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Inactivation of nitric oxide synthases and cellular nitric oxide formation by N^6-iminoethyl-L-lysine and N^5-iminoethyl-L-ornithine

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Abstract

The kinetics of inactivation of affinity-purified nitric oxide synthase isoforms by N^6-iminoethyl-L-lysine (NIL) and N^5-iminoethyl-L-ornithine (NIO) has been examined. Each of the agents produced a time and concentration dependent first order inactivation of the nitric oxide synthase isoforms that required exposure of the NO synthase to drug under conditions that supported catalysis, consistent with the proposal that these agents act as alternate substrate, mechanism-based inactivators. As measured at 100 μM arginine, NIL and NIO were equally efficient as inactivators of the cytokine-inducible nitric oxide synthase exhibiting apparent second order inactivation rate constants of 31.5 and 32.0 mM^{-1} min^{-1} respectively. By contrast, NIL and NIO were less efficient as inactivators of the constitutive neuronal nitric oxide synthase isoform exhibiting apparent second order inactivation rate constants of 0.79 and 8.4 mM^{-1} min^{-1} respectively. As measured at 100 μM extracellular arginine, NIL and NIO produced a time and concentration dependent inactivation of the NO synthetic capability of cytokine-induced murine macrophage RAW 264.7 cells exhibiting apparent second order inactivation rate constants of 3.1 and 1.8 mM^{-1} min^{-1}. The inactivated RAW cell NO synthetic capability was restored to 30% of its pretreatment value over a 3-h period despite the presence of cycloheximide.

Keywords: N^6-Iminoethyl-L-lysine; N^5-Iminoethyl-L-ornithine; Kinetics; Mechanism-based inactivation; Nitric oxide (NO) synthesis

1. Introduction

Nitric oxide is a free radical gas that readily crosses cell membranes and is able to exert biological actions both in the cell of synthetic origin and in neighboring cells (Nathan, 1992). Nitric oxide plays a role as a neurotransmitter in the central and peripheral nervous system (Bredt and Snyder, 1994); is a regulator of blood pressure and blood flow (Rees et al., 1989); and provides a defense mechanism in the non-specific efferent arm of the immune system (MacMicking et al., 1997). Under selected pathophysiological circumstances overproduction of nitric oxide synthesized by one or a combination of nitric oxide synthase isoforms exerts harmful effects. Nitric oxide synthase exists as three isoforms termed either I, II or III (based on the order of their successful cloning) or as neuronal, inducible and endothelial NO synthases.

The overproduction of nitric oxide implicated in the pathology of septic and hemorrhagic shock (Szabo, 1995; Thiemermann et al., 1993) as well as autoimmune disorders (Weinberg et al., 1994; Connor et al., 1995) is generated by the cytokine-inducible nitric oxide synthase isoform. The overproduction of nitric oxide implicated in ischemia-reperfusion injury following stroke is generated initially by the neuronal nitric oxide synthase isoform and subsequently by the inducible isoform (Samdani et al., 1997). It is thus a desirable goal to develop inhibitors selective for the inducible and neuronal NO synthase isoforms that spare the endothelial isoform since inhibition of the latter produces hypertension (Moncada and Higgs, 1995) and potentially adversely affects memory consolidation (Bohme et al., 1993). Thus to be useful therapeutic agents these inhibitors will need to be isoform selective, cell permeable and non toxic in vivo.

Accordingly during the past several years many laboratories including our own have directed their attention to examining at the cellular and molecular level the properties and mechanisms of agents that act as inhibitors or

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inactivators of nitric oxide synthase isoforms (Griffith and Stuehr, 1995). Among the agents that have been studied are the arginine-mimics N^5-iminoethyl-L-ornithine (NIO) and N^6-iminoethyl-L-lysine (NIL). Initial studies of NIO identified that this agent inhibits NO production by endothelial cells (Rees et al., 1990) and by the cytokine-induced murine macrophage cell-line J774 (McCall et al., 1991). In these studies the effects of NIO could be prevented by the presence of increased arginine concentrations but were not reversible subsequent to treatment. More recent studies have examined the properties of the structurally-related agent NIL and have identified that this agent exhibits selectivity as an inhibitor of the inducible as compared to the neuronal and endothelial NO synthase isoforms (Stenger et al., 1995; Moore et al., 1996). In these studies the effects of NIL and NIO on enzyme activity in incubations were conducted for fixed times and did not explore issues of mechanism or examine time-dependent changes of enzyme activity in the presence of these agents. We were interested in extending the current understanding of the actions of NIO and NIL by exploring the kinetic basis for their action and their apparent 'irreversibility'. This manuscript identifies that similarly to aminoguanidine (Wolff and Lubeskie, 1995), NIL and NIO act as alternate substrate, mechanism based inactivators of nitric oxide synthases and characterizes both in intact cells and with isolated affinity-purified enzyme the kinetic basis for the isoform selectivity of NIL.

2. Materials and methods

2.1. Materials

NIO and bovine hemoglobin were obtained from Sigma (St. Louis, MO). NIL was obtained from Alexis, San Diego, CA. Oxyhemoglobin was prepared and freed of methemoglobin contamination as described previously (Wolff et al., 1997). All other reagents were obtained as described previously (Wolff et al., 1997).

2.2. Preparation and characterization of nitric oxide synthase isoforms

Ca^{2+}-calmodulin-dependent nitric oxide synthase was prepared from GH_3 cell extracts by adsorption to ADP-agarose and elution with NADPH and was characterized as described previously (Wolff and Datto, 1992). A typical preparation of GH_3 nitric oxide synthase exhibited a specific activity of approximately 0.6 μmol of citrulline formed/min mg at saturating concentrations of arginine and co-factors and was stable to storage at -70°C for periods up to 4 months. The GH_3 nitric oxide synthase is identical physically, kinetically and immunologically to the bovine brain nitric oxide synthase (Wolff and Datto, 1992) but routinely contains substoichiometric quantities (0.15 mol/mol) of bound (6*R*)-5,6,7,8 tetrahydro-L-biopterin (BH_4) such that it commonly displays a 6- to 10-fold stimulation by addition of exogenous BH_4.

Interferon-γ-inducible nitric oxide synthase from murine macrophages was prepared from cultured RAW 264.7 cells by adsorption to ADP-agarose and elution with NADPH and was characterized as described previously (Wolff and Gribin, 1994). A typical preparation exhibited a specific activity of 0.3 to 0.8 μmol of citrulline formed/min mg as measured at saturating concentrations of arginine and co-factors and lost less than 50% of activity when stored at -70° for periods up to 9 months.

Bovine arterial endothelial NO synthase was prepared and characterized as described previously (Wolff et al., 1994). These preparations of endothelial NOS routinely possessed a specific activity of 0.2 ± 0.1 μmol citrulline formed/min mg protein when assayed at saturating substrate and co-factor concentrations. As measured by formation of citrulline, the enzyme exhibited a 20-fold stimulation by the concurrent presence of Ca^{2+} and calmodulin, a K_m for arginine of 5 μM, and a K_{act} for BH_4 of 200 nM. Diverse preparations of enzyme apparently contained somewhat variable quantities of co-purified bound BH_4 co-factor as evidenced by stimulations by added co-factor that varied from 2- to 6-fold.

2.3. Assay of nitric oxide synthase activity by citrulline formation

Nitric oxide synthase activity was measured by a modification of the procedure of Bredt and Snyder (1990), as described previously (Wolff and Datto, 1992). Standard incubations for the measurement of citrulline formation by either endothelial constitutive nitric oxide synthase or GH_3 constitutive nitric oxide synthase contained 30 mM *N*-[2-hydroxyethyl]piperaine-*N*′[2-ethanesulfonic acid), (HEPES), pH 7.4, 1 mM dithiothreitol, 120 nM [^{3}H]-arginine (a sub-saturating concentration), 1 mM EGTA, 0.85 mM Ca^{2+}, calmodulin 6 μM, 100 μM NADPH and 100 μM BH_4. Standard incubations for the measurement of citrulline formation by the interferon-γ-inducible macrophage nitric oxide synthase contained 30 mM HEPES, pH 7.4, 1 mM dithiothreitol, 120 nM ^{3}H-arginine, 1 mM EGTA, 100 μM NADPH and 300 μM BH_4. Incubations were conducted at 30°C for 30 min in duplicate and the mean values were calculated. Variability of values about the mean routinely averaged ± 3% of the mean. Routinely assays were conducted at dilutions of enzyme that provided 5–10% of total substrate consumption. At these conditions of measurement product formation was linear over time.

2.4. Growth of, and measurement of nitric oxide formation by, cytokine-induced murine RAW 264.7 cells

Murine RAW 264.7 cells were grown to confluency in six-welled (9 cm^2) polystyrene dishes in 3 ml of Dul-

becco's modified Eagle's medium supplemented with 10% fetal bovine serum. Nitric oxide synthase was induced by treating the cells with 75 units murine interferon-γ/25 μg lipopolysaccharide for 16 h. Growth medium was removed and replaced with 4 ml of modified Ham's F-10 containing either 100 μM (physiological conditions) or 1 mM arginine (maximal rate) and 5 μM oxyhemoglobin. The release of NO from the cells was assessed by measuring the formation of methemoglobin as the absorbance difference at 401 (absorbance maximum) and 411 nm (isosbestic point) over time as described by Noack et al. (1992). The nanomoles of NO formed was calculated using an extinction coefficient for methemoglobin of 38 mM^{-1} cm^{-1}. When NO formation was measured in the presence of drug or by drug-treated cells, rates were adjusted for interference generated from the slow autoxidation of oxyhemoglobin.

2.5. Assay of the NO synthase catalyzed formation of NO by measurement of conversion of oxyhemoglobin to methemoglobin

Standard reaction mixtures were constructed in 1 ml disposable polystyrene cuvettes containing 50 mM HEPES, pH 7.4, 100 μM NADPH, 6 μM calmodulin, 1 mM EGTA, when present 0.85 mM Ca^{2+}, 0.5 μM BH_4, 5 μM oxyhemoglobin, and unless otherwise indicated 100 μM arginine. Reactions mixtures were added to both a sample and a reference cuvette and the instrument zeroed at 401 nm. Reactions were initiated by addition of NO synthase enzyme source to the sample cuvette and time dependent formation of methemoglobin measured at 401 nm. NO formation was calculated using the known extinction coefficient for methemoglobin.

2.6. Miscellaneous procedures

Protein was determined by the method of Bradford (1976) with bovine serum albumin as the reference standard.

3. Results

3.1. Inactivation of the nitric oxide synthase activity of neuronal NO synthase by NIL and NIO

In incubations measuring nitric oxide formation by neuronal NO synthase (Fig. 1) it was observed that the concurrent addition of NIL and Ca^{2+} resulted in a time dependent loss of NO synthetic activity. This loss of activity was not due to the time-dependent accumulation in solution of an inhibitor of NOS since the addition of a second aliquot of neuronal NO synthase was initially as fully active as the first aliquot and underwent an identical time-dependent loss of activity as had been observed when

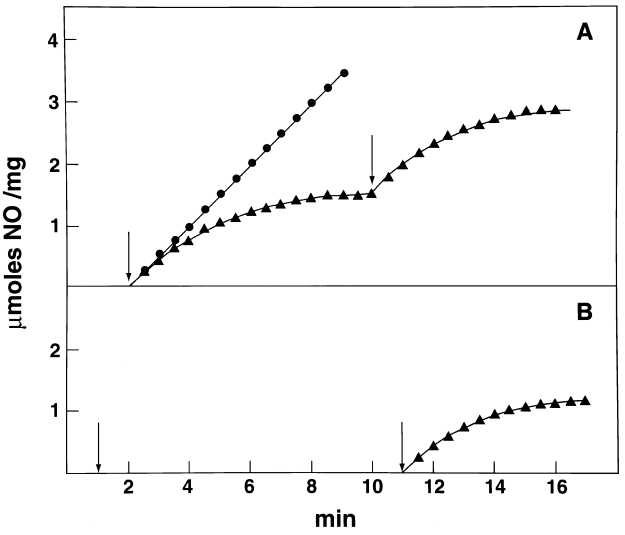

Fig. 1. The effect of NIL on NO formation by GH_3 pituitary neuronal NO synthase. Panel A: Standard incubations were constructed in 1 ml polystyrene cuvettes containing 50 mM HEPES, pH 7.4, 100 μM NADPH, 6 μM calmodulin, 1 mM EGTA, 100 μM arginine and 5 μM oxyhemoglobin. The reaction was initiated at zero time with 14 μg of affinity-purified neuronal NO synthase. At 2 min post initiation, the incubation was adjusted to contain 0.85 mM Ca^{2+}, either alone (●) or in combination with 1 mM NIL (▲). At 11 min post initiation, a second 14 μg portion of neuronal NO synthase was added to the NIL-containing incubation. Panel B: A standard incubation was constructed as described for Panel A containing 14 μg of neuronal NO synthase added at zero time and adjusted to contain 1 mM NIL at 1 min. At 11 post initiation the incubation was adjusted to contain 0.85 mM Ca^{2+}. Throughout NO-generated formation of methemoglobin was measured as the increase in light absorbance at 401 nm.

the first aliquot of nNOS had been exposed to NIL and Ca^{2+} concurrently. At a concentration of 100 μM arginine, 1 mM NIL produced a 50% loss of NO synthetic activity in 1.6 min and a complete loss of activity within 6 min. In order to explore the issue of whether the inactivation of nNOS by NIL occurred because NIL served as an alternate substrate, mechanism-based inactivator converted during catalytic turnover to a 'suicide' intermediate, we exposed the neuronal NO synthase (Fig. 1, Panel B) to 1 mM NIL for 10 min under conditions identical to those described for Panel A but without the Ca^{2+} necessary to convert neuronal NO synthase to its catalytically active form. After 10 min exposure to NIL, neuronal NO synthase was activated by the addition of Ca^{2+}. Upon addition of Ca^{2+}, NO synthesis occurred at a rate identical to neuronal NO synthase never exposed to NIL. Thus, no inactivation of nNOS by NIL occurred unless and until the enzyme was converted to a catalytically active form by addition of Ca^{2+}. A time and Ca^{2+}-dependent loss of neuronal NO synthase activity was also observed when NIO was examined in an identical paradigm, however the loss of activity occurred more rapidly and at a lower concentration (not shown). In further experiments, preincubation of either neuronal NO synthase with either NIL or NIO under anaerobic conditions but otherwise containing

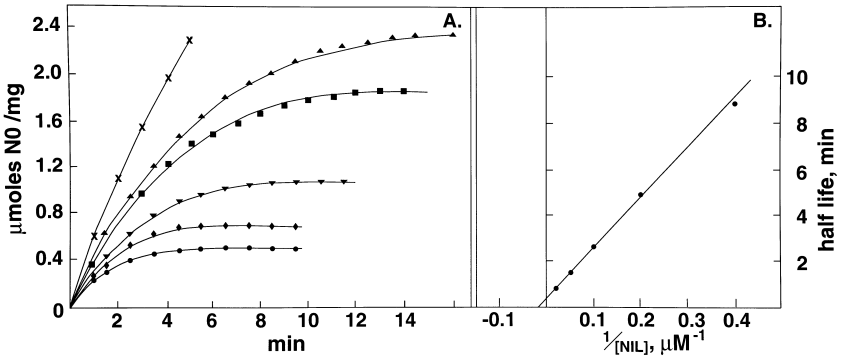

Fig. 2. The effect of NIL concentration on time-dependent NO formation by ADP-agarose purified RAW 264.7 inducible NO synthase. Panel A: Standard incubations were constructed in 1 ml polystyrene cuvettes containing 50 mM HEPES, pH 7.4, 100 μM NADPH, 1 mM EGTA, 2 μM BH_4, 5 μM oxyhemoglobin and 10 μM arginine without (X) or containing 2.5 (▲), 5 (■), 10 (▼), 20 (♦) or 50 (●) μM NIL. Reactions were initiated with 2.3 μg of ADP-agarose purified inducible NO synthase and methemoglobin formation was assessed by measuring continuously the increase in absorbancy at 401 nm. NO formation rate was calculated using the known extinction coefficient for methemoglobin. Panel B: The half time of inactivation of NO forming activity is plotted vs. the reciprocal of the NIL concentration in Kitz–Wilson format.

the co-factors necessary to support catalytic activity, prevented inactivation by these agents. The requirement for a catalytically active form of neuronal NO synthase in the presence of oxygen in order to observe its inactivation by either NIL or NIO, supports the proposal that these agents act as alternate substrate, mechanism based inactivators of nitric oxide synthase.

3.2. Determination of the kinetic constants for inactivation of inducible NO synthase and neuronal NO synthase by NIL and NIO

Since our observations (Fig. 1) had indicated that NIL and NIO were mechanism-based inactivators producing inhibitions of NO synthase that progress with time of incubation, we undertook to measure the time and concentration dependence of the inhibition of NO formation using a continuous spectrophotometric assay measuring the NO-mediated conversion of oxyhemoglobin to methemoglobin. Incubations were conducted at 100 μM arginine, a saturating concentration of substrate comparable to that found in normal human extracellular fluid (Moncada and Higgs, 1995; Meyer et al., 1997). The NO formation rate by inducible NO synthase was measured in incubations without drug or at concentrations of NIL ranging from 2.5 to 50 μM (Fig. 2). A time and concentration dependent loss of NO synthetic rate was observed. The half time of inactivation was determined at each NIL concentration as the time required for the initial rate (first 20 s) to decline to a rate one-half this initial rate. The half times of inactivation were plotted in Kitz–Wilson format (Kitz and Wilson, 1962) vs. the reciprocal of the inactivating concentration of NIL. A maximal inactivation rate of 1.73 min^{-1} = 0.029 s^{-1} was determined from the ordinal intercept ($k_{inact\ max} = 0.693/t_{1/2}$) while the concentration of NIL providing the half maximal inactivation rate (55 μM) was

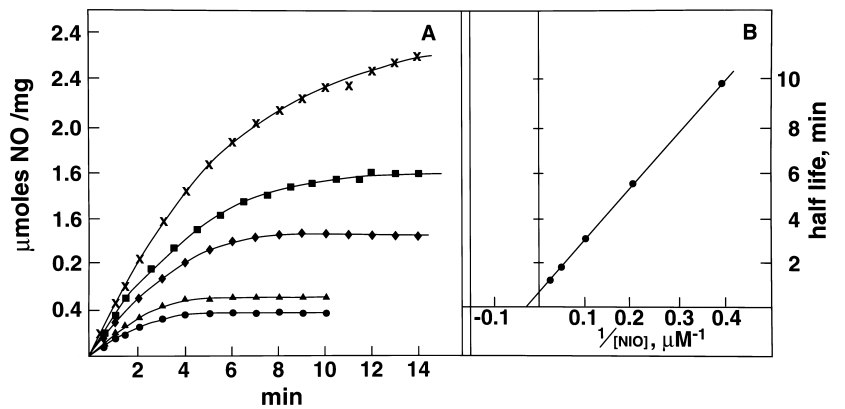

Fig. 3. The effect of NIO concentration on time dependent NO formation by ADP-agarose purified RAW 264.7 inducible NO synthase. Panel A: Standard incubations were constructed as described in the legend to Fig. 5 containing 2.5 (X), 5 (■), 10 (♦), 20 (▲) or 50 (●) μM NIO. Reactions were initiated with 2.3 μg of ADP-agarose purified inducible NO synthase and methemoglobin formation was assessed by measuring continuously the increase in absorbance at 401 nm. Panel B: The half time of inactivation of NO forming activity is plotted vs. the reciprocal of the NIO concentration.

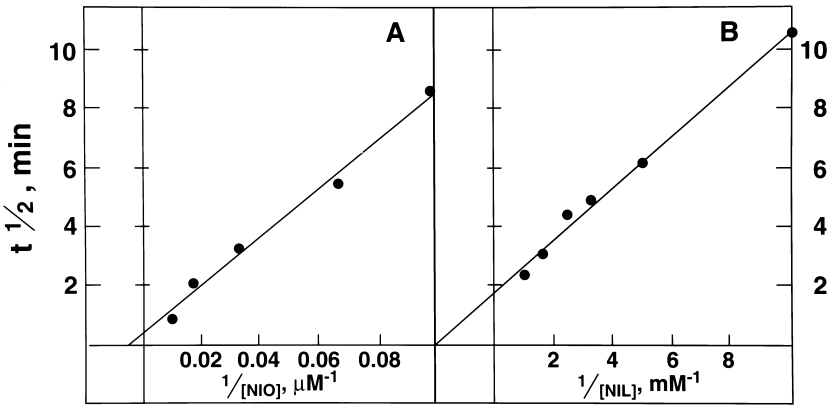

Fig. 4. The effect of NIO and NIL on the time dependent inactivation of GH_3 pituitary neuronal NO synthase. Standard incubations were constructed in 1 ml polystyrene cuvettes containing 50 mM HEPES, pH 7.4, 100 μM NADPH, 6 μM calmodulin, 1 mM EGTA, 0.85 mM Ca^{2+}, 100 μM arginine and 5 μM oxyhemoglobin without or containing either NIO at concentrations ranging from 10 to 100 μM (as indicated in Panel A) or NIL at concentrations ranging from 100 to 1000 μM (as indicated in Panel B). Reactions were initiated with 14 μg of affinity-purified GH_3 neuronal NO synthase and NO formation was assessed at 401 nm continuously over a 30-min period. The half times of inactivation were determined as previously (Figs. 2 and 3) and are plotted in Kitz–Wilson format.

determined from the abscissal intercept ($=-1/K_i$). From these data the apparent (determined at 100 μM arginine) second order rate constant for inactivation ($k_{\text{inact max}}/K_i$) was calculated to be 31.5 mM^{-1} min^{-1}. The second order rate constant for inactivation is a formal measure of the efficiency of a mechanism-based inactivator (Bandyapadhyah et al., 1993; Fitzpatrick and Villafranca, 1986; Rando, 1984; Silverman, 1988). In a paradigm identical to that used for NIL, the effects of concentrations of NIO ranging from 2.5 to 50 μM on NO formation rate by inducible NO synthase was determined (Fig. 3). In the presence of NIO, a time and concentration dependent inactivation of inducible NO synthase activity was observed exhibiting a $k_{\text{inact max}}$ of 1.44 $min^{-1} = 0.024$ s^{-1} and a K_i of 45 μM. From these data an apparent second order rate constant for inactivation of 32.0 mM^{-1} min^{-1} was calculated.

In order to allow an assessment of the relative efficiency of NIL and NIO as inactivators of neuronal NO synthase as compared to inducible NO synthase we examined the kinetics of their inactivation of the neuronal NO synthase isoform under conditions identical to those described above for inducible NO synthase. In incubations containing 100 μM arginine and NIL at concentrations ranging from 100 to 1000 μM, a time and concentration dependent inactivation of neuronal NO synthase was observed and the data were plotted in Kitz–Wilson format (Fig. 4, Panel B). A maximal inactivation rate of 0.45 min^{-1} was observed with a K_i of 570 μM NIL. From these data an apparent second order rate constant for inactivation of 0.79 mM^{-1} min^{-1} was calculated. Similarly, in incubations containing 100 μM arginine and concentrations of NIO ranging from 10 to 100 μM, a time and concentration dependent inactivation of neuronal NO synthase was observed and provided a linear Kitz–Wilson plot (Fig. 4, Panel A). A maximal inactivation rate of 2.1 $min^{-1} = 0.035$ s^{-1} was observed with a K_i of 250 μM. From these data an apparent second order rate constant for inactivation of 8.4 mM^{-1} min^{-1} was calculated.

3.3. Determination of the nitric oxide synthase isoform selectivity of NIL and NIO in fixed-time incubations

The studies described above allowed calculation of the second order rate constant for inactivation of the neuronal NO synthase and inducible NO synthase isoforms by NIL and NIO and provided a formal measure of the relative efficiency of these drugs as inactivators of the NO syn-

Table 1
Determination of the IC_{50} values for NIL and NIO vs. the three nitric oxide synthase isoforms

Compound	Inducible NO synthase (μM)	Neuronal NO synthase (μM)	Endothelial NO synthase (μM)
NIL	0.5	7.0	8.0
NIO	0.3	0.3	0.08
Aminoguanidine	5.0	41.0	255.0

Standard incubations were constructed for citrulline formation containing 120 nM arginine as described in Section 2 in the absence and presence of the indicated agents at concentrations ranging from 10 nM to 10 mM. The concentration of agent providing a 50% inhibition of activity in a 30-min incubation is indicated above. Incubations were initiated with an enzyme dilution that provided less than 10% consumption of arginine substrate as measured in the absence of added agent.

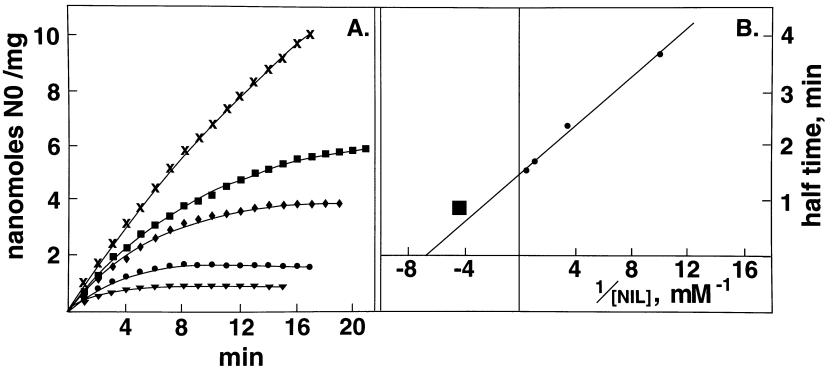

Fig. 5. The effect of NIL concentration on time dependent NO formation by cytokine-induced RAW 264.7 cells. Panel A: Cells grown to confluency in 6-welled (9 cm^2) polystyrene dishes were treated with interferon-γ/LPS for 16 h and the medium was replaced with modified Ham's F-10 containing 100 μM arginine and 5 μM oxyhemoglobin without further additions (X) or with 100 (■), 300 (♦), 1000 (●), or 3000 μM (▼) NIL. Samples were withdrawn at 1-min intervals and methemoglobin formation was assessed as the absorbance difference measured at 401 nm as compared to 411 nm (the isosbestic point). The amount of NO formed was calculated following adjustment for time dependent oxyhemoglobin autoxidation using an extinction coefficient of 38 mM^{-1} cm^{-1} and is expressed as nmol NO formed/min mg. Panel B: the half times of inactivation of NO forming capability are plotted vs. the reciprocal of the NIL concentration in Kitz–Wilson format.

thase isoforms. We were interested in extending these studies to the endothelial isoform which is difficult to purify from natural sources in the quantities necessary for detailed kinetic studies. In order to obtain a measure of inhibitory effectiveness that allows comparison of all three isoforms and to allow comparison of our studies to studies already available in the literature for the human NO synthase isoforms, we measured the IC_{50} values for NIL and NIO vs. the three NO synthase isoforms murine inducible, rat neuronal and bovine endothelial in fixed time (30 min) incubations (Table 1). NIL was found to be most potent in inhibiting the inducible isoform exhibiting an IC_{50} value (as measured at 120 nM arginine) of 0.5 μM, a value 16-fold and 14-fold lower than that observed for the endothelial and neuronal NO synthase isoforms respectively. NIO was found to be most potent in inhibiting the endothelial isoform with an IC_{50} value of 0.08 μM, a value approximately 4-fold lower than that observed for the inducible and neuronal isoforms.

3.4. Inactivation by and recovery from NIL and NIO in cytokine-induced RAW 264.7 cells

NO formation by cytokine-induced RAW 264.7 cells was measured in confluent 6-well plates as the ability of NO released from cells to convert oxyhemoglobin to methemoglobin measured spectrophotometrically. NO formation was measured in Ham's F-10 medium containing 100 μM arginine (its normal extracellular concentration) without or containing NIL at concentrations ranging from 25 to 3000 μM. A rapid, time and concentration dependent loss of NO synthetic rate was observed (Fig. 5, Panel A). The

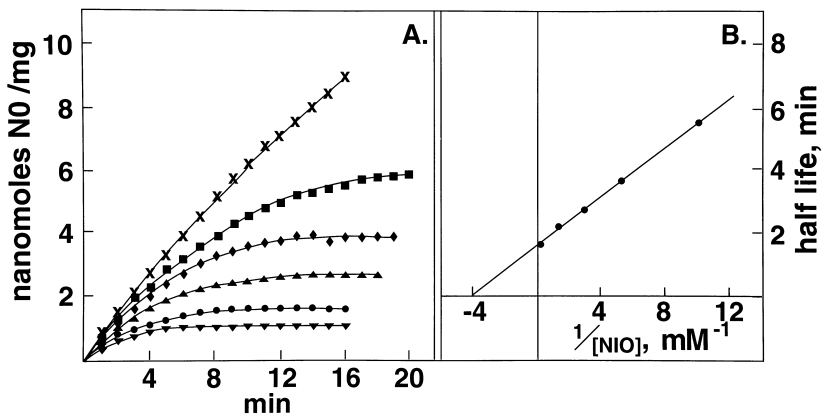

Fig. 6. The effect of NIO concentration on time-dependent NO formation by cytokine-induced RAW 264.7 cells. Cells grown to confluency in 6-welled polystyrene plates were treated with interferon-γ/LPS as described in Section 2 for 16 h and the medium was replaced with modified Ham's F-10 containing 100 μM arginine and 5 μM oxyhemoglobin without further additions (X) or containing 100 (■), 200 (♦), 300 (▲), 750 (●) or 3000 (▼) μM NIO. Samples were withdrawn at 1-min intervals and methemoglobin formation was assessed as the difference in absorbance at 401 nm relative to 411 nm (isosbestic point). The amount of NO formed was calculated as described previously. Panel B: The half times of inactivation of NO forming capability are plotted vs. the reciprocal of the NIO concentration in Kitz–Wilson format.

Table 2
Kinetic constants for the mechanism-based inactivation by NIL and NIO of the inducible and neuronal NO synthase isoforms and of NO synthesis in cytokine-induced RAW 264.7 cells

Compound	Inducible NO synthase			Neuronal NO synthase			RAW 264.7 cells		
	K_i (mM)	$k_{inact\ max}$ (min^{-1})	$k_{inact\ max}/K_i$ ($mM^{-1}\ min^{-1}$)	K_i (mM)	$k_{inact\ max}$ (min^{-1})	$k_{inact\ max}/K_i$ ($mM^{-1}\ min^{-1}$)	K_i (mM)	$k_{inact\ max}$ (min^{-1})	$k_{inact\ max}/K_i$ ($mM^{-1}\ min^{-1}$)
NIL	0.055	1.73	31.5	0.57	0.45	0.79	0.16	0.49	3.06
NIO	0.045	1.44	32.0	0.25	2.1	8.4	0.25	0.45	1.80

half times of inactivation were determined at each NIL concentration and were plotted in Kitz–Wilson format (Fig. 5, Panel B). A linear Kitz–Wilson plot was obtained with a $k_{inact\ max}$ of 0.49 min^{-1} and a K_i of 160 μM. From these data an apparent second order rate constant for inactivation of 3.1 $mM^{-1}\ min^{-1}$ was calculated.

The effect of NIO on NO formation by cytokine-induced RAW 264.7 cells was also measured in incubations

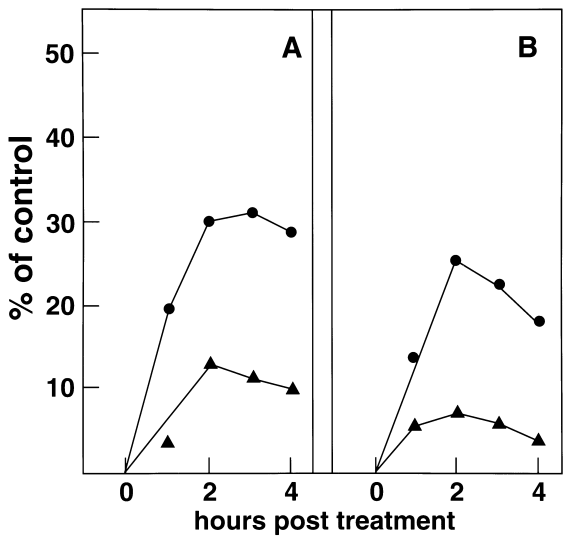

Fig. 7. The recovery of NO synthetic capability and inducible NO synthase activity in cytokine-induced RAW 264.7 cells treated with NIL or NIO. Cells grown to confluency in duplicate 6-well plates were treated with interferon-γ/LPS for 16 h. The medium was replaced with modified Ham's F-10, 1 mM arginine, 5 μM oxyhemoglobin and maximal NO formation rate was determined for each of the wells. A control initial NO formation rate was determined as 8 nmol NO formed/min mg and varied less than 5% amongst the 12 wells. Well 1 from each plate served as a control and was retained in Ham's F-10 containing 1 μM cycloheximide throughout. Wells 2–6 of Plate 1 (Panel A) were treated for 30 min with modified Ham's F-10 containing 300 μM NIL, while wells 2–6 of Plate 2 were treated in parallel with 300 μM NIO. Immediately following treatment the cells were rinsed twice and the medium replaced with recovery medium containing modified Ham's F-10, 1 mM arginine, 1 μM cycloheximide and NO formation was assessed following adjustment to 5 μM oxyhemoglobin immediately (zero time) or after 1, 2, 3, or 4 h of 'recovery'. Immediately following measurement of NO formation rate the cells were rinsed and lysed in a buffer containing 0.5% CHAPSO and 30 μM arginine and citrulline formation rate was assessed in standard incubations for inducible NO synthase activity as described in Section 2. Values for NO formation rate (●) and inducible NO synthase activity (▲) are expressed as the percentage of the control samples of well 1 never exposed to the drug. The control inducible NO synthase activity was 323 nmol citrulline formed/min mg lysate protein.

ranging in concentration from 100 to 3000 μM NIO. A rapid, time and concentration dependent inactivation of NO synthetic capability was observed (Fig. 6, Panel A). The half time of inactivation at each NIO concentration was determined and the half times were plotted in Kitz–Wilson format (Fig. 6, Panel B). A linear Kitz–Wilson plot was obtained with a $k_{inact\ max}$ of 0.45 min^{-1} and a K_i value of 250 μM NIO. From these data an apparent second order rate constant for inactivation of 1.8 $mM^{-1}\ min^{-1}$ was calculated. For ease of comparison the second order rate constants for inactivation observed using isolated NO synthase isoforms as well as in intact cells are provided in Table 2.

Our laboratory has reported previously, (Wolff et al., 1997) that upon exposure to aminoguanidine, cytokine induced RAW 264.7 cells show a complete loss of NO synthetic capability and inducible NO synthase activity measured in lysates. NO synthetic capability and inducible NO synthase activities recover partially over a 4-h period following drug removal. This recovery occurred in the presence of 10 μM cycloheximide, a concentration of cycloheximide sufficient to inhibit cellular inducible NO synthase synthesis by more than 99% (Wolff et al., 1997). We examined whether cytokine-induced RAW 264.7 cells behaved similarly following treatment with either NIL or NIO at concentrations sufficient to inactivate completely cellular NO synthesis (Fig. 7). Cytokine-induced RAW 264.7 cells were treated with arginine-free Ham's F-10 medium containing either 300 μM NIL (Panel A) or 300 μM NIO (Panel B) for 30 min. Following treatment cells were transferred to Ham's F-10 medium containing 100 μM arginine and 10 μM cycloheximide and NO synthetic capability and cellular inducible NO synthase activity was measured over a 4-h 'recovery' period. A partial recovery of both NO synthetic activity and inducible NO synthase activity was observed for both the NIO and NIL treated cells with restored activities peaking following 2 to 3 h of recovery. The recovery of activities was incomplete, with only a 25–30% restoration of NO synthetic activity and a 7–12% restoration of cellular inducible NO synthase activity being observed.

4. Discussion

The agents NIL and NIO produce a time and concentration dependent inactivation of nitric oxide synthase that

follows first order kinetics as revealed by linear Kitz–Wilson plots. The inactivation of neuronal NO synthase by NIL and NIO occurred only when the enzyme was exposed to drug under conditions that supported catalytic activity (presence of Ca^{2+}) consistent with the proposal that these agents act as alternate substrate, mechanism-based inactivators. The efficiency of NIL and NIO as inactivators of the inducible and neuronal NO synthase isoforms has been evaluated by determining their kinetic constants for inactivation under identical conditions and at an arginine concentration (100 μM) identical to that measured in the normal human extracellular fluid (Table 2). The relative efficiency of mechanism-based inactivators are indicated by their second order rate constants for inactivation and have units of reciprocal concentration reciprocal time, e.g., mM^{-1} min^{-1}.

As measured using the inducible NO synthase, NIL and NIO were found to be virtually identically effective as inactivators, providing second order inactivation rate constants of 31.5 and 32.0 mM^{-1} min^{-1} respectively (Table 2) and were similar both with respect to their K_i values and their maximal inactivation rates. Both NIL and NIO were found to be approximately 4-fold more efficient in inactivating inducible NO synthase (32.0, 31.5 mM^{-1} min^{-1}) than in inactivating neuronal NO synthase (8.4 mM^{-1} min^{-1}). The higher efficiency of NIO and NIL in inactivating the inducible NO synthase as compared to the neuronal isoform is not attributable to a higher maximal inactivation rate vs. this isoform, indeed the maximal inactivation rate of NIO vs. neuronal NO synthase (2.1 min^{-1}) was higher than that exhibited by either NIL (1.73 min^{-1}) or NIO (1.44 min^{-1}) vs. the inducible NO synthase isoform. Rather the diminished efficiency of NIO in activating neuronal NO synthase is attributable to a 5 to 6-fold elevated K_i value. Thus, higher concentrations of NIO (measured in the presence of a competing, saturating concentration of arginine = 100 μM) are required to drive the inactivation of this isoform and suggest a poorer 'fit' of NIO as compared to the normal arginine substrate into the catalytic site of this isoform.

The striking difference among efficiencies of inactivation of NO synthase isoforms by NIL and NIO in our studies was observed for the inactivation of the neuronal NO synthase isoform by NIL. NIL exhibited an efficiency of inactivation for neuronal NO synthase 40-fold lower (0.79 mM^{-1} min^{-1}) than that observed for either NIL or NIO vs. the inducible NO synthase isoform. This diminished efficiency was attributable to differences in both the K_i and $k_{inact\ max}$ values. The K_i value for inactivation of neuronal NO synthase by NIL (0.57 mM) was elevated approximately 10-fold relative to the K_i value of either NIL (0.055 mM) or NIO (0.045 mM) vs. the inducible isoform. Thus, neuronal NO synthase required concentrations of NIL one order of magnitude higher than inducible NO synthase in order to drive its inactivation, consistent with a poorer capability to bind NIL to the catalytic site of neuronal NO synthase in the presence of competing, saturating concentrations of arginine. Further, the maximal inactivation rate achieved by NIL vs. the neuronal NO synthase isoform (0.45 min^{-1}) was approximately 4- to 5-fold slower than that observed for the inactivation of inducible NO synthase by either NIL (1.73 min^{-1}) or NIO (1.44 min^{-1}). Similarly, the maximal inactivation rate produced by NIL vs. neuronal NO synthase (0.45 min^{-1}) is 4.7-fold slower as compared to NIO (2.1 min^{-1}). This relatively inefficient inactivation of neuronal NO synthase by NIL would appear to reflect an increased partition ratio. The partition ratio for a mechanism-based inactivator (Silverman, 1988; Rando, 1984) describes the ratio of the number of latent inactivator molecules converted and released as product relative to each turnover that leads to enzyme inactivation. The partition ratio is a measure of the efficiency of the inactivating reactive intermediate generated from the alternate substrate precursor during catalysis. The inactivation efficiency of the intermediate depends on the intrinsic chemical reactivity of the intermediate, its rate of diffusion from the active site and its proximity when formed to its active site target, which for NO synthase may be the heme residue(s) or a critical reactive site amino acid. The mechanism-based inactivation of NO synthase by aminoguanidine has been shown to require oxygen which presumably 'oxidizes' aminoguanidine to the 'suicide' intermediate (Wolff and Lubeskie, 1995). NIL and NIO behave similarly. Assuming that the neuronal and inducible NO synthase isoforms generate an identical reactive intermediate from NIL, the diminished maximal inactivation of the neuronal vs. the inducible NO synthase isoform would appear to result from a difference in reactive site geometry such that a reduced efficiency of active site 'capture' of the reactive intermediate occurs, that is, a larger number of turnovers occur per inactivation event (an increased partition ratio).

NO formation by and release from cytokine-induced murine macrophage RAW 264.7 cells has been studied in the absence and presence of either NIL or NIO. NIL and NIO each produced a time and concentration dependent inactivation of the NO-synthesizing capability of the cells that followed first order kinetics as revealed by linear Kitz–Wilson plots. Determination of the second order rate constant for inactivation of NO synthesizing activity of the cells revealed that similarly to the isolated inducible NO synthase enzyme, NIL (3.06 mM^{-1} min^{-1}) and NIO (1.80 mM^{-1} min^{-1}) were comparably effective to one another as inactivators in the intact cell. However, the efficiency of inactivation in the intact cells was 10- to 20-fold less efficient than when isolated inducible NO synthase enzyme was employed. This decreased efficiency was reflected both by increased K_i values (for NIL 160 vs. 55 μM, for NIO 250 vs. 45 μM) and by decreased maximal inactivation rates (for NIL 0.49 min^{-1} vs. 1.73 min^{-1}, for NIO 0.45 min^{-1} vs. 1.44 min^{-1}). The increased concentration of NIL and NIO required to drive the inactivation

of the inducible NO synthase in the intact cells relative to the isolated enzyme may be attributable to an increased intracellular, relative to extracellular, arginine concentration as well as by a diminished intracellular as compared to extracellular ratio of NIL and NIO to arginine. The uptake of arginine by RAW 264.7 cells is mediated by the cationic amino acid transporter system y^+ (Schmidt et al., 1994). The uptake of NIO by macrophages also employs the y^+ transporter and is competitive vs. arginine (Baydoun and Mann, 1994). Presumably NIL, a close structural homolog of NIO, would employ the same transport system. The cationic amino acid y^+ transporter system is a Na^+-independent, pH-insensitive, voltage-dependent, ATP-independent, carrier-mediated transport system (White, 1985; Kavanaugh, 1993). Since arginine is cationic and the intracellular environment of macrophages electronegative relative to the cellular exterior, intracellular concentrations should be elevated (>100 μM) relative to the extracellular concentration (100 μM) equilibrating against an electrochemical, rather than merely a chemical concentration gradient. Further, the efficiency of binding to and 'carrying' of NIL or NIO by the protein porter may be reduced relative to arginine. Accordingly cytokine-induced RAW 264.7 cells may have, relative to the extracellular concentrations, both an elevated intracellular arginine concentration and a diminished NIL and NIO to arginine concentration ratio. These conditions would elevate the K_i values observed for inactivation of cellular NO synthesis by NIL and NIO in intact cells as compared to the K_i values observed for inactivation of the isolated inducible NO synthase enzyme.

The inactivation of cellular NO synthesis by NIL and NIO occurred at virtually identical inactivation rates (NIL $= 0.49\ min^{-1}$; NIO $= 0.45\ min^{-1}$) reduced approximately 3-fold from that observed using isolated enzyme. The reduced maximal inactivation rate observed in intact cells may reasonably be attributable to an access delay incurred by uptake of drug into the cell, whereas with isolated enzyme, drug access to the enzyme is essentially immediate.

In an attempt to evaluate further the isoform selectivity of NO synthase with respect to NIL and NIO we measured the IC_{50} values for inhibition of citrulline formation by each of the isoforms under subsaturating concentrations of arginine (120 nM) and compared them to the IC_{50} values determined for a previously characterized, isoform-selective mechanism-based inactivator aminoguanidine (Table 1). Clearly, both NIL and aminoguanidine exhibited specificity for inducible as compared to either the neuronal or endothelial NO synthase isoforms, however NIL was able to exert its effects on inducible NO synthase at 10-fold lower concentrations. NIO by contrast was most potent in inhibiting the endothelial isoform. The ability of NIL to discriminate as an inhibitor of the human NO synthase isoforms has been reported previously (Moore et al., 1996). These workers identified that the IC_{50} values of the endothelial and neuronal NO synthase isoforms were 30-fold and 13-fold higher than for the inducible NO synthase isoform. These values are similar to those reported here using NO synthase isoforms from diverse species (murine inducible, rat neuronal, bovine endothelial NO synthase) and suggest only a modest variation in NIL sensitivity of the NO synthase isoforms amongst species.

In previous studies from our laboratory (Wolff et al., 1997) treatment of cytokine-induced RAW 264.7 cells with either N^G-methyl-L-arginine or aminoguanidine both of which drugs are mechanism-based inactivators, lead to the complete loss of cellular NO synthesizing capability and inducible NO synthase activity. Upon transfer of the treated cells to a drug-free medium containing arginine and cycloheximide, recovery of up to 60% of the pretreatment NO synthesizing activity was observed. This recovery appeared to be attributable to the assembly of drug-undamaged inducible NO synthase monomers into functionally competent dimers following drug removal and restoration to an arginine-containing medium. Since our data (Fig. 1) support the proposal that both NIL and NIO are mechanism-based inactivators we wished to examine whether recovery from inactivation produced by these agents also occurred following drug removal. Similarly to the observations with aminoguanidine and N^G-methyl-L-arginine, NIL and NIO (Fig. 7) treated cells also underwent a partial recovery of NO synthesizing capability and inducible NO synthase activity following drug removal despite the presence of cycloheximide sufficient to inhibit inducible NO synthase synthesis by more than 99%. The quantitative degree of recovery from NIL and NIO (25–30% of NO synthesizing activity) was less than that observed previously for aminoguanidine and N^G-methyl-L-arginine (approximately 60%). In experiments not shown we observed that exposure of cytokine-induced RAW cells to NIO or NIL continuously for periods ranging from 15 min to 3 h prior to drug removal, produced a degree of recovery that diminished progressively with exposure time, such that no recovery was observed in cells exposed to either NIL or NIO for 3 h. By contrast when cells were exposed to aminoguanidine for 3 h, the degree of recovery was unaltered as compared to a 1-h exposure.

The ability of arginine substrate analogs to promote the formation of inducible NO synthase dimers from monomers has been studied by Stuehr and colleagues (Sennequier and Stuehr, 1996; Stuehr, 1997). In these studies aminoguanidine was unable to support dimer formation whereas other arginine analogs possessed this capability. Thus, it is possible that NIL and NIO, in contrast to aminoguanidine, are able to support dimer formation during prolonged treatment (up to 3 h) supporting the slow formation of functional dimers with subsequent drug-induced dimer inactivation. This process would progressively deplete the pool of 'undamaged' monomers necessary to support the partial recovery process and thus the degree of recovery would diminish with drug exposure time. In aminoguanidine-

treated cells the monomeric enzyme form could not assemble into dimers throughout the drug exposure period. Since only dimeric enzyme can generate the suicide intermediate, the monomeric inducible NO synthase would be protected from damage throughout this period. The monomer unable to dimerize would not be depleted by prolonged aminoguanidine exposure and would assemble to dimers following aminoguanidine removal and arginine restoration to catalyze NO formation.

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