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Selective Inhibition of Nitric Oxide Synthase Type I by Clonidine, an Anti-hypertensive Drug

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ABSTRACT. Clonidine, clinically used in the treatment of hypertension, is a central α_2-adrenergic agonist that reduces blood pressure and slows heart rate by reducing sympathetic stimulation. Considering the structural similarity between clonidine and hydrophobic heterocyclic nitric oxide synthase (NOS) inhibitors, the effect of clonidine on the nitric oxide (NO) pathway was investigated. This was verified by determination of NOS activity *in vitro* and by analysis of inducible Ca^{2+}-independent NOS (NOS-II) mRNA expression and measurement of nitrite levels in rat C6 glioma cells, taken as a cellular model. Clonidine inactivated neuronal Ca^{2+}-dependent NOS (NOS-I) competitively without affecting NOS-II and endothelial Ca^{2+}-dependent NOS (NOS-III) activity. However, the value of K_i for clonidine binding to NOS-I depended on tetrahydrobiopterin (BH_4) concentration, as reported for NOS inhibition by other nitrogen heterocyclic compounds. In particular, the value of K_i for clonidine binding to NOS-I increased (from $[7.9 \pm 0.4] \times 10^{-5}$ M to $[8.0 \pm 0.4] \times 10^{-3}$ M) as BH_4 concentration was increased (between 3.0×10^{-7} M and 1.0×10^{-3} M), at pH 7.5 and 37.0°. In addition, clonidine (1.0×10^{-4} M) enhanced NOS-II mRNA expression in rat C6 glioma cells, as induced by *Escherichia coli* lipopolysaccharide (LPS) plus interferon-γ (IFN-γ). Finally, clonidine (1.0×10^{-4} M to 1.0×10^{-3} M) dose dependently increased the levels of LPS/IFN-γ-induced nitrites, the breakdown product of NO, in supernatants of rat C6 glioma cells. As reported for other NOS inhibitors, clonidine was also able to regulate NOS-I and NOS-II inversely. BIOCHEM PHARMACOL **60**;4:539–544, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. clonidine; α_2-adrenergic agonist; anti-hypertensive drug; nitric oxide; NO synthase; glial cells

Starting from the terminal guanidino nitrogen atom of L-arginine, the synthesis of NO† is catalysed by at least three distinct isoforms of NOS. Two enzymes, the neuronal and the endothelial Ca^{2+}-dependent isoforms (NOS-I and NOS-III, respectively) are constantly expressed and termed constitutive NOS. The third enzyme is an inducible Ca^{2+}-independent isoform (iNOS or NOS-II), which is expressed after stimulation with endotoxins and/or cytokines, such as IFN-γ or tumour necrosis factor-α. NOS is a homodimeric enzyme consisting of a reductase domain with FAD and flavin mononucleotide prosthetic groups, and an oxygenase domain that binds arginine, BH_4, and heme. Moreover, NOS activity requires O_2 as a co-substrate and NADPH-derived electrons [1]. Nitrogen heterocyclic compounds represent an important group of NOS inhibitors by binding at the sixth co-ordination position of the heme-iron atom [2]. In particular, it has been demonstrated that indazole, 5-nitro-, 6-nitro-, and 7-nitroindazole as well as 1-phenyl-, 2-phenyl-, and 4-phenylimidazole are potent NOS-I inhibitors [3–5].

Clonidine (2-(2,6-dichloroanilino)-2-imidazoline), a central α_2-adrenergic agonist, is used in the treatment of hypertension, in the prophylaxis of migraine, and as part of the treatment of opioid withdrawal. Considering the structural similarity between clonidine (Fig. 1) and nitrogen heterocyclic compounds, it may be hypothesised that this drug interferes with NOS activity. In the present study, the interference of clonidine on the NO pathway was investigated by assessment of NOS activity *in vitro* and by analysis of NOS-II mRNA expression and measurement of nitrite levels in rat C6 glioma cells, taken as a cellular model.

MATERIALS AND METHODS

Materials

[^{3}H]L-Arginine was obtained from NEN™ Life Science Products. Clonidine was purchased from Sigma Chemical Co. Recombinant rat IFN-γ was obtained from Genzyme Corp. All other products were purchased from Sigma Chemical Co. and Merck AG. All chemicals were of analytical grade and used without further purification.

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† *Abbreviations:* BH_4, tetrahydrobiopterin; GAPDH, glycerol-3-phosphate dehydrogenase; IFN-γ, interferon-γ; L-NAME, L-N^G-nitroarginine methyl ester; LPS, *Escherichia coli* lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; NOS-I, neuronal Ca^{2+}-dependent NOS; NOS-II, inducible Ca^{2+}-independent NOS; and NOS-III, endothelial Ca^{2+}-dependent NOS.

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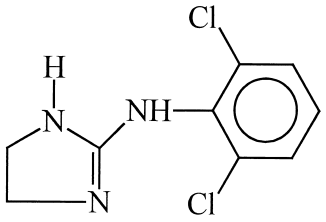


FIG. 1. Chemical structure of clonidine.

Cell Cultures

Rat C6 glioma cells were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Life Technologies Italia) supplemented with 10% foetal bovine serum (GIBCO BRL, Life Technologies Italia). Bovine aortic endothelial cells were harvested from the internal surface of aortas using collagenase XI according to a procedure described elsewhere [6] and grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 30 $\mu g \times mL^{-1}$ of endothelial cell growth supplement (Sigma Chemical Co.), 100 $U \times mL^{-1}$ of penicillin, 100 $\mu g \times mL^{-1}$ of streptomycin, and 50 $\mu g \times mL^{-1}$ of gentamicin (GIBCO BRL, Life Technologies Italia) at 37.0° under an atmosphere of 5% CO_2 in air. Endothelial cells were characterised by immunofluorescence staining using antibodies to Factor VIII. The confluent monolayers were subcultured by conventional trypsinisation and cells were used at passages 5–8.

NOS Preparation

NOS-I was prepared from rat brain homogenates, as already reported [7]. NOS-II was obtained from lung homogenate of rats treated with *Escherichia coli* LPS (10 $mg \times kg^{-1}$) [7]. NOS-III was prepared from the homogenate of bovine aortic endothelial cells treated with 2.0×10^{-2} M 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate for 20 min in ice, as previously described [7]. All samples were homogenised at pH 7.5 (5.0×10^{-2} M HEPES), 5.0×10^{-4} M EGTA, 1.0×10^{-3} M dithiothreitol, and 0.1 $mg \times mL^{-1}$ of phenylmethylsulphonyl fluoride [7]. Then, homogenates were desalted by chromatography over disposable PD-10 columns packed with Sephadex G-25 medium (Pharmacia Biotec).

NOS Activity Assay

NOS activity was assessed by evaluating the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, at pH 7.5 (5.0×10^{-2} M HEPES) and 37.0°. For NOS-I and NOS-III activity, an enzyme aliquot was added to a reaction mixture containing 1.0×10^{-3} M NADPH, 1.2×10^{-3} M $CaCl_2$, 1.0 $\mu g \times mL^{-1}$ of calmodulin, 1.0×10^{-5} M FAD, 1.0×10^{-5} M flavin mononucleotide, [^{3}H]L-arginine (from 12 kBq to 185 kBq), and L-arginine (from 2.0×10^{-6} M to 5.0×10^{-5} M) in the presence or absence of clonidine (ranging between 4.0×10^{-4} M and 1.0×10^{-2} M). For NOS-II, $CaCl_2$ and calmodulin were omitted, and 1.0×10^{-3} M EGTA was added. NOS-I, NOS-II, and NOS-III activity was assayed as a function of BH_4 concentration (ranging between 3.0×10^{-7} M and 1.0×10^{-3} M). After 15 min of incubation, the reaction was stopped with ice-cold 2.0×10^{-2} M HEPES solution, pH 5.5, containing 2.0×10^{-3} M EDTA. [^{3}H]L-Citrulline was separated from [^{3}H]L-arginine by ion-exchange chromatography on Dowex 50WX8 (Fluka Chemie AG) [7–9]. The enzyme activity was linear up to 30 min of incubation, and results were expressed as nmol citrulline $\times$ $min^{-1} \times mg^{-1}$. In addition, NO production was monitored spectrophotometrically following the NO-mediated conversion of human oxyhaemoglobin, added to the NOS-I, NOS-II, and NOS-III preparations, to methaemoglobin, at pH 7.5 and 37.0° [10, 11].

Determination of K_i for Clonidine Binding to NOS-I

Values of the inhibition dissociation equilibrium constant (K_i) for clonidine binding to NOS-I were determined under conditions where $0.2 \times K_m < [S] < 5 \times K_m$ and $0.2 \times K_i < [I] < 5 \times K_i$, [12], according to the Dixon graphical method [13]:

$$v_i^{-1} = K_m \times V_{max}^{-1} \times [S]^{-1} + V_{max}^{-1} + K_m \times V_{max}^{-1} \times [S]^{-1} \times [I] \times K_i^{-1} \quad (1)$$

where v_i is the initial velocity, K_m is the Michaelis constant, V_{max} is the maximal velocity, [S] is the substrate (i.e. L-arginine) concentration, and [I] is the inhibitor (i.e. clonidine) concentration. According to Eqn 1, if v_i^{-1} is plotted against [I], keeping [S] constant, a straight line will be obtained. If this is done at different substrate concentrations (i.e. $[S_1]$, $[S_2]$, . . . $[S_n]$), the lines will cut one another at a point on the left of the vertical axis. If competitive enzyme inhibition occurs, this point lies at $-K_i$ and V_{max}. Therefore, $-K_i$ can be read off directly. Furthermore, K_m can also be determined from the Dixon plot when K_i is found. In fact, each straight line cuts the horizontal axis at a value of [I], corresponding to $-K_i \times ([S] \times K_m^{-1} + 1)$ (see Fig. 2).

Reverse Transcriptase–Polymerase Chain Reaction

Reverse transcriptase–polymerase chain reaction was carried out to analyse the effect of clonidine on NOS-II mRNA expression in rat C6 glioma cells, as induced by LPS (10 $\mu g \times mL^{-1}$) plus IFN-γ (100–1000 $U \times mL^{-1}$), for 4 hr. Briefly, total cellular RNA was purified from 1×10^6 rat C6 glioma cells by the method of Chomczynski and Sacchi [14] and reverse-transcribed into cDNA, as previously reported [14]. cDNA was amplified for the NOS-II gene (450 bp) using rat NOS-II-specific primers, as described elsewhere [15]. The mRNA for the constitutive GAPDH enzyme was examined as the reference cellular transcript.

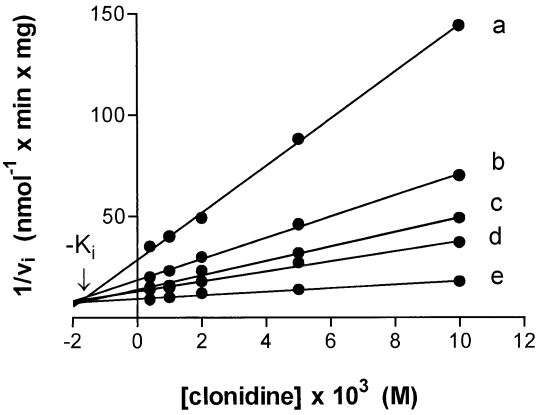


FIG. 2. Dixon plot [12] for rat brain NOS-I inhibition by clonidine. The analysis of data allowed the value of K_i (= [1.8 ± 0.1] × 10^{-3} M) to be evaluated. BH_4 concentration was 5.0 × 10^{-6} M. L-Arginine concentration was: 2.0 × 10^{-6} M (a), 5.0 × 10^{-6} M (b), 1.0 × 10^{-5} M (c), 2.5 × 10^{-5} M (d), and 5.0 × 10^{-5} M (e). The initial velocity (i.e. v_i) was expressed as nmol citrulline × min^{-1} × mg^{-1}. Data were obtained at pH 7.5 (5.0 × 10^{-2} M HEPES buffer) and 37.0°. For further details, see text.

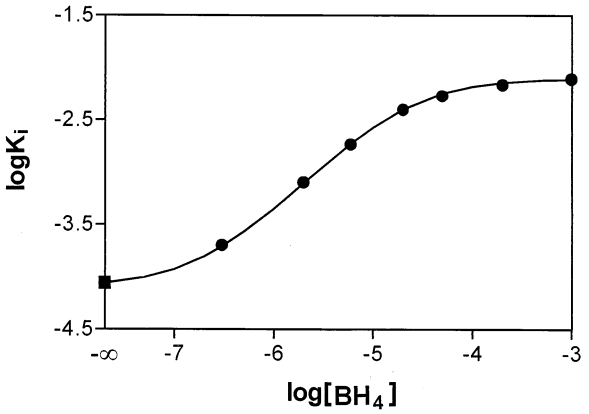


FIG. 3. Effect of BH_4 concentration (i.e. log[BH_4]; M) on logK_i values (M) for clonidine binding to NOS-I. The symbol on the ordinate (filled square) indicates the value of logK_i for clonidine binding to desalted NOS-I (i.e. logK_i'). The continuous line was calculated according to Eqn 2 with values of K_i' (= [7.9 ± 0.4] × 10^{-5} M), K_{BH4O} (= [2.4 ± 0.2] × 10^{-7} M) and K_{BH4L} (= [2.0 ± 0.1] × 10^{-5} M). Data were obtained at pH 7.5 (5.0 × 10^{-2} M HEPES buffer) and 37.0°. For further details, see text.

GAPDH mRNA amplification products (195 bp) were present at equivalent levels in all cell lysates. Estimates of the relative NOS-II mRNA amounts were obtained by dividing the area of the NOS-II band by the area of the GAPDH band (Bio-Rad Multi-Analyst™/PC Version 1.1). Setting the value of LPS (10 μg × mL^{-1}) plus IFN-γ (1000 U × mL^{-1}) as equal to 1 unit, values for the other samples were calculated relative to it.

Nitrite Determination

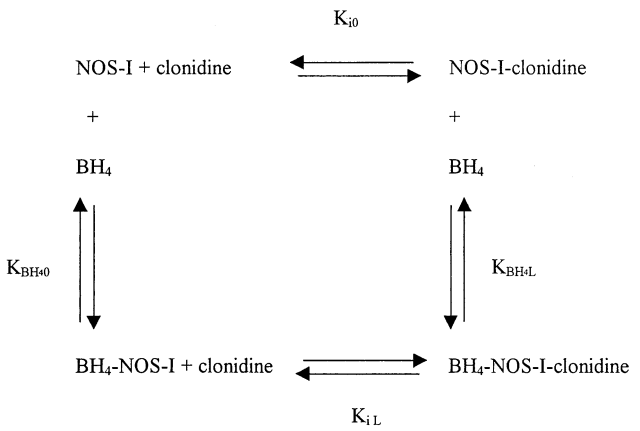
In order to evaluate the effect of clonidine (1.0 × 10^{-4} M to 1.0 × 10^{-3} M) on the total NO production, nitrite (the breakdown product of NO) levels were measured [16]. Briefly, 2 × 10^5 rat C6 glioma cells were treated with LPS (10 μg × mL^{-1}) plus IFN-γ (10–1000 U × mL^{-1}) for 48 hr. Either in the presence or absence of clonidine, the nitrite levels present in the cell supernatant were determined by using the Griess reagent (1.0 × 10^{-4} M sulphanilamide, 1.0 × 10^{-4} M naphthylenediamine dihydrochloride, and 1.0 × 10^{-2} N HCl). The absorbance was measured at 540 nm, and nitrite concentration was determined using sodium nitrite as a standard. Results were expressed as nmol × mL^{-1}.

RESULTS

Effect of Clonidine on NOS Activity

Here, clonidine was shown to competitively inhibit NOS-I activity (see Fig. 2). Moreover, the value of K_i for clonidine binding to NOS-I was dependent on the BH_4 concentration (see Fig. 3). According to the 'linked functions' [17], the decrease in clonidine affinity for NOS-I (i.e. the increase in K_i values) on increasing BH_4 concentration reflected the higher affinity of BH_4 for the clonidine-free NOS-I rather than for the clonidine-bound (i.e. inhibited) enzyme.

Scheme I represents the apparent minimum model accounting for the effect of BH_4 concentration on clonidine binding to NOS-I [17]. In this scheme, K_{i0} is the competitive inhibition dissociation equilibrium constant for clonidine binding to desalted NOS-I; K_{iL} is the competitive inhibition dissociation equilibrium constant for clonidine binding to NOS-I in the presence of saturating amounts of BH_4; K_{BH4O} is the dissociation equilibrium constant for BH_4 binding to clonidine-free NOS-I; and K_{BH4L} is the dissociation equilibrium constant for BH_4 binding to NOS-I in the presence of saturating amounts of clonidine. In the case of K_{i0}, it is to be noted that one BH_4 molecule remained tightly bound to dimeric NOS-I during enzyme purification, not being replaced by BH_4-based NOS inhibitors. This finding reflects the extremely low dissociation equilibrium constant, in the order of 10^{-11} M (i.e. very



SCHEME 1.

high affinity), of the first BH_4 molecule for the BH_4-free NOS-I [17].

Applying Scheme I, a relation between values of K_i for clonidine binding to NOS-I and BH_4 concentration (i.e. $[BH_4]$) may be expressed according to the following equation [18]:

$$\log K_i = \log K_i' + \log \{(K_{BH40} + [BH_4])/(K_{BH4L} + [BH_4])\} + \log (K_{BH4L}/K_{BH40}) \quad (2)$$

Eqn 2 was used to generate the continuous line shown in Fig. 3 with the following values of K_i' (= [7.9 ± 0.4] × 10^{-5} M), K_{BH40} (= [2.4 ± 0.2] × 10^{-7} M), and K_{BH4L} (= [2.0 ± 0.1] × 10^{-5} M). The agreement with the experimental data was fully satisfactory, strongly suggesting that the assumptions underlying Eqn 2 were correct. The value of K_{BH40} (= [2.4 ± 0.2] × 10^{-7} M) obtained by the analysis of data given in Fig. 3 according to Eqn 2 was in good agreement with the value of (2.4 ± 0.1) × 10^{-7} M, as reported elsewhere [19]. On the contrary, clonidine affected neither NOS-II nor NOS-III activity even at the highest drug concentration used (5.0 × 10^{-3} M) (data not shown). Therefore, as expected for a simple system [20, 21], values of K_i for clonidine binding to NOS-II and NOS-III exceeded 5 × 10^{-2} M. Moreover, clonidine was not an NO precursor. In fact, oxyhaemoglobin added to NOS-I, NOS-II, and NOS-III preparations was not converted to methaemoglobin in the presence of clonidine instead of L-arginine (data not shown).

Effect of Clonidine on NOS-II mRNA Expression in Glial Cells

Using reverse transcriptase–polymerase chain reaction, clonidine was observed to enhance NOS-II mRNA expression in rat C6 glioma cells treated with the NOS-II inducers LPS and IFN-γ. As shown in Fig. 4, untreated cells did not express NOS-II mRNA (*lane 1*). Incubation of 1.0 × 10^6 cells with a suboptimal concentration of a mixture of LPS (10 μg × mL^{-1}) plus IFN-γ (100 U × mL^{-1}) for 4 hr induced a less pronounced NOS-II mRNA expression (*lane 2*) than did treatment with the optimal concentration of LPS (10 μg × mL^{-1}) plus IFN-γ (1000 U × mL^{-1}) (*lane 5*). When cells were incubated with the suboptimal concentration of LPS and IFN-γ, clonidine (1.0 × 10^{-4} M) caused a NOS-II mRNA overexpression (*lane 4*). The same effect was obtained by the NOS inhibitor L-NAME (1.0 × 10^{-3} M) (*lane 3*). When cells were incubated with the optimal concentration of LPS and IFN-γ, clonidine (1.0 × 10^{-4} M) did not provoke a significant NOS-II mRNA overexpression (*lane 6*).

Effect of Clonidine on Nitrite Production in Glial Cells

Stimulation of 2 × 10^5 cells with LPS (10 μg × mL^{-1}) plus IFN-γ (100 U × mL^{-1} and 1000 U × mL^{-1}) for 48 hr caused a dose-dependent generation of nitrite (Fig. 5). The increase in nitrite levels was abolished by L-NAME (1.0 × 10^{-3} M) treatment, indicating that nitrite production was dependent on L-arginine metabolism. When cells were incubated with the suboptimal concentration of LPS (10 μg × mL^{-1}) plus IFN-γ (100 U × mL^{-1}), clonidine (ranging between 1.0 × 10^{-4} M and 1.0 × 10^{-3} M) increased NO production dose dependently, this increase being complete at the highest dose (see Fig. 5).

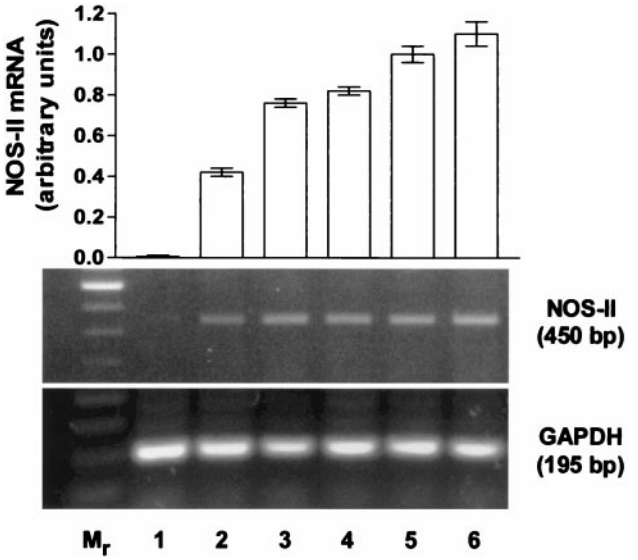


FIG. 4. Analysis of NOS-II gene expression in rat C6 glioma cells. *Lane 1*, untreated cells; *lane 2*, a 4-hr treatment of 1 × 10^6 cells with a mixture of LPS (10 μg × mL^{-1}) plus IFN-γ (100 U × mL^{-1}) suboptimally induced NOS-II mRNA expression; *lane 3*, the same sample as in lane 2, incubated with L-NAME (1.0 × 10^{-3} M), showed a NOS-II mRNA overexpression; *lane 4*, the same effect was obtained by clonidine (1.0 × 10^{-4} M) treatment; *lane 5*, LPS (10 μg × mL^{-1}) plus IFN-γ (1000 U × mL^{-1}) optimally induced NOS-II mRNA expression; *lane 6*, the same sample as in lane 5, incubated with clonidine (1.0 × 10^{-4} M), did not show a significant NOS-II mRNA overexpression. PCR product (195 bp) for the GAPDH gene was taken as the reference cellular transcript. Estimates of the relative NOS-II mRNA amounts were obtained by dividing the area of the NOS-II band by the area of the GAPDH band. Setting the value of LPS (10 μg × mL^{-1}) plus IFN-γ (1000 U × mL^{-1}) as equal to 1 unit, values for the other samples were calculated relative to it. Relative molecular mass (M_r) is the 100-bp DNA ladder (Life Technologies, Inc.). Bars represent the means ± SEM of 3 experiments and the gel shows a typical experiment. Student's unpaired *t*-test was used to determine the significant differences: $P \leq 0.001$ between clonidine (1.0 × 10^{-4} M) or L-NAME (1.0 × 10^{-3} M) plus LPS (10 μg × mL^{-1}) + IFN-γ (100 U × mL^{-1}) and LPS (10 μg × mL^{-1}) + IFN-γ (1000 U × mL^{-1}); $P \leq 0.0001$ between LPS (10 μg × mL^{-1}) + IFN-γ (100 U × mL^{-1}) and LPS (10 μg × mL^{-1}) + IFN-γ (1000 U × mL^{-1}); not significant between clonidine (1.0 × 10^{-4} M) plus LPS (10 μg × mL^{-1}) + IFN-γ (1000 U × mL^{-1}) and LPS (10 μg × mL^{-1}) + IFN-γ (1000 U × mL^{-1}).

DISCUSSION

The present results indicate that clonidine competitively inhibits NOS-I activity (see Fig. 2), this effect depending

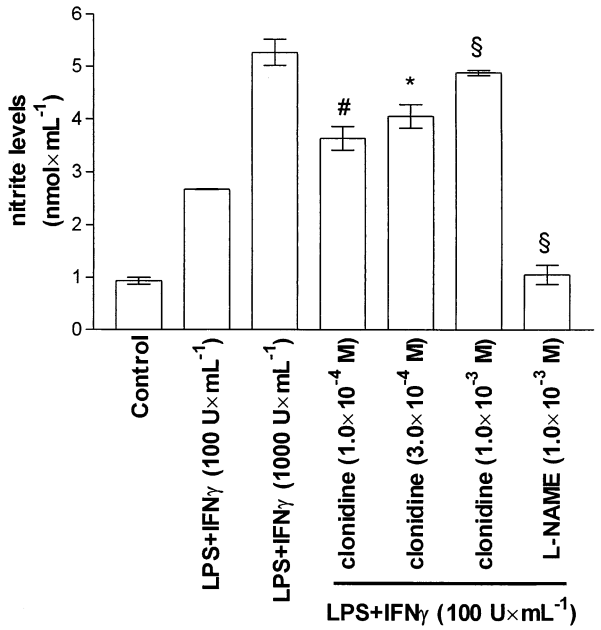


FIG. 5. Determination of nitrite levels in supernatants of 2 × 10^5 rat C6 glioma cells. LPS (10 μg × mL^{-1}) plus IFN-γ (100 U × mL^{-1} and 1000 U × mL^{-1}) increased nitrite production dose dependently, this effect being abolished by L-NAME (1.0 × 10^{-3} M). Clonidine (1.0 × 10^{-4} M to 1.0 × 10^{-3} M) dose dependently increased LPS/IFN-γ-mediated nitrite levels in supernatants of C6 cells. Bars represent the means ± SEM of 3 experiments. Student's unpaired *t*-test was used to determine the significant differences: $^{\#}P \leq 0.001$ between clonidine (1.0 × 10^{-4} M) plus LPS + IFN-γ (100 U × mL^{-1}) and LPS + IFN-γ (100 U × mL^{-1}): $^{*}P \leq 0.0005$ between clonidine (3.0 × 10^{-4} M) plus LPS + IFN-γ (100 U × mL^{-1}) and LPS + IFN-γ (100 U × mL^{-1}); $^{\S}P \leq 0.0001$ between clonidine (1.0 × 10^{-3} M) or L-NAME (1.0 × 10^{-3} M) plus LPS + IFN-γ (100 U × mL^{-1}) and LPS + IFN-γ (100 U × mL^{-1}). For further details, see text.

on the BH_4 concentration (see Fig. 3). On the contrary, clonidine affects neither NOS-II nor NOS-III activity, accounting for structural differences in the heme-, BH_4-, and L-arginine-binding domains of NOS isoforms [22, 23]. Although the three-dimensional structure of the NOS-I: clonidine complex is not available, clonidine may be postulated to inhibit NOS-I by binding at the sixth (axial) co-ordination position of the heme-iron atom, as observed for a number of nitrogen heterocycles [22, 23]. The heme-iron-bound positively charged clonidine may be stabilised by the negatively charged NOS-I Glu^{597} residue that is required for L-arginine binding [22, 23].

The affinity of clonidine for NOS-I is similar to that observed for inhibitor binding to swine kidney and *Lens culinaris* copper amine oxidase ($K_i = 9.0 \times 10^{-4}$ M and 5.0×10^{-4} M, respectively) [24]. Furthermore, aprotinin, gabexate mesylate, *p*-aminobenzamidine, amiloride, and 4,6-diamidino-2-phenylindole inhibit NOS isoforms, trypsin-like serine proteinases, and copper amine oxidases [9, 10, 15, 24–27]. In addition, agmatine, a competitive inhibitor and/or poor substrate of NOS isoforms [28, 29], is a substrate for copper amine oxidases [27, 30]. Furthermore, gabexate mesylate, *p*-aminobenzamidine, amiloride, 4,6-diamidino-2-phenylindole, and agmatine may also affect arginase, L-arginine-glycine transaminase, kyotorphine synthase, and L-arginine decarboxylase, all using L-arginine as the substrate [9, 10, 15, 24–27, 31]. Therefore, as already reported for aprotinin, gabexate mesylate, *p*-aminobenzamidine, amiloride, 4,6-diamidino-2-phenylindole, and agmatine, clonidine may affect (un)related functions, representing a further case of cross-reactivity.

Recently, NOS inhibitors as well as NO-trapping agents have been reported to up-regulate NOS-II gene expression in glial cells [15, 32]. This effect reflects the inhibitory action of 'pre-existing' NO at physiological levels (e.g. as produced by constitutive NOS) on nuclear factor kappaB activation, which leads to the suppression of NOS-II mRNA induction [33–35]. As observed for other inhibitors of constitutive NOS activity [15, 32], clonidine is able to increase NOS-II gene expression, as induced by LPS and IFN-γ in glial cells (Fig. 4). In order to verify whether the effect of clonidine also occurs on NO release, levels of nitrites, the breakdown product of NO, were measured in the supernatant of rat C6 glioma cells. Unlike other non-selective NOS inhibitors (e.g. L-NAME), clonidine has been found to increase NO production dose dependently, this increase being complete at the highest dose (Fig. 5), i.e. under those conditions where NOS-I (but not NOS-II) activity *in vitro* was affected by clonidine (Fig. 2). The present findings suggest that clonidine may enhance NOS-II gene expression and NOS-II-mediated nitrite production in rat C6 glioma cells by selectively inhibiting NO produced by NOS-I, thus explaining why inducible and constitutive NOSs are inversely regulated.

In conclusion, the present results indicate that clonidine can be added to the ever-increasing list of NOS inhibitors, *in vitro*, with a high selectivity for NOS-I. Moreover, clonidine is able to increase NOS-II-mediated NO production in glial cells.

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References

1. Mayer B and Hemmens B, Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem Sci* **22:** 477–481, 1997.
2. Griffith OW and Gross SS, Inhibitors of nitric oxide synthases. In: *Methods of Nitric Oxide Research* (Eds. Feelisch M and Stamler JS), pp. 187–208. J Wiley, Chichester, 1996.
3. Moore PK, Babbedge RC, Wallace P, Gaffen ZA and Hart SL, 7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* **108:** 296–297, 1993.
4. Wolff DJ, Datto GA, Samatovitz RA and Tempsick RA, Calmodulin-dependent nitric-oxide synthase. Mechanism of inhibition by imidazole and phenylimidazoles. *J Biol Chem* **268:** 9425–9429, 1993.
5. Wolff DJ and Gribin BJ, The inhibition of the constitutive

and inducible nitric oxide synthase isoforms by indazole agents. *Arch Biochem Biophys* **311:** 300–306, 1994.
6. Gimbrone MA Jr, Culture of vascular endothelium. *Prog Hemost Thromb* **3:** 1–28, 1976.
7. Stuehr DJ and Griffith OW, Purification, assay and properties of mammalian nitric oxide synthases. In: *Methods of Nitric Oxide Research* (Eds. Feelisch M and Stamler JS), pp. 177–186. J Wiley, Chichester, 1996.
8. Colasanti M, Persichini T, Fabrizi C, Cavalieri E, Venturini G, Ascenzi P, Lauro GM and Suzuki H, Expression of a NOS-III-like protein in human astroglial cell culture. *Biochem Biophys Res Commun* **252:** 552–555, 1998.
9. Venturini G, Colasanti M and Ascenzi P, Aprotinin, the first competitive protein inhibitor of NOS activity. *Biochem Biophys Res Commun* **249:** 263–265, 1998.
10. Venturini G, Menegatti E and Ascenzi P, Competitive inhibition of nitric oxide synthase by *p*-aminobenzamidine, a serine proteinase inhibitor. *Biochem Biophys Res Commun* **232:** 88–90, 1997.
11. Feelisch M, Kubitzek D and Werringloer J, The oxyhemoglobin assay. In: *Methods of Nitric Oxide Research* (Eds. Feelisch M and Stamler JS), pp. 455–478. J Wiley, Chichester, 1996.
12. Dixon M, The determination of enzyme inhibitor constants. *Biochem J* **55:** 170–171, 1953.
13. Purich DL (Ed.), *Enzyme Kinetics and Mechanisms*, Part A. *Methods Enzymol* **63:** 3–513. Academic Press, New York, San Francisco, London, 1979.
14. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidine thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162:** 156–157, 1987.
15. Colasanti M, Persichini T, Venturini G, Menegatti E, Lauro GM and Ascenzi P, Effect of gabexate mesylate (FOY®), a drug for serine proteinase-mediated diseases, on the nitric oxide pathway. *Biochem Biophys Res Commun* **246:** 453–456, 1998.
16. Tracey WR, Spectrophotometric detection of nitrogen oxides using azo dyes. *Neuroprotocols* **1:** 125–131, 1992.
17. Gorren AC, Schrammel A, Schmidt K and Mayer B, Thiols and neuronal nitric oxide synthase: Complex formation, competitive inhibition and enzyme stabilization. *Biochemistry* **36:** 4360–4366, 1997.
18. Wyman J, Linked functions and reciprocal effects in hemoglobin: A second look. *Adv Protein Chem* **19:** 223–286, 1964.
19. Gorren AC, List BM, Schrammel A, Pitters E, Hemmens B, Werner ER, Schmidt K and Mayer B, Tetrahydrobiopterin-free neuronal nitric oxide synthase: Evidence for two identical highly anticooperative pteridine binding sites. *Biochemistry* **35:** 16735–16745, 1996.
20. Anderson SR and Antonini E, The binding of carbon monoxide by human hemoglobin. Proof of validity of the spectrophotometric method and direct determination of the equilibrium. *J Biol Chem* **243:** 2918–2920, 1968.
21. Antonini E and Brunori M, *Hemoglobin and Myoglobin in Their Reactions with Ligands*, pp. 153–187. North-Holland Publishing Company, Amsterdam and London, 1971.
22. Crane BR, Arvai AS, Gachhui R, Wu C, Ghosh DK, Getzoff ED, Stuehr DJ and Tainer JA, The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **278:** 425–431, 1997.
23. Fischmann TO, Hruza A, Niu XD, Fossetta JD, Lunn CA, Dolphin E, Prongay AJ, Reichert P, Lundell DJ, Narula SK and Weber PC, Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat Struct Biol* **6:** 233–242, 1999.
24. Ercolini L, Angelini R, Federico R, Rea G, Venturini G and Ascenzi P, Competitive inhibition of *Lens culinaris* L. copper amine oxidase by amiloride, *p*-aminobenzamidine, clonidine, 4′,6-diamidino-2-phenylindole and gabexate mesylate: A comparative study. *J Enzyme Inhib* **13:** 465–471, 1998.
25. Casale E, Collyer C, Ascenzi P, Balliano G, Milla P, Viola F, Fasano M, Menegatti E and Bolognesi M, Inhibition of bovine beta-trypsin, human alpha-thrombin and porcine pancreatic beta-kallikrein-B by 4′,6-diamidino-2-phenylindole, 6-amidinoindole and benzamidine: A comparative thermodynamic and X-ray structural study. *Biophys Chem* **54:** 75–81, 1995.
26. Ascenzi P, Federico R, Menegatti E and Venturini G, Competitive inhibition of mouse brain nitric oxide synthase by amiloride: A case for enzyme cross-inhibition. *Biochemistry Mol Biol Int* **43:** 507–511, 1997.
27. Federico R, Angelini R, Ercolini L, Venturini G, Mattevi A and Ascenzi P, Competitive inhibition of swine copper amine oxidase by drugs: Amiloride, clonidine, and gabexate mesylate. *Biochem Biophys Res Commun* **240:** 150–152, 1997.
28. Galea E, Regunathan S, Eliopoulos V, Feinstein DL and Reis DJ, Inhibition of mammalian nitric oxide synthases by agmatine, an endogenous polyamine formed by decarboxylation of arginine. *Biochem J* **316:** 247–249, 1996.
29. Grant SK, Green BG, Stiffey-Wilusz J, Durette PL, Shah SK and Kozarich JW, Structural requirements for human inducible nitric oxide synthase substrates and substrate analogue inhibitors. *Biochemistry* **37:** 4174–4180, 1998.
30. Holt A and Baker GB, Metabolism of agmatine (clonidine-displacing substance) by diamine oxidase and the possible implications for studies of imidazoline receptors. *Prog Brain Res* **106:** 187–197, 1995.
31. Nakaki T and Kato R, Beneficial circulatory effect of L-arginine. *Jpn J Pharmacol* **66:** 167–171, 1994.
32. Park SK, Lin HL and Murphy S, Nitric oxide limits transcriptional induction of nitric oxide synthase in CNS glial cells. *Biochem Biophys Res Commun* **201:** 762–768, 1994.
33. Colasanti M, Persichini T, Menegazzi M, Mariotto S, Giordano E, Caldarera GM, Sogos V, Lauro GM and Suzuki H, Induction of nitric oxide synthase mRNA expression. Suppression by exogenous nitric oxide. *J Biol Chem* **270:** 26731–26733, 1995.
34. Togashi H, Sasaki M, Frohman E, Taira E, Ratan RR, Dawson TM and Dawson VL, Neuronal (type I) nitric oxide synthase regulates nuclear factor kappaB activity and immunologic (type II) nitric oxide synthase expression. *Proc Natl Acad Sci USA* **94:** 2676–2680, 1997.
35. Taylor BS, Kim Y-M, Wang Q, Shapiro RA, Billiar TR and Geller DA, Nitric oxide down-regulates hepatocyte-inducible nitric oxide synthase gene expression. *Arch Surg* **132:** 1177–1183, 1997.