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Research report

Nitric oxide synthase inhibitors protect cholinergic neurons against $AlCl_3$ excitotoxicity in the rat brain

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ABSTRACT

The present experiment was carried out to determine the effectiveness of nitric oxide synthase inhibitors: 7-nitroindazole and aminoguanidine in modulating the toxicity of aluminium chloride on acetylcholine esterase activity, as well as behavioural and morphological changes of Wistar rats. For biochemical analysis the animals were killed 10 min, 3 h, 3 days and 30 days after the treatment and forebrain cortex, striatum, basal forebrain and hippocampus were removed. The biochemical changes observed in neuronal tissues show that nitric oxide synthase inhibitors exert as protective action in aluminium chloride-treated animals. In the present study, active avoidance learning was significantly impaired after aluminium chloride injection, while pretreatment with nitric oxide synthase inhibitors prevented the behavioural deficits caused between 26th and 30th day after intrahippocampal application of neurotoxin. Our data suggest that aluminium may cause learning and memory deficits, while the treatment with specific nitric oxide synthase inhibitors may prevent learning and memory deficits caused by aluminium chloride. We have also applied immunohistochemical techniques to identify neuronal- and inducible-nitric oxide synthase expression 30 days after aluminium chloride and nitric oxide synthase inhibitors injections. Our data suggest that 7-nitroindazole and aminoguanidine can be effective in the protection of toxicity induced by aluminium chloride.

1. Introduction

Aluminium compounds are neurotoxic and have been shown to induce experimental neurodegeneration although the mechanism of this effect is unclear [9]. Aluminium is transported by the iron-carrier protein, transferrin that enters the brain by binding to transferrin receptors [39]. Aluminium exposure may exacerbate underlying events associated with brain aging and thus could contribute to progression of neurodegeneration [6]. Aluminium has the ability to produce neurotoxicity by many mechanisms, promoting formation and accumulation of insoluble beta-amyloid peptide (Abeta) and hyperphosphorylated tau [15]. Aluminium induces conformational changes of Abeta protein enhancing its aggregation and leading to progressive neuronal degeneration and death [24,26]. The senile plaques are generated by brain deposition of fibrils of Abeta, a fragment derived from the proteolytic processing of the amyloid precursor protein (APP) [32]. Tau protein is the major component of paired helical filaments, which form a compact filamentous network described as neurofibrillary tangles [20]. Also, to some extent, aluminium mimics the deficit of cortical cholinergic neurotransmission [25].

Aluminium exerts its toxic effects by altering cholinergic transmission, which is ultimately reflected in neurobehavioral deficits [14]. The dysfunction of cholinergic neurons is believed to be primarily responsible for cognitive deficits after intracerebral aluminium intoxication [37]. Cholinergic neurons, unlike other brain cells utilize acetyl-CoA not only for energy production but also for acetylcholine (ACh) synthesis. Therefore, suppression of acetyl-CoA metabolism by different neurotoxic inputs may be particularly harmful for this group of cells [36]. Studies *in vitro* have suggested that acetylcholine esterase (AChE), a marker of cholinergic system function, may interact with Abeta to promote deposition of amyloid plaques in the brain after aluminium intoxication [28].

Acetylcholine and nitric oxide (NO) are important neuromodulators implicated in brain plasticity and disease [30]. The use of NO donors and NO synthase (NOS) inhibitors as pharmacological tools revealed that this free radical is probably implicated in the regulation of excitability and firing, in long-term potentiation and long-term depression, as well as in memory processes [11]. Moreover, NO modulates neurotransmitter release. *In vivo* and *in*

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vitro studies have shown that, in all brain structures investigated, endogenous NO modulates the release of several neurotransmitters, such as ACh, catecholamines, excitatory and inhibitory amino acids, serotonin, histamine, and adenosine [27].

Nitric oxide is an enzymatic product of NOS, which exists in three isoforms, two constitutive enzymes (i.e., neuronal, nNOS, and endothelial eNOS) and one inducible enzyme (iNOS) [33]. Expression of both iNOS and eNOS is increased in activated astrocytes under experimental conditions associated with elevated expression of APP or Abeta-deposition [19]. Cell death and changes in neurite morphology were partly reduced when NO production was inhibited by NOS inhibitors [3].

Our previous results demonstrated positive effects of NOS inhibitors on the development of neurotoxicity [13,34,35]. In view of the above, the present investigation was undertaken to examine whether activity of AChE and the task of active avoidance after receiving intracerebral injections of aluminium chloride ($AlCl_3$) can be modulated by the pretreatment with 7-nitroindazol (7-NI), the specific neuronal NOS inhibitor and aminoguanidine (AG), the specific inducible NOS inhibitor.

2. Materials and methods

2.1. Animals

Male adult Wistar rats, with body mass 500 ± 50 g, were used for experiments. Groups of two or three rats per cage (Erath, FRG), were housed in an air-conditioned room at a temperature of 23 ± 2 °C with 55 ± 10% humidity and with lights on 12 h/day (07.00–19.00 h). The animals were given a commercial rat diet and tap water ad libitum.

Animals used for procedure were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

2.2. Experimental procedure

Animals were anesthetized by intraperitoneal injections of sodium pentobarbital (0.04 g/kg b.w.). A single dose of aluminium chloride ($AlCl_3$) (Sigma, USA) (3.7×10^{-4} g/kg b.w. in 0.01 ml of deionizied water), was injected into CA1 sector of the hippocampus, by using a Hamilton microsyringe, using stereotaxic instrument for small animals (coordinates: 2.5 A; 4.2 L; 2.4 V) [17]. The second and third groups were treated with 7-NI (Sigma Chemical Co., USA; 1×10^{-4} g dissolved in olive oil) + $AlCl_3$ and 7-NI + saline solution. The fourth and fifth groups were treated with AG (Sigma Chemical Co., USA; 1×10^{-4} g dissolved in saline solution) + $AlCl_3$ and AG + saline solution. Both, 7-NI and AG were immediately used before neurotoxin. The sixth group ($n = 10$) received the same volume of 0.9% saline solution only and it served as control (sham-operated). In all treated animals the injected intracerebral volume was 10 μl and it was always injected into the same-left side.

For biochemical analysis the rats were divided into six basic groups (according to drug treatment). Each basic group consisted of four different subgroups (according to survival times—10 min, 3 h, 3 days and 30 days) and each subgroup consisted of 10 animals. All animals were decapitated and heads were immediately frozen in liquid nitrogen and stored at −70 °C until use. Then ipsi- and contralateral forebrain cortex, striatum, basal forebrain and hippocampus were quickly isolated and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580 × *g* for 15 min at 4 °C. The supernatant (crude mitochondrial fraction) obtained by this procedure was then frozen and stored at −70 °C [10].

For the test of acquisition and expression of active avoidance, 26th day after the treatment (saline solution, $AlCl_3$, NOS inhibitors + $AlCl_3$, NOS inhibitors), animals were subjected to behavioral tests (two-way active avoidance) over the five consecutive days. Animals were then sacrificed by decapitation 30 days after surgery, their brains were removed and flash-frozen in liquid nitrogen.

For immunohistochemical analysis the animals were decapitated 30 days after the treatment. Brains were removed from the skull, fixed in 4% paraformaldehyde (TAAB Laboratory Equipment, Aldermaston, UK) for at least 24 h and cryoprotected in graded sucrose at 4 °C. Brains were frozen in methylbutane and stored at −70 °C until cryosectioning (CRIOCUT-E Reichert-Yung).

2.3. Biochemical analyses

The determination of acetylcholine esterase (True cholinesterase; Acetylcholine acetylhydrolase EC 3.1.1.7, AChE) activity was based on degradation of acetyl tiocholine iodide by AChE into a product which binds to 5,5-dithiobis-2-nitrobezoic acid (DTNB), forming yellow colour [22]. Kinetics of the enzymatic reaction was followed over 3–5 min at 412 nm. Values of AChE activity were calculated from the linear part of the reaction curve and were expressed as μmol acetyl tiocholine/min/g prot.

The protein content in the rat brain homogenates (forebrain cortex, striatum, basal forebrain and hippocampus, ipsi- and contralateral) was measured by the method of Lowry using bovine serum albumin (Sigma) as standard [18].

Chemicals were purchased from Sigma (St. Louis, MO, USA). All used chemicals were of analytical grade. All drug solutions were prepared on the day of experiment.

2.4. Active avoidance test

2.4.1. Apparatus

Acquisition of two-way active avoidance (AA) was studied in a series of automatically operated commercial shuttle-boxes and programming-recording units (Campden Instruments, USA). Boxes (48 cm × 21 cm × 22.5 cm) were used without the central partition.

2.4.2. Procedure

The acquisition of AA responses was achieved using spaced trials behavioral procedure (20-trial sessions daily for five consecutive days). A conventional two-way AA schedule was used with trials starting at 30 s intervals. Each trial began with a conditioned signal (CS) (broad-band noise of 68 dB lasting seven seconds), followed by an unconditioned stimulus (US) (foot shock of 1.5 mA, 3 s duration) which was delivered through the grid floor. Crossing responses during the conditioned stimulus (AA response) terminated the conditioned stimulus and prevented the onset of unconditioned stimulus. A response after the onset of unconditioned stimulus (escape response) terminated both conditioned and unconditioned stimuli. Inter-trial crossings were not punished.

2.5. Processing of brain tissue and immunohistochemistry

Frozen, 8 μm thick sections were deposited on poly-L lysine coated slides and allowed to air dry. DakoCytomation EnVision + System-HRP kit was used a two-step IHC staining technique. Criostat sections were fixed in acetone and endogenous peroxidase activity was blocked by peroxidase blok (0.03% hydrogen peroxide containing sodium azide) (Dako Cytomation) for 15 min. Slides were incubated with appropriate dilutions of mAb (mouse monoclonal nNOS antibody 1:50 (Santa Cruz Biotechnology, Inc.) and mouse monoclonal iNOS antibody 1:25 (Santa Cruz Biotechnology, Inc.) for 60 min. After that slides were incubated with the labelled polymer (DakoCytomation) conjugated to goat anti-mouse immunoglobulins in Tris–HCl buffer containing stabilizing protein and an anti-microbial agent with addition of 5% normal rat serum for 30 min. Staining is completed by a 5–10 min incubation with 3,3′-diaminobenzidine (DAB) + substrate-chromogen (DakoCytomation) which results in a brown-colored precipitate at the antigen site. Finally, slides were counterstained with hematoxylin and mounted with Kaiser gel (Merck). Control slides were incubated in the same way, using mouse isotype-matched irrelavant Ab (produced in MMA, Belgrade).

2.6. Data presentation and analysis

Data are expressed as means ± S.D. Statistical significance was determined as $p < 0.05$ using either the Student's *t*-test or ANOVA followed by Tukey's *t*-test.

3. Results

3.1. Acetylcholine esterase activity in the rat brain

The results presented in Table 1 show the activity of acetylcholine esterase-AChE (μM acetyltiocholine/min/g proteins), ipsilaterally in the rat forebrain cortex, striatum, basal forebrain and hippocampus homogenates respectively, at 10 min, 3 h, 3 days and 30 days after the treatment. Both selective NOS inhibitors partly recovered AChE activity following $AlCl_3$ treatment. Similar results were obtained in contralateral site of brain (data not shown).

3.2. Behavioral changes after the treatment

As a part of active avoidance model, we determined the number of active avoidance (AA) of aversive unconditioned stimulus during 5 consecutive days (26–30 days after the treatment), at 20 trials per day, as a measure of acquisition of positive reactions. Difference in the number of correct responses first became evident 28 days after $AlCl_3$ injection (3rd day of examination) and progressively widened over the subsequent 3 days. At the end of 30th day (5th day of examination), $AlCl_3$-treated animals showed two-fold reduction in correct responses compared to control group. $AlCl_3$