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Nitric oxide synthase inhibitors have antidepressant-like properties in mice
2. Chronic treatment results in downregulation of cortical β-adrenoceptors

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Abstract

Down-regulation of cortical β-adrenoceptors is observed in rodents following chronic treatment with many clinically effective antidepressant therapies. [^{3}H]dihydroalprenolol binding to cortical β-adrenoceptors was examined in mice treated with the nitric oxide (NO) synthase antagonist N^G-nitro-L-arginine (L-NNA). Administration of L-NNA (0.1, 0.3 mg/kg) for 21 days produced a significant reduction (28%, 31%, respectively, $P < 0.05$) in [^{3}H]dihydroalprenolol binding to cortical membranes without affecting K_d. Dose 1 mg/kg of L-NNA given chronically also produced a 20% decrease in β-adrenoceptor density, but this effect was not statistically significant. While chronic treatment with imipramine (15 and 30 mg/kg) produced respectively a 30% and 25% ($P < 0.05$) reduction in the density of [^{3}H]dihydroalpenolol, single injection of either imipramine (15 and 30 mg/kg) or L-NNA (0.1, 0.3, and 1 mg/kg) had no effect on [^{3}H]dihydroalprenolol binding. These findings are consistent with the hypothesis that drugs which can affect the Ca^{2+}-calmodulin/nitric oxide synthase/guanylyl cyclase signaling pathway may represent a novel approach to the treatment of depression and are congruent with our previous observation, which has demonstrated the antidepressant-like properties of NO synthase inhibitors in the forced swim test.

Keywords: β-Adrenoceptor; N^G-nitro-L-arginine; Imipramine; Nitric Oxide (NO) synthase; Cortex; (Mouse)

1. Introduction

Chronic, but not acute treatment of rodents with many clinically effective antidepressant therapies which include tricyclics, monoamine oxidase (MAO) inhibitors, atypical antidepressants, electroconvulsive shock, and rapid eye movement (REM) sleep deprivation reduce the density of cortical β-adrenoceptors without altering ligand affinity (reviewed in Heninger and Charney, 1987). Conversely, procedures that result in antidepressant-sensitive behavioral changes are often accompanied by increased cortical β-adrenoceptor density (Papp et al., 1994). Finally, some laboratories have reported alterations in β-adrenoceptor density in post-mortem cortical samples from humans diagnosed with major depressive disorders (Mann et al., 1986; De Paermentier et al., 1989, 1990, 1991).

Previous studies have reported that competitive *N*-methyl-D-aspartate (NMDA) receptor antagonists reduce the duration of immobility in forced swim test and tail suspension tests (Porsolt et al., 1977; Steru et al., 1985) with efficacies comparable to clinically effective antidepressants (Trullas and Skolnick, 1990). Likewise NMDA receptor antagonists normalized the reduction in sweetened water consumption of animals subjected to chronic mild stress (Papp and Moryl, 1994). Moreover, chronic treatment with NMDA receptor antagonists down-regulates cortical β-adrenoceptors with a magnitude comparable to that observed following chronic imipramine treatment (Paul et al., 1992; Klimek and Papp, 1994).

The NMDA receptor complex gates Ca^{2+} entry into neurons, which can interact with calmodulin to subsequently activate nitric oxide (NO) synthase. NO synthase

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catalyzes the conversion of L-arginine to L-citruline with subsequent release of NO.

NO synthase exists in two major forms: constitutive, which is constitutively expressed in various cells including neurons, and inducible, that is expressed only after gene induction. The constitutive form of NO synthase becomes activated in the presence of calmodulin and increased intracellular [Ca^{2+}] and is associated with the function of NMDA receptors. Stimulation of the NMDA receptor complex opens the ionophore and facilitates Ca^{2+} entry into the neuron. Therefore, activation of NO synthase is part of the cascade of subcellular events leading from activation of the NMDA receptor to stimulation of guanylyl cyclase and postsynaptic activation.

Several studies have demonstrated that antagonists of voltage operated Ca^{2+} channel produce antidepressant-like effects in forced swim test (Mogilnicka et al., 1987; Czyrak, 1993). Moreover, co-administration of the calcium channel antagonist nifedipine with electroconvulsive treatment increased the efficacy of electroconvulsive treatment to downregulate β-adrenoceptors system (Antkiewicz-Michaluk et al., 1993). Conversely, chronic administration of citalopram, chlorprothixene or electroconvulsive treatment increased the density of voltage operated Ca^{2+} channels (Antkiewicz-Michaluk et al., 1991, 1994).

We hypothesized that NO synthase inhibitors might display antidepressant- and anxiolytic-like properties similar to voltage-operated calcium channel blockers and NMDA receptor antagonists. Recently we demonstrated that acute administration of NO synthase inhibitors of NO synthase have antidepressant and anxiolytic-like properties in the forced swim test and elevated plus maze test (Harkin et al., 1999). In the present study, we examined whether chronic administration of the NO synthase inhibitor N^G-nitro-L-arginine (L-NNA) would down-regulate β-adrenoceptors to an extent comparable to that of clinically active antidepressants such as imipramine.

Now we report that chronic but not acute treatment with the NO synthase inhibitor L-NNA in mice reduces [^{3}H]dihydroalprenolol binding to cortical β-adrenoceptors with a magnitude comparable to that observed following administration of imipramine. These findings are consistent with the hypothesis that the Ca^{2+}-calmodulin/nitric oxide synthase/guanylyl cyclase signaling pathway is involved in the pathophysiology of affective disorders and that interruption of this cascade at any point will result in antidepressant-like activity.

2. Materials and method

2.1. Animals and treatment

Male NIH Swiss–Webster mice (Harlan Sprague–Dawley, Indianapolis, IN) were housed under standard laboratory conditions, in groups of five per cage at room temperature (~20°C), with a 12 h light/dark cycle (lights on at 0600 h). Mice weighed 20–25 g at the start of the experiment and had free access to food and water throughout treatment.

Imipramine (RBI, Natick, MA) 15 and 30 mg/kg, L-NNA (RBI) 0.1, 0.3 and 1 mg/kg or vehicle (0.9% NaCl) were injected intraperitoneally once or for 21 days. All drugs were prepared in 0.9% NaCl and given in a volume of 0.2 ml.

Twenty-four hours after the last injection, the animals were sacrificed by decapitation and the cerebral cortices were dissected on an ice-chilled culture plate. The dissected tissue was frozen on aluminum foil over solid CO_2 and stored at −70°C until assayed.

2.2. Membrane preparation and receptor binding assay

β-Adrenoceptors were labeled with [^{3}H]dihydroalprenolol (DHA, NEN-Dupont: specific activity 120 Ci/mmol). Cerebral cortical tissue was thawed and homogenized in 50 volumes 50 mM Tris buffer solution (pH 7.8) using a Brinkmann Polytron (setting 6 for 20 s). The homogenate was centrifuged at 32,000 × *g* for 20 min and the resulting supernatant discarded. The pellet was resuspended in 50 volumes fresh Tris and again centrifuged at 32,000 × *g* for 20 min. The resulting supernatant was discarded and the membrane suspensions (0.2–0.3 mg protein/assay) were incubated with 6–10 concentrations of [^{3}H]dihydroalprenolol (from 0.06–2.5 nM) for 30 min at room temperature (21–24°C). Non-specific binding was defined with 100 μM isoprotenerol bitartrate (RBI-Natick, MA). To exclude the serotoninergic component of [^{3}H]dihydroalprenolol binding, 10 nM serotonin creatinine sulfate and 20 μM pargyline were included in all assays (Stockmeier and Kellar, 1989). Assays were terminated by rapid filtration through Whatman GF/C filters presoaked with Tris buffer. The filters were rinsed twice with 5 ml ice-cold Tris. Filters were soaked in 0.5 ml Microscint™ 20 (Packard, Meriden, CT).

Table 1
The effect of chronic imipramine and L-NNA treatment on β-adrenoceptors in mouse cortex

Drug (mg/kg, i.p.)	B_{max} (fmol/mg protein)	K_d (nM)
Saline	140 ± 14	0.25 ± 0.02
Imipramine 15	98 ± 5[a]	0.26 ± 0.01
Imipramine 30	106 ± 9[a]	0.25 ± 0.01
L-NNA 0.1	101 ± 7[a]	0.25 ± 0.01
L-NNA 0.3	97 ± 5[a]	0.25 ± 0.01
L-NNA 1	111 ± 8	0.25 ± 0.01

Experiment was carried out 24 h after last injection.
Values represent the mean ± S.E.M. of 9–16 subject/group.
B_{max} and K_d values of [^{3}H]dihydroalprenolol binding were determined using LIGAND program Version 5.0.3.
Data were analyzed using a one-way ANOVA followed by LSD test.
[a] $P < 0.05$ difference from saline control.

Table 2
The effect of acute imipramine and L-NNA treatment on β-adrenoceptors in mouse cortex

Drug (mg/kg, i.p.)	B_{max} (fmol/mg protein)	K_d (nM)
Saline	91 ± 5	0.36 ± 0.014
Imipramine 15	104 ± 8	0.32 ± 0.013
Imipramine 30	96 ± 9	0.41 ± 0.030
L-NNA 0.1	109 ± 7	0.40 ± 0.020
L-NNA 0.3	115 ± 9	0.37 ± 0.017
L-NNA 1	99 ± 6	0.42 ± 0.017

Experiment was carried out 24 h after acute injection.
Values represent the mean ± S.E.M. of six subject/group.
B_{max} and K_d values of [^{3}H]dihydroalprenolol binding were determined using LIGAND program Version 5.0.3.
Data were analyzed using a one-way ANOVA followed by LSD test.

The radioactivity retained by the filters was measured in a Packard Top Count scintillation counter. Protein determinations were made using the bicinchoninic acid method (Smith, 1985) with kits supplied by Pierce (Rockford, IL). B_{max} and K_d were determined using the LIGAND program Version 5.0.3. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Student–Neuman–Keuls and Fisher's Least Significant Difference (LSD) test.

3. Results

Binding of [^{3}H]dihydroalprenolol at concentrations ranging from 0.06 to 2.5 nM in mouse cerebral cortices yielded a K_d value of 0.25 ± 0.02 nM and B_{max} of 140 ± 14 fmol/mg protein in control mice treated chronically with saline for 21 days. Chronic treatment with imipramine (15 and 30 mg/kg) for 21 days resulted in a significant (30% and 25%, $P < 0.05$) reduction in the density of [^{3}H]dihydroalprenolol binding sites without affecting the K_d. Administration of L-NNA (0.1, 0.3 mg/kg) for 21 days produced a comparable, significant reduction (28%, 31%, respectively, $P < 0.05$) in [^{3}H]dihydroalprenolol binding to cortical membranes. Chronic administration of 1 mg/kg of L-NNA also produced a 20% decrease in β-adrenoceptor density, but this effect was not statistically significant (Table 1).

A single injection of either imipramine (15 and 30 mg/kg) or L-NNA (0.1, 0.3, 1 mg/kg) was without effect on [^{3}H]dihydroalprenolol binding: the B_{max} in saline-treated animals was 91 ± 5 fmol/mg protein; after acute injection of imipramine (15 mg/kg) 104 ± 8 fmol/mg protein and after single dose of L-NNA (0.3 mg/kg) 115 ± 9 fmol/mg protein (Table 2).

4. Discussion

These data demonstrate that chronic, but not acute, treatment of mice with NO synthase inhibitor L-NNA down regulates cortical β-adrenoreceptors with a magnitude comparable to that observed following chronic imipramine treatment. These findings are congruent with previous observations which have demonstrated the antidepressant-like properties of NO synthase inhibitors in the forced swim test, and that such an antidepressant effect was reversed by pretreatment with the NO synthase substrate, L-arginine (Harkin et al., 1997, 1999).

Down-regulation of the β-adrenoceptor system following chronic antidepressant administration, was observed initially by Vetulani and Sulser (1975). They demonstrated that chronic (21 days) administration of antidepressants resulted in a reduction in activity of adenylate cyclase and a reduction in the number of β-adrenoceptors in the rat brain (Vetulani et al., 1976; Banerjee et al., 1977; Wolfe et al., 1978; Okada et al., 1986). In recent years this phenomenon was also observed after chronic treatment with NMDA receptor antagonists (Paul et al., 1992; Klimek and Papp, 1994). Our present study demonstrates that down-regulation of β-adrenoceptors can also be observed following NO synthase inhibitor administration. Moreover, chronic administration of NMDA receptor antagonists and NO synthase antagonists not only down regulates β-adrenoceptors but are also active in preclinical behavioral screening procedures. These data suggest that NMDA receptor antagonists and NO synthase inhibitors may have antidepressant properties.

NMDA receptors may be involved in the mechanism of action of antidepressant drugs, and may play an important role in the pathogenesis of depression. Previous studies have demonstrated that antidepressant drugs bind to NMDA receptors (Reynolds and Miller, 1988; Sills and Leo, 1989) and after chronic administration, can cause substantial adaptive changes in the NMDA receptor complex (Nowak et al., 1993). There is also evidence that non-competitive ((+)-5methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imime maleate-MK-801, dizocilpine), and competitive (DL-(*E*)-2-amino-4-methyl-5-phosphono-3-pentonoic acid, CGP 37849 and its (*R*)-enantiomer CGP 40116) NMDA receptor antagonists can reduce the sucrose drinking deficit in an animal model of depression with a magnitude comparable to that observed following similar administration of imipramine (Papp and Moryl, 1994).

Several studies have demonstrated that antagonists of NMDA receptors are as efficacious as tricyclic antidepressant in acute preclinical antidepressant screening procedures (Trullas and Skolnick, 1990; Trullas et al., 1991; Skolnick et al., 1992). Moreover, NMDA receptor agonists enhance the release of norepinephrine from hippocampal slices and synaptosomal preparations, while antagonists block those effects (Jones et al., 1987; Ransom and Deschenes, 1988; Schmidt and Taylor, 1988; Keith et al., 1989; Pittaluga and Raiteri, 1990). In addition, one study indicates that co-incubation of forebrain-derived synaptosomes with competitive NMDA receptor antagonists inhibit basal turn-over of norepinephrine (Yee et al., 1989). It is likely

that an increase in synaptic cleft concentration of norepinephrine can account for the down-regulation of β-adrenoceptors observed after chronic treatment with NMDA receptor antagonists and NO synthase inhibitors. The present findings are consistent with our earlier report indicating that drugs which modify transmission through NMDA-coupled cation channels may represent a novel approach to the treatment of endogenous depression (Trullas and Skolnick, 1990; Paul et al., 1992).

Activation of NMDA receptors on neurons in the vertebrate central nervous system is important for excitatory synaptic transmission (Thomson, 1986), synaptic plasticity (Tsumoto et al., 1987), neurotoxicity (Choi, 1988), and leads to the production of NO (Garthwaite et al., 1988, 1989). NO is a membrane-permeable molecule involved in signaling processes and cellular communication in a variety of systems. Previous studies have suggested that NO may be able to stimulate the release of neurotransmitters such as dopamine, and norepinephrine (Zhu and Luo, 1992), and that effect is dependent on NMDA receptor stimulation. NMDA receptors, when stimulated, can open ion channels to admit extracellular Ca^{2+}. Ca^{2+} binds to calmodulin, which then activates NO synthase. NO synthase is a Ca^{2+}-dependent enzyme, therefore Ca^{2+} entry through the NMDA channel is likely to be necessary for NO production (Garthwaite, 1991). Moreover, recent data suggest that NMDA-mediated neurotransmitter release is linked to NO production inasmuch as NO synthase inhibitors L-NNA and 7-nitroindazole blocked the NMDA-mediated release of neurotransmitters (Montague et al., 1994). The mechanism by which NO synthase inhibitors induce β-adrenoceptor down-regulation is still speculative, although, several studies indicate that NO modulates dopamine and norepinephrine release. In fact, both an increase (Zhu and Luo, 1992; Strasser et al., 1994) and decrease (Bowyer et al., 1995; Lin et al., 1995) in basal monoamine efflux after pretreatment with NO precursors or donors have been reported. Recent reports indicate that NO plays the role of an inhibitory endogenous substance in discriminative effects of psychostimulants in rats, because inhibition of NO synthase enhances the effects of amphetamine and cocaine while an increased [NO] attenuates them (Filip and Przegalinski, 1998). Moreover, microdialysis data have shown that NO has an inhibitory influence on dopamine release in the rat striatum (Guevara-Guzman et al., 1994). Likewise, Silva et al. (1995) demonstrated an increased dopamine release in vivo following intrastriatal administration of 7-nitroindazole, which was antagonized by coperfusion with L-arginine.

The first evidence of biochemical linkage between antidepressant medication and NO synthase activity was described by Mitchell et al. (1996). They observed that selective serotonin reuptake inhibitor—paroxetine inhibits constitutive isoform of NO synthase activity in the laboratory animals and in humans. Clinical depression has been associated with a variety of physiological and biochemical abnormalities including dysfunction in circadian rhythm, sexual impotence, altered serotonergic activity and enhanced platelet aggregation (Meltzer and Lwy, 1987; Arora and Meltzer, 1989; Kusumi et al., 1991; Kirby, 1994). Serotonin dysfunction underlies various physiological abnormalities characteristic of clinical depression. Serotonin has been shown to mediate effects through NO (Verbeuren et al., 1991) and NO has been implicated as a signaling molecule in each of the above mentioned physiological processes that are manifestly dysfunctional in patients with clinical depression (Radomski et al., 1990; Burnett et al., 1992; Balligand et al., 1993; Ding et al., 1994).

Our present observations that a NO synthase inhibitor exhibits antidepressant-like activity can provide new avenues for the identification and development of novel antidepressant therapies. Developing well tolerated and selective NO synthase inhibitors for the various isoforms of NO synthase may be useful for the treatment of central nervous system disorders in which a significant role of NO is implicated.

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