation of cyclic GMP (see Ignarro, 1987*a,b* for reviews).

Deguchi & Yoshioka (1982) determined that L-arginine iso-

lated from the synaptosomal soluble fraction of rat brain is the endogenous activator of soluble guanylate cyclase. Although the mechanism of activation of soluble guanylate cyclase by L-arginine was not elucidated, these investigators suggested that this process is similar to that triggered by nitroso compounds (Deguchi & Yoshioka, 1982). More recently, studies using the combinations of ¹⁵N labelling at the terminal guanidino group of L-arginine, gas chromatography and mass spectrometry have demonstrated that NO is derived from the terminal guanidino moiety of L-arginine, with the resulting formation of L-citrulline (Palmer *et al.*, 1988; Marletta *et al.*, 1988). Several studies in different laboratories (Bredt & Snyder, 1989; Knowles *et al.*, 1989; Mayer & Bohme, 1989; Mayer *et al.*, 1989; Palmer & Moncada, 1989) have indicated that the enzyme (or enzymes) that catalyses the formation of NO and L-citrulline from L-arginine is Ca²⁺- and NADPH-dependent, stereospecific for L-arginine and located in the cytoplasm of the cell. In fact, Bredt & Snyder (1990) used the formation of radioactive L-citrulline as an indicator to isolate the enzyme which catalyses this reaction from rat brain. However, the precise biochemical pathway (or pathways) for the generation of NO from L-arginine is not known, although several mechanisms have been postulated. Hibbs *et al.* (1987*b*) proposed the involvement of an arginine deiminase which converts L-arginine to L-citrulline and ammonia, which in turn can be further oxidized to nitrite. In contrast, it has been suggested that the initial step involves a two-electron oxidation of one of the guanidine nitrogens of L-arginine to the corresponding hydroxylamine, which can be tautomerized to an oxime guanidine (see Fig. 1), followed by a series of one-electron oxidations to generate NO and L-citrulline (Marletta *et al.*, 1988; Tayeh & Marletta, 1989). An alternative mechanism (Fig. 1) involves the hydrolysis of the oxime arginine to L-citrulline and *N*-hydroxylamine (NH₂OH), which in turn is converted by

Abbreviations used: SOD, superoxide dismutase; EDRF, endothelium-derived relaxing factor; ED₅₀, concentration having half-maximal effect.

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Fig. 1. Proposed pathways for the conversion of L-arginine into NO

The proposed pathways are through a nitroso intermediate (Marletta *et al.*, 1988) or through a NH_2OH intermediate (DeMaster *et al.*, 1989).

catalase and H_2O_2 to NO (DeMaster *et al.*, 1989). It should be noted that the enzymes found in the brain, endothelial cells and the adrenal gland are Ca^{2+} - and calmodulin-dependent and do not require tetrahydrobiopterin (Mayer *et al.*, 1989; Palacios *et al.*, 1989; Bredt & Snyder, 1990), whereas the macrophage enzyme does not require Ca^{2+} but is tetrahydrobiopterin-dependent (see Nathan & Stuehr, 1990, for a review). Earlier we reported on the role of intracellular Ca^{2+} mobilization in muscarinic- and histamine-receptor-mediated activation of guanylate cyclase in N1E-115 neuroblastoma cells (Surichamorn *et al.*, 1990). In the present study we have examined the activation of guanylate cyclase by NH_2OH and L-arginine in N1E-115 neuroblastoma cells. We provide evidence which suggests that NH_2OH is not a direct intermediate in the conversion of L-arginine to an activator of guanylate cyclase. In fact, NH_2OH appears to act as an inhibitor of guanylate cyclase in the absence of enzymes that catalyse its conversion to NO.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, Hank's balanced salt solution and calf serum were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). $[8\text{-}^3\text{H}]\text{Guanosine}$ was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.). $[^{32}\text{P}]\text{GTP}$ was purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). Ionomycin and A23187 were purchased from Calbiochem (San Diego, CA, U.S.A.). Superoxide dismutase (SOD) and catalase were obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Cell culture conditions

Murine neuroblastoma clone N1E-115 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum as described previously (Surichamorn *et al.*, 1990). Confluent cells (15–20 days after subculture) from passages 10–16 were utilized in experiments.

Preparation of the cytosolic fraction from N1E-115 cells

Cells were harvested using Puck's D1 solution (Honegger &

Richelson, 1976) and washed once with the same buffer. Detached cells $[(30\text{--}40) \times 10^6 \text{ cells}]$ were resuspended in 10 ml of TEM buffer (50 mM-Tris/HCl, 1 mM- MgCl_2 and 0.1 mM-EGTA, pH 7.4) and homogenized on ice in a glass homogenizer (40 strokes). The homogenate was centrifuged at 1000 g for 10 min. The supernatant fraction was collected and recentrifuged for 30 min at 17000 g. The pellet was discarded and the supernatant was centrifuged at 100000 g for 60 min at 4 °C. The supernatant from the high-speed spin was collected and then concentrated and dialysed with TEM buffer three times by ultrafiltration through a CentriCell (30 kDa cut-off; Polysciences, Inc. Warrington, PA, U.S.A.). The cytosolic fractions were stored in portions at -70°C and were used for experiments within 2–3 days, as losses of enzyme activity were observed upon storage for longer periods (one week).

Assay of guanylate cyclase activity in the cytosolic fraction

Guanylate cyclase activity in cytosolic fractions was determined by measuring the conversion of $[^{32}\text{P}]\text{GTP}$ to cyclic $[^{32}\text{P}]\text{GMP}$. All drugs studied were added to TEM buffer containing 1 mM-NADPH, 1 mM-cyclic GMP, 0.75 mM-sodium phosphocreatine, 25 units of creatine kinase/ml, 1 mM-isobutylmethylxanthine, 100 nM free Ca^{2+} , 0.1 mM-GTP and 1 μCi of $[^{32}\text{P}]\text{GTP}$ for 5 min at 37 °C. In some cases, various concentrations of Ca^{2+} were added in the presence of 0.1 mM-EGTA to give the desired free Ca^{2+} concentration at pH 7.4, which was calculated by using the computer program SKIN-1a provided by Dr. Chi-Ming Hai (University of Virginia School of Medicine). To initiate assays, cytosolic fractions from N1E-115 cells (approx. 0.05 mg of protein/assay) were added to the mixture in a final volume of 100 μl . In all experiments where H_2O_2 was required, it was added immediately before activation to avoid its destruction by catalase. Incubations were performed at 37 °C for 10 min and terminated by adding 250 μl of 5% trichloroacetic acid. Cyclic $[^{32}\text{P}]\text{GMP}$ was separated by ion-exchange chromatography as described in detail elsewhere (Surichamorn *et al.*, 1990). It was noted during the course of this work that the levels of cyclic GMP varied among different preparations. Therefore experiments were done using the same cytosolic preparations when comparisons between experimental conditions were performed.

Cyclic GMP measurements in cell suspensions

Harvested cells were labelled with 10 μCi of $[^3\text{H}]\text{guanosine/ml}$ (12 Ci/mmol) in Hepes buffer (110 mM-NaCl, 5.3 mM-KCl, 1.8 mM- CaCl_2 , 1 mM- MgSO_4 , 25 mM-glucose and 20 mM-Hepes supplemented with sucrose to bring up to 340 mosm, pH 7.4). Labelled cells were washed and distributed into multiwell trays $[(0.1\text{--}0.2) \times 10^6 \text{ cells/well}]$. Cells were preincubated for 15 min at 37 °C before being stimulated with activators for the indicated times. The reaction was stopped by using 5% trichloroacetic acid, and cyclic $[^3\text{H}]\text{GMP}$ was isolated by ion-exchange chromatography as described above.

Data analysis

Cells were counted using a Coulter Counter (Model Zm). Dose-response curves of activator-induced cyclic GMP formation and competition profiles of NH_2OH were fitted according to a four-parameter logistic function by non-linear regression analysis using the GRAPHPAD computer program (ISI, Philadelphia, PA, U.S.A.). Protein was determined by using the method of Bradford (1976). K_i values were calculated according to the following equation: $K_i = [\text{B}]/(\text{DR} - 1)$, where [B] is the concentration of NH_2OH (0.1 mM) and DR is the dose ratio of the concentrations of L-arginine which give the same response in the presence and the absence of NH_2OH .

Fig. 2. Effect of NH_2OH on cyclic GMP accumulation in N1E-115 cells

(a) Time course and effects of short- and long-term preincubation with 5 mM-EGTA on NH_2OH -induced cyclic [^3H]GMP formation. Cells were incubated with [^3H]guanosine for 60 min at 37 °C and pretreated with and without 5 mM-EGTA for 2 min or 30 min and then activated with 100 μM - NH_2OH . Error bars correspond to S.D. ○, No EGTA (2 min); ●, +EGTA (2 min); △, no EGTA (30 min); ▲, +EGTA (30 min) (b) Dose-response curve of cyclic [^3H]GMP formation induced by NH_2OH . Labelled cells were stimulated with increasing concentrations of NH_2OH for 2 min. Cyclic [^3H]GMP was isolated as described in the Materials and methods section. Data shown are representative of three independent experiments; error bars correspond to S.D.

RESULTS

Formation of cyclic GMP in response to NH_2OH in intact cells

NH_2OH caused a time- and concentration-dependent increase in the formation of cyclic GMP in N1E-115 cells (Fig. 2). The time course of NH_2OH -mediated cyclic GMP formation was rapid, with a maximum between 2 and 3 min, followed by a slow decrease after 5 min. Since Ca^{2+} is required for receptor-mediated cyclic GMP synthesis (Surichamorn *et al.*, 1990), experiments were designed to determine the effects of changing the extracellular Ca^{2+} concentration on the formation of cyclic GMP induced by NH_2OH . This was accomplished by adding a final concentration of 5 mM-EGTA to normal buffer (1.8 mM- Ca^{2+}) for 2 min to adjust the extracellular Ca^{2+} concentration to 40 nM, which is below the resting intracellular free Ca^{2+} level (~ 100 nM) in these cells (Surichamorn *et al.*, 1990). As shown in Fig. 2, the cyclic GMP response was not changed relative to the control (no EGTA). Similar findings were observed when cells were incubated for 30 min with 5 mM-EGTA to deplete intracellular Ca^{2+} pools (Surichamorn *et al.*, 1990). These results suggest that the ac-

Fig. 3. Effects of Ca^{2+} on the activation of guanylate cyclase in the presence of NADPH in the cytosol of N1E-115 cells by different activators

A portion of the cytosol was incubated in assay medium as described in the Materials and methods section in the presence of L-arginine (100 μM ; ●) or NH_2OH (100 μM ; ■), or without activators (○), in the presence of increasing concentrations of free Ca^{2+} (see text) at 37 °C for 10 min. Cyclic [^{32}P]GMP formation was measured as described in the Materials and methods section. Data shown are representative of three independent experiments; error bars correspond to S.D.

tivation of guanylate cyclase by NH_2OH is independent of Ca^{2+} , as in the case of NaN_3 -induced activation (Surichamorn *et al.*, 1990).

Effects of L-arginine and NH_2OH on cytosolic guanylate cyclase

It is known that Ca^{2+} and NADPH are required for the enzymic conversion of L-arginine into a metabolite which activates soluble guanylate cyclase (Bredt & Snyder, 1989; Knowles *et al.*, 1989; Mayer & Bohme, 1989; Mayer *et al.*, 1989; Palmer & Moncada, 1989). Fig. 3 shows the dose-response curves of cyclic GMP formation which were obtained when the cytosolic fractions were incubated in the absence or presence of NH_2OH or L-arginine, and in the presence of NADPH and increasing concentrations of free Ca^{2+} . The basal guanylate cyclase activity was increased 2–3-fold when the concentration of free Ca^{2+} was increased from 10 to 100 nM, but declined at higher Ca^{2+} concentrations. The increase in the formation of cyclic GMP in response to L-arginine (100 μM) was about 4–7-fold within the same range of Ca^{2+} . In contrast, NH_2OH (100 μM) inhibited basal guanylate cyclase at the same concentrations of Ca^{2+} (Fig. 3). To further characterize the activation and the inhibition of guanylate cyclase by L-arginine and NH_2OH respectively, the effects of each on cytosolic guanylate cyclase activity were investigated in the presence of fixed concentrations of Ca^{2+} (100 nM) and NADPH (1 mM). Furthermore, since catalase and the combination of catalase plus H_2O_2 are known to convert NH_2OH to NO (Craven *et al.*, 1979), the effects of these additions were also studied. H_2O_2 (10 μM and 100 μM) had no effect on the inhibition of basal cyclic GMP formation by NH_2OH (Fig. 4b, and results not shown). However, in the presence of catalase (0.1 mg/ml), NH_2OH increased rather than decreased guanylate cyclase activity, but still inhibited cyclic GMP formation at concentrations higher than 100 μM (Fig. 4b). Furthermore, the combination of catalase (0.1 mg/ml) and H_2O_2 (100 μM) resulted in a further enhancement of cyclic GMP

Fig. 4. Effect of catalase (0.1 mg/ml) and H₂O₂ (100 μ M) on cyclic GMP formation in response to different activators in the presence of 100 nM free Ca²⁺ and 1 mM-NADPH in the cytosol

(a) A portion of the cytosol was incubated with catalase (●), H₂O₂ (■) or the combination of catalase and H₂O₂ (□) with increasing concentrations of L-arginine at 37 °C for 10 min (○, control). Error bars correspond to S.D. (b) As in (a), except that L-arginine was replaced with NH₂OH. ●, Control; ○, catalase; □, H₂O₂; ■, catalase + H₂O₂.

formation by 10–100 μ M-NH₂OH. The maximum activation by NH₂OH (100 μ M) of the enzyme in this preparation was about 3–4-fold over basal activity in the presence of catalase (0.1 mg/ml) and H₂O₂ (100 μ M) (Fig. 4b). These results are in agreement with those reported by Craven *et al.* (1979), who demonstrated that catalase, and the combination of catalase and H₂O₂, can oxidize NH₂OH to NO, which in turn activates guanylate cyclase.

L-Arginine stimulated cytosolic guanylate cyclase activity in a dose-dependent manner and produced a maximal response at 100 μ M. The maximal stimulation by L-arginine in this cytosolic preparation was 12-fold over the basal activity (Fig. 4a). In contrast with the effects of NH₂OH, additions of increasing concentrations of H₂O₂ (1–100 μ M) led to a slight decrease in the accumulation of cyclic GMP in response to L-arginine (Fig. 4a, and results not shown). Similarly, addition of catalase (0.1 mg/ml) and a combination of catalase (0.1 mg/ml) and H₂O₂ (100 μ M) also caused a small decrease in the activation of guanylate cyclase by L-arginine (Fig. 4a).

Since NH₂OH appears to inhibit basal activity in the absence of catalase and H₂O₂, its effect on the stimulation of guanylate cyclase by L-arginine was examined in cytosolic preparations. Fig. 5(a) shows the formation of cyclic GMP induced by L-arginine (100 μ M) as a function of increasing concentrations of

Fig. 5. Effects of NH₂OH on cyclic GMP formation induced by different activators in the presence of 100 nM free Ca²⁺ and 1 mM-NADPH in the cytosol

(a) A portion of the cytosol was incubated with (□, ■) and without (○, ●) L-arginine (100 μ M) in the presence (●, ■) or absence (○, □) of SOD, in the presence of various concentrations of NH₂OH, at 37 °C for 10 min. Error bars correspond to S.D. (b) A portion of the cytosol was incubated with L-arginine (100 μ M; ●) or sodium nitroprusside (100 μ M; ○) in the presence of various concentrations of NH₂OH. Error bars correspond to S.E.M. The value of 100% was taken for stimulation by L-arginine without NH₂OH.

NH₂OH. NH₂OH inhibited L-arginine-induced cyclic GMP formation significantly at 1 mM, and completely abolished it at 10 mM (Fig. 5a). The effects of SOD on this inhibition were also investigated. In the presence of SOD (100 units/ml) a 3-fold increase in basal cyclic GMP formation was observed. However, SOD (100 units/ml) did not prevent the inhibition of basal cyclic GMP formation by NH₂OH. Similarly, SOD (100 units/ml) did not prevent the inhibition by NH₂OH of L-arginine (100 μ M)-induced cyclic GMP formation (Fig. 5a). To determine whether NH₂OH inhibited cyclic GMP formation by scavenging NO generated through the metabolism of L-arginine, the above-described experiment was performed by substituting sodium nitroprusside (100 μ M) which is known to spontaneously generate NO, in the place of L-arginine. As shown in Fig. 5(b), NH₂OH had the same effect on NO generation induced by sodium nitroprusside as on that induced by L-arginine, both qualitatively and quantitatively. To further characterize this inhibition, the effects of increasing the concentration of NH₂OH on the dose-response relationship of cyclic GMP formation mediated by L-arginine was investigated. As shown in Fig. 6, NH₂OH (0.1 mM) significantly ($P < 0.05$) inhibited cyclic GMP formation induced by L-arginine in a competitive manner, as it shifted the EC₅₀ (concn. causing half-maximal response) from $0.93 \pm 0.03 \mu$ M to $2.1 \pm 0.2 \mu$ M without altering the maximum response, with a K_i of

Fig. 6. Effects of NH_2OH on the dose-response of cyclic GMP formation induced by L-arginine

NH_2OH concentrations used were (mM) $\circ$, 0; $\bullet$, 0.10; $\blacksquare$, 0.25; $\blacktriangle$, 0.50; $\square$, 1.0. Error bars correspond to S.D.

$47 \pm 3 \mu\text{M}$. At higher concentrations (between 0.25 and 1 mM), NH_2OH inhibited this process in an uncompetitive manner.

DISCUSSION

The main purpose of the present study was to demonstrate whether or not NH_2OH is a direct intermediate in the activation of guanylate cyclase by L-arginine, as has been proposed by some investigators (DeMaster *et al.*, 1989; Thomas & Ramwell, 1989). The dose-response relationship for cyclic GMP formation in intact N1E-115 cells showed that NH_2OH is an activator of guanylate cyclase. These findings are consistent with earlier reports (Deguchi, 1977; Katsuki *et al.*, 1977). Furthermore, NH_2OH induced the formation of cyclic GMP independent of the concentration of Ca^{2+} , similar to the effects of NaN_3 (Surichamorn *et al.*, 1990). It has been shown that NH_2OH does not activate guanylate cyclase directly, but rather requires oxidation to NO to exert its effect (Waldman & Murad, 1987). Catalase and the combination of catalase and H_2O_2 are known to catalyse this reaction (Craven *et al.*, 1979). In contrast with the findings in intact cells, NH_2OH inhibited guanylate cyclase activity in the cytosolic fraction (Fig. 3). This is in spite of the fact that this preparation contained the enzymes necessary for the conversion of L-arginine into an activator of guanylate cyclase, as indicated by the increase in the formation of cyclic GMP induced by L-arginine (Figs. 4 and 5a). While the present work was nearing completion, Gorsky *et al.* (1990) reported the production of an EDRF-like activity in the cytosol of N1E-115 cells. The activity of L-arginine was dependent on the Ca^{2+} concentration, with a maximum at 100 nM- Ca^{2+} (Fig. 3). It has been shown that the conversion of L-arginine into an activator of guanylate cyclase requires Ca^{2+} (Bredt & Snyder, 1989; Knowles *et al.*, 1989; Mayer & Bohme, 1989; Mayer *et al.*, 1989; Palmer & Moncada, 1989). However, higher Ca^{2+} concentrations lowered L-arginine-induced activation of guanylate cyclase. This effect is most likely due to direct inhibition of guanylate cyclase by Ca^{2+} , since enzyme activation by sodium nitroprusside was also suppressed at these concentrations of Ca^{2+} (results not shown). The same observation has been reported by other investigators (Palacios *et al.*, 1989; Knowles *et al.*, 1989). Similarly, basal guanylate cyclase activity exhibited the same Ca^{2+} -dependence,

suggesting that our cytosol preparation contained some endogenous L-arginine which could not be washed out (Fig. 3).

In the mechanism proposed by DeMaster *et al.* (1989), NH_2OH is formed through the hydrolysis of the oxime of L-arginine followed by its oxidation to NO by catalase and H_2O_2 (Fig. 1). This pathway would lead to the generation of superoxide anions, which we have confirmed by spin-trapping experiments (results not shown). Therefore we compared the effects of catalase, H_2O_2 and SOD on cyclic GMP formation in response to L-arginine and NH_2OH in the cytosol. Our results, as shown in Fig. 4, are consistent with those of others which showed that the activation of guanylate cyclase by NH_2OH requires oxidation of NH_2OH to NO (Craven *et al.*, 1979; Waldman & Murad, 1987). Intact N1E-115 cells might possess oxidases which can oxidize NH_2OH to NO *in situ*, which in turn activates guanylate cyclase. Deguchi (1977) has shown that the crude mitochondrial fraction is necessary for the activation of soluble guanylate cyclase by NH_2OH in the cytosol fractions of rat cerebral cortex. Thus it is possible that the appropriate oxidases are also located in the mitochondria of N1E-115 cells.

The fact that L-arginine induced cyclic GMP synthesis in the cytosolic fraction without the addition of catalase and H_2O_2 , which is different from the case of NH_2OH , suggests that the bioconversion of L-arginine into an activator of guanylate cyclase does not involve the formation of NH_2OH as an intermediate. Recently, Kwon *et al.* (1990) have demonstrated that the ureido oxygen of the citrulline product of macrophage NO synthase is derived from dioxygen and not from water. Their results excluded any reaction mechanisms that require water to provide the ureido oxygen of L-citrulline. Thus it is also likely that the bioconversion of L-arginine into NO in macrophages does not involve the hydrolysis of the oxime arginine to L-citrulline and NH_2OH .

To study further the inhibitory role of NH_2OH on cytosolic guanylate cyclase activity, its effects on the activation of the enzyme by L-arginine and sodium nitroprusside were compared. The latter produces NO spontaneously without sharing the metabolic pathways involved in the bioactivation of L-arginine. As shown in Fig. 5(a), NH_2OH , at concentrations higher than 0.1 mM, inhibited cyclic GMP formation induced by L-arginine. SOD (100 units/ml) did not prevent this inhibition and that of basal cyclic GMP formation, indicating that superoxide is not involved in this process. However, SOD (100 units/ml) increased the basal activity by 2-fold (Fig. 4a), similar to the findings of Gorsky *et al.* (1990). NH_2OH also inhibited guanylate cyclase activation by both L-arginine and sodium nitroprusside with equal potency (Fig. 5b). It is interesting to note that Deguchi *et al.* (1978) have reported that another hydroxylamine, namely N-methylhydroxylamine, also inhibits cyclic GMP formation induced by nitrosoguanidine. These findings suggest that NH_2OH directly inhibits the activation of guanylate cyclase by NO rather than interfering with the metabolism of L-arginine. However, the possibility also exists that NH_2OH might function as a scavenger for NO, since NO is a highly reactive chemical species. In support of this hypothesis, Deguchi *et al.* (1978) have also speculated that N-methylhydroxylamine inhibits the formation of cyclic GMP by scavenging NO. Finally, it appears that NH_2OH at 0.1 mM inhibited guanylate cyclase competitively, whereas at higher concentrations (0.25–1.0 mM) it inhibited the enzyme uncompetitively, since the inhibition was more apparent at higher concentrations of substrate (Fig. 6). Further testing of this hypothesis and the details of the mechanistic aspect of the inhibitory effects of NH_2OH should be investigated in future studies.

In summary, we have shown that NH_2OH is a strong activator of guanylate cyclase in intact cells, whereas in cytosol pre-

parations NH_2OH is an inhibitor of basal and L-arginine-induced cyclic GMP formation. The activation of guanylate cyclase by L-arginine in the cytosol preparations from N1E-115 cells requires the presence of NADPH and free Ca^{2+} . This biochemical pathway converting L-arginine into an activator of guanylate cyclase does not involve the formation of NH_2OH as a putative intermediate.

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