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Short communication

Minimal amidine structure for inhibition of nitric oxide biosynthesis

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Abstract

Pharmacological modulation of nitric oxide synthase activity has been achieved using structural analogs of arginine. In the present studies, we demonstrated that the minimal amidine structure required for enzymatic inhibition is formamidine. We found that the production of nitric oxide by primary cultures of rat hepatocytes and several mouse and human cell lines, including RAW 264.7 macrophages, PAM 212 keratinocytes, G8 myoblasts, S180 sarcoma, CX-1 human colon cells, and GH3 rat pituitary cells, was inhibited in a concentration- and time-dependent manner by formamidine. Formamidine was 2- to 6-fold more effective in inhibiting nitric oxide production in cells expressing inducible nitric oxide synthase (NOS2) than in a cell line expressing calcium-dependent neuronal nitric oxide synthase (NOS1). Whereas formamidine had no effect on γ-interferon-induced expression of nitric oxide synthase protein, its enzymatic activity was blocked. Kinetic analysis revealed that formamidine acts as a simple competitive inhibitor with respect to arginine (K_i formamidine ~ 800 μM). Using a polarographic microsensor to measure real-time flux of nitric oxide release from RAW 264.7 macrophages, formamidine was found to require 30–90 min to inhibit enzyme activity, suggesting that cellular uptake of the drug may limit its biological activity. Our data indicate that formamidine is an effective inhibitor of nitric oxide production. Furthermore, its low toxicity may make it useful as a potential therapeutic agent in diseases associated with the increased production of nitric oxide.

Keywords: Nitric oxide; Formamidine; Aminoguanidine; NOS2

1. Introduction

With the recognition that nitric oxide is a critical mediator of a variety of physiological processes and pathologic conditions, it is clear that manipulation of its production by pharmacological means would be of significant therapeutic benefit [1]. Nitric oxide is formed via the NADPH-dependent enzyme nitric oxide synthase, using L-arginine as the substrate [2]. The earliest inhibitors of the enzyme characterized were the N^{ω}-substituted L-arginines [1,3]. Non-amino acid inhibitors were based on extending the structural definition of arginine mimics to bioisosteres of the terminal guanidino group and included compounds such as *S*-alkyl isothioureas, isoselenoureas, and amidines [4–6]. Substituted amidines, including both linear and cyclic amidines, have been reported to be potent inhibitors of nitric oxide synthase, although many, but not all of these derivatives are unable to discriminate between the inducible and the constitutive forms of the enzyme [6–10]. In the present studies, we evaluated formamidine (Fig. 1), the minimal amidine structure, for activity as an inhibitor of nitric oxide formation in mammalian cells and as an inhibitor of nitric oxide synthase. We found that this arginine analog is an effective inhibitor of nitric oxide production in a variety of cell types and exhibits greater activity against cells expressing the inducible form of nitric oxide synthase (NOS2). Unlike the close structural analog aminoguanidine, a mechanism-based inhibitor of NOS2, formamidine, despite its small size, is an effective competitive inhibitor of the enzyme. In addition, formamidine does not function by inhibiting the uptake of arginine into cells, thus limiting its potential for impairing

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Abbreviations: NOS1, neuronal nitric oxide synthase; NOS2, inducible nitric oxide synthase; L-NMMA, N^{G}-monomethyl-L-arginine; and NIO, L-N^{5}-(iminoethyl)ornithine.

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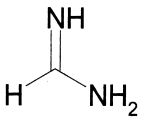
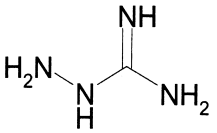
formamidine



aminoguanidine



L-arginine

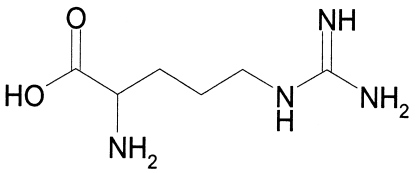


Fig. 1. Chemical structures of formamidine, aminoguanidine, and L-arginine. Comparison of the structure of formamidine with those of L-arginine, the natural substrate of nitric oxide synthase, and aminoguanidine, the well-characterized NOS2 inhibitor, reveals that formamidine is a structural approximation of the metabolically reactive end of arginine. Note that formamidine may also be viewed as a nor-azaguanidine.

other arginine-dependent processes. Although formamidine is not as potent as many other linear and cyclic amidines [7,8,11], it is efficacious and demonstrates very low cytotoxicity. Thus, this compound may be useful as a selective inhibitor of nitric oxide production *in vivo*.

2. Materials and methods

2.1. Chemicals

Dowex AG 50W-X8 resin and all chemicals, unless otherwise indicated, were purchased from the Sigma Chemical Co. L-[2,3,4,5-^{3}H]Arginine hydrochloride (sp. act. 42 Ci/mmol) was purchased from New England Nuclear. Recombinant mouse and human γ-interferons were provided by Dr. Sidney Pestka, UMDNJ-Robert Wood Johnson Medical School. Antibodies against inducible nitric oxide synthase were from Santa Cruz Biotechnology or Transduction Labs. Bay K-8644 was from Research Biochemicals International.

2.2. Cells and treatments

PAM 212 mouse keratinocytes were obtained as described previously [12]. CX-1 human colon cells were from the Cancer Institute of New Jersey. RAW 264.7 mouse macrophages and GH3 rat pituitary cells were provided by Dr. Donald Wolff, UMDNJ-Robert Wood Johnson Medical School. G8 mouse myoblasts and S180 mouse sarcoma cells were purchased from the American Type Culture Collection. Primary cultures of freshly isolated rat hepatocytes were prepared as previously described [13]. All cells were grown in phenol-free Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Culture medium for primary hepatocytes was also supplemented with 26 U/mL of insulin.

2.3. Assays for nitric oxide production, Western blotting, and arginine uptake

Nitric oxide biosynthesis was induced in GH3 cells following treatment with the potassium ionophore Bay K-8644 (2.8 μM, 8–24 hr) [14]. Induction of nitric oxide biosynthesis by all other cells was achieved following treatment with γ-interferon (100 U/mL, 24–48 hr). In addition to γ-interferon stimulation, hepatocytes were also treated with lipopolysaccharide (1 μg/mL). Nitric oxide production by cells was quantified spectrophotometrically by measuring the accumulation of nitrite in the culture medium using the Greiss reagent as previously described [15]. No cytotoxicity of formamidine was observed at concentrations up to 10 mM after incubation periods up to 48 hr, as determined by trypan blue dye exclusion and the ability of actively respiring cells to convert a tetrazolium compound into a colored formazan product, which was then detected spectrophotometrically (MTT assay, data not shown).

For western blotting, equal amounts of total cellular protein were electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking non-specific antibody binding, membranes were incubated with a 1:1000 dilution of antibodies to NOS2 followed by a 1:15,000 dilution of horseradish peroxidase conjugated secondary antibodies. A chemiluminescence-based detection kit was used to visualize the enzyme (ECL Western Blotting Kit, Amersham Life Sciences). Protein content in cell lysates was determined using the DC protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Uptake of [^{3}H]arginine into cells was measured as previously described [15].

2.4. Nitric oxide synthase enzyme assays

The production of L-citrulline by lysates of cytokine-stimulated RAW 264.7 macrophages was measured using a modification of the assay described by Bredt and Schmidt [16]. Briefly, in the presence or absence of various inhibitors, 100 μg of cell extract protein was added to a reaction buffer containing 25 mM Tris base (pH 7.4), 1 μCi/mL of [^{3}H]arginine, 10 μM unlabeled arginine, 3 μM tetrahydrobiopterin, 1 μM FAD, 1 μM FMN, and 1 mM NADPH in a total reaction volume of 1 mL. At specified times following addition of the enzyme, 100-μL aliquots were removed

Table 1
Effect of formamidine on nitric oxide production in various cell types

Cell type	Description	Enzyme	IC_{50} (10^{-3} M)
Hepatocytes	Rat primary	NOS2	1.6 ± 0.5
RAW 264.7	Mouse macrophage	NOS2	1.4 ± 0.4
PAM 212	Mouse keratinocyte	NOS2	1.1 ± 0.1
CX-1	Human adenocarcinoma of colon	NOS2	3.1 ± 1.0
S180	Mouse sarcoma	NOS2	1.4 ± 0.3
G8	Mouse myoblast	NOS2	1.3 ± 0.2
GH3	Rat pituitary	NOS1	5.9 ± 1.1

Cells were induced to produce nitric oxide as described in "Materials and methods". Each value represents the mean ± SEM of four different experiments.

and added to 4 mL of stop buffer comprised of 50 mM HEPES (pH 5.5). To measure total [^{3}H]citrulline formation, 1 mL of AG 50W-X8 cation exchange resin was then added to the stop buffer solution. After thorough mixing, the resin was allowed to settle (2–4 hr), and aliquots of the buffer containing [^{3}H]citrulline were then transferred to plastic scintillation vials and counted for radioactivity.

2.5. Measurement of nitric oxide flux from cells

A nitric oxide specific microelectrode was used to measure real-time flux of nitric oxide gas from cytokine-stimulated cells.[1] Before and after use, the electrode was calibrated using known concentrations of nitric oxide and tested for specificity with ascorbic acid. Voltages detected by the electrode increased linearly with increasing concentrations of nitric oxide.

3. Results and discussion

In initial studies, we examined the inhibitory effects of formamidine on nitric oxide production by cells expressing NOS1 or NOS2. We found that formamidine was active in blocking nitric oxide production in all cell types including primary cultures of rat hepatocytes (Table 1). Cells expressing NOS2 were 2–6 times more sensitive to nitric oxide inhibition by formamidine when compared with a cell line expressing NOS1 (IC_{50} values from ~1–3 mM vs ~6 mM for GH3 cells). Fig. 2A shows a typical concentration–response and time–course for inhibition of nitric oxide production by formamidine using RAW 264.7 macrophages. Washing out the formamidine rapidly restored nitric oxide production by the cells, indicating that the inhibition was reversible (data not shown). Formamidine had no effect on the expression of NOS2 in macrophages (Fig. 2B). Similar results were observed in primary cultures of rat hepatocytes (Fig. 2 and data not shown). Using a nitric oxide electrode, we found that the effects of formamidine on cellular nitric oxide production by the cells were rapid, and complete inhibition of nitric oxide flux was observed within 90 min (Fig. 2C).

The endogenous substrate for nitric oxide synthase is L-arginine. This amino acid is transported into cells via the y^+ basic amino acid transporter [17,18]. Several arginine analogs including L-NMMA, NIO, and nitroarginine compete for arginine uptake into cells. Decreased arginine uptake limits substrate availability for the enzyme, thus indirectly inhibiting the activity of nitric oxide synthase and other arginine-dependent processes [15]. Therefore, the ability of formamidine to compete with arginine for uptake into cells was examined as a potential mechanism of inhibition of nitric oxide production. We found that at concentrations that inhibited nitric oxide production, L-NMMA, but not formamidine, blocked arginine uptake into RAW 264.7 macrophages (Fig. 2D). A small increase in arginine uptake was observed at high concentrations of formamidine (10 mM), possibly due to trans-stimulation mechanisms [17]. Taken together, our data demonstrate that formamidine inhibits nitric oxide production by cells without blocking arginine uptake or altering the expression of nitric oxide synthase.

The finding that relatively high concentrations of formamidine (3–10 mM) were required to inhibit nitric oxide production *in vitro* would not be expected to limit the use of the drug *in vivo*. Millimolar concentrations of inhibitors of nitric oxide synthase are needed to block nitric oxide production in almost all cell culture studies, despite the fact that the reported IC_{50} values for nitric oxide synthase inhibition using isolated enzymes are in the micromolar concentration range [8,10,19]. Experimental animals were found to tolerate very high concentrations of formamidine (LD_{50} in mice >0.2 g/kg, unpublished observations), presumably because its minimal structure reduces the number of metabolic processes it can inhibit, and this drug is predicted to have a high therapeutic index. In this regard, a dose of 0.1 g/kg of 2-iminopiperidine, a cyclic amidine, is required to completely suppress plasma nitrite/nitrate in a rat model of LPS-induced endotoxemia, despite the fact that the ic_{50} for inhibition of human inducible nitric oxide synthase enzyme activity is ~1 μM [8,10].

In further studies, we examined the ability of formamidine to inhibit nitric oxide synthase activity as measured by the formation of L-citrulline from L-arginine. Formamidine was found to readily block enzymatic activity in a concentration-dependent manner (Fig. 3, A and C). This inhibitory activity remained constant with time up to at least 80 min (Fig. 3B). The production of citrulline from arginine in the presence or absence of formamidine followed Michaelis–Menten kinetics (Fig. 3C). Lineweaver–Burk analysis (Fig. 3D) revealed that formamidine acts as a simple competitive inhibitor of nitric oxide synthase with respect to the arginine

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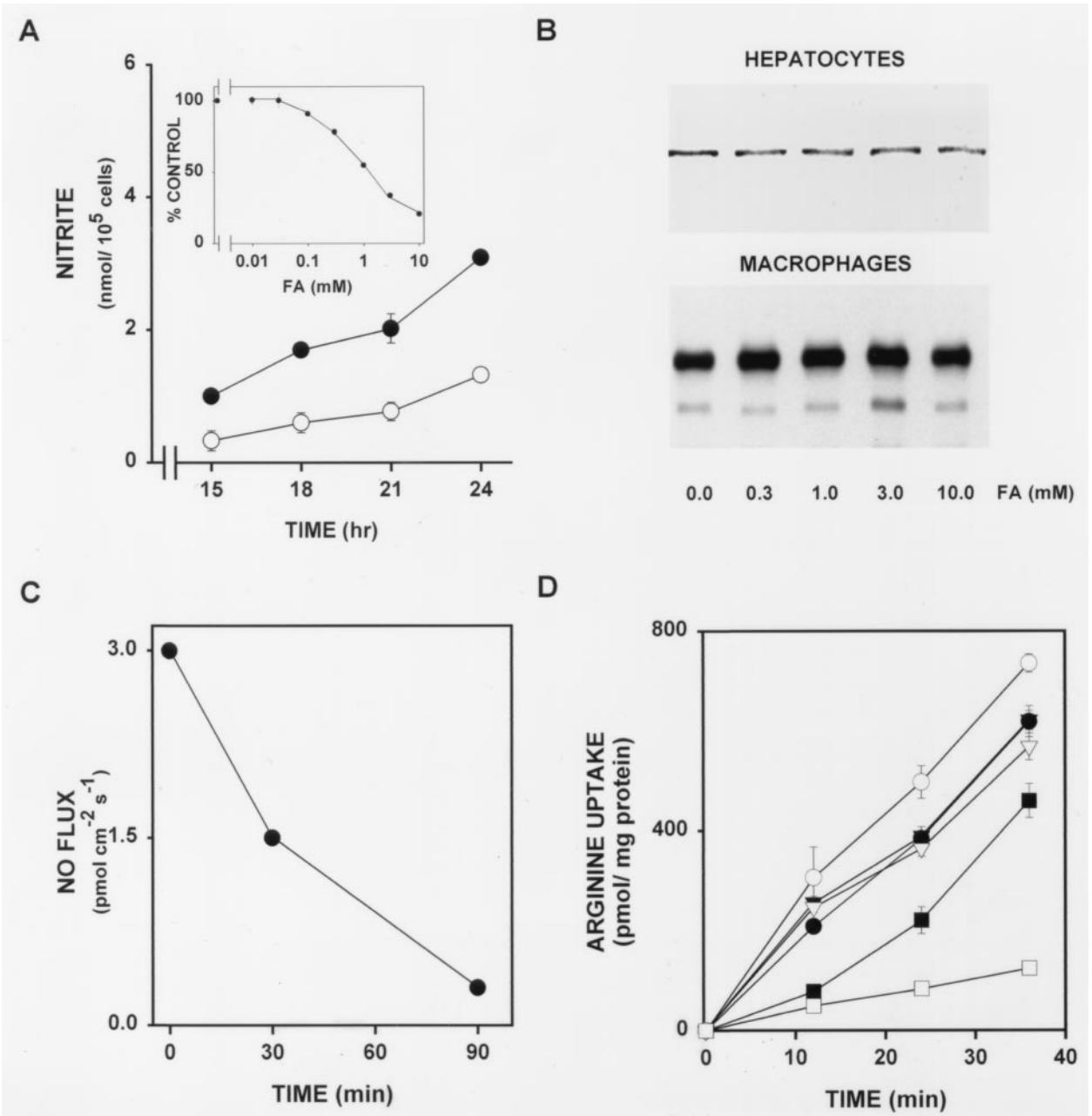

Fig. 2. Effects of formamidine on nitric oxide production and arginine uptake. Cells were stimulated to produce nitric oxide as described in "Materials and methods." (A) Time-dependent generation of nitrite by RAW 264.7 macrophages in the presence (○) or absence (●) of 1 mM formamidine. *Inset*: Concentration-dependent inhibition of nitrite accumulation from macrophages by formamidine (FA). Control value (100%) = 3.09 nmol/10^5 cells. (B) Representative western blots showing NOS2 expression in extracts of stimulated hepatocytes and RAW 264.7 macrophages treated without or with increasing concentrations of formamidine. The relative molecular weight (M_r) of NOS2 was 130,000 in both the hepatocyte and macrophage blots. The second lower band in the macrophage blot is a degradation product. (C) Time-dependent inhibition of nitric oxide flux by formamidine (10 mM). (D) [^{3}H]Arginine uptake in the absence (●) or presence of 1.0 (▽), 3.0 (▼), or 10.0 (○) mM formamidine, 1.0 mM L-NMMA (■), or 0.5 mM unlabeled arginine (□). In panels A, C and D, data represent the means ± SEM of triplicate samples. Note that for some points the SEM fell within the symbols on the graph.

substrate: V_{max} ~ 200 pmol/min/mg protein, K_m (arginine) ~ 10 μM, and K_i (formamidine) ~ 800 μM. Thus, both cyclic and linear amidines appear to act by similar mechanisms [8,11].

Non-amino acid mimics of arginine that are inhibitors of nitric oxide synthase have various mechanisms of action. For example, whereas aminoguanidine acts as a mechanism-based inhibitor, both linear and cyclic amidines have been reported to be competitive inhibitors of the enzyme [5–8]. Aminoguanidine acts via multiple pathways including covalent modification of inducible nitric oxide synthase protein as well as the heme residue at the active site [20]. In contrast, formamidine appears to act by reversibly competing with the guanidino portion of arginine for binding to

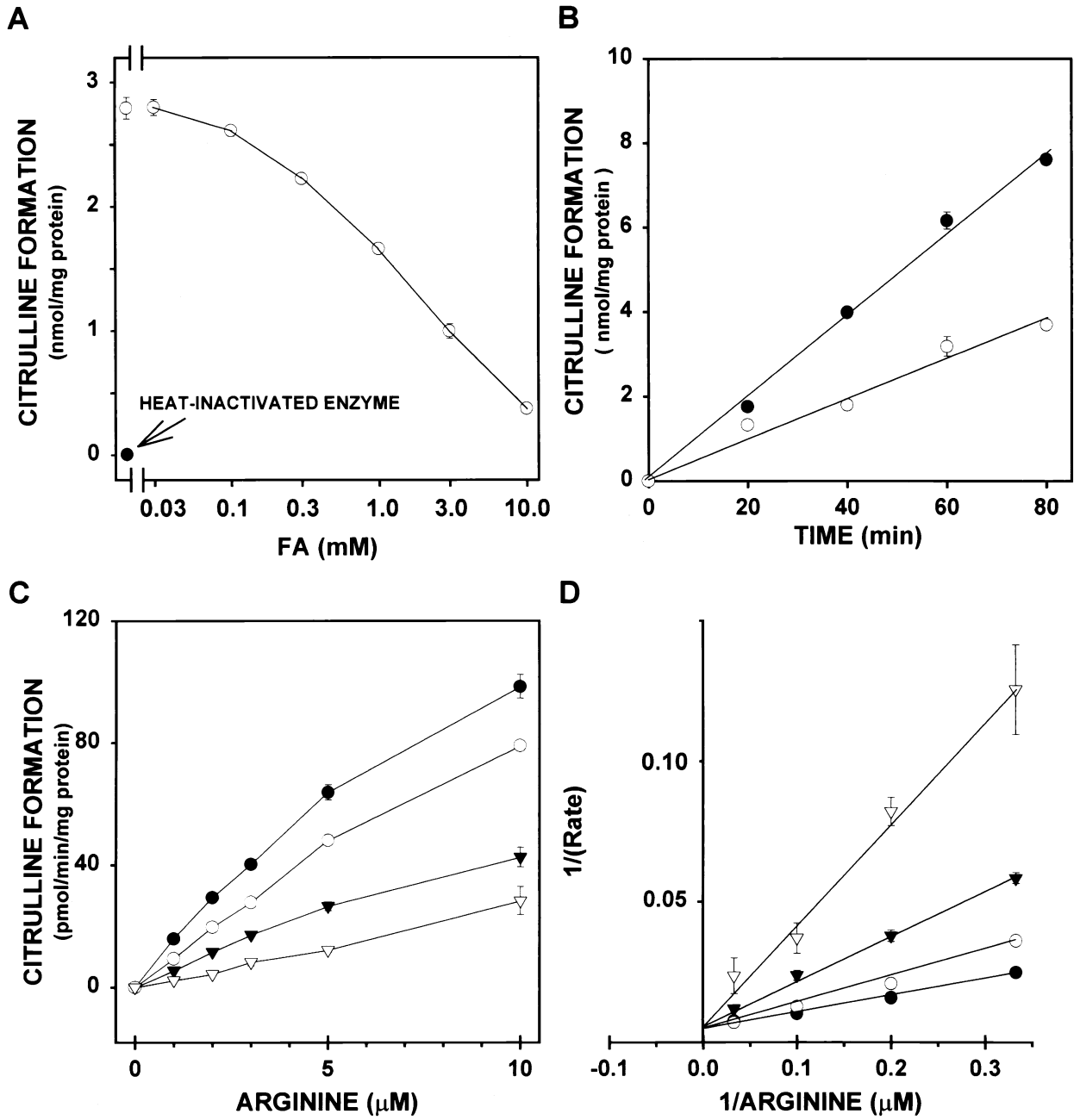

Fig. 3. Effects of formamidine on nitric oxide synthase activity. Nitric oxide synthase activity was assayed in the presence or absence of formamidine. (A) Concentration-dependent inhibition of NOS2 activity by formamidine (FA). NOS2 activity was measured after 60 min. Note that heat inactivation of the enzyme completely suppressed citrulline formation. (B) Time-dependent inhibition of NOS activity in the absence (●) or presence (○) of 1 mM formamidine. (C) Rate of citrulline production in the absence (●) or presence of 1.0 (○), 3.0 (▼), or 10.0 (▽) mM formamidine. (D) Lineweaver–Burk analysis of citrulline formation in the absence (●) or presence of 1.0 (○), 3.0 (▼), or 10.0 (▽) mM formamidine. Values in panels A-D are the means ± SEM of triplicate samples.

nitric oxide synthase. Apparently, a mimic for this site on the enzyme is sufficient to inhibit enzymatic activity, a situation analogous to the development of inhibitors of acetylcholinesterase [21]. One can speculate that binding of other linear and cyclic amidines is also selective for this region of the enzyme, and this remains to be determined. Compounds such as formamidine will be important for understanding the precise structural features of the nitric oxide synthases that are targeted by distinct classes of inhibitors.

Acknowledgments

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