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Molsidomine modulates the cNOS activity in an experimental model of cholinergic damage induced by 192-IgG saporin

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ABSTRACT

The aim of this work was to study the effect of molsidomine (MOLS), a nitric oxide (NO) donor, on the nitrergic system changes in an experimental model of cholinergic damage induced by 192 IgG saporin (SAP). Male rats were injured by intraseptal administration of SAP (0.22 μg), after seven days, rats were administered with MOLS (4 mg/kg, i.p.) 60 min before sacrifice. Prefrontal cortex (PC), striatum (S) and hippocampus (HC) were dissected out. Results showed significant recovery of the constitutive NOS activity (cNOS) in PC and S regions by MOLS but not in HC compared against controls. SAP reduced the cellular population in the lesion site and MOLS was able to avoid the progression of damage in this area. NO donor is able to modulate the nitrergic status in an experimental model induced by SAP.

Immunotoxin 192 IgG saporin (SAP) model has been used in order to analyze the role of basal forebrain (BF) in learning and memory processes as well as the cognitive deficits associated to cholinergic denervation in experimental models. SAP produces selective damage in p75 receptor containing cholinergic neurons of the medial septum nucleus (MS), which provide the major cholinergic innervations to hippocampus (HC) [5]. It has been extensively demonstrated that intraventricular or intraseptal administration of SAP is related to disturbances in cholinergic transmission and also to biochemical and physiological changes *in situ* and in hippocampal areas [10,14,20]. The cholinergic denervation induced by SAP results in the evident depletion of cholinergic markers such as choline acetyltransferase (ChAT) in the presynaptic terminals and also a marked failure in several cognitive tasks [20]. Likewise in humans, the loss of basal forebrain (BF) cholinergic neurons (CBF) has been associated with deficits in cognition in early stages of Alzheimer's disease [5].

The cognitive decline resulting from the injury with SAP is associated with a neurotrophic malfunction. Nerve growth factor (NGF) is a signaling molecule that regulates the survival and neuronal maintenance of CBF neurons. NGF signaling in MS neurons is thought to occur after the binding to and activation of both TrkA and p75 receptors. Thus, the NGF/TrkA-p75 complex is then internalized to soma by retrograde transport. The down regulation of TrkA expression alters the retrograde transport of NGF to target MS neurons and may have important consequences related to apoptosis [8].

In addition to NGF, nitric oxide (NO) is also required for CBF neurons survival and the delivery of NGF seems to be related to modulation of excessive NO production in order to prevent excitotoxicity. The role of NO as a neuromodulator in BF and as regulator of cerebral blood flow suggests a relationship between cholinergic and nitrergic systems. SAP reduces the cortical blood flow and alters the catalytic activity and substrate binding of hippocampal neuronal nitric oxide synthase isoform (nNOS) [12].

The relationship between NO and cholinergic system has been evidenced by blocking cognition through the use of NOS inhibitors, while NO donors can facilitate it [7,21]. The NO donor molsidomine (MOLS) has been used as a pharmacological tool in order to antagonize the cognitive deficit associated to cholinergic hypofunction produced by scopolamine (SCO). The successful of MOLS on the recovery of cognitive impairment could be associated to its high

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bioavailability, long-lasting duration of action and the lack of overt side effects at doses displaying an antiamnesic action [21]. Nevertheless, the participation of NO in the recovery of cholinergic deficit caused by SAP administration has not been analyzed.

In the present study we used the experimental cholinergic damage model induced by SAP in rats, in order to evaluate the effect of the NO donor MOLS. We also evaluated the nitrergic system in rats, determining calcium-dependent and calcium-independent NOS activities, as well as the expression of nNOS in HC region.

All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the local guidelines on the ethical use of animals from the Ministry of Health in Mexico. All efforts were made to minimize animal suffering.

To carry-out the present experiments, we used 132 adult male Wistar rats (250–300 g) for the different evaluations. Under sodium pentobarbital anesthesia (40 mg/kg i.p.), animal groups were injected in MS with 1 μl of SAP (0.22 μg, Chemicon International Inc., CA, USA) or 1 μl of vehicle (PBS 0.1 M pH 7.4) both at 0.25 μl/min according to the coordinates: AP +0.6, L 0.0, V −7.0 from bregma [18]. After seven days, SAP group was divided into isotonic saline solution (SS, i.p.) and MOLS (4 mg/kg i.p.) (N-[etoxycarbonil]-3-[4-morpholynosydnonimine] Sigma–Aldrich, Mexico). Parallely, PBS groups were also divided in SS and MOLS subgroups. Rats were allowed to recover from anesthesia and surgical procedures, keeping them warmed and then were housed into acrylic cages with sterile sawdust, grouping five animals per cage in a regulated environment (temperature, humidity, light, etc.) with free access to food and water.

In order to discard some motor disturbances or sedation effects related to SAP, MOLS or vehicles, the total motor activity task was evaluated 7 days after the administration of SAP and 60 min after MOLS injection, into an automated cage (Opto-Varimex Minor; Columbus Instruments, Ohio). Considering that the amnesic SCO is widely used as a control of hyperactivity [21], a group of 16 rats was used as a positive control of hyperactivity, and was injected with SCO (0.5 mg/kg s.c.) 60 min before evaluation. This procedure was performed according to the protocol described by Pitsikas et al. and Reddy and Kulkarni [21,22]. Each rat was randomly chosen and placed in the automatic cage for a 30 min habituation period. Thereafter, horizontal, vertical and ambulatory activity was monitored for 10 min.

Constitutive NOS (cNOS) comprises the calcium-dependent endothelial (eNOS) and neuronal (nNOS) isoforms. While inducible NOS (iNOS) refers to calcium-independent isoform.

In order to assess the NOS activity and expression, and considering also that at 7th day posterior to SAP lesion the evident decay of cognition has been observed [5], 28 animals (7 rats per experimental condition) were sacrificed by decapitation 7 days after the surgery, and 60 min after the injection of MOLS or SS [21].

The NOS activity was evaluated by the indirect measuring method described by Bredt and Snyder (1990) and modified by Pérez-Severiano et al. for brain tissue [19]. This technique quantifies the stequiometric conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline plus NO. Prefrontal cortex (PC), striatum (S), and hippocampus (HC) areas were dissected out over ice and frozen in liquid nitrogen. The samples were stored at −70 °C until NOS activity and expression analyses were performed. Tissues were homogenized at 4 °C in a glass homogenizer using a teflon pestle, with 250 μl of a buffer containing: 50 mM Tris–HCl and pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol pH 7.5, containing a cocktail of protease inhibitors (100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml of soybean trypsin inhibitor and 0.1% v/v Noninet P-40).

Sample protein content in PC, S and HC regions was measured by Lowry method [15]. After protein determination, volumes containing 500 μg of protein were incubated during 30 min at 37 °C in the presence of 10 μM L-arginine-HCl, 1 mM NADPH, 100 nM calmodulin, 30 μM tetrahydrobiopterin, 2.5 mM $CaCl_2$, and 0.2 μCi of [^{3}H]-L-arginine (Amersham Pharmacia Biotech, Buckinghamshire, UK) in order to evaluate cNOS activity. The maximum volume used for each reaction was 100 μl.

The activity of iNOS was tested by the samples incubation in the presence of EGTA without $CaCl_2$. Reaction was stopped by adding a buffer containing 2 mM EGTA, 2 mM EDTA, 20 mM HEPES, pH 5.5. The reaction mixture was then transferred into a cation exchange resin column (Dowex-50W-200, Sigma–Aldrich Co. St. Louis Mis., USA) which had previously been equilibrated with the stop buffer. This column retains labeled arginine and allows [^{3}H]-L-citrulline to flow through itself. Labeled L-citrulline amount was measured using a Beckman LS6500 scintillation counter. Results were expressed as ng of L-citrulline/500 μg protein/30 min.

With the aim to determine the nitrergic status in HC, which is the target region of the cholinergic innervation from MS, the expression of hippocampal nNOS was carried-out using the homogenized hippocampal region ($n = 3$ per treatment and control): samples containing 30 μg of protein were aligned and carried out on a 10% Tris/glycine-sodium dodecyl sulfate-polyacrylamide gel and then were transferred onto hybond polyvinylidene fluoride membrane (PVDF, Amersham Biosciences, UK). Blots from each sample were blocked with a PBS/0.05% Tween 20 buffer solution containing 5% of non-fat milk, during 1 h at room temperature, and then were incubated overnight at 4 °C with two antibodies: polyclonal mouse antibody against nNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a final dilution of 1:300 as well as with a monoclonal mouse antibody against β-actin, using the same dilution. After incubation, membranes were then washed and incubated with their respective secondary peroxidase goat antibodies, diluted 1:3000. Blots were washed again and the protein was developed using the ECL detection system (Perkin Elmer Inc, Waltham MA, USA).

The presence of nNOS was normalized by β-actin, the reference protein. The film images were digitally acquired with a BioDoc-It System (UVP, CA, USA). The densitometric analysis was performed using the LabWorks 4.0 Image Acquisition and Analysis Software (UVP, CA, USA). The densitometric scanning protein density data were expressed as optic density arbitrary units (OD).

Histological procedures were performed according to Gridley [9]. Rats from the experimental groups were anesthetized with sodium pentobarbital anesthesia (40 mg/kg i.p.), and transcardially perfused with phosphate buffered saline containing heparin (5000 IU/ml), diluted 1/500, v/v followed by 4% (v/v) paraformaldehyde solution (in 0.1 M phosphate buffer, pH 7.4) at 4 °C. Brains were removed, postfixed in the same fixative solution for 24 h and immersed in paraffin. Tissue was sectioned on a microtome, and sections (7 μm of thickness) were obtained from the lesioned zone. All sections were stained with cresyl violet 0.2% in order to identify cell bodies and to verify changes in the population density at 20× magnification. The sections were observed under a wide-field microscope (Olympus H2).

All results are expressed as mean ± S.E.M. Statistical analysis was achieved by one way analysis of variance (ANOVA) followed by the Tukey's *post hoc* test. For all cases, values of $p < 0.05$ were considered as statistically significant.

With the aim to demonstrate that SAP and MOLS did not show any effect over motor activity, we evaluated the motor activity in PBS control group and also in groups treated with SAP or MOLS; the results demonstrated no significant differences between treatments for the ambulatory ($F = 19.81_{0.95;\,5,84}$), horizontal ($F = 20.18_{0.95;\,5,84}$) and vertical activities ($F = 14.23_{0.95;\,5,84}$). Nevertheless, the administration of SCO caused significant hyperactivity (ambulatory: 760.2 ± 97.94, horizontal: 1199 ± 113.5; vertical: 409.7 ± 63.16) in comparison with PBS group (ambulatory: