Short communication

Anesthetic concentrations of riluzole inhibit neuronal nitric oxide synthase activity, but not expression, in the rat hippocampus

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Abstract

We hypothesized that anesthetic dose of riluzole, an inhibitor of glutamate neurotransmission, may affect the activity and/or expression of neuronal NOS (nNOS). Riluzole, 1G-nitro-L-arginine-methyl ester (L-NAME) and 7-nitro indazole (7-NI) produced a concentration-related inhibition of nNOS activity in vitro. Riluzole competed with 7-NI for inhibition of nNOS activity, but had no effect on nNOS or endothelial NOS (eNOS) protein expression. Also, nNOS activity was significantly decreased in riluzole-anesthetized rats (40 mg kg\(^{-1}\) i.p., \(-32\pm6\%\) from controls, \(P<0.05\)). Therefore, blockade of nNOS activity may be involved in the anesthetic effects of riluzole in vivo.

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Riluzole (2-amino-6-trifluoromethoxy benzothiazole) is a sedative/anesthetic [18] and neuroprotective agent in vitro and in vivo [7,17]. Also, it prolongs survival in patients with neurodegenerative diseases such as amyotrophic lateral sclerosis [15]. Some of the target sites of riluzole have been identified yet: riluzole depresses glutamate release in the striatum [4], it slows down conduction velocity of presynaptic glutamate fibers [16] and blocks some postsynaptic responses mediated by glutamate-gated ionic channels [5] as well as by voltage-gated calcium and sodium channels [12,13]. It has also been recently shown to stimulate neuronal two P domain K\(^+\) channels [8].

The mechanisms underlying the action of anesthetics in the central nervous system remain still to be clarified. Anesthetics are known to enhance inhibitory GABA\(_A\)-mediated neurotransmission and depress the excitatory, glutamate-mediated neurotransmission [11]. Several lines of evidence suggest, however, that the NO-cGMP pathway could also be one of the potential targets of anesthetics in the mammalian brain [14,19,20]. We have previously shown that riluzole, used at concentrations greater than those for which neuroprotective properties are observed, is an anesthetic agent [18]. Here, we hypothesized that anesthetic concentrations of this agent could also directly affect nNOS activity. The aim of the present study was first, to examine the effects of riluzole on hippocampal nNOS and eNOS protein expression, and second, on hippocampal nNOS activity both in vitro and in riluzole-anesthetized rats.

All experiments were conducted on male Sprague–Dawley rats strictly according to the recommendations of the European Communities Council Directive of 24 November 1986 and the NIH Guide for the Care and Use of Laboratory Animals.

In order to verify that constitutive NOS was predominantly represented by nNOS, the nNOS:eNOS ratio was determined in the preparation. Tissue samples were homogenized in a 1.5 ml lysis buffer (Tris-HCl 50 mM (pH 7.4), EDTA 0.1 mM, Leupeptin 1 \(\mu\)M, PMSF 1 \(\mu\)M, aprotinin 1 \(\mu\)M) and incubated with riluzole (200 \(\mu\)M, 60
min) or without any pharmacological agent (control). Proteins (75 μg per lane) and the tissue homogenates were denatured by boiling for 5 min in sample buffer (0.5 M Tris–HCl (pH 6.8), 10% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-β-mercaptoethanol, 0.05% (w/v) bromophenol blue) and separated by electrophoresis on precoated 7.5% sodium dodecyl sulfate–polyacrylamide gel (Bio-Rad, Richmond, CA). They were transferred overnight at 4°C to a PVDF membrane (Bio-Rad) in 25 mM CAPSO buffer (pH 10) and 20% methanol. Subsequent steps were performed at room temperature. The membranes were then blocked with 10% non-fat dry milk in Tris-buffered saline (25 mM Tris (pH 7.5), 150 mM NaCl) +0.05% (v/v) Tween-20 (TBST solution). After washing in TBST, the membranes were incubated 1 h with a monoclonal anti-nNOS or anti-eNOS antibody used at 1:1000 dilution (Transduction Laboratories, Lexington, KY). After washing, the membranes were incubated for 1 h with 1:3000 dilution of a goat anti-mouse IgG or a goat anti-mouse conjugated to alkaline phosphatase (Bio-Rad). The blots were washed with TBST, followed by detection of immunoreactive proteins by the enhanced chemiluminescence method using AMPPD (Tropix) as substrate (Bio-Rad). Results were expressed as the nNOS/β actin ratio.

NOS activity was measured by the conversion of L-[3H]arginine to L-[3H]citrulline [3]. Briefly, a 25-μl homogenate of hippocampal tissue corresponding to 50 μg total cellular proteins was incubated for 60 min at 37°C in the reaction buffer (150 μl final volume) containing 50 mM Hepes (pH 7.40), 0.5 mM NADPH, 5 mM FAD, and 5 mM tetrahydrobiopterin (B. Schricks Laboratories), 1.25 mM CaCl₂, 10 mM calmodulin and 39 nM L-[3H]arginine (specific activity 35.7 Ci/mmol). The enzymatic reaction was stopped by the addition of 2 ml ice-cold mM Hepes (pH 5.5)/2 mM EDTA, and the total volume was applied to Dowex-50W X8 column (Bio-Rad) preequilibrated with the same buffer. L-[3H]Arginine was retained on the column, whereas L-[3H]citrulline was eluted with 2 ml deionized water. L-[3H]Citrulline concentrations were determined by liquid scintillation counting after subtracting the blank values, which gave the non-specific radioactivity in the absence of the enzyme. Various concentrations of riluzole (10⁻⁵–10⁻³ M) or its vehicle dimethylsulfoxide (DMSO), 7-nitro indazole (7-NI) and N⁶-nitro-L-arginine-methyl ester (L-NAME) (10⁻⁷–10⁻³ M) were added to the incubation buffer. Concentration–response curves, IC₅₀ values and Hill coefficients were computer-generated using GraphPAD software (Intuitive Software for Science, San Diego, CA).

Animals anesthetized with riluzole were given either riluzole (40 mg kg⁻¹) dissolved in 0.5 ml in DMSO or DMSO intraperitoneally. Anesthesia was defined by the loss of righting reflex, which is the inability of the animal to right itself when being placed in a lateral recumbent position. Animals were killed 30 min after the i.p. injection, since this delay corresponds to the time of maximal riluzole plasma concentrations achieved following i.p. injection [2].

Results were considered reliable only if they had been reproduced in at least four independent experiments (each run in triplicate). Data are presented as mean±S.D. Normality of distributions was first assessed by the Fisher test for equality of variances. Statistics were performed by analysis of variance followed by post hoc Scheffe’s test. A P value of less than 0.05 was considered the threshold for significance. nNOS was the predominant NOS isoform present in the hippocampal homogenates (Fig. 1). Indeed, 92% of the expression of NOS was represented by nNOS.
while eNOS was only 8%. Riluzole (200 μM) was not found to significantly alter either nNOS or eNOS protein expression (Fig. 1). nNOS basal activity was 16.1 ± 3.2 pmol (mg protein)^{-1} min^{-1}, in the hippocampus. Removing CaCl₂ from the buffer resulted an almost complete (more than 90%) reduction in nNOS activity in both structures. Both l-NAME and 7-NI induced a potent, concentration-related, inhibition of the calcium-dependent NOS activity, the IC₅₀ values being 0.5 μM for l-NAME and 4 μM for 7-NI (Fig. 2). The best fit of the curves to the data was always obtained when the Hill coefficient was forced to 1. Riluzole caused a concentration-related inhibition of nNOS activity in the hippocampus (IC₅₀ = 200 μM, Fig. 2). The vehicle, DMSO (5 mM) did not produce any change in nNOS activity. The kinetic profile of nNOS inhibition induced by riluzole was characterized by a lack of change in Vₐₘₙ (2493 ± 131 vs. 2667 ± 102 pmol (mg protein)^{-1} min^{-1}) and a significant increase in Kₐₜₚ (9.5 ± 0.9 vs. 6.4 ± 0.8 μM, P < 0.05). In the presence of an IC₅₀ concentration of 7-NI (4 μM) there was a marked decrease in the potency of riluzole inhibition as assessed by a more than 1000-fold increase in the IC₅₀ value (500 000 μM), while no change in IC₅₀ value was observed for l-NAME under these experimental conditions.

All rats treated with riluzole i.p. (n=8) had loss of righting reflex, while this was not observed in the DMSO-treated animals (control group, n=8). The onset time of riluzole action after injection was 4.7 ± 2.3 min. nNOS activity was significantly decreased in rats given riluzole (40 mg/kg i.p.) vs. controls (−32 ± 6%, P < 0.05).

The present study indicates that riluzole blocks nNOS activity, but not protein expression, in homogenates of the rat hippocampus. Riluzole competes with 7-NI, a NOS inhibitor structurally distinct from l-arginine analogues, for nNOS inhibition. These findings may have potential physiological relevance, since hippocampal nNOS activity is significantly decreased in riluzole-anesthetized rats as well.

NOS activity measured in the present study was constitutive NOS, since it was clearly dependent on external calcium. Theoretically, both nNOS and eNOS were likely to contribute to the NOS signal, since these two isoforms are present in both the hippocampus [10]. Our results clearly indicate that the main isoform of NOS expressed in the hippocampus was the nNOS. Both eNOS and nNOS are sensitive to competitive inhibitors of the class of arginine analogues, such as l-NAME. However, 7-NI appears to be a potent and somewhat selective inhibitor of nNOS [1]. We found that both l-NAME and 7-NI potently inhibited NOS activity in the hippocampus. The efficacy of 7-NI at blocking NOS in the present study supports that NOS played a major role in the NOS activity measured in our study. 7-NI exhibited a lower potency in our study than in the cerebellum in vitro while l-NAME had the same IC₅₀ as that reported previously in this area [1]. This might be due to the presence of a different NOS:eNOS ratio in the cerebellum in comparison with the hippocampus.

The ability of riluzole to block nNOS activity has not been reported yet and was consistent with a competitive profile. The kinetic parameters associated with riluzole effects were supportive of a competitive action, since no change in Vₐₘₙ was observed whilst a significant decrease in riluzole affinity for the enzyme was reported. Cooperative mechanisms with conformational changes of the enzyme are unlikely, since the best fit of the curves to the data was always obtained when Hill slope was forced to one. Further, we observed that the potency of riluzole effect on nNOS was dramatically decreased in the presence of 7-NI. In contrast, the same 7-NI concentration failed to significantly affect l-NAME potency for NOS inhibition. These observations, together with the lack of effect of riluzole on NOS protein expression, are supportive of a competition between riluzole and 7-NI for the substrate site of nNOS, since l-NAME is an irreversible inhibitor of nNOS [6]. Since 7-NI is known to bind to the heme site on NOS, thereby hindering access of both the arginine and the tetrahydrobiopterin to their respective bindings sites, explaining the apparent competitive kinetics for 7-NI at both sites, it might be speculated that riluzole likely acts on the same heme moiety.

The concentrations for which nNOS inhibition by riluzole was observed (IC₅₀ = 200 μM) were higher than those reported to be neuroprotective. We did not directly measure plasma concentrations of riluzole at the moment of sacrificing the rats for processing of nNOS activity measurement. However, owing to the pharmacokinetic properties of riluzole and the anesthetic dose selected, such concentrations were very likely to be present in the plasma of anesthetized animals [18]. In addition to its properties on glutamate transmission, the inhibitory effect of riluzole on NOS activity may be relevant to the mechanisms whereby riluzole induces anesthesia. Decreasing the activity of the NO–cGMP pathway has been proposed to contribute to the effects of anesthetics on the mammalian brain [14,19,20]. As anesthesia is a reversible phenom-
enon, any reported neurobiological action likely to contribute to the production of unconsciousness should not induce irreversible damage. Both the competitive profile of riluzole-induced nNOS inhibition and the lack of effect of riluzole on expression of nNOS supports that riluzole does not irreversibly alter the structure of the enzyme. The fact that nNOS activity was inhibited both in vitro by riluzole and in rats anesthetized with this agent strongly supports physiological relevance of the reported effects. Finally, it has been recently proposed that processes controlled by the cortical NMDA synapse (and thereby the NO–cGMP pathway) play a crucial role for consciousness and are the final common pathway of anesthetic action [9]. Our findings are consistent with this hypothesis.

In summary, we have shown a concentration-related, competitive, inhibition of basal hippocampal nNOS activity by riluzole in the rat hippocampus. This action may contribute to the anesthetic effect of riluzole in vivo.

References


