

EVALUATION OF CORTICAL AND HIPPOCAMPAL CHOLINERGIC SYSTEMS IN RATS TREATED WITH SPARTEINE

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ABSTRACT

Sparteine was administered subcutaneously to male neonate rats, and the effects on muscarinic subunit receptor types m-m⁴ from different brain regions were studied. A semi-quantitative reverse transcription-polymerase chain reaction and western blot was used to measure expression levels in the cerebral cortex and hippocampus. Sparteine (25 mg/kg body weight, on postnatal days 1 and 3) produced an important reduction of m¹, m² and m³ of mRNA and protein during the first three weeks of age in those animals under sparteine treatment compared to control group in cerebral cortex and hippocampus. Changes in the expression of the subunit m⁴ were increased during the first two weeks of age in those animals under sparteine treatment compared to control group in cerebral cortex and hippocampus. We propose that an early excessive activation of muscarinic receptors could modify subunit expression and its structural composition on postnatal development. This, as part of a compensatory response by an altered neuronal circuit, suggests that these subunits will have an important role in the mechanisms of neuronal death.

KEYWORDS

sparteine, muscarinic receptor, hippocampus, cerebral cortex, neonatal

INTRODUCTION

Quinolizidine (lupine) alkaloids are characteristic secondary metabolites in many taxa of the subfamily Papilionidae (Family Leguminosae) and over 150 structures have been described (Kinghorn and Balandrin, 1984; Wink, 1993). Quinolizidine alkaloids (QA) are important for the well being of the plants producing them (Wink, 1993; Wink and Hartmann 1982; Wink and Witte, 1991; Schmeller *et al.* 1994), since as insects and grazing mammals. Various pharmacological and toxicological properties have been attributed to particular lupine alkaloids such as antipyretic, anti-inflammatory, central nervous system

(CNS) depressant, anti-arrhythmic, respiratory depressant and stimulant, uterotonic, diuretic, hypoglycemic, hypotensive, hallucinogenic, antidiabetic and mutagenic properties (Tsiodras *et al.* 1999). QA exhibit both general vertebrate and insect toxicity (Kinghorn and Balandrin, 1984; Wink, 1993; Schmeller and Wink, 1988; Strasburger *et al.* 1994). Modulation of acetylcholine receptors and ion channels (Na⁺, K⁺) by QA has been suggested a mechanism and explanation for toxicity and some of their pharmacological properties (Schmeller *et al.* 1994; Roberts and Wink, 1998; Tsiodras *et al.* 1999).

Works done in our laboratory have demonstrated that the AQ of extracts of *Lupinus montanus* and *L. exaltatus* provoke several injuries in various cerebral regions of the CNS of the rat, the more susceptible are, the thalamus, hypothalamus, and hippocampus.

Therefore, with the purpose to have a better understanding about the action mechanism of sparteine on CNS, this work was performed to establish its neurotoxic activity on cerebral cortex and hippocampus its toxicity neuronal using commercially purified sparteine. Different acetylcholine (Ach) receptor (m¹, m², m³ and m⁴) subunits expression, were measured in cerebral cortex and hippocampus of rats neonatally treated with sparteine and during their development. These results may improve a better understanding about the neurotoxic susceptibility involved in brain injury and its potential pharmacological preventive strategies on CNS.

MATERIALS AND METHODS

Male Wistar rats were used in this study. Eight animals, used as the experimental group, were injected subcutaneously with sparteine (25 mg/kg body weight) in aqueous solution (100 µl total volume per rat) on postnatal days 1 and 3 and sacrificed at different postnatal ages. A group of untreated animals was used as controls. All animals were used on PD 7, 14, 21, and 60, some treated and control animals were killed by

decapitation and the cerebral cortex and hippocampus was dissected. Animal care and handling were in accordance with Mexican General Health Laws and their corresponding chapters (1987).

Unfixed cerebral cortices and hippocampus collected from each group were dissected out at 4°C to be used for molecular biology studies.

EXTRACTION AND QUANTIFICATION OF RNA

Extraction and quantification of total RNA from different brain regions was carried out according to the method described by Chomczynski and Sacchi (1987). Briefly, brain tissue was homogenised in the presence of Trizol, chloroform was added, the aqueous phase was obtained and the RNA precipitated with isopropanol at 48 hr overnight. Determining a 260/280 routinely tested the quantity and intactness of RNA and ethidium bromide fluorescence of RNA electrophoresed in agarose gels containing 1% formaldehyde (Armendariz-Borunda *et al.* 1997). We standardised a semi-quantitative RT-PCR method based on the co-amplification of the target gene to the Gene expression of mACh receptor subunits. Primers sequences and the size of the PCR product were as follows: **m¹**, 5'-gca cag ccc acc aag cag-3' and 5'-aga gca gca gca ggc gg aa cg-3' having a PCR product of 373 bp; **m²**, 5'-cac gaa acc tct gac cta ccc-3' and 5'-tct gac ceg acg acc caa cta-3' having a PCR product of 686 bp; **m³**, 5'-gtc tgg ctt ggg tea tct cct-3' and 5'-gct gct gct gtg gtc ttg gtc-3' having a PCR product of 434 bp; **m⁴**, 5'-att ctc tat gaa cct cta cac ttt gt-3' and 5'-tgg ctg ctg ggc gca tac cag ctg ga-3' having a PCR product of 865, and β -actina, 5'-cac cac agc tga gag gga aat cgt gcg tga-3' and 5'-att tgc ggt gca cga tgg agg ggc cgg act-3' having a PCR product of 517 pb.

The optical density of the bands was determined by revising the Kodak Digital Science Scanner and analysis was performed using the same system. Optical density measurements were normalised to the optical density of the β -actin band used as an internal standard.

GENE EXPRESSION OF MACH RECEPTOR SUBUNITS BY WESTERN BLOT

Some animals of 14 and 60 days of age were used for this study. The region cerebral cortex and hippocampus was removed rapidly and frozen. Tissue was sonicated on ice in lysis buffer. Tissue lysates (in 10 mmol/L Tris-HCl, pH 7.0, 1% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were rinsed in TBS, incubated in 10% nonfat dry milk dissolved in TBS for 24 hours, and then incubated with rabbit anti-m¹ and anti-m² antibodies 1:1000 for 24 hr After five washes in TBS with 0.05% Tween-20 (TTBS), blots were incubated for 24 hr with biotinylated goat anti-rabbit IgG (1:1,000) as secondary antibody. After five TTBS washes, blots were incubated with ABC Elite kit for 1 hr. After a final three TTBS washes, membranes

were developed with diaminobenzidine. For data collection, blots were photographed using a Kodak Digital Science Scanner and analyses were carried out using the same system. Data obtained corresponded to values from three experiments with determinations made by duplicate.

Molecular studies were analysed using ANOVA and a Student's t test using the Biostat computer software and a $p < 0.05$ was considered to be significant. Values are expressed as the means \pm S.D.

RESULTS AND DISCUSSION

CEREBRAL CORTEX: MACH RECEPTOR SUBUNITS MRNA (M¹, M², M³ AND M⁴) EXPRESSION

Results showed an important reduction in m¹ subunit expression of the mAChR during the first three weeks of age in those animals under sparteine treatment compared to control group. However, neonatal exposure of sparteine did not modify the m¹ subunits of muscarinic receptor expression in the cerebral cortex at adulthood compared with the control group. The atropine administered 1 hr before sparteine reverted the reduction of subunit m¹ to the 7, 14 and 21 days of age. On the other hand, sparteine induced a significant reduction in gene expression of m² subunit type of mAChR in all ages studied compared with control. Respect to m³ subunit type of mAChR expression, it was reduced by sparteine treatment during the first two weeks of life, since at PD21 and adulthood age (PD60) no differences was found between both groups studied. Changes in the gene expression of muscarinic acetylcholine receptor (mAChR) subunit type m⁴ were increased during the first two weeks of age in those animals under sparteine treatment compared to control group. However, neonatal exposure of sparteine did not modify the expression of m⁴ subunit of muscarinic receptor in the cerebral cortex to the 21 and 60 days of age. The effect of atropine on expression of m² and m³ in cerebral cortex administered 1 hr before sparteine produced a protective effect against changes in all ages studied. The atropine alone did not produce any effect on expression of m¹, m², m³ and m⁴ in cerebral cortex in all ages studied.

M¹ AND M² IMMUNOBLOT STUDIES

At 14 day following injury, there was significant reduction in the protein m¹ and m² signal compared to control animals. At 4 weeks post injury, the decrement seen at 14 day was no longer evident to the subunits m¹ and m² (Fig. 1). The effect of atropine on expression of the protein m¹ and m² in cerebral cortex administered 1 hr before sparteine produced a protective effect against changes to the 14 days of age. The atropine alone did not produce any effect on expression of m¹ and m² in cerebral cortex in all ages studied (Fig. 1).

HIPPOCAMPUS: mACh RECEPTOR SUBUNITS MRNA (M¹, M², M³ AND M⁴) EXPRESSION

Results showed an important reduction in m¹ subunit expression of the mAChR in all ages studied. The atropine administered 1 hr before sparteine reverted the reduction of subunit m¹ to the 7, 14, 21 and 60 days of age. On the other hand, sparteine induced a significant reduction in gene expression of m² subunit type of mAChR during the first three weeks of age in those animals under sparteine treatment compared to control group. However, neonatal exposure of sparteine did not modify the m² subunits of muscarinic receptor expression in the hippocampus at adulthood. The atropine administered 1 hr before sparteine reverted the reduction of subunit m² to the 7, 14 and 21 days of age. Respect to m³ subunit type of mAChR expression, it was reduced by sparteine treatment to the 7, 14 and 21 days of age in relation to control group, at adulthood age (PD60) no differences was found between both groups studied. Changes in the gene expression of muscarinic acetylcholine receptor (mAChR) subunit type m⁴ were increased during the first two weeks of age in those animals under sparteine treatment. However, neonatal exposure of sparteine did not modify the expression of m⁴ subunit of muscarinic receptor in the hippocampus to the 21 and 60 days of age. The effect of atropine on expression of m² and m³ in the hippocampus administered 1 hr before sparteine produced a protective effect against changes in all ages studied. The atropine alone did not produce any effect on expression of m¹, m², m³ and m⁴ in the hippocampus in all ages studied.

M¹ AND M² IMMUNOBLOT STUDIES

Fig. 2 shows a graph representative of m¹ and m² levels in control animals to the 14 and 60 days PD and of animals treated with sparteine after injury to the 14 and 60 PD. At 14 day following injury, there was significant reduction in m¹ and m² signal. At 4 weeks post injury, the decrement seen at 14 day was no longer evident to the subunits m¹ and m². The effect of atropine on expression of m¹ and m² in cerebral cortex administered 1 hr before sparteine produced a protective effect against changes to the 14 days of age. The atropine alone did not produce any effect on expression of m¹ and m² in cerebral cortex in all ages studied (Fig. 2).

Current knowledge on the mechanism of action of sparteine in cells animals describes the interaction with acetylcholine receptors in individual with a high affinity to muscarinic receptors (Schmeller, 1994; Schmeller and Wink, 1988), or the data reported on the inhibition of channels of Na⁺ and K⁺ of the neuronal membrane by sparteine (Agid *et al.* 1997). Nevertheless they lack studies in live animals that show that nervous cells are sensible and the originated mechanism of cellular death. The neurons affected after treatment with sparteine showed lesions typical of those caused by ischemia, hypoxia (eosinophilic neurons) and some neurotoxins (data not shown). This type of damage is associated

with an acute stage of cell death. Although in our case there was a selective way to ascertain the type of neurons. This process of neuronal death seems to be influenced by differential activation of the muscarinic receptor subtypes. It may be due possibly to high variation in expression, localisation, and functions of the mACh receptor subtypes and signalling systems (Hamilton *et al.* 1998). It is therefore possible to infer that susceptibility to neuronal death induced by sparteine differs between brain regions and in various developmental stages of the nervous system. Reduction in the expression of m¹ and m³, particularly significant at PD7 and 14 and 21 is especially important, due mainly to its postulated role of both m¹ and m³ would be predicted to be excitatory. The initial decrease might be a compensatory mechanism designed to decrease cell excitability and therefore prevent further seizures (Mingo *et al.* 1998). These reductions in muscarinic mRNA of subunit m¹, m² and m³ expression seem likely to be related to cell loss, while cell loss has been reported (data not shown). In addition, the increment in expression found at 60 days would not be compatible with the hypothesis that the changes at 7, 14 and 21 PD were due to cell loss. It would seem that the changes observed reflect a genuine change in gene expression.

On the other hand the increase in the expression of the subunit m⁴ could indicate the development of compensatory mechanisms, like part of the phenomenon of neuronal plasticity, since studies made by Vizi and Kiss (1998) they demonstrated that the m⁴ auto-receptors are the inhibitors of the liberation of acetylcholine in some cerebral regions. The mechanism involved in the liberation process of acetylcholine must be mainly the inhibition of the formation of AMPc and the opening of channels of Ca⁺⁺.

In conclusion, these results suggest the possibility that the not neurons damaged by the effect induced by the administration of sparteine could increase their efficacy to replace the damaged neurons. Thus, an up-regulation of this subunit, in surviving neurons, could favor the re-establishment of synaptic connections compensatory like a plastic answer of the brain (Südhof, 1995; Struckmann *et al.* 2003; Zhan *et al.* 2002).

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Fig. 1. Expression levels of protein m¹ (A) and m² (B) the muscarinic acetylcholine receptor subunit to the 14 and 60 days of age postnatal, in cerebral cortex from control animals and those that received neonatal treatment (1 and 3 days of age) with sparteine (25 mg/g of body weight,). A third group treated with atropine 10 mg /kg, administered s.c. 1 hr before sparteine and a fourth group was treated with sparteine alone at the same doses.



Fig. 2. Expression levels of protein m¹ (A) and m² (B) the muscarinic acetylcholine receptor subunit to the 14 and 60 days of age postnatal, in hippocampus from control animals and those that received neonatal treatment (1 and 3 days of age) with sparteine (25 mg/g of body weight,). A third group treated with atropine 10 mg/kg, administered s.c. 1 hr before sparteine and a fourth group was treated with sparteine alone at the same doses.