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Increased brain nitric oxide levels following ethanol administration

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ABSTRACT

Nitric oxide is a ubiquitous messenger molecule, which at elevated concentrations has been implicated in the pathogenesis of several neurological disorders. Its role in oxidative stress, attributed in particular to the formation of peroxynitrite, proceeds through its high affinity for the superoxide radical. Alcoholism has recently been associated with the induction of oxidative stress, which is generally defined as a shift in equilibrium between pro-oxidant and anti-oxidant species in the direction of the former. Furthermore, its primary metabolite acetaldehyde, has been extensively associated with oxidative damage related toxic effects following alcohol ingestion. The principal objective of this study was the application of long term *in vivo* electrochemistry (LIVE) to investigate the effect of ethanol (0.125, 0.5 and 2.0 g kg^{-1}) and acetaldehyde (12.5, 50 and 200 mg kg^{-1}) on NO levels in the nucleus accumbens of freely moving rats. Systemic administrations of ethanol and acetaldehyde resulted in a dose-dependent increases in NO levels, albeit with very differing time courses. Subsequent to this the effect on accumbal NO levels, of subjecting the animal to different drug combinations, was also elucidated. The nitric oxide synthase inhibitor L-NAME (20 mg kg^{-1}) and acetaldehyde sequestering agent D-penicillamine (50 mg kg^{-1}) both attenuated the increase in NO levels following ethanol (1 g kg^{-1}) administration. Conversely, the alcohol dehydrogenase inhibitor 4-methylpyrazole (25 mg kg^{-1}) and catalase inhibitor sodium azide (10 mg kg^{-1}) potentiated the increase in NO levels following ethanol administration. Finally, dual inhibition of aldehyde dehydrogenase and catalase by cyanamide (25 mg kg^{-1}) caused an attenuation of ethanol effects on NO levels. Taken together these data highlight a robust increase in brain NO levels following systemic alcohol administration which is dependent on NO synthase activity and may involve both alcohol- and acetaldehyde-dependent mechanisms.

1. Introduction

Nitric oxide (NO) is a pleiotropic signaling molecule with important roles in both the central and peripheral nervous systems [1]. It can also contribute to cellular toxicity from increased formation of oxidative species such as peroxynitrite ($ONOO^-$) [2] which can be highly detrimental to proteins and cell membranes. The latter effect is more likely in conditions with chronic increases in NO synthesis or a pro-oxidant environment.

There is a growing interest in the link between aberrations in nitric oxide signaling and neurodegenerative brain disorders such as Parkinson's disease [3] and multiple sclerosis [4]. In particular real-time measurements of NO in the ventral striatum of freely moving rats show the effect of exposure to PD risk factors and their link to theoretically predicted changes in reactive oxygen species [5]. Alcoholism is another example of a disorder where aspects of the pathophysiology of the disorder are linked to increased formation of oxidative species [6,7]. Further, alcohol dependent individuals have been shown to manifest increased plasma levels of NO precursors and metabolites [8] and mice lacking the inducible form of nitric oxide synthase do not develop signs of alcohol-induced liver injury [9]. Thus, nitric oxide may be a key component in the mechanism relating alcohol intake to oxidative stress.

Alcohol is mainly metabolized to acetaldehyde by alcohol dehydrogenase 2 in the liver, but other organs, including the brain, possess the catalytic capacity for alcohol metabolism [10]. Acetaldehyde is a highly toxic substance that is rapidly metabolized to less toxic species. However, the production of acetaldehyde resulting from alcohol ingestion has been associated with oxidative stress related toxic effects such as inhibited mitochondrial respiration [11]. Interestingly, recent studies suggest that acetaldehyde can exert neurobehavioral effects similar to alcohol in rodents. Blood acetaldehyde levels after alcohol intake are generally considered too low to effectively penetrate the blood–brain barrier [12,13], but as

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mentioned alcohol can be metabolized to acetaldehyde within the brain itself.

The aim of the present study was to investigate the effect of ethanol and acetaldehyde on *in vivo* brain NO levels using a NO sensitive electrochemical sensor which has been extensively characterized in a number of different brain regions [14].

2. Materials and methods

2.1. Animals

Male Wistar rats (Taconic, Denmark and Charles River, U.K., 250–300 g) were used. All animals were housed, with a maximum of four per cage, in a colony room under constant temperature (20 ± 1 °C) and humidity (50 ± 5%). Food and water were available *ad libitum*. The daylight cycle was maintained artificially (lights on from 0600 to 1800 hours), and the experiments were conducted during the light phase. The animals were allowed to acclimatize for at least one week prior to surgery. All experimental procedures were performed under license in accordance with the European Communities Regulations 2002 and was approved by the Ethics Committee for Animal Experiments, Göteborg, Sweden.

2.2. Drugs

Ethanol (VWR International AB, Stockholm, Sweden/Merck Chemicals, Ireland), acetaldehyde (SigmaAldrich), L-N^G-Nitroarginine methyl ester hydrochloride (L-NAME) (SigmaAldrich), D-penicillamine (SigmaAldrich), 4-methylpyrazole (SigmaAldrich), cyanamide (SigmaAldrich) and sodium azide (SigmaAldrich) were used in the present study. L-NAME, D-penicillamine, 4-methylpyrazole, cyanamide and sodium azide were dissolved in saline (0.9% NaCl) and injected subcutaneously (s.c.) in a volume of 2 ml kg^{-1}. Ethanol (95%) was diluted in saline to a final concentration of 15% and injected s.c. Similarly, acetaldehyde was diluted to 10% in saline and injected s.c.

2.3. Surgical procedure

The rats were anesthetized with isoflurane, placed in a Kopf stereotaxic instrument and kept on a heating pad to prevent hypothermia. An incision was placed down the midline of the skull and the bone was exposed. Four holes for the anchor screws, two holes for the reference (8T Ag wire, 200-μm bare diameter; Advent Research Materials, UK) and auxiliary (8T Ag wire) electrodes and two holes for the NO sensor were drilled. Electrodes were then implanted bilaterally. The coordinates used for the nucleus accumbens cortex relative to bregma were as follows: anterior +1.85 mm, lateral to midline ±1.3 mm, and ventral –6.8 mm from the brain surface. The electrodes were inserted into the brain and connected to a pedestal that was secured to the anchor screws with dental cement. During surgery, the rats were administered 2.0 ml of saline, to reduce postoperative dehydration and an analgesic (buprenorphine or carprofen) to reduce post-operative pain. The animals were allowed to recover for 2–3 days prior to the experiments. They were housed individually in standard plastic cages.

2.4. Electrochemical detection of nitric oxide

Brain NO levels were monitored in real-time using a NO selective amperometric microsensor. The microsensor is a Nafion-modified Pt disk electrode which has been extensively validated for *in vitro* and *in vivo* NO sensitivity [15,16] and *in vitro* selectivity against ascorbic acid, uric acid and dopamine [17]. The NO oxidation current (electrode potential of +0.90 V against an Ag reference electrode) was detected using a low-noise potentiostat (ACM Instruments, United Kingdom) and converted using an A/D converter (PowerLab, ADInstruments, United Kingdom). The digital signal was then recorded using Chart software (v5, ADInstruments) running on a PC or Apple computer. Individual sensors were tested for ascorbic acid interference and calibrated to ensure NO sensitivity *in vitro* prior to surgery.

Each rat was connected to the *in vivo* voltammetry equipment on the day before the experiment to allow the NO oxidation current to reach a stable baseline. All experiments were carried out with the animal in its home cage. For the dose–response experiments with ethanol and acetaldehyde animals were first administered saline (2 mL kg^{-1}) to control for effects related to the injection procedure followed by either ethanol (0.125, 0.5 and 2.0 g kg^{-1}) or acetaldehyde (12.5, 50 and 200 mg kg^{-1}) in a cumulative dosing regimen with a 3 hour dosing interval. In all other experiments, each animal was subjected to four drug treatment combinations with a two-day washout period between each treatment. The drug combinations were saline + saline, inhibitor + saline, saline + ethanol (1 g kg^{-1}) and inhibitor + ethanol (1 g kg^{-1}), where the inhibitor was one of L-NAME (20 mg kg^{-1}), D-penicillamine (50 mg kg^{-1}), 4-methylpyrazole (25 mg kg^{-1}), sodium azide (10 mg kg^{-1}) or cyanamide (25 mg kg^{-1}). The drug combinations were administered with a 15-minute interval between the two injections and the order of the drug combinations was varied between animals in a semi-randomized manner. The ethanol dose–response experiment, 4-methylpyrazole + ethanol and sodium azide + ethanol experiments were performed at the Department of Chemistry, National University of Ireland Maynooth. The acetaldehyde dose–response experiment, L-NAME + ethanol, D-penicillamine + ethanol and cyanamide + ethanol experiments were performed at the Department of Pharmacology, University of Gothenburg.

2.5. Probe placement verification

After termination of the experiments the rats were decapitated. The brains were removed and frozen at –80 °C. Sensor placement was verified by sectioning the brains with an atlas of the rat brain for reference. All sensors were verified to be positioned within the nucleus accumbens at 1.85 ± 0.2 mm anterior of bregma.

2.6. Statistical analysis

Unless otherwise stated, data represent mean ± S.E.M. with n = number of sensors implanted in 4 animals per group. Statistical analysis was carried out using Graphpad Prism 4. Dose/response data and ethanol response after inhibitor pre-treatment were evaluated using two-way ANOVA followed by Bonferroni post hoc test where appropriate. The response to inhibitor pre-treatment *per se* was evaluated as the change in the current from the baseline calculated as the area under the curve (nA × time) by integrating the current over the time period of the change. AUC analysis was carried out using unpaired t-tests. Two tailed levels of significance were used and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ethanol (0.125, 0.5 and 2.0 g kg^{-1}) and nucleus accumbens NO levels

Systemic administration of ethanol resulted in a dose-dependent increase in NO levels in the nucleus accumbens (Fig. 1) as demonstrated by the two-way ANOVA (effect of dose, $F(2,198) = 15.38$, $p < 0.001$). Further, the increase in NO levels was time dependent (effect of time, $F(11,198) = 11.32$, $p < 0.0001$) and the NO current over time profile differed between doses (dose × time interaction, $F(22,198) = 13.10$, $p < 0.0001$). The Bonferroni post hoc test indicated that the 2.0 g kg^{-1} dose differed significantly from the 0.125