Original Article

Upregulation of the Inducible Nitric Oxide Synthase in Rat Hippocampus in A Model of Alzheimer’s Disease: A possible Mechanism of Aluminium Induced Alzheimer’s

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ABSTRACT

Background: Alzheimer’s disease attacks the brain causing gradual memory loss. Alzheimer’s brain showed excess beta amyloid protein and neurofibrillary tangles, containing deposition of aluminium. Increasing evidence suggests that many neurons may die through apoptosis in Alzheimer’s. Inducible nitric oxide synthase (iNOS) derived nitric oxide (NO) has been implicated in this process of neuronal cell death and apoptosis. Aluminium is considered a potential etiological factor in Alzheimer’s disease and was used to produce an animal model of Alzheimer’s. However, the exact mechanisms of aluminium induced Alzheimer’s and neurotoxicity remain largely unknown.

Aim of the Work: The present study was carried out to investigate the profile of the expression of iNOS in the hippocampus in an animal model of Alzheimer’s produced by aluminium administration.

Materials and Methods: Twenty four adult male albino rats were divided equally into four groups. Group I was the untreated control, groups II, III and IV were given aluminium chloride (300 mg/kg body weight) orally daily for one week, two and four weeks, respectively. At the end of the experiment, rats were killed by decapitation under brief anaesthesia. The brains were removed and processed for immunohistochemistry using antibody raised against iNOS.

Results: By comparison to the untreated control, aluminium treated rats showed significant (P<0.05) increase in the expression of iNOS in the hippocampus. The expression was mainly neuronal and was seen in all areas. Additionally, administration of aluminium for four weeks caused marked histological changes with significant (P <0.05) reduction in hippocampus neuronal number and distortion of neuronal morphology.

Conclusion: These data provide further evidence that exposure to aluminium may contribute to pathogenesis of Alzheimer’s and neurotoxicity by induction of iNOS with subsequent increase in NO production that potentiate neuronal cell death in hippocampus.

Key Words: Alzheimer’s, hippocampus, aluminium, iNOS, nitric oxide.

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INTRODUCTION

Alzheimer’s disease is a common neurodegenerative disease occurring in the elderly. The first and most severely affected brain area is the hippocampus1,2. Aluminium is considered a potential etiological factor in Alzheimer’s disease3,4. Neurotoxicity from excess brain exposure to aluminium is well documented from both clinical observations and animal experiments5,6. Several studies have used aluminium to produce an animal model of neurotoxicity and Alzheimer’s disease5,6. Chronic aluminium toxicity was achieved during four weeks by daily administration of aluminium chloride7,8. Inflammatory mediators such as nitric oxide (NO) play an important role in pathogenesis of Alzheimer’s8,9. NO is an endogenous water and lipid soluble gas generated by nitric oxide synthase (NOS). It has been shown that NO is critical for the normal physiological regulation of the nervous system10 and that it plays an important role in synaptic plasticity, learning and memory11,12. Three isoforms of NOS have been identified; neuronal NOS and endothelial NOS are constitutively expressed and generate low levels of NO for homeostatic regulation. In contrast, iNOS is hardly expressed in and usually induced under pathological disorders. It can generate high, concentrations of NO that are associated with apoptosis in a variety of cells11,13. Previously, we have shown the contribution of iNOS in pathogenesis of several disorders including chronic leg ulcer, liver cirrhosis and wound healing14,15. Recently, significant increase in iNOS expression was reported in aged hippocampus and frontal cortical tissues16. These findings...
have strongly supported the idea that iNOS could be responsible for the increased NO in brain in neurodegenerative disorders. It is hypothesised that iNOS generated NO could be one of the free radicals produced in Alzheimer’s. Therefore, in this study aluminium was used to produce an animal model of Alzheimer’s in order to investigate the possible contribution of iNOS to pathogenesis of this disorder. This study might also explain the mechanism of aluminium neurotoxicity.

MATERIALS AND METHODS

Tissue harvesting and preparation: 24 Adult male albino rats weighing 300-400g were used. Rats were divided equally into four groups. Group I was the untreated control; groups II, III and IV were given aluminium chloride (300 mg/kg body weight) once daily by stomach tube for one, two and four weeks, respectively. At the end of the experiment, animals were killed by decapitation under brief halothane anaesthesia. Brains were removed and fixed in 4% paraformaldehyde then processed for paraffin sectioning. Brains were sectioned at 6 μm thickness, mounted on poly-L-Lysine coated slides and used for immunohistochemistry.

Immunohistochemical staining was performed according to a previously published protocol. Sections were deparaffinized, hydrated then washed in 0.1M phosphate buffer saline (PBS). Sections were then treated with trypsin 0.01% for 10 minutes at 37°C then washed with PBS for 5 minutes. Endogenous peroxidases were quenched by treatment with 0.5% H2O2 in methanol and non-specific binding was blocked in normal goat serum diluted 1:50 in 0.1M PBS. Sections were incubated in the primary antibody (polyclonal rabbit anti-iNOS, Transduction laboratories), diluted 1:1000 overnight at 4°C. Sections were washed and incubated in biotinylated goat anti-rabbit secondary antibody (Vector laboratory:1:2000) for 30 minutes. Following further 30 minutes incubation in Vectastain ABC reagent, the substrate amino ethylcarbazole (AEC) was added for 6 minutes, this substrate gives red colour at the immunoreactive sites where iNOS is expressed. Slides were counterstained with haematoxylin.

Image capture: Tissue sections were viewed by light microscopy and images were digitally captured on a computerized image analysis system consisting of a color analogue camera connected to a computer and dealt with using adobe Photoshop.

Counting cells in the hippocampus: Haematoxylin counterstained sections were used for counting cells. Cells were counted in ten adjacent non overlapping fields in each zone (CA1, 2 and 3). Cells were counted in the field of a 40X objective using light microscopy. Only cells with evident nucleus were included in the counts. In each animal six sections were counted and the distance between sections were 300 μm to scan through the depth of the hippocampus.

Counting iNOS positive cells in the hippocampus: The changes in the number of iNOS positive cells in the hippocampus were monitored over the time course of aluminium administration using the same counting method as above. The sections used for this counting were serial sections to those used for counting the total cell number in the hippocampus.

Statistical analysis: Statistical analysis was performed using the Statistics Package instat. Statistical significance of the experiments was determined using one way ANOVA test followed by Tukey-Cramer post-hoc test. P< 0.05 was considered as statistically significant.

RESULTS

Hippocampus was identified as a C shaped structure in coronal section of the brain. The major three areas of the hippocampus CA1, 2 and 3 were defined (Fig.1). Hippocampus cells were arranged into three layers; molecular, pyramidal and polymorphic layers. The main cellular layer was the pyramidal layer which was formed of many large pyramid-shaped neurons (Fig.1). The three layers were well defined in CA1 and CA2 which had the most compact layer of pyramidal cells (Figs.1-7). Dentate gyrus appeared as a dark C shaped structure enclosing CA3 (Fig.1).

I. Changes in hippocampus cell number:

The changes in the number of cells in the hippocampus were monitored over the time course of aluminium administration. The thickness of the pyramidal cell layer varied according to the depth of the sectioning (Figs. 2 & 3). Thus, counting cells was done on several sections taken at different levels of the hippocampus to scan through the whole structure. Number of cells counted per field in the hippocampus showed that aluminium administration caused gradual reduction in cell number over the time course but this reduction reached significance (P<0.05) only following four weeks aluminium administration.

Graph 1: Shows reduction in the total hippocampus cell number in aluminium treated groups but this reduction reached significance in group IV only.
II. iNOS expression in the hippocampus from group I:

The control rats showed little iNOS immunoreactivity in few cells scattered all over the hippocampus. The expression was mainly in the pyramidal cell layer in CA1, 2 and 3 and in the granular cell layer in the dentate gyrus (Figs. 2 & 3).

III. iNOS expression in the hippocampus from aluminium treated groups:

Aluminium administration induced changes in iNOS expression in the hippocampus. The expression was confined to the pyramidal cell layer in CA1, 2 and granular cell layer in the dentate gyrus (Figs. 4-8). The expression was higher than in the control (Graph 2) and increased with aluminium administration for longer period.

A. Group II: Aluminium administration for one week caused increase in iNOS expression in the hippocampus (Figs. 4 & 5) by comparison to the control group. The upregulation was in all areas of the hippocampus but highest expression was in CA3 and dentate gyrus (Fig. 4).

B. Group III: Aluminium administration for two weeks induced iNOS expression in all areas of the hippocampus; in CA1, 2 and 3 and in the dentate gyrus (Fig. 7). Cells in the hilus also showed immunoreactivity.

C. Group IV: Aluminium administration for fours weeks caused increase in iNOS immunoreactivity in all areas of the hippocampus in CA1, 2 and 3 and in the dentate gyrus (Fig. 8).

IV. Changes in iNOS positive cell count in the hippocampus:

Counting was done on several sections taken at different levels to scan through the whole hippocampus. By comparison to the control aluminium administration for one and two weeks caused significant (P<0.05) increase in the number of iNOS positive cells in the hippocampus. However, the four weeks treated group showed insignificant change in the number of iNOS positive cells. Comparing (Graph 1 and Graph 2) showed that aluminium caused induction of iNOS in most of hippocampal cells in the three treated groups. In the four weeks aluminium treated group the reduction in the number of iNOS positive cells was associated with overall reduction in the total number of hippocampal cells.

V. Cellular changes in the hippocampus

Along the course of aluminium administration there were histological changes in the hippocampus. High magnification photographs taken for CA2 illustrated these changes (Figs. 8-11). In control group, intact cells with rounded nuclei were seen with iNOS immunoreactivity seen in few cells (Fig. 8). However, in one week aluminium treated rats, cells showed increase in the number of cells expressing iNOS with extension of the expression into the neuronal processes (Fig. 9). With aluminium administration for longer time the changes became more prominent (Figs. 10 & 11) and this was confirmed by finding reduction in overall cell numbers in the hippocampus (Graph 1) and finding increase in the number of cells expressing iNOS (Graph 2).

Graph 2: Shows significant increase in the number of iNOS positive cells in the hippocampus in the one and two weeks aluminium treated groups.
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Fig. 2: Showing iNOS immunoreactivity in the hippocampus in few cells scattered in CA1, 2 and 3 (arrowhead). It is also seen in the granular cell layer of the dentate gyrus (DG, arrows).
Control, iNOS immunostaining X 100.

Fig. 3: Showing iNOS immunoreactivity mainly in CA3 and in few cells in CA1. Control, section of the hippocampus taken at a different.
iNOS immunostaining X 100.

Fig. 4: Showing increased iNOS immunoreactivity in the three areas mainly CA3. iNOS positive cells are seen scattered in CA2 (arrows). Immunoreactivity is also seen in the dentate gyrus (DG). Group II, section of the hippocampus taken at almost the same level as in Fig. 3.
iNOS immunostaining X 100.

Fig. 5: Showing iNOS immunoreactivity in pyramidal cell layers in CA1, 2 and 3 and also in the dentate gyrus (DG). Group II, section of the hippocampus taken at a level at which pyramidal cells appear densely packed particularly in CA1 (arrows).
iNOS immunostaining X 100.

Fig. 6: Showing iNOS immunoreactivity in the pyramidal cell layer in CA1, 2 and 3 and also in the dentate gyrus (DG). Notice the densely packed cells in the pyramidal cell layer in CA1 (arrows). Group III, this section of the hippocampus appears to be at almost the same level as in Fig. 5.
iNOS immunostaining X 100.

Fig. 7: Showing iNOS immunoreactivity in the densely packed pyramidal cells in CA1, C2 and 3 and also in the dentate gyrus. Group IV, section of the hippocampus taken at almost the same level as in Figs. 5 and 6.
iNOS immunostaining X 100.
DISCUSSION

In this study the hippocampus showed evident histological changes and significant reduction in cell number following chronic aluminium administration. This effect was an indicator for using the proper aluminium dose and administration time in order to cause neurotoxicity and to obtain the proper Alzheimer’s model. Aluminium administration caused upregulation of iNOS in rat hippocampus.

Aluminium is neurotoxic and long-term administration of aluminium to rats result in a neuropathological, neuroanatomical and neurochemical alterations in the brain with neurofilament accumulation, condition in which selective neuronal loss was evident. Moreover, aluminium was found to be an etiologic factor in Alzheimer’s and aluminium was believed to represent a key element for senile plaque formation. High density of senile plaques in brain was a characteristic feature of Alzheimer’s and aluminium was believed to represent a key element for senile plaque formation. However, accumulating evidence suggested that aluminium could potentiate prooxidative and inflammatory events, leading to tissue damage. In vitro and in vivo experimental studies have implicated the formation of reactive oxygen species in the potential neurotoxic effect of aluminium. Therefore, a possible role of oxygen radicals in Alzheimer’s has been a topic of burgeoning research. In Alzheimer’s disease, oxidative damage could lead to the formation of amyloid plaques and hyperphosphorylated tau that polymerizes to form neurofibrillary tangles which are hallmarks of Alzheimer’s. Nitric oxide is a free radical that has a role in neurodegenerative disorders. iNOS mediate the vast majority of pathophysiological effects attributed
to NO. Consequently, this isoform was believed to be of fundamental importance to the inflammatory process associated with neurodegenerative disorders. Therefore, aluminium was used to produce an animal model of Alzheimer in order to be able to investigate the possible contribution of iNOS in pathogenesis of Alzheimer’s disease. This study showed that aluminium upregulated iNOS in hippocampus. In agreement with this finding several studies have shown change in iNOS expression in the CNS under pathological conditions. Injection of amyloid beta in the hippocampus induced high expression of iNOS. Also iNOS showed high expression in the CNS during inflammatory states. iNOS is responsible for the increased NO in the aged brain and iNOS itself plays an important role in the aging process. Therefore, with the potential involvement of iNOS in the neurodegenerative process, upregulation of iNOS in the hippocampus following aluminium administration could indicate a critical role for iNOS in the pathogenesis of aluminium induced Alzheimer’s. It would provide the diffusible messenger NO which stimulates nuclear cell death. In Alzheimer’s, NO might act as a junction point between beta amyloid, caspase activation and tau aggregation.

iNOS-derived NO is a mediator of neurotoxicity. It was widely held that neurons do not express functional levels of iNOS and that the source of cytotoxic levels of NO is from neighbouring glial cells in neurodegenerative disorders and this serves as a source of NO which could diffuse into surrounding neurons to initiate apoptosis. However, this study showed iNOS upregulation in neurons following aluminium administration. Recently, in vitro study showed expression and induction of iNOS in rat cortical neuronal cells and this is in agreement with our findings. Therefore, neuronal iNOS induction could lead to generation of high concentrations of NO which might execute apoptotic death in neurons by activation of the apoptotic cascade. Mitochondria have been reported to be a primary target of oxidative-free radicals such as NO. To initiate apoptosis, NO can induce mitochondrial dysfunction and can upregulate pro-apoptotic factors or downregulate anti-apoptotic factors. Additionally, NO can directly react with superoxide to produce peroxynitrite. Peroxynitrite serves as a strong oxidizing agent that react with all classes of biomolecules causing oxidative stress inducing damage of DNA, proteins and lipids. In conclusion, NO that is generated by excessive iNOS may participate directly or through production of peroxynitrite in the mechanisms triggering neurodegeneration and Alzheimer’s induced by aluminium.

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REFERENCES


مقدمة

بهلامي مرض الزهايمر الدماغ ويسبب فقدان ذاكرة تدريجي. وبالتحليل النسيجي لمرض الزهايمر يتم وجد زيادة NO (نيتركس أكسيد) لمرض الزهايمر. وينتج عنه نموذج مرض الزهايمر، وقد وجد أن آمون الناتج من آمون النتيك (iNOS) يساهم في عملية الموت المبرمج لخلايا الدماغية. وي(coll) الذي فيه الغلوبية المسببة لمرض الزهايمر وقد استخدم لإنتاج نموذج مرض الزهايمر بالحيوانات. ولكن ما زالت الآليات التي يسبب بها آمون النتيك العصبية ومرض الزهايمر غير معلومة على وجه الدقة إلى حد كبير.

الهدف: الدراسة الحالية تهدف إلى تحديد مدى انتشار iNOS في قرن أمون في نموذج مرض الزهايمر بعد إعطاء الحيوانات كلوريد الآمون.

الطريقة: قد أجربت هذه الدراسة على أربع وعشرين من الفئران البالغة تقسيمتها بالتساوي إلى أربع مجموعات. المجموعة الأولى كانت المجموعة الضابطة. وأعطى كلودي الألومينيوم للمجموعات الثانية والثالثة والرابعة عن طريق الفم يومياً لمدة أسبوع وسبع عشرة أسابيع على التوالي. وفي نهاية الدراسة خذت الفئران ودُرست بالرئيسي الفم على الرأس ثم أخذت الأدمغة وُضعت باستخدام طريقة هستوكيميائية مناعية باستخدام الأجسام المضادة التي أثيرت ضد آمون iNOS.

النتائج: بالمقارنة بالمجموعة الضابطة قد وجدت زيادة ملحوظة في انتشار آمون في الخلايا العصبية بجميع أجزاء قرن أ몬. وقد ظهرت أيضا تغييرات ملحوظة بالخلايا العصبية بالمجموعة المعالجة لمدة أربعة أسابيع. في بالإضافة إلى زيادة في آمون (iNOS) نقص عدد الخلايا وظهور تغيرات بشكل الخلايا.

الخلاصة: هذه البيانات تقدم دليلاً إضافياً على أن التعرض للألومينيوم قد يساهم في آلية مرض الزهايمر العصبية عن طريق زيادة في آمون النتيك (iNOS) مما ينتج عنه زيادة في آمون النتيك (iNOS) الذي يدورة بسبب الموت المبرمج للخلايا العصبية في قرن أمون.