



Acute effect of methylprednisolone on the brain in a rat model of allergic asthma

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ABSTRACT

Asthma affects not only the airways but also the central nervous system (CNS). Corticosteroids are an effective therapeutic agents for asthma. In our study, we investigated the acute effect of ovalbumin (OVA) on the brain and the effectiveness of methylprednisolone (MP) in both the periphery and CNS in a rat model of allergic asthma. Rats sensitized to OVA and exposed to OVA aerosol challenge to induce allergic asthma were compared with control rats and rats sensitized to OVA and pretreated with MP before OVA exposure. In response to OVA stimulation, the amount of c-Fos and glial fibrillary acidic protein (GFAP) increased, while that of neuronal nitric oxide synthase (nNOS) decreased in the nucleus tractus solitarius (NTS). In addition, the c-Fos, GFAP, and nNOS levels in the hippocampus and the nNOS levels in the olfactory bulb increased. However, the expression of these proteins in the frontal and cerebellar cortices was not affected by OVA stimulation. In contrast, pretreatment with MP before OVA exposure decreased the protein expression of c-Fos in the CA1 area, GFAP in NTS, and nNOS in CA1 and olfactory bulb, and while it increased the nNOS content in the NTS. These findings suggest that the brain responds to OVA stimulation in a rat model of allergic asthma and that MP treatment cannot only ameliorate airway inflammation but also OVA-induced effects.

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Introduction

Asthma is an inflammatory disease of the airways, and corticosteroids are the most effective anti-inflammatory therapeutic agents available for the treatment of asthma in both human and animals [1,16]. In mammals, respiration is controlled by the central nervous system (CNS) – specifically the nucleus tractus solitarius (NTS) – as well as by peripheral pathways. Sensory receptors and neurons and neurogenic inflammation are involved in the pathogenesis of asthma [10]. In addition, since airway neurons project to the nuclei of the brainstem, neurogenic inflammation and the activation of neural pathways in response to asthmatic stimuli can sensitize the CNS.

Functional magnetic resonance imaging studies have demonstrated that the insula and anterior cingulate cortex, which are essential for the processing of emotion-congruent information, are activated in asthmatic patients [13]. In addition, asthmatic patients have a high incidence of neuropsychiatric symptoms that can also trigger and exacerbate asthma attacks [9]. Therefore, asthma itself may not only sensitize the CNS but also in turn be affected by it. However, biochemical evidence of the CNS response to sensitization is limited. Further, the areas of the CNS that are activated and responsible for modulating asthma attacks remain unclear.

It has been demonstrated that glial fibrillary acidic protein (GFAP), c-FOS, and neuronal nitric oxide synthase (nNOS) are up-regulated at the injury site following CNS injury caused by various insults [4,7,8]. These proteins also play important roles in the development of neuroinflammation. The aims of our present study were as follows: (1) to examine this up-regulation by studying protein (including GFAP, c-FOS, and nNOS) expression in various regions of the brain by using a rat model of allergic asthma and (2) to determine whether this effect is ameliorated by treatment with methylprednisolone (MP).

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Materials and methods

Animals

Male Brown Norway rats (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) weighing 300 ± 20 g were housed in cages, provided with *ad libitum* access to food and tap water, and maintained under a standard 12:12 h light:dark cycle. Our experimental protocols conformed to the guidelines approved by the Animal Care and Use Committee of Taipei Veterans General Hospital and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Antigen challenge and sensitization and asthma induction in rats

Control group

A subgroup of control rats was intraperitoneally (i.p.) injected with 1 ml of 0.9% saline on days 0 and 7. From days 8 to 17, these rats were placed in a plastic chamber (45 cm $\times$ 45 cm $\times$ 15 cm) and exposed to an aerosol of 0.9% saline nebulized using an ultrasonic nebulizer (V-16, Sumo, Japan) once a day for 30 min (Fig. 1A).

Establishment of asthmatic rat model

On days 0 and 7, a subgroup of rats in the asthma group was actively sensitized by i.p. injection of 2 mg ovalbumin (OVA) emulsified with 200 mg aluminum hydroxide (alum) in a total volume of 1 ml 0.9% sterile saline and 0.5 ml *Bordetella pertussis* vaccine (containing 1×10^{10} heat-killed bacilli). To elicit an allergic response in the airways, these rats were placed in a plastic chamber and exposed to an OVA aerosol (1% (w/v) in saline; particles, 0.5–5 μ m in diameter) once a day for 30 min from days 8 to 17 (Fig. 1B). The sensitization protocol was modified from that of Martin et al. [12].

Methylprednisolone pretreatment of asthmatic rats

Another subgroup of the rats in the asthma group was similarly actively sensitized by i.p. injection of OVA emulsified with alum and pertussis vaccine on days 0 and 7. These rats were also exposed to the OVA aerosol once a day for 30 min from days 8 to 17. However, unlike the first subgroup, these rats were subjected to pretreatment with i.p. injection of 5 mg MP sodium succinate at 30 min before

the start of the OVA aerosol challenge every day from days 8 to 17 (Fig. 1C).

Control rats treated with MP

Another subgroup of control rats was i.p. injected with 1 ml of 0.9% saline on days 0 and 7, and these rats were also exposed to an aerosol of 0.9% saline once a day for 30 min from days 8 to 17. However, unlike the first subgroup of control rats, these rats were subjected to pretreatment with i.p. injection of 5 mg MP at 30 min before the exposure to 0.9% saline aerosol every day, from days 8 to 17 (Fig. 1D).

Histological assessment of the airway

Rats ($n=6$, each group) were sacrificed via exsanguination 3 h after the last aerosol challenge by cutting the abdominal aorta and inferior vena cava under deep inhaled isoflurane (5%) anesthesia. Immediately after death, the lungs were removed and fixed in a buffered 10% formalin solution for 24 h. Tissues were then sampled, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or Masson's trichrome.

Western blot analysis

At the end of each experiment on day 17, the animals ($n=5$, each group) were decapitated 3 h after the last aerosol challenge; their brains were removed and briefly cooled in isotonic phosphate-buffered saline at a temperature of approximately 4 °C. The brain was coronally sectioned at a thickness of 2 mm by using a rodent brain matrix (Harvard Apparatus). The CA1, CA2, and NTS of the brain were identified based on "The atlas of the rat brain" by Swanson, 1992 [14]. The dissection of the CA1, CA2, cerebral cortex, cerebellar cortex, and NTS in a coronal section is illustrated in Fig. 2.

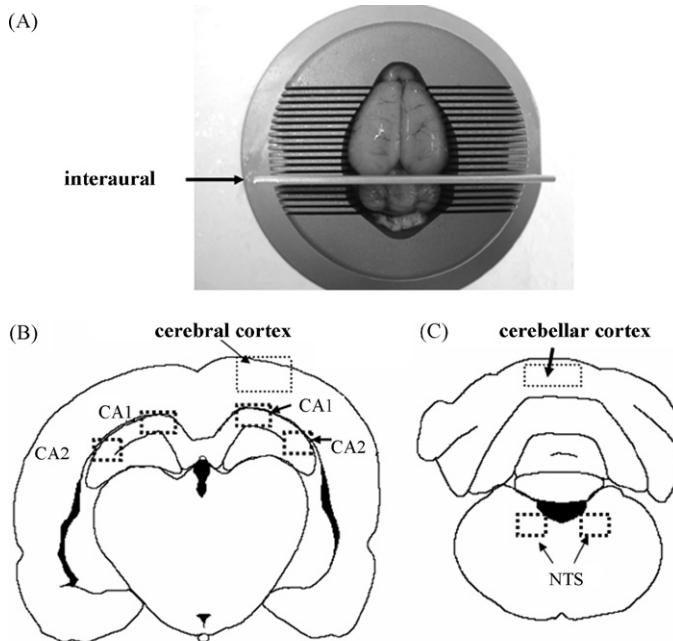


Fig. 2. Schematic drawing of coronal section illustrating the CA1, CA2, cerebral cortex, NTS, and cerebellar cortex of the rat brain. (A) The rat brain was sliced on a rodent-brain matrix. (B) At the level 5 mm anterior in relation to interaural to show a representative region of the CA1, and CA2 area and cerebral cortex. (C) At the level 4 mm posterior in relation to interaural to show a representative region of the NTS and cerebellar cortex.

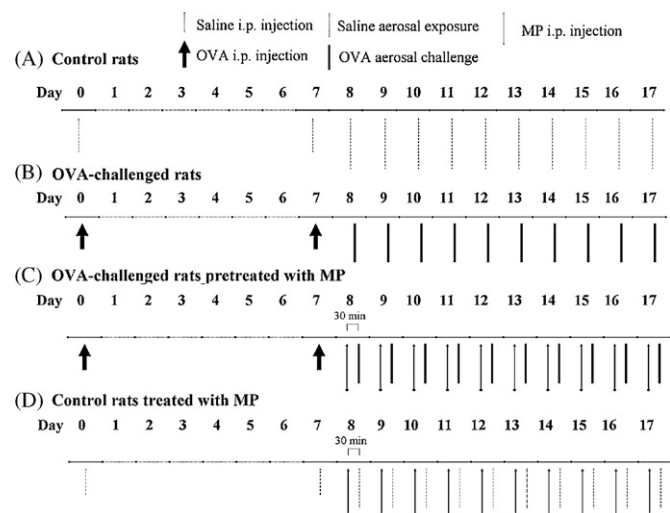


Fig. 1. Experimental designs. A, control rats received both intraperitoneal (i.p.) injection and aerosol exposure of normal saline; B, ovalbumin (OVA)-sensitized rats received both i.p. injection and aerosol exposure of OVA; C, OVA-sensitized rats received both i.p. injection and aerosol exposure of OVA but were pretreated with methylprednisolone (MP) before OVA aerosol exposure; and D, control rats were pretreated with MP before exposure to 0.9% saline aerosol.

The tissue samples were homogenized in a cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2% Triton X-100, 0.1 mM EDTA, 0.1 mM EGTA, 100 μ g/ml of PMSF, and 1 μ g/ml of aprotinin) and centrifuged at 13,000 $\times$ g at 4°C for 35 min. The protein content in the supernatant was measured; electrophoresis was then conducted, and the bands were transferred to a polyvinylidene difluoride (PVDF) membrane. The bound antibody was detected by chemiluminescence using an X-ray film. The antibodies used in this study were mouse nNOS monoclonal antibody (1:1000 dilution; Transduction Laboratories), GFAP monoclonal antibody (1:1000 dilution; Transduction Laboratories), and c-Fos polyclonal antibody (1:1000 dilution; Calbiochem). A β -actin blot was used as an internal control for protein content. The density of the bands was expressed in relation to the background density.

Chemicals

OVA (Grade V), alum, and the *B. pertussis* vaccine were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA), Pierce Chemical Company (Rockford, IL, USA), and Wako Pure Chemical Industries (Wako, Osaka, Japan), respectively. MP was purchased from Pharmacia & Upjohn Company (Kalamazoo, MI, USA) and reconstituted in the supplied diluent.

Data analysis

All data are presented as mean $\pm$ standard error of mean (S.E.M). Data were analyzed using a non-paired *t*-test. A *p* value of <0.05 was considered statistically significant.

Results

MP ameliorates the OVA-induced allergic and inflammatory responses in the airway of rats

Compared to the lung tissue from control rats (Fig. 3A and D), that from the OVA-challenged rats (the asthma group) contained fewer of eosinophils, a moderate number of neutrophils (Fig. 3B), remarkable inflammatory infiltrates around the bronchioles with destruction of the epithelium and inflammatory debris, and significant collagen deposition in the submucosal and peribronchial areas (Fig. 3E). In contrast, in the lung tissue of the MP-treated asthmatic rats, there were significantly fewer inflammatory infiltrates without debris or mucous plugging in the bronchioles compared to that of the non-treated asthmatic rats; however, the tissue exhibited a mild increase in the number of collagen fibers compared to those of the control rats (Fig. 3C and F).

MP pretreatment inhibits OVA-up-regulated c-Fos protein expression in the brain

The c-Fos protein content has been used as a marker of neuronal activation in the CNS. Western blot analysis showed that following OVA challenge, the c-Fos content increased in the CA1 area and NTS compared to that in these areas of the control rats (Fig. 4). MP treatment of the control rats did not affect c-Fos expression; however, pretreatment with MP before OVA exposure decreased its expression in the CA1 area (40% reduction) and NTS (50% reduction) compared to that in these areas of the OVA-challenged rats (Fig. 4).

MP can inhibit the OVA-up-regulated GFAP protein expression in the NTS but not in the CA1 and CA2 areas

The content of GFAP, an astrocyte marker, increased in the CA1 and CA2 areas and in the NTS after OVA stimulation as compared to

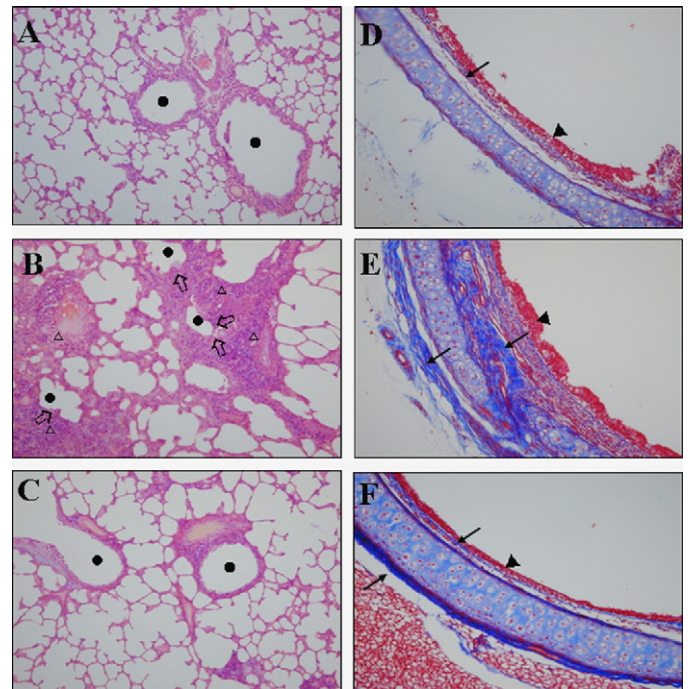


Fig. 3. Photomicrographs of airways showing representative morphological changes in each group. (A), (B), and (C) represent H&E staining of samples from the control, asthma, and asthma with MP treatment groups, respectively; bronchioles are indicated by (●) (magnification, 100 $\times$). (D), (E), and (F) represent Masson's trichrome staining for epithelium (closed arrowheads) and collagen deposition (closed arrows) in samples from the control, asthma, and asthma with MP treatment groups, respectively. Asthma group: (B) remarkable infiltration of inflammatory cells (including eosinophils and neutrophils, open arrowheads) around the bronchioles with the destruction of epithelium, accumulation of inflammatory debris (open arrows), and significantly increased collagen deposition (E, closed arrows) in the submucosal and peribronchial areas; MP significantly decreased inflammatory cell infiltration (C) and caused a mild increase in collagen fibers (F, closed arrows) as compared to that in the asthma group.

the levels in both subgroups of the control rats either with or without MP treatment (Fig. 5). MP pretreatment of the OVA-sensitized rats inhibited the up-regulation of GFAP expression (24% reduction) in the NTS compared to the OVA-challenged rats (Fig. 5). However, this pretreatment did not reduce the OVA-up-regulated GFAP protein expression in the CA1 and CA2 areas (Fig. 5).

MP ameliorated the OVA-affected nNOS protein expression in the brain

After OVA challenge, the amount of nNOS protein increased in the CA1 area, CA2 area, and olfactory bulb but decreased in the NTS (*p*=0.007) compared to the level in the control rats either treated or untreated with MP (Fig. 6). Similarly, MP pretreatment of the OVA-sensitized rats decreased the OVA-up-regulated expression in the CA2 area (20% reduction) and olfactory bulb (21.4% reduction) and increased the down-regulated expression in the NTS (21.4% increase) compared to these expressions in the OVA-challenged rats (Fig. 6). However, this pretreatment did not reduce the OVA-up-regulated nNOS protein expression in the CA1 area (Fig. 6).

Discussion

The present study demonstrates that the OVA challenge also resulted in an influx of inflammatory cells into the lungs and

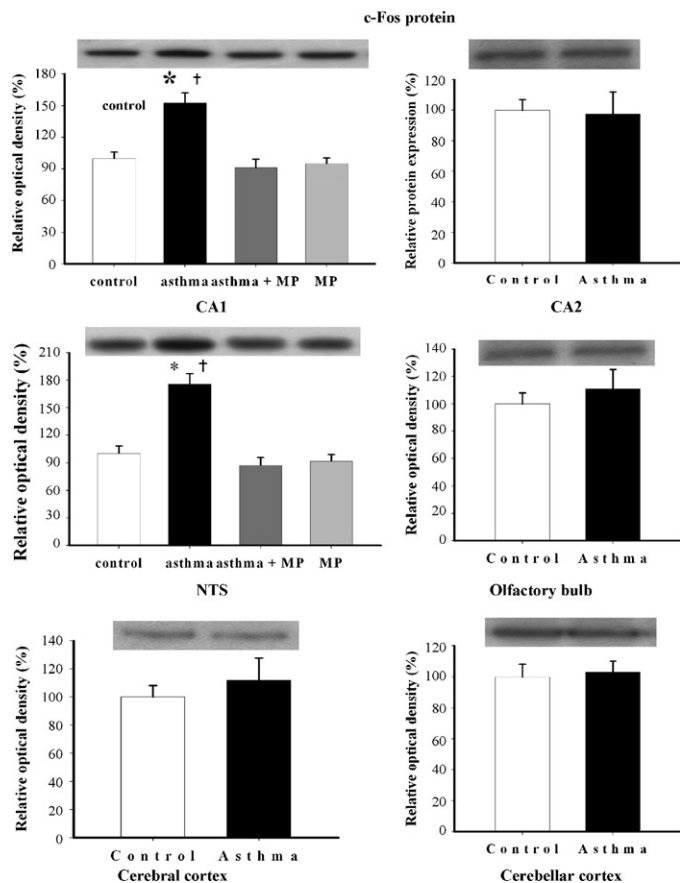


Fig. 4. c-Fos protein expression in selected areas of the CNS in OVA- and saline-treated rats. Data of the densitometric analysis is provided below each Western blot image. The bars indicate mean $\pm$ S.E.M. ($n=6$). * $p \leq 0.05$ versus control rats either treated or untreated with MP, † $p \leq 0.05$ versus asthmatic rats pretreated with MP.

increased the levels of GFAP in the hippocampus and NTS, c-Fos in the CA1 area and NTS, and nNOS in the hippocampus and olfactory bulb. In addition, OVA decreased the nNOS protein content in the NTS. Pretreatment with MP cannot only decrease inflammatory infiltration in the airways but also ameliorate the induced protein expression in the CNS of OVA-challenged rats. Taken together, these results suggest that in rats, OVA induces both peripheral and central effects that can be inhibited by MP treatment.

The mammalian CNS is considered to be an immunologically privileged site because of the blood–brain barrier. Although the pathophysiology of asthma has been well characterized, little is known about the effect of peripheral allergy on neuroinflammation in the CNS. Recently, Costa-Pinto et al. [6] indicated that there is a direct interaction between allergic reactions and behavioral changes. Moreover, our present study also shows that a peripheral allergy contributes to the changes in inflammatory mediators in various regions of the CNS.

The vagus nerves in the lungs send information to the brain, especially to the NTS [11]. NTS neurons play a pivotal role in regulating the lung–CNS reflex and respiratory responses. Repeated exposure to an allergen induces neuroplasticity in the NTS in monkeys, and the stimulation of NTS neurons initiates extensive CNS responses that precipitate asthma-like symptoms [3,5]. In the present study, the elevation of the c-Fos protein levels (in the CA1 area and NTS) observed after OVA challenge suggests that OVA acti-

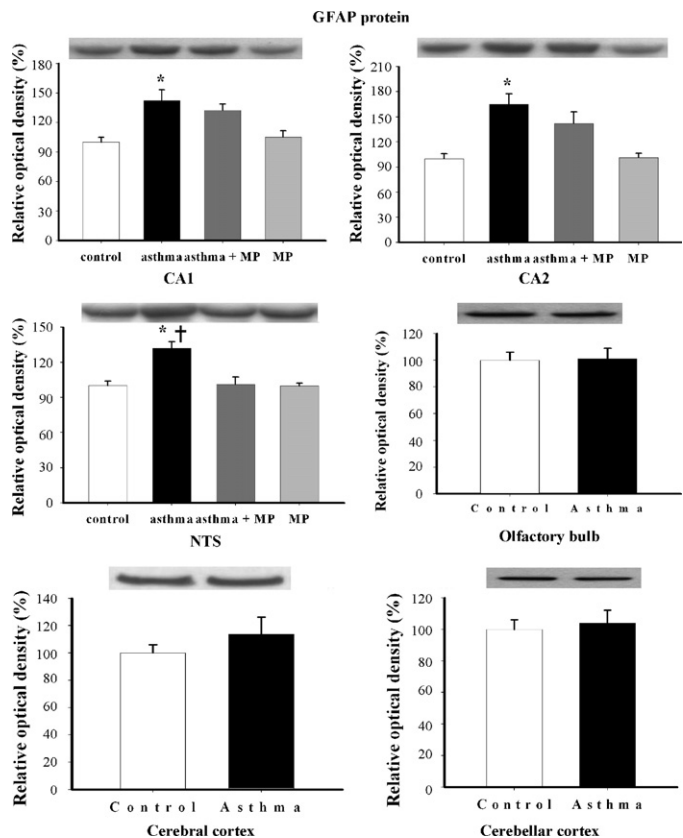


Fig. 5. Glial fibrillary acidic protein (GFAP) protein expression in the selected areas of the CNS in OVA- and saline-treated rats. Data of the densitometric analysis is shown below each Western blot photo. The bars indicate mean $\pm$ S.E.M. ($n=6$). * $p \leq 0.05$ versus control rats either treated or untreated with MP, † $p \leq 0.05$ versus asthmatic rats pretreated with MP.

vates the neurons in these areas. This is similar to the results of Basso et al. [2], who have demonstrated that OVA challenge induced the up-regulation of c-Fos protein expression in the CNS. Furthermore, the elevated GFAP protein content in the hippocampus and NTS suggests that astrocytes are also activated after OVA stimulation. Thus, OVA challenge induces both airway inflammation and activation of neurons and astrocytes in the brain, especially in the NTS. Further studies are necessary to delineate the relationship between the protein expression and functional changes in the brain after OVA stimulation.

OVA challenge also causes inflammation and mucopitthelial metaplasia in the nasal mucosa of rats [15]. Olfactory information is sent from the nasal mucosa to the olfactory bulb and then through the lateral and accessory olfactory tracts to the frontal cortex, thalamus, hypothalamus, amygdala, and hippocampus; therefore, the olfactory bulb might also be a target of OVA challenge. In the present study, the elevation of the nNOS level but not the GFAP level in the olfactory bulb suggests that OVA activates neurons but not astrocytes in the olfactory bulb.

From the results of a previous study [2] and present study, we conclude that asthma may affect the protein levels in the CNS, especially in the hippocampus, NTS, and olfactory bulb, even after a short-term exposure to OVA, as observed in a rat model of allergic asthma. The finding that MP inhibits not only airway inflammation but also protein expression in the CNS of asthmatic rats may provide a rationale for early and constant intervention using steroids for the treatment of asthma.

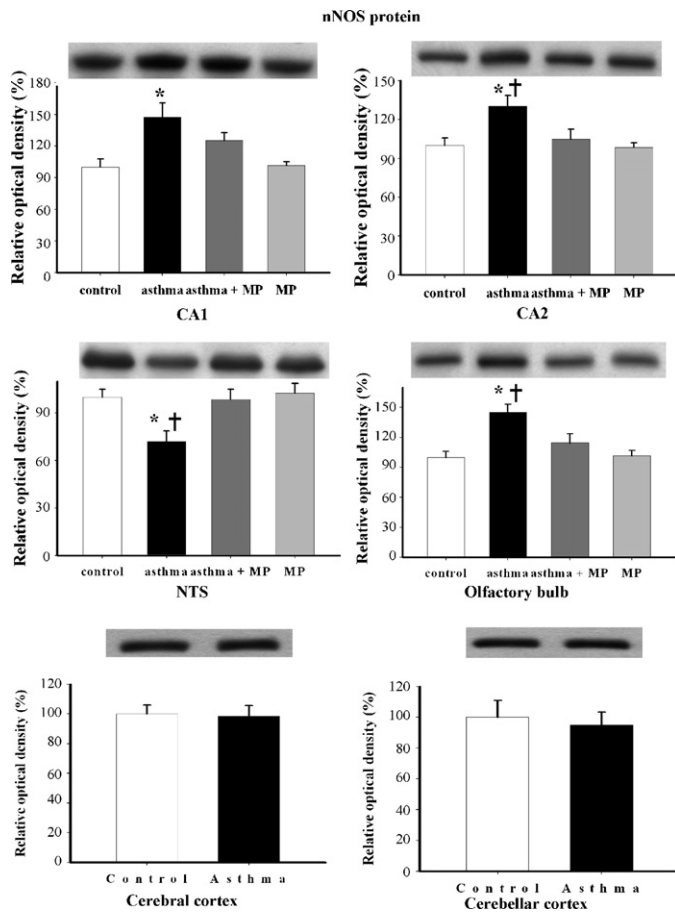


Fig. 6. Neuronal type nitric oxide synthase (nNOS) protein expression in the selected areas of the CNS in OVA- and saline-treated rats. The bars indicate mean $\pm$ S.E.M. ($n=6$). Data of the densitometric analysis is shown below each Western blot photo. * $p \leq 0.05$ versus control rats either treated or untreated with MP, † $p \leq 0.05$ versus asthmatic rats pretreated with MP.

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