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Inhibition of nitric oxide synthase by straight chain and cyclic alcohols

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Abstract

Nitric oxide appears to mediate some of the central effects of alcohols. However, the direct effects of alcohols on brain nitric oxide synthase have not been determined. In the present study, we tested on purified nitric oxide synthase from rat brain *n*-alkan-1-ols, *n*-alkan-2-ol enantiomers, cycloalkanols, and cycloalkanemethanols. In general, the alcohols inhibited nitric oxide synthase activity noncompetitively. Enzyme inhibitory potencies increased with increasing lipophilicity (increasing carbon number) up to the point of 'cutoff', which was C7 for *n*-alkan-1-ols and C13 for cycloalkanemethanols, indicating that the alcohol binding site on nitric oxide synthase accommodates a maximum chain length of 6–7 carbon atoms. Before the point of 'cutoff', K_i values for the cyclic alcohols and short chain *n*-alkanols on nitric oxide synthase were less than their respective anesthetic EC_{50} values. As reported for tadpole anesthesia, there was no stereoselectivity between enantiomeric pairs of secondary alcohols for inhibition of nitric oxide synthase. These results indicate that the nitric oxide synthase inhibitory potency of alcohols of diverse structure is directly related to lipophilicity and length of the alcohols and that direct inhibition of nitric oxide synthase mediates some of the effects of alcohols even at subanesthetic concentrations.

Keywords: Alcohol; Nitric oxide (NO) synthase; Brain, rat

1. Introduction

Nitric oxide synthase is a heme-containing enzyme that catalyzes the formation of nitric oxide (NO) from arginine. NO is a novel neurotransmitter and second messenger which affects virtually all cells. NO has been implicated in a large number of pathologies (such as neurotoxicity in Alzheimer's disease and Huntington's disease, cerebral ischemia, stroke and anxiety); also in normal physiological functions, such as memory and learning, regulation of the cerebrovascular system, modulation of wakefulness, mediation of nociception, and regulation of neurotransmitter release (Bruhwyler et al., 1993).

Two lines of evidence suggest that alcohols and other anesthetics interfere with the arginine-nitric oxide synthase-NO systems. Firstly, nitric oxide synthase inhibitors modify the pharmacological effects of alcohols and anesthetics. For example, inhibition of nitric oxide synthase blocked the development of rapid tolerance to the motor incoordinating effects of alcohols (Khanna et al., 1993) and alcohol-induced suppression of testosterone secretion (Adams et al., 1993) in rats, suggesting that alcohol interacts with NO pathways regulating alcohol-induced tolerance and testicular effects. A recent study (Adams et al., 1995) indicated that the arginine-nitric oxide synthase-NO system is involved in mediating alcohol dependence and withdrawal. Not only did the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (NAME) consistently decrease the severity of withdrawal signs if given during withdrawal, but the NO donor, isosorbide dinitrite, increased the severity of withdrawal signs. It has been suggested that NO mediates chronic alcohol-induced neurotoxicity caused by the excess activity of excitatory amino acids (Lancaster, 1992). In rabbits, alcohol anesthesia was accompanied by decreased levels of exhaled NO (Persson and Gustafsson, 1992). Pretreatment with NAME, 40 min before alcohol treatment, delayed the onset and increased the duration of alcohol-induced loss of righting reflex. Simultaneous treatment with the nitric oxide synthase substrate, L-arginine methyl ester, and NO donor, isosorbide dinitrite, blocked the effects of NAME (Adams et al., 1994). Inhibition of NO production enhanced, and isosorbide dinitrite inhibited, alcohol-induced narcosis (Boucher et al., 1992).

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Secondly, cytochrome *P*-450 enzymes are sensitive to clinically relevant concentrations of diverse alcohols and anesthetics (LaBella and Queen, 1993). The cloning of nitric oxide synthase and cytochrome *P*-450 enzymes shows that these two enzyme classes are similar in several ways. Both are heme-containing oxygenases and possess binding sites for NADPH (nicotinamide adenine dinucleotide phosphate), FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide). In addition, nitric oxide synthase catalyses a *P*-450-like reaction (Bredt et al., 1991). Nitric oxide synthase shows 58% homology with cytochrome *P*-450 reductase. Therefore, nitric oxide synthase may share with cytochrome *P*-450 a similar sensitivity to inhibition by alcohols and other anesthetics.

This indirect evidence suggests a potential role of perturbation of the arginine-nitric oxide synthase-NO system in contributing to the effects of alcohols and anesthetics. However, direct effects of alcohols and other anesthetics on nitric oxide synthase remain uncertain. In unpublished work we found that short chain alcohols and several anesthetics with relatively lower lipophilicity, e.g., paraldehyde, diethyl ether, phenobarbital, acetone, methyl ethyl ketone, inhibited nitric oxide synthase activity from rat brain at concentrations below or close to their anesthetic EC_{50} values, while the highly lipophilic anesthetics, enflurane, pentobarbital, thiopental and propofol inhibited nitric oxide synthase activity only at concentrations higher than their anesthetic EC_{50} values. These findings raised a further question as to whether the inhibitory potency on nitric oxide synthase is determined solely by the lipophilicity of a compound or that, in addition, there are specific structural requirements.

The alcohols have been among the most widely studied CNS (central nervous system) depressants in experimental animals. They have been applied for probing the molecular dimensions of the anesthesia site because of the availability of different chain lengths, molecular volumes, cyclic series and enantiomers. In the present study, *n*-alkan-1-ols, *n*-alkan-2-ols, cycloalkanols, and cycloalkanemethanols (Fig. 1) were tested on nitric oxide synthase. The polar heads on both the *n*-alkan-1-ol and cycloalkanemethanol series are identical, but the cycloalkanemethanols and cycloalkanols are approximately one-half the length of their respective *n*-alkan-1-ol and *n*-alkan-2-ol analogs.

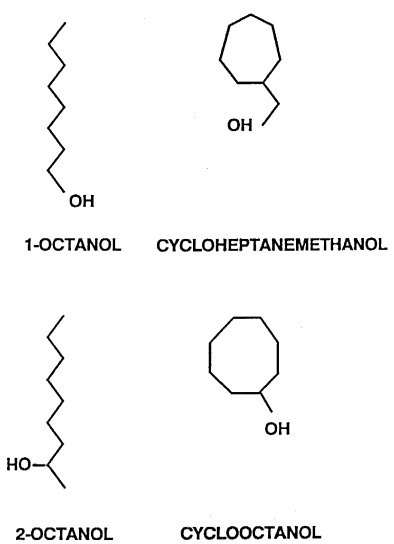

Fig. 1. Structures of 8-carbon alcohols: cycloheptanemethanol, 1-octanol, cyclooctanol and 2-octanol.

2. Materials and methods

2.1. Materials

[^{3}H]Arginine (35.7 Ci/mmol) was purchased from New England Nuclear. Common buffer salts and biochemicals were purchased from Sigma (St. Louis, MO, USA). Dowex AG50 X-8 (Na form) resin was purchased from Bio-Rad (Hercules, CA, USA) and packed into glass pipette tubes (1 ml resin/tube).

2.2. Preparation of rat brain supernatant (Bredt and Snyder, 1989)

Male Sprague-Dawley rats (250–275 g) were killed by decapitation. Cerebellum plus olfactory bulb were homogenized in 20 vols. of 0.32 mM sucrose/20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ehanesulfonic acid), pH 7.4/0.5 mM EDTA (ethylenediaminetetraacetic acid)/1 mM dithiothreitol and centrifuged at $175\,000 \times g$ for 30 min. The supernatant was kept on ice prior to assay of nitric oxide synthase. Protein was determined according to Lowry et al. (1951).

2.3. Enzyme assays

Nitric oxide synthase activity was measured using the method of Bredt and Snyder (1989) with some modifications. After preincubation of the brain supernatant at 37°C for 5 min, alcohol or vehicle control was added with vigorous vortexing, followed by 20 min incubation at room temperature with constant mixing. The reaction was started by the addition of arginine (0.8–20 μM) and [^{3}H]arginine (0.2 μCi/ml) to buffer containing (final concentrations) 2 mM NADPH, 0.45 mM Ca^{2+} and tissue supernatant (0.2 mg protein/ml). After 6 min incubation at 37°C, the reaction was terminated by pouring the reaction mixture into 2 ml of 20 mM HEPES (pH 5.5)/2 mM EDTA. [^{3}H]Citrulline was isolated by chromatography on a Dowex AG50 X-8 (Na form) column and radioactivity measured in the effluent fractions. K_m and K_i were determined with the IBM-PC program ENZYME by a weighted non-linear squares curve-fitting procedure (Lutz et al., 1986).

3. Results

The inhibition of nitric oxide synthase activity by 5- and 8-carbon members of each of the five alcohol series is compared in Fig. 2. Alcohol concentrations used in this study were chosen on the basis of their anesthetic EC_{50} values (Table 1). Comparison of the effects of *n*-alkan-1-ols and cycloalkanemethanols, *n*-alkan-2-ols ($S(+)$ and $R(-)$) and cycloalkanols on nitric oxide synthase activity showed that 10 mM of 5-carbon members of each alcohol series inhibited nitric oxide synthase activity significantly (Fig. 2, upper panel). The order of inhibitory potency was: 1-pentanol > 2-pentanol = cyclobutanemethanol > cyclopentanol, in accordance with the order of lipophilicity for these alcohols (McCreery and Hunt, 1978). However, for the 8-carbon member, the order was: cycloheptanemethanol > cyclooctanol > 1-octanol > 2-octanol (Fig. 2, bottom panel). The order of inhibition on nitric oxide synthase cannot be accounted for by the lipophilicity of the alcohols. There was no significant difference between $R(-)$ and $S(+)$ secondary alcohols for both 5- and 8-carbon members (Fig. 2), suggesting lack of stereoselectivity for inhibition of nitric oxide synthase.

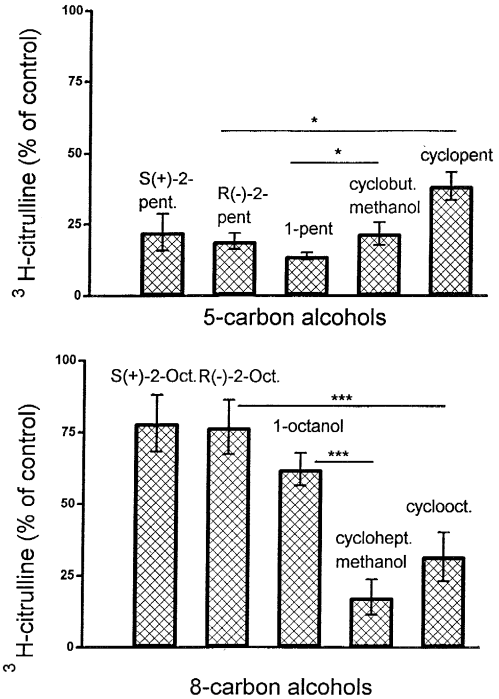

Fig. 2. Inhibition of nitric oxide synthase activity by 5- and 8-carbon alcohols. Nitric oxide synthase activity was expressed as amount of [^{3}H]citrulline formed as percent of control. The results are mean ± S.E. of three experiments, each with a single determination. For both 5- and 8-carbon alcohols, 1-alkanols were compared with their respective cycloalkanemethanol analogs, while 2-alkanols were compared with their respective cycloalkanol analogs. Significant difference between the two groups: * $P < 0.05$; *** $P < 0.0001$. Upper panel: each of the 5-carbon alcohols was tested at 10 mM. Bottom panel: each of the 8-carbon alcohols was tested at 0.3 mM.

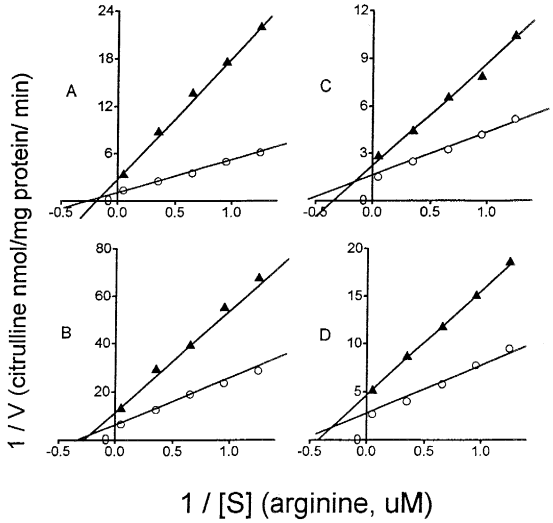

Fig. 3. Lineweaver-Burk plots of nitric oxide synthase as affected by 1-butanol, cyclopropanemethanol, 1-octanol, and cycloheptanemethanol. Open circles, controls; solid triangles, alcohols; A, 1-butanol, 18 mM; B, cyclopropanemethanol, 27 mM. C, 1-octanol, 0.78 mM; D, cycloheptanemethanol, 0.107 mM. All alcohols inhibited nitric oxide synthase noncompetitively.

To further provide evidence for the molecular dimensions of the alcohol binding pocket on nitric oxide synthase, we determined K_i values for the cycloalkanemethanol and *n*-alkan-1-ol series. Typical Lineweaver-Burk plots for 1-butanol and cyclopropanemethanol are shown in Fig. 3A and B, and for 1-octanol and cycloheptanemethanol in Fig. 3C and D. Each of these alcohols inhibited nitric oxide synthase noncompetitively. The concentrations for alkanol and cycloalkanemethanol series to inhibit rat brain nitric oxide synthase (K_i) in vitro and to effect general anesthesia (EC_{50}) are compared in Table 1. In the alkanol series, K_i values for the short chain alcohols (C3, C4) were at or below their anesthetic EC_{50} values, while those for 1-heptanol and 1-octanol were 3.47 and 14.3 times their EC_{50} values, respectively (Table 1). In the cycloalkanemethanol series, the K_i values were less than their anesthetic EC_{50} values, except for C13 which gave a K_i value 9.69 times its EC_{50} (Table 1). The K_i values for C9–C12 members of the cycloalkanemethanol series were not determined because these alcohols were not available commercially. However, the fact that K_i/EC_{50} ratio of cycloalkanedodecanol was 9.69, even lower than that for 1-octanol (14.3), suggests that the cutoff for the cycloalkanemethanol and *n*-alkan-1-ol series occurs at about the same molecular length. In both series, the inhibitory potency increases with each additional methylene group (and increased lipophilicity) up to the point of cutoff, which occurred at about C7 to C8 for the straight chain alcohols and C13 for cycloalkanemethenols, respectively. Before the point of cutoff, inhibition of nitric oxide synthase by both alcohol series occurred at concentrations lower than their anesthetic concentrations. Comparing the

Table 1
Concentrations of alcohols to inhibit rat brain nitric oxide synthase (K_i) in vitro and to effect general anesthesia (EC_{50})

Alcohol	No. of carbons	K_i (μM)	EC_{50} [a] (μM)	K_i/EC_{50}
n-Alkan-1-ol	3	30 000 ± 5 579	73 000 ± 2 400	0.41
	4	5 490 ± 140	10 800 ± 770	0.51
	7	797 ± 53	230 ± 11	3.47
	8	816 ± 11	57 ± 2.5	14.3
n-Alkan-2-ol	4	17 000 ± 2 400	17 100 ± 110	1.01
Cycloalkanemethanol	4	19 800 ± 2 015	54 000 ± 3 200	0.37
	6	727 ± 62	2 700 ± 190	0.27
	8	65 ± 14	320 ± 14	0.20
	13	126 ± 27	13 ± 2	9.69

[a] EC_{50} for general anesthesia (reversible loss of righting reflex) in the tadpole: data for *n*-alkan-1-ols from Alifimoff et al. (1989), for *n*-alkan-2-ol from Alifimoff et al. (1987) and for cycloalkanemethanols from Raines et al. (1993). K_i values are from the mean ± S.E. of three experiments, each with a single determination.

relative inhibitory potency of the *n*-alkan-1-ols and their cycloalkanemethanol analogs with the same carbon number, it can be seen that at C4 the *n*-alkan-1-ol is about 3.6 times as potent as its cycloalkanemethanol analog, which may be attributed to greater lipophilicity, but for C8 the alkanol is 12.6 times weaker than the latter, which cannot be explained on the basis of lipophilicity.

4. Discussion

4.1. Characterization of the alcohol binding site on nitric oxide synthase with the use of straight chain and cyclic alcohols

By comparing the effects of *n*-alkan-1-ols and cycloalkanemethanols, *n*-alkan-2-ols and cycloalcohols, and the enantiomeric pairs of secondary alcohols on nitric oxide synthase, the alcohol binding site on the enzyme can be partially characterized. The rank order of potency to inhibit nitric oxide synthase by the 5-carbon members was: 1-pentanol > 2-pentanol = cyclobutanemethanol > cyclopentanol, a rank that relates to the order of lipophilicity. However, for the 8-carbon members, the cyclic alcohols were more potent than the straight chain alcohols. This difference may be related to molecular length; the lengths of the cyclic alcohols are about one-half that of their straight chain analogs (Raines et al., 1993). When the length of the straight chain alcohols exceeds the depth of the pocket, the carbon atoms extending outside the pocket do not contribute to the binding energy, and therefore do not enhance the inhibitory potency. On the contrary, the shorter cycloalcohols fit within the binding pocket and, with increasing number of carbons, there is a corresponding higher binding energy, and consequently, greater inhibitory potency. From Table 1, K_i values for *n*-alkan-1-ols and cycloalkanemethanols start to level off at about carbon 7 and 13, respectively, suggesting that the alcohol binding pocket on nitric oxide synthase accommodates a chain up to 7 carbons. Up to a certain molecular length, the inhibitory potency increased with the number of carbon atoms in the hydrocarbon chain, indicating that the alcohol binding site on nitric oxide synthase is hydrophobic in nature.

The alcohol binding site on nitric oxide synthase seems to contain a hydrophilic region which interacts with the –OH head of the alcohols. In both short and long chain alcohols, the secondary alcohols are slightly weaker than the primary alcohols with the same carbon number, while cycloalcohols are slightly weaker than cycloalkanemethanols, suggesting that the position of the –OH group affects the potency.

Comparison of the effects of enantiomeric pairs of secondary alcohols on nitric oxide synthase provided additional information on the alcohol binding site on this enzyme. When stereoselective binding occurs, an enantiomer interacts at three points with a chiral center in its target molecule. Nonselectivity will be observed if the binding site does not have a chiral center, or if binding involves only two points of attachment. There was no stereoselectivity between enantiomeric pairs of secondary alcohols (Fig. 2), a phenomenon also seen for tadpole anesthesia (Alifimoff et al., 1987).

4.2. The possible location of the alcohol binding site on nitric oxide synthase

Nitric oxide synthase is a heme enzyme with several binding domains. It has homologous domains to cytochrome *P*-450 reductase, substrate sites that reflect the oxidative mechanism of NO biosynthesis and a consensus binding site for calmodulin. The finding that all alcohol series tested inhibited nitric oxide synthase noncompetitively suggests that the alcohol binding site is distinct from the L-arginine binding site. It is known that the guanidino nitrogen and the gaunidino carbon of the substrate arginine must be in close proximity to the heme iron (Griffith and Stuehr, 1995). The finding that the alcohol series and most of the anesthetics also inhibit, at relevant concentrations, heme enzymes cytochrome *P*-450 and cyclooxygenase

(Chen et al., 1994a,b) suggests that alcohols and other anesthetics have an affinity for the environment of a heme moiety.

4.3. Functional consequences of inhibition of nitric oxide synthase by anesthetics

Our results indicate that nitric oxide synthase is a pharmacological target for diverse alcohols. Inhibition of nitric oxide synthase by alcohols would interfere with NO-mediated biological functions. In the central nervous system, inhibition of nitric oxide synthase obtunds the stimulation of cGMP (cyclic guanosine monophosphate) levels and, presumably, results in changes in the activity of protein kinases, cyclic nucleotide phosphodiesterase, ion channels and, consequently, neurotransmitter release (Garthwaite, 1991). Neurotransmission in response to acetylcholine, glutamate, and glycine has long been associated with increased brain cGMP concentrations (Garthwaite, 1990). Garthwaite et al. (1988) first demonstrated that the increase in cGMP concentrations in rat cerebellum in response to *N*-methyl-D-aspartate (NMDA) stimulation is the result of NO release. In addition to this indirect regulatory role through cGMP, NO directly modulates plasmalemmal and intracellular calcium channels and pumps, phospholipase C, protein phosphatase, and various protein kinases (Schmidt and Walter, 1994). Therefore, NO appears to serve both as second messenger and neurotransmitter to mediate the effects of alcohols and other anesthetics on multiple cell components. An increasing body of data indicates that NO is a physiological modulator of consciousness, analgesia and anesthesia (Nakamura and Mori, 1993). 7-Nitro indazole, an inhibitor of central but not endothelial nitric oxide synthase, induced a dose-related and prolonged (several hours) sedative/narcotic effect in rats, associated with loss of righting reflex, a decrease of desynchronized electrocorticogram amplitude and disruption of normal sleep stages (Dzoljic and Vries, 1994). N^G-Monomethyl-L-arginine, another nitric oxide synthase inhibitor, reduces the wakefulness response which can be abolished by administration of L-arginine, suggesting that central NO promotes vigilance (Dzoljic and Vries, 1994). Also, NO appears to control the functional state of the thalamocortical network (Pape and Mager, 1992), and to contribute to nociceptive processing within the spinal cord (Meller and Gebhart, 1993). In experimental pain in the rat, NO promotes spinal hyperalgesia, resulting in facilitation, expansion of receptive fields, and central sensitization (Meller and Gebhart, 1993). A variety of NO-related biological and physiological functions is affected by alkanols and general anesthetics. These functions include regulation of receptor-mediated ion channels, neurotransmitter release, synaptic transmission, synaptic plasticity, long-term potentiation, learning and memory, wakefulness, nociception and others (Bruhwyler et al., 1993; Garthwaite et al., 1988; Franks and Lieb, 1994).

Inhibition of nitric oxide synthase, together with the inhibition of other enzymes or functional proteins, would produce widespread perturbation of central nervous system functions. Inhibition of nitric oxide synthase by subanesthetic concentrations of alcohols may explain the lower dose (nonanesthetic) effects of alcohols, such as reinforcement, anxiolytic effects, incoordination, cognition effects, as well as the development of tolerance and dependence.

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