

Influence of chronic unpredictable stress on the allergic responses in rats

Fernanda Datti, Marcelo Datti, Edson Antunes, Nancy A. Teixeira*

Faculty of Medical Sciences, Department of Pharmacology, UNICAMP, P.O. Box 6111, 13081-970, Campinas (SP), Brazil

Received 13 November 2001; received in revised form 19 February 2002; accepted 24 May 2002

Abstract

The effects of chronic unpredictable stress on airway leukocyte infiltration and plasma extravasation in female rats have been investigated. The chronic stress lasted for 14 days and consisted of transitory and variable changes in the living conditions of the animals. Concomitant to the stress procedure, the animals were sensitized (Day 0) and challenged with ovalbumin (OVA; 200 µg) at Day 14. Bronchoalveolar lavage (BAL) was performed 48 h after intratracheal challenge with OVA (0.4 ml of a 0.25% solution). The increase in plasma extravasation was assessed by the rat paw oedema induced by OVA (0.1 mg/paw) or the mast cell degranulator compound 48/80 (5 µg/paw). A significant increase ($P < .05$) in the total leukocyte influxes into the airways was observed in the stressed (sensitized) group compared to nonstressed (sensitized) animals, and this was associated with a marked recruitment of eosinophils and mononuclear cells in the BAL fluid. In OVA-sensitized rats, intraplantar injection of OVA induced a marked paw oedema that was significantly higher in stressed compared to nonstressed groups. In contrast to OVA, the compound 48/80 (5 µg/paw)-induced oedema did not significantly differ between nonstressed and stressed groups. Our results indicate that chronic unpredictable stress exacerbates the vascular and cellular inflammatory responses.

© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Allergy; Chronic stress; Inflammation; Antigen; Plasma extravasation; Leukocytes

1. Introduction

It is well established that the inflammatory reaction is controlled by the central nervous system, but the communication between the brain and the inflammatory processes has been proven to be complex provided this involves bidirectional signals connecting central nervous system with peripheral and endocrine systems, as well as with immune responses [1,2]. It is known that stress perturbs this equilibrium leading to alterations of the immunological functions and inflammatory reactions, a phenomenon that depends on the type, intensity and duration of the stress [3]. Among the various stress models employing experimental animals, the unpredictable stress causes profound and long-lasting alterations in rodents, including behavioral changes [4]. This is believed to reflect the incapacity of the animal to deal with the different and unavoidable types of experimentally imposed aggression. The resulting imbalance elicits biochemical changes in the central and peripheral systems that impair an adequate immunological

regulation in the body [5–7]. In humans, psychological stress precipitates or aggravates mental diseases including affective disorders, and this is often associated with immunological and inflammatory diseases, such as bronchial asthma and other allergic manifestations, rheumatoid arthritis, ulcerative colitis and inflammatory bowel disease [8–10]. Individuals presenting alterations of the immunological system also frequently show emotional depression [8–10]. In experimental animals, the effects of either acute or chronic unpredictable stress on the allergic inflammatory responses have been poorly investigated. Therefore, in the present study, a chronic inescapable unpredictable stress was applied to adult rats [11], and the vascular and cellular inflammatory responses were investigated in an allergic model of inflammation.

2. Materials and methods

2.1. Animals

Female nulliparous Wistar rats weighing 160–210 g (aged 10–12 weeks) provided by the Biology Institute Laboratory Animal Center (CEMIB) of the State University

* Corresponding author. Tel.: +55-19-37887185; fax: +55-19-32892968.

E-mail address: airoldi@obelix.unicamp.br (N.A. Teixeira).

of Campinas (UNICAMP) were used. They were individually housed at $26 \pm 2^\circ\text{C}$ with food and water ad libitum on a 12-h light:dark cycle with the lights turned on at 6:00 a.m. All experiments were carried out in accordance with the guidelines of the State University of Campinas (UNICAMP).

2.2. Experimental protocols with actively sensitized rats

The rats were initially randomly divided into non-stressed and stressed groups. In the first day of chronic unpredictable stress, each of these groups was further divided into another two groups, namely nonsensitized and sensitized rats. The latter refers to rats actively sensitized with ovalbumin (OVA), whereas the former refers to rats receiving only the OVA vehicle (aluminium hydroxide). Thus, these protocols resulted in four experimental groups of 13 rats each: (1) nonstressed, nonsensitized; (2) nonstressed, sensitized; (3) stressed, nonsensitized and (4) stressed, sensitized. Fourteen days later, all of these groups were employed for either the leukocyte counts in bronchoalveolar lavage (BAL) fluid (eight rats each) or the OVA-induced paw oedema (five rats each) experiments (see details below).

2.3. Chronic unpredictable stress

The stressed group underwent a schedule of chronic unpredictable stress that was based on a previously published method [12], and consists of different types of stressors presented randomly over a period of 14 days. Briefly, the stress regime (per week) consisted of 60 h of food and 78.5 h of water deprivation, 35 h of continuous lighting, 24.5 h of cage tilting, 17 h of paired housing, 17 h in a soiled cage, 2 h of reduced temperature, 10 h of exposure to intermittent white noise (85 dB), 1 h of stroboscopic lighting, 0.5 h of exposure to empty water bottles following a period of water deprivation, 2 h of restricted access to food, 17 h of exposure to a new odor and 17 h of exposure to objects in the home cage. All stressed rats were exposed to the stressors in the same order. Control rats were left undisturbed throughout the experimental period (14 days) except for the routine animal care.

2.4. Sensitization procedure and BAL fluid analysis

The animals were actively sensitized against OVA chicken egg (Grade III) according to a previous study [13], with slight modifications. Sensitization was performed by subcutaneous injection of OVA (0.15 ml) solution containing 200 μg of OVA and 8 mg of aluminium hydroxide prepared in saline. Nonsensitized rats received only 8 mg of aluminium hydroxide. Fourteen days later, all the animals (nonstressed nonsensitized, nonstressed sensitized, stressed nonsensitized, stressed sensitized groups) were anaesthetized with chloral hydrate (300 mg/kg ip) and submitted to a minor surgical procedure to inject OVA

into the airways. Briefly, the trachea was exposed through a midline ventral incision of approximately 0.5 cm length in the neck. With the aid of a 26.5-gauge needle, 0.4 ml of 0.25% OVA solution was injected into the airways. The animal sensitisation and challenging were carried out between 08:00 and 10:00 h for all studied groups. BAL was performed 48 h after intratracheal injection of OVA. Briefly, the animals were again anaesthetized with chloral hydrate (300 mg/kg ip) and exsanguinated by cutting the abdominal aorta. The trachea was exposed and cannulated with a polyethylene tube (1 mm diameter) connected to a syringe. The lungs were washed by flushing with phosphate buffered saline (PBS) solution containing heparin (20 IU/ml) and serum albumin (0.03%). The PBS buffer was instilled through the tracheal cannula as one 10-ml aliquot followed by three 5-ml aliquots. The fluid recovered after each aliquot instillation was combined and centrifuged ($1000 \times g$ for 10 min at 20°C). The cell supernatant was discarded and the cell pellet was resuspended in 2 ml of PBS buffer. Total cell counts were done with an automated cell counter (CELL-DYN, 1600, USA). A cytocentrifuge (Revan, São Paulo, Brazil) was used to prepare slides that were stained with May-Grunwald-Giemsa. A minimum of 400 cells were counted and classified as neutrophils, eosinophils and mononuclear cells based on normal morphological criteria.

2.5. Plasma protein extravasation

The animals were subcutaneously sensitized with OVA (or received the OVA vehicle alone) as described above. Fourteen days later, all the animals were anaesthetized with inhaled halothane. A subplantar injection of OVA (0.1 mg/paw) was made into one paw of the rat in a final volume of 0.1 ml. The paw volume was assessed immediately before (basal volume) and at selected time intervals (15, 30, 60, 120, 180 and 240 min) thereafter, using a hydroplethysmometer (model 7150, Ugo Basile, Italy). A separate group of stressed and nonstressed rats was subplantarily injected with mast cell degranulator compound 48/80 (5 μg /paw; $n=5$ each), and the paw volume was assessed at 15, 30, 60, 120 and 180 min postinjection. The increase in paw volume (ml) was obtained by subtracting the paw volume measured prior to the injection of stimuli from the volumes for the different time-points. When appropriate, the area under the time-course curve (AUC; ml min) was calculated using a trapezoidal method. The results were expressed as percent increase of paw volume or AUC.

2.6. Statistical analysis

All data are expressed as means $\pm$ S.E.M. The program InStat (GraphPad Software) was used for statistical analysis. Data were analysed by analysis of variance (ANOVA) followed by Duncan's test to determine differences between

Table 1

Body weight in nonstressed (nonsensitized and sensitized) and stressed (nonsensitized and sensitized) rats

Groups	Body weight (g)		
	Day 1	Day 15	% variation
Nonstressed, nonsensitized	184 ± 3.4	204 ± 4.5 *	+ 11
Nonstressed, sensitized	176 ± 3.8	197 ± 4.5 *	+ 12
Stressed, nonsensitized	184 ± 2.2	181 ± 3.1 [#]	– 1.6
Stressed, sensitized	176 ± 6.8	172 ± 5.8 [#]	– 2.3

Data represent media ± S.E.M. of 13 rats.

* $P < .05$ compared to Day 1.

[#] $P < .05$ compared to respective nonstressed group at Day 15.

groups, or *t*-test for two independent samples. Significance was defined at the $P < .05$ level.

3. Results

3.1. Body weight

The nonstressed rats (nonsensitized and sensitized groups) presented an 11–12% increase ($P < .05$) in body weight, as evaluated at the last day of the stress procedure. In contrast, the stressed rats (nonsensitized and sensitized groups) did not show any significant body weight gain during the stress procedure (Table 1).

3.2. Leukocyte counts in BAL fluid

The BAL fluid recovered from the nonsensitized rats revealed the presence of 3.8 ± 1.2 and $3.6 \pm 0.7 \times 10^6$ leukocytes/BAL for the nonstressed and stressed groups,

respectively. This was constituted by $> 97\%$ of mononuclear cells, while neutrophils (0.03 ± 0.01 and $0.07 \pm 0.02 \times 10^6$ cells/BAL, respectively) and eosinophils (0.06 ± 0.05 and $0.02 \pm 0.01 \times 10^6$ cells/BAL, respectively) were very few. No statistical differences were observed among both of these nonsensitized groups. However, the BAL fluid recovered from OVA (48 h)-sensitized rats in both nonstressed and stressed groups showed a significant increase in the eosinophil counts (0.8 ± 0.2 and $1.2 \pm 0.2 \times 10^6$ cells/BAL, respectively; $P < .05$) compared to nonsensitized rats, thus, confirming that sensitization with OVA was efficient. In addition, the counts of total leukocytes, eosinophils and mononuclear cells in OVA-sensitized rats were significantly higher ($P < .05$) in the stressed group compared to nonstressed animals (Fig. 1).

3.3. Plasma protein extravasation

In order to investigate whether the vascular component of the inflammatory reaction was modified by the stress procedure, two different oedematogenic agents were used, namely OVA and the mast cell degranulating substance compound 48/80. Intraplantar injection of OVA (100 μ g/paw) into the nonsensitized animals (nonstressed or stressed groups) did not evoke a significant oedema formation, as expected (Fig. 2). In OVA-sensitized rats, intraplantar injection of OVA induced a marked oedema of rapid onset and long duration in both nonstressed and stressed groups. However, the response in the stressed group was significantly higher compared to nonstressed animals, as detected at 2–4 h post-OVA injection ($P < .05$; Fig. 2). The area under the time course curve ($AUC_{1-4 \text{ h}}$) showed a 41% increase ($P < .05$) in the stressed group. In contrast to OVA, the paw

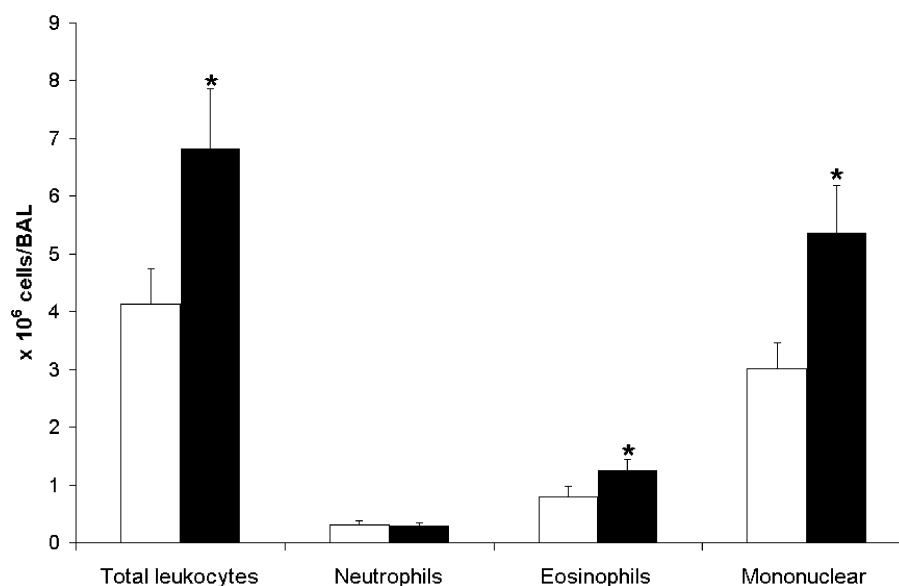


Fig. 1. Effects of chronic stress on total and differential (neutrophils, eosinophils and mononuclear cells) leukocyte counts in BAL fluid from OVA-sensitized female rats after 48 h following intratracheal injection of OVA. Open columns represent nonstressed, whereas solid columns represent stressed rats. Each column represents the mean ± S.E.M. ($n = 7-8$). * $P < .05$ compared to nonstressed rats.

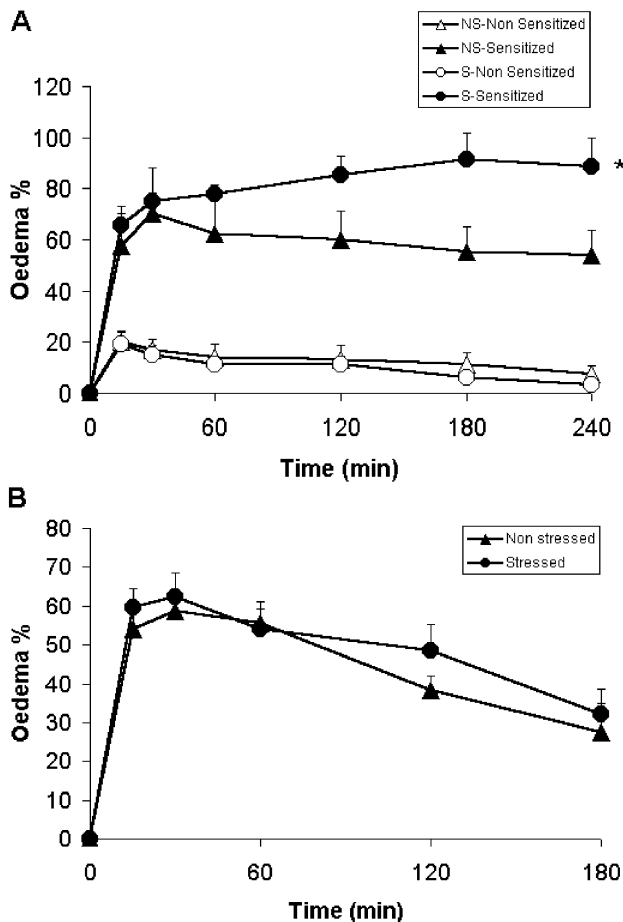


Fig. 2. Rat paw oedema induced by OVA (Panel A) or compound 48/80 (Panel B). OVA was injected at the dose of 100 μ g/paw in nonstressed nonsensitized, nonstressed sensitized, stressed nonsensitized and stressed sensitized rats ($n=5$). Compound 48/80 (5 μ g/paw) was injected in both nonstressed and stressed rats ($n=5$). The rat paw oedema in the rat was expressed as percent increase in volume of the injected paw compared to basal volume. * $P<.05$ compared to nonstressed rats.

oedema induced by compound 48/80 (5 μ g/paw) did not significantly differ between nonstressed and stressed groups throughout the time observed.

4. Discussion

Using the chronic unpredictable stress model, our results showed an exacerbation of the allergic vascular and cellular inflammatory reaction in rats, as evaluated by the higher airway leukocyte influxes and increased vascular permeability in sensitized animals exposed to the antigen OVA.

In humans, although no direct influence of the stress on pulmonary disorders has been demonstrated yet, it is well known that chronic stress and depression influences bronchial asthma attacks [14–16]. Using different experimental approaches to induce stress in rodents, controversial results have been documented, a feature that seems to reflect the time, duration and controllability of the stress

as well as the immune/inflammatory model used [3]. Although early studies showed attenuation of inflammatory reactions by psychological stress due to release of adrenocortical hormones, more recent studies showed that stress can determine bidirectional effects (enhancement or reduction) on the immune/inflammatory reaction depending on whether stressors are acutely or chronically applied to animals [17–20]. For instance, while acute stress exacerbates delayed type hypersensitivity reactions in the rat, chronic stress attenuates this allergic immune reaction [19,20]. Additionally, chronic stress can attenuate or aggravate autoimmune encephalomyelitis in rats depending on whether animals were stressed before or after the immune challenge, respectively [21].

Our present study showed an exacerbation of both vascular (OVA-induced paw oedema) and cellular (leukocyte influx in BAL fluid) responses in sensitized rats submitted to chronic unpredictable stress. Different cell types have been implicated in the allergic inflammatory reactions in the airways, including eosinophils, lymphocytes and mast cells [22,23]. Compound 48/80 is known to directly stimulate G-proteins by a nonimmunological mechanism causing massive mast cell degranulation [24]. This leads to the release of vasoactive amines such as histamine and serotonin causing, in turn, increased plasma extravasation and oedema formation [25]. Our findings that compound 48/80-induced plasma extravasation was unchanged by the chronic stress exclude the possibility that an IgE-independent mechanism takes place on rat mast cells exposed to the stress paradigm. Moreover, preformed mast cell-derived mediators have been shown to play minor roles in antigen-induced increase in vascular permeability [26,27]. Interestingly, repeated exposures of rats to restraint stress markedly inhibit the bradykinin-induced plasma extravasation in the knee joint, an effect suppressed by adrenalectomy [28]. The discrepancy between this latter study and our findings are unclear, but one may postulate that this reflects the plasma corticosterone levels of the stressed animals. Glucocorticoids can shift the cytokine production from a primarily proinflammatory TH1 pattern (through release of IL-2 and IFN γ) to an antiinflammatory TH2 pattern (through release of IL-4 and IL-10) [29]. Moreover, lower glucocorticoid plasma levels have been shown to exacerbate the inflammatory process, whereas high plasma glucocorticoid levels attenuate the inflammatory process [30]. However, the corticosterone level in rats exposed to chronic unpredictable stress has been shown to be controversial since it can be unaltered [12] or augmented [31] compared to nonstressed animals. Further studies are thus required to elucidate the role of the hypothalamus–pituitary–adrenal (HPA) axis and cytokines in the allergic responses of chronically stressed rats.

In airways of rat offspring sensitized with OVA at adult ages, the chronic unpredictable stress increases the recruitment of eosinophils in BAL fluid [32], which is consistent

with our present findings showing that eosinophil counts are significantly higher in BAL fluid from the stressed group. Indeed, eosinophils accumulation into inflamed tissues plays a critical role in allergic diseases, particularly bronchial asthma, since they synthesize and release a number of proinflammatory substances [22]. Mononuclear cells such as macrophages and T lymphocytes have also been implicated in the pathogenesis of allergic diseases. In agreement with the rat offspring [32], a higher influx of mononuclear cells in the stressed rats was observed in our study. Of interest, isolated alveolar macrophages obtained from stressed rats showed a marked increase of IL-1 β and TNF- α secretion [33], an effect unrelated to corticosterone and ACTH levels [34,35]. One may speculate therefore that aggravation of airway inflammatory responses by chronic unpredictable stress is a consequence of higher influx and activity of both eosinophils and mononuclear cells into the lungs, two cell types that may play a critical role in the stress paradigm.

Acknowledgements

Fernanda Datti was supported by CAPES, Brazil.

References

- [1] Rabin BS, Cohen S, Ganguli R, Lysle DT, Cunnick JE. Bidirectional interaction between the central nervous system and the immune system. *Crit Rev Immunol* 1989;9:279–312.
- [2] Rabin BS. Stress, immune function, and health: the connection. New York: Wiley-Liss, 1999.
- [3] Kusnecov AW, Rabin BS. Stressor-induced alterations of immune function: mechanisms and issues. *Int Arch Allergy Immunol* 1994;105:107–21.
- [4] Willner P. Animal models of depression. In: Willner P, editor. Behavioral models in psychopharmacology. Cambridge: Cambridge Univ Press, 1991. p. 91–125.
- [5] Stefanski V, Engler H. Effects of acute and chronic social stress on blood cellular immunity in rats. *Physiol Behav* 1998;64:733–41.
- [6] Stefanski V, Engler H. Social stress, dominance and blood cellular immunity. *J Neuroimmunol* 1999;94:144–52.
- [7] Chover-Gonzalez AJ, Harbuz MS, Tejedor-Real P, Gibert-Rahola J, Larsen PJ, Jessop DS. Effects of stress on susceptibility and severity of inflammation in adjuvant-induced arthritis. *Ann NY Acad Sci* 1999;876:276–86.
- [8] Hurwitz EL, Morgenstern H. Cross-sectional associations of asthma, hay fever, and other allergies with major depression and low-back pain among adults aged 20–39 years in the United States. *Am J Epidemiol* 1999;150:1107–16.
- [9] Heim C, Ehler U, Hellhammer DH. The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinology* 2000;25:1–35.
- [10] O'Connor TM, O'Halloran DJ, Shanahan F. The stress response and the hypothalamic–pituitary–adrenal axis: from molecule to melancholia. *QJM* 2000;93:323–33.
- [11] Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology* 1997;134:319–29.
- [12] Willner P, Towell A, Sampson D, Sophokleas S, Muscat R. Reduction of sucrose preference by chronic unpredictable mild stress and its restoration by tricyclic antidepressants. *Psychopharmacology* 1987;93:358–64.
- [13] Vianna EO, Garcia-Leme J. Allergen-induced airway inflammation in rats. Role of insulin. *Am J Respir Crit Care Med* 1995;151:809–14.
- [14] Sandberg S, Paton JY, Ahola S, McCann DC, McGuinness D, Hillary CR, Oja H. The role of acute and chronic stress in asthma attacks in children. *Lancet* 2000;356:982–7.
- [15] Ritz T, Steptoe A. Emotion and pulmonary function in asthma: reactivity in the field and relationship with laboratory induction of emotion. *Psychosom Med* 2000;62:808–15.
- [16] Goebel MU, Mills PJ. Acute psychological stress and exercise and changes in peripheral leukocyte adhesion molecule expression and density. *Psychosom Med* 2000;62:664–70.
- [17] Monjan AA, Collector MI. Stress-induced modulation of the immune response. *Science* 1977;196:307–8.
- [18] Jessop JJ, Gale K, Bayer BM. Enhancement of rat lymphocyte proliferation after prolonged exposure to stress. *J Neuroimmunol* 1987;16:261–71.
- [19] Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav Immun* 1997;11:286–306.
- [20] Dhabhar FS. Stress-induced enhancement of cell-mediated immunity. *Ann NY Acad Sci* 1998;840:359–72.
- [21] Correa SG, Rodriguez-Galan MC, Rivero VE, Riera CM. Chronic varied stress modulates experimental autoimmune encephalomyelitis in Wistar rats. *Brain Behav Immunol* 1998;12:134–48.
- [22] Kroegel C, Virchow JR, Luttmans J, Walker C, Warner JA. Pulmonary immune cells in health and disease: the eosinophil leukocyte (Part I). *Eur Respir J* 1994;7:519–43.
- [23] Warner JA, Kroegel C. Pulmonary immune cells in health and disease: mast cells and basophils. *Eur Respir J* 1994;7:1326–41.
- [24] Mousli M, Bueb JL, Bronner C, Rouot B, Landry Y. G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom peptides. *Trends Pharmacol Sci* 1990;11:358–62.
- [25] Garland LG, Payne AN. The role of cell-fixed calcium in histamine release by compound 48/80. *Br J Pharmacol* 1979;65:609–13.
- [26] Amorim CZ, Cordeiro RS, Vargaftig BB. Interference of antihistamines and anti-allergic drugs with antigen-induced paw edema in boosted and unboosted mice. *Eur J Pharmacol* 1992;216:429–34.
- [27] Motta JIB, Cunha FQ, Vargaftig BB, Ferreira SH. Drug modulation of antigen-induced paw oedema in guinea-pigs: effects of lipopolysaccharide, tumor necrosis factor and leukocyte depletion. *Br J Pharmacol* 1994;112:111–6.
- [28] Strausbaugh HJ, Dallman MF, Levine ID. Repeated, but not acute, stress suppresses inflammatory plasma extravasation. *Proc Natl Acad Sci USA* 1999;96:14629–34.
- [29] Sternberg EM. Neuroendocrine regulation of autoimmune/inflammatory disease. *J Endocrinol* 2001;169:429–35.
- [30] Potter PT, Zautra AJ. Stressful life events' effects on rheumatoid arthritis disease activity. *J Consult Clin Psychol* 1997;65:319–23.
- [31] Haile CN, GrandPre T, Kosten TA. Chronic unpredictable stress, but not chronic predictable stress, enhances the sensitivity to the behavioural effects of cocaine in rats. *Psychopharmacology* 2001;154:213–20.
- [32] Nogueira PJ, Ferreira HHA, Antunes E, Teixeira NA. Chronic mild prenatal stress exacerbates the allergen-induced airway inflammation in rats. *Med Inflamm* 1999;8:119–22.
- [33] Persoons JHA, Schornagel K, Brevé J, Berkenbosch F, Kraal G. Acute stress affects cytokines and nitric oxide production by alveolar macrophages differently. *Am J Respir Crit Care Med* 1995;152:619–24.
- [34] Broug-Holub E, Persoons JHA, Schornagel K, Mastbergen SC, Kraal G. Effects of stress on alveolar macrophages: a role for the sympathetic nervous system. *Am J Respir Cell Mol Biol* 1998;19:842–8.
- [35] Persoons JHA, Moes NM, Broug-Holub E, Schornagel K, Tilders FJH, Kraal G. Acute and long-term effects of stressors on pulmonary immune functions. *Am J Respir Cell Mol Biol* 1997;17:203–8.