

Mechanisms Involved in the Enhancement of Allergic Airways Neutrophil Influx by Permanent C-Fiber Degeneration in Rats

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ABSTRACT

This study was undertaken to clarify the mechanisms by which C-fiber degeneration at neonatal stages exacerbates the inflammatory responses of rat airways. Rats were treated with capsaicin at neonatal stages and immunized with ovalbumin (OVA) at adult ages. Challenge of capsaicin-pretreated rats with OVA promoted a higher influx of neutrophils in bronchoalveolar lavage (BAL) fluid compared with the vehicle group. No significant differences were found for the other cell types. The increased adhesion of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 0.1 μ M)- and phorbol myristate acetate (PMA; 1 μ M)-treated neutrophils to fibronectin-coated wells did not differ among vehicle- and capsaicin-pretreated rats. Additionally, fMLP (10 μ M), platelet-activating factor (0.1 μ M), and substance P (50 μ M) induced a significant neutrophil chemotaxis, but no differences were found among vehicle and capsaicin groups. Increased levels of tumor necrosis factor (TNF)- α , in-

terleukin (IL)-6, IL-10, and leukotriene B₄ in BAL fluid as well as higher expression of cytokine-induced neutrophil chemoattractant (CINC)-3 in lung homogenates were detected in the capsaicin group compared with vehicle group. In the capsaicin group, chronic treatment with compound 48/80 restored the TNF- α levels to control values and prevented the neutrophil influx in BAL fluid. The enhanced production of TNF- α , superoxide anion, and nitrite by isolated alveolar macrophages in response to lipopolysaccharide (3 μ g/ml), PMA (10 nM), and/or zymosan (100 particles/cell) did not differ between vehicle- and capsaicin-pretreated rats. In conclusion, chronic neuropeptide depletion promoted by neonatal capsaicin treatment up-regulates airways mast cells, which upon activation by antigen at adult ages, release large amounts of cytokines such as TNF- α and CINC-3 that accounts for the massive airways neutrophil infiltration.

The airways are densely innervated by efferent and afferent autonomic nerves, which regulate many aspects of airway functions. The parasympathetic nervous system is the dominant neural efferent pathway in the control of airway smooth muscle, but afferent inhibitory and excitatory nonadrenergic noncholinergic (NANC) nerves also play important roles in the lung functions. The inhibitory NANC system is the only bronchodilator pathway in the airways, whereas the excitatory NANC system mediates the bronchoconstrictor responses via the release of neuropeptides (substance P and neurokinin A) from a subpopulation of nonmyelinated sensory

C fibers (Barnes et al., 1996). Studies performed with capsaicin indicate that this substance is the best known pharmacological agent that releases neuropeptides from sensory C fibers since it interferes with the synthesis and intraneuronal transport of the peptides (Jancsó et al., 1977). Therefore, capsaicin, given to adult or neonate animals, has largely been used to identify capsaicin-sensitive neuronal pathways and to explore their contributions to evaluate sensory neuron mechanisms. Experimental studies have shown that acute treatment of adult animals with capsaicin to deplete the neuropeptide stores generally lead to anti-inflammatory effects characterized by reduction of microvascular permeability, bronchoconstriction, and/or leukocyte influx in rats, guinea pigs, mouse, and rabbit upon exposition to different inflammatory conditions (Cambridge and Brain, 1993; Buckley and Nijkamp, 1994; Herd et al., 1995; Tibério et al., 1997;

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ABBREVIATIONS: NANC, nonadrenergic noncholinergic; LPS, lipopolysaccharide; OVA, ovalbumin; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline; MEM, Eagle's minimum essential medium; MPO, myeloperoxidase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; iNOS, inducible nitric oxide synthase; CINC, cytokine-induced neutrophil chemoattractant; COX, cyclooxygenase; PCR, polymerase chain reaction; LTB₄, leukotriene B₄; TNF- α , tumor necrosis factor- α ; IL, interleukin; O₂⁻, superoxide anion; PAF, platelet-activating factor; AM, alveolar macrophage; CPS, capsaicin.

Ferreira et al., 2000; MacLean et al., 2000). On the other hand, when administered at neonatal stages of the rat, capsaicin produces extensive and irreversible lesions of sensory fibers resulting in a permanent loss by degeneration of 50 to 90% of all unmyelinated afferent fibers (Jancsó et al., 1977). Using this model of neonatal capsaicin treatment, a marked enhancement of the airways hyperresponsiveness and/or inflammatory responses to *Mycoplasma pulmonis* infection (Bowden et al., 1996), ozone (Sterner-Kock et al., 1996), lipopolysaccharide (LPS), and SO₂ gas (Long et al., 1996, 1997) have been reported. More recently, the neonatal capsaicin treatment has also been shown to exacerbate both the neutrophil recruitment into the airways and tracheal reactivity of ovalbumin-sensitized adult rats by mechanisms independent of IgE responses (Medeiros et al., 2001, 2003), suggesting a protective role for C-fibers in attenuating cell infiltration into tissues. Likewise, in certain organs such as the rat and human stomach, capsaicin-sensitive sensory fibers, via the release of calcitonin gene-related peptide, tachykinins, and NO, have been shown to play a pivotal role in maintaining the gastric mucosal integrity against injurious interventions (Wallace et al., 1992; Szolcsanyi and Bartho, 2001). The present study was designed to further clarify the mechanisms involved in the protective role exerted by C-fibers in the rat lungs. To achieve this, rats were treated with capsaicin at neonatal stages and immunized with ovalbumin (OVA) at adult ages. The production of inflammatory mediators in bronchoalveolar lavage (BAL) fluid and mRNA expression in lung tissue, as well as in vitro neutrophilic functions (adhesion and chemotaxis), were investigated in the OVA-challenged rats.

Materials and Methods

Neonatal Capsaicin Treatment. The experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation. Both male and female Wistar rats were treated on the 2nd day of life with capsaicin (30 mg/kg s.c.) or the vehicle [10% ethanol and 10% Tween 80 in 0.9% (w/v) NaCl solution], as previously described (Jancsó et al., 1977). The animals were then used at adult ages (8–10 weeks after capsaicin pretreatment). The levels of substance P in bronchoalveolar lavage fluid of the capsaicin-pretreated rats were reduced by 75% at adult ages (9.3 ± 1.2 and 2.3 ± 0.5 pg/ml for control and capsaicin groups, respectively), indicating that neonatal treatment of rats with capsaicin was efficient to promote the neuropeptide depletion.

Sensitization Procedure and Antigen Challenge. Adult rats (180–250 g) were sensitized with OVA chicken egg (grade III). Sensitization was performed by subcutaneous injection of OVA (0.15 ml) solution containing 200 μ g of OVA and 8 mg of Al (OH)₃ prepared in saline. Nonsensitized rats received only Al (OH)₃. Fourteen days later, both sensitized and nonsensitized rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.), and 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% solution of OVA was injected into airways. Our experimental protocols resulted in four groups: vehicle pretreated, nonsensitized; capsaicin pretreated, nonsensitized; vehicle pretreated, OVA sensitized; and capsaicin pretreated, OVA sensitized. In a separate group of OVA-sensitized rats (vehicle- and capsaicin-pretreated), compound 48/80 was chronically administered intraperitoneally to animals twice a day for 4 days. The dose employed was 0.6 mg/kg for the first six injections and 1.2 mg/kg for the last two injections (Di Rosa et al., 1971).

Leukocyte Counts in BAL Fluid. BAL was performed 6 h after OVA challenge. Briefly, the animal trachea was exposed and cannulated with a polyethylene tube (1-mm diameter) connected to a syringe. The lungs were washed by flushing with PBS solution containing heparin (20 UI/ml). The fluid recovered was centrifuged (1000g 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 1700), whereas differential counts were carried out on a minimum of 200 cells using cytopsin preparation stained with May-Grünwald. The cells were classified as neutrophils, eosinophils, and mononuclear cells based on normal morphological criteria.

Isolation of Peripheral Blood Neutrophils. Neutrophils were separated from peripheral blood collected in 3.13% (w/v) sodium citrate (10:1) and obtained by Dextran sedimentation followed by Ficoll gradient (1.077 g/liter). After separation of monocytes and granulocytes by centrifugation at 400g for 30 min, the granulocyte layer was washed once in Eagle's minimum essential medium (MEM; pH 7.2) before performing a hypotonic lysis to lyse contaminating red cells. Cells were washed once again in MEM before resuspending, finally, in MEM/0.1% ovalbumin. Total cell number was calculated (cells were diluted 1:20 and counted in an improved Neubauer hemocytometer), and cells were cytopspinned onto slides and a cell differentiation count performed. The final cell suspension contained 95% of neutrophils. Cell viability (>95%) was assessed by Trypan blue dye exclusion test.

In Vitro Neutrophil Chemotaxis. Cell migration assays were performed using a 96-well chemotaxis chamber (ChemoTx; Neuro Probe, Gaithersburg, MD). Twenty-five microliters of cell suspension in MEM/ovalbumin (8×10^6 cells/ml) were added to the upper compartment of the chamber and separated from the lower chamber containing 29 μ l of chemotactic agents by a polycarbonate filter (5- μ m pore). The chambers were incubated at 37°C in a 5% CO₂ atmosphere for 120 min. The wells of the upper compartment were emptied by aspiration and then disassembled. To detach adherent neutrophils from the filter, the microtiter plate with attached filter was centrifuged at 1200 rpm for 5 min at room temperature. Plates were then stored frozen overnight before measuring the myeloperoxidase (MPO) content. Plates were defrosted on ice before extracting with 0.5% (w/v) hexadecyltrimethyl ammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. Twenty microliters of each well sample was mixed with 200 μ l of *o*-dianisidine solution (0.167 mg/ml *o*-dianisidine dihydrochloride, 0.0005% hydrogen peroxide in 50 mM phosphate buffer, pH 6.0) immediately prior to reading the absorbance at 460 nm over 5 min in a microplate (Multiscan MS; Labsystems, Helsinki, Finland). Migrated neutrophils were calculated by comparing absorbance changes of unknown samples with those of the standard curve, which was performed measuring the MPO values of different neutrophil number (2.0×10^5 to 20×10^5 cells/ml).

Neutrophil Adhesion Assays. Ninety-six-well plates were prepared by coating individual wells with 60 μ l of fibronectin (20 μ g/ml) overnight at 4°C. Wells were then washed twice with PBS before blocking noncoated sites with 0.1% (w/v) bovine serum albumin for 60 min at 37°C. Wells were washed twice again with PBS before allowing plates to dry. Neutrophils (50 μ l of 0.5×10^6 cells/ml in MEM/ovalbumin) were seeded onto the coated wells alone or with fMLP (10^{-7} M) or PMA (10^{-6} M), and cells were allowed to adhere for 30 min at 37°C, 5% CO₂. Neutrophils were treated with fMLP and PMA alone or with antibodies against Mac-1 (ICRF 44, 1:12) or VLA-4 (HP21, 20 μ g/ml) and a nonspecific control monoclonal antibody (IgG, 10 μ g/ml). Following incubation, nonadherent cells were washed twice with PBS. Fifty microliters of MEM was added to each well, and varying concentrations of the original neutrophil cell suspension were added to empty wells to form a standard curve. Plates were then stored frozen overnight before measuring the MPO content of adherent cells and calculating percentage neutrophil adhesion. The MPO activity was evaluated as described above. Adherence

was calculated by comparing absorbance changes of unknowns to those of the standard curve.

Analysis of CINC-1, CINC-2, CINC-3, COX-2, and iNOS Gene Expression by Reverse Transcriptase-Polymerase Chain Reaction (PCR). Total RNA from whole lung was extracted by the Trizol reagent method, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 15 µg of total RNA using Superscript II (Invitrogen), and the obtained material was stored at -20°C until use. PCR reactions were performed in a final volume of 50 µl, containing 5 µl of cDNA solution, 5 µl of 10× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 units of *Taq* DNA polymerase (Invitrogen), and variable concentrations of each oligonucleotide primer pair [CINC-1, 1 µM; CINC-2, 2 µM; CINC-3, 3 µM; COX-2 and iNOS, 5 µM; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control, 3 µM]. The nucleotide sequences of the used primers are shown in Table 1.

Amplification cycle for CINC-1 was done with denaturation for 5 min at 94°C followed by 23 cycles of amplification consisting of a denaturation step 95°C for 1 min, a primer-annealing step at 56°C for 2 min, and an extension step at 72°C for 3 min. After the last amplification cycle, samples were incubated at 72°C for 7 min for extension. Thirty-three cycles were performed for the amplification of the COX-2 (1 min of denaturation at 94°C, 2 min of annealing at 56°C, and 1 min of extension at 72°C), and 30 cycles were performed for the iNOS (1 min of denaturation at 94°C, 45 sec of annealing at 65°C, and 1.5 min of extension at 72°C). Lungs samples obtained from rats 6 h after an LPS injection (0.3 mg/kg i.v.) were run in parallel and served as positive controls for COX-2 and iNOS expression. PCR products were separated on 1.8% agarose gels containing ethidium bromide. Band fluorescence images were acquired and digitalized using a ChemiImager 5500 system (Alpha Innotech Corporation, San Leandro, CA), and the band intensities were determined by densitometry using the equipment software. For each sample, the ratios between the densitometry value for each specific gene and that corresponding GAPDH were calculated and statistically analyzed for comparison among the experimental groups.

Measurement of LTB₄, TNF-α, IL-6, IL-10, and Substance P Levels in BAL Fluid. Rat TNF-α, LTB₄, IL-6, IL-10, and substance P levels were measured in BAL supernatants using commercially available enzyme-linked immunosorbent assays according to the manufacturer's instructions for rat TNF-α (BD Biosciences Pharmingen, San Diego, CA) and rat LTB₄, IL-6, IL-10, and substance P (R&D Systems, Minneapolis, MN).

Measurement of Nitrite and TNF-α Production by Alveolar Macrophages in Vitro. Bronchoalveolar lavage fluid obtained from capsaicin- and vehicle-pretreated rats were centrifuged (10 min, 470g), and cells recovered from pellets were pooled. The cell pellets were resuspended in culture medium (RPMI 1640) supplemented with bovine serum albumin (0.1%) and transferred to 35-mm-diameter tissue culture wells (1 ml/well, six wells). They were allowed to adhere for 2 h at 37°C in a humidified atmosphere containing 5% CO₂. Nonadherent alveolar macrophages were discarded, and the

remaining cells corresponded to >95%. Cells were incubated for 6 h in the presence of LPS (3 µg/ml) or OVA (10 µg/ml), then the supernatant from each well was collected and stored at -70°C. The concentrations of both TNF-α and nitrite were assayed using Griess reagent and enzyme-linked immunosorbent assay (see above), respectively. For the nitrite production, absorbance was measured at 540 nm, and amount of nitrite produced was determined using sodium nitrite as a standard.

Measurement of Superoxide Anion (O₂⁻) Production by Alveolar Macrophages in Vitro. Alveolar macrophages from capsaicin- and vehicle-pretreated rats were isolated as described above. Cells were resuspended with Hank's balanced salt solution supplemented with 10 mM HEPES (pH 7.4), and the isolated cells were counted. Cell suspensions were diluted to a concentration of 2 × 10⁶ cells/ml. The production of O₂⁻ by alveolar macrophages was measured based on the superoxide dismutase-inhibitable spectrophotometric detection of reduced cytochrome *c*. The production of O₂⁻ was studied after 2 h of incubation with zymosan (100 particles/cell), PMA (10 nM), and OVA (10 µg/ml) in comparison with nonstimulated cells. Spectrophotometric measurements at 550 nm were performed to determine the degree of reduction of ferri-cytochrome *c*.

Materials. Antibodies were purchased from Serotec (Raleigh, NC) (HP2/1, ICRF 44, and isotype control monoclonal antibodies), Rockland (Gilbertsville, PA). All other products were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. Data are presented as the means ± S.E.M. and were analyzed by analysis of variance for multiple comparisons followed by Bonferroni test or Student's unpaired *t* test where appropriate. In both cases, the level of significance was set at *P* < 0.05.

Results

Number of Leukocytes in BAL Fluid. The number of total and differential leukocyte in BAL fluid was evaluated in vehicle- and capsaicin-pretreated rats at 6 h after OVA challenge. The number of total leukocytes and neutrophils in the capsaicin group was significantly higher (*P* < 0.05) than the vehicle group, particularly in the OVA-sensitized rats (Table 2). The mononuclear cell counts did not differ significantly among groups (Table 2).

Neutrophil Adhesion Induced by fMLP and PMA in Fibronectin-Coated Plates: Effect of VLA-4- and Mac-1-Blocking Monoclonal Antibodies. Isolated neutrophils obtained from vehicle- or capsaicin-pretreated rats were incubated (15 min, 37°C, 5% CO₂) with fMLP (10⁻⁷ M) or PMA (10⁻⁶ M) to evaluate the stimulated adhesion. Under these conditions, fMLP (10⁻⁷ M) significantly increased the neutrophil adhesion to fibronectin-coated plates independently if cells were isolated from vehicle or capsaicin group (Fig. 1). A similar result was observed using PMA (10⁻⁶ M) to stimulate

TABLE 1
Nucleotide sequences of the used primers

Primer	Sequence (5' → 3')
iNOS (651 bp)	ACA ACA GGA ACC TAC CAG CTC A GAT GTT GTA GCG CTG TGT GTC A
COX-2 (1158 bp)	AGA CAG ATC ATA AGC GAG GAC C CAC TTG CAT TGA TGG TGG CTG T
CINC-1 (298 bp)	TGGAGAAAGAGATAGATTGC TTCTTCCCGCTCAACACCTTC
CINC-2 (309 bp)	CACTGAAGAGTTACGATGTCA TGAGGCTCCATAAATGAAAGA
CINC-3 (300 bp)	CCTGGAAGGAAGAACATGGG ACCTCCCAACTACATAAGTAA
GAPDH (400 bp)	GGT GCT GAG TAT GTC GTG GA TTC AGC TCT GGG ATG ACC TT

TABLE 2
Number of total leukocytes (TLs), neutrophils (NE), and mononuclear cells (MN) in BAL fluid from rats neonatally treated with capsaicin (CPS) or vehicle

	BAL Fluid (×10 ⁶ /BAL)			
	Nonsensitized		OVA-sensitized	
	Vehicle	CPS	Vehicle	CPS
TL	1.4 ± 0.4	2.3 ± 0.3	2.6 ± 0.4*	6.1 ± 1.3***
NE	0.4 ± 0.1	0.8 ± 0.2	1.1 ± 0.3*	3.9 ± 1.4***
MN	1.0 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	2.1 ± 0.3

* *P* < 0.05 compared with respective nonsensitized rats.

** *P* < 0.05 compared with OVA-sensitized vehicle group.

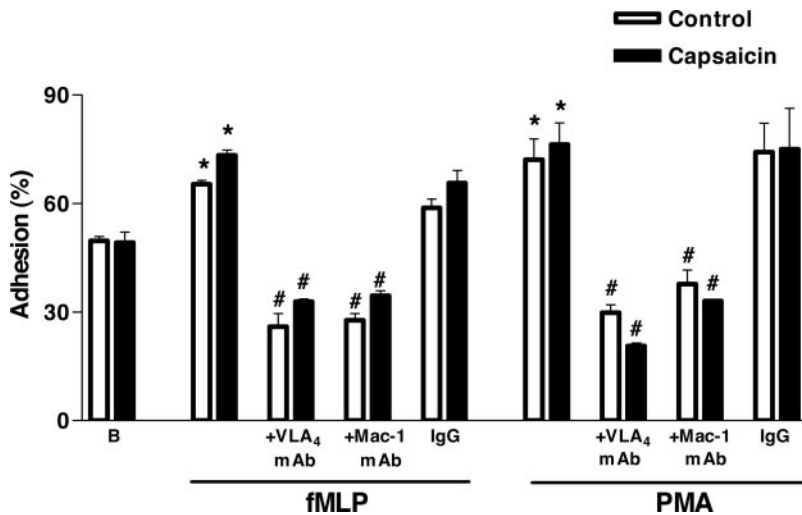


Fig. 1. Increased adhesion of stimulated neutrophils to fibronectin-coated plates. Peripheral neutrophils obtained from either vehicle- or capsaicin-pretreated rats were incubated with fMLP (1×10^{-7} M) or PMA (1×10^{-6} M) for 30 min (37°C , 5% CO_2), in the absence or presence of anti-VLA-4 or anti-Mac-1 monoclonal antibodies (mAb). A nonspecific monoclonal antibody (IgG) was used as negative control. Results are expressed as percent adhered cell values $\pm$ S.E.M. of three independent experiments with three replicates in each. *, $P < 0.05$ compared with basal adhesion (B). #, $P < 0.05$ compared with stimulated cells in absence of mAb.

adhesion (Fig. 1). In addition, the increased adhesion to fibronectin seen when neutrophils were incubated with fMLP was abolished upon coincubation with either anti-VLA-4 or anti-Mac monoclonal antibodies, irrespective if cells were obtained from vehicle or capsaicin groups. Similarly, the anti-VLA-4 or anti-Mac monoclonal antibodies prevented the increased adhesion of PMA-treated neutrophils in fibronectin-coated plates, as observed in both vehicle and capsaicin groups (Fig. 1). The nonspecific monoclonal antibody had no significant effect on the neutrophil adhesion in any of the conditions used.

In Vitro Neutrophil Chemotaxis. In preliminary experiments, a concentration-response curve for fMLP (0.1 to 10 μM), PAF (0.01 to 10 μM), and substance P (10 to 50 μM) on neutrophil chemotaxis was made, and a single concentration of each agent that yielded submaximal responses was then routinely used. Our results showed that fMLP (10 μM), PAF (0.1 μM), and SP (50 μM)-induced neutrophil chemotaxis did not significantly differ between vehicle and capsaicin groups, as observed in both nonsensitized and OVA-sensitized rats (Table 3).

COX-2, iNOS, CINC-1, CINC-2, and CINC-3 mRNA Expression in the Lung Tissues. The expressions of COX-2, iNOS, CINC-1, CINC-2, and CINC-3 were studied in the lung tissues obtained from vehicle (nonsensitized and OVA-sensitized)- and capsaicin (nonsensitized and OVA-sensitized)-pretreated rats. The expression of COX-2 and iNOS expression was not significantly increased in any condition used (data not shown). Using LPS (6 h) as a positive

control, a marked increase in both COX-2 and iNOS expression was detected (data not shown). The expression of CINC-1 in lung tissues of OVA-sensitized animals did not significantly differ among vehicle and capsaicin groups, whereas the CINC-2 expression was decreased ($P < 0.05$) in capsaicin-pretreated rats (Fig. 2). On the other hand, CINC-3 mRNA expression in the lung tissue of OVA-sensitized rats was significantly increased in the capsaicin group compared with the vehicle group (Fig. 2).

Measurement of LTB_4 , $\text{TNF-}\alpha$, IL-6, and IL-10 in BAL Fluid. The levels of LTB_4 , $\text{TNF-}\alpha$, IL-6, and IL-10 were measured in the BAL fluid from vehicle and capsaicin groups (nonsensitized and OVA-sensitized rats) at 6 h after OVA challenge. In both groups, increased levels of $\text{TNF-}\alpha$ were detected in OVA-sensitized rats, but the increase in the capsaicin group was 168% higher ($P < 0.01$) compared with vehicle group (Fig. 3). In vehicle-pretreated rats, the concentrations of LTB_4 , IL-6, and IL-10 did not significantly differ among nonsensitized and OVA-sensitized animals. However, in the capsaicin-pretreated rats, significantly higher concentrations of LTB_4 , IL-6, and IL-10 were detected in OVA-sensitized rats in comparison with the respective nonsensitized animals or the OVA-sensitized rats belonging to vehicle group (Fig. 3).

Rats Treated Chronically with Compound 48/80: Number of Leukocyte Counts and $\text{TNF-}\alpha$ Levels in BAL Fluid. In OVA-sensitized rats, compound 48/80 was given chronically to both vehicle and capsaicin groups, and neutrophil counts and $\text{TNF-}\alpha$ levels were evaluated in BAL fluid at 6 h after OVA challenge. In the capsaicin group, treatment with compound 48/80 reversed the increased counts of neutrophils, without significantly affecting the cell counts in the vehicle group (Fig. 4). Moreover, the increased levels of $\text{TNF-}\alpha$ seen in BAL fluid of the capsaicin group were markedly reduced by pretreatment with compound 48/80, whereas in the vehicle group, a moderate (but nonsignificant) reduction was observed (Fig. 4).

Measurement of $\text{TNF-}\alpha$, Nitrite, and O_2^- in Stimulated Alveolar Macrophages (AMs). Alveolar macrophages obtained from vehicle- and capsaicin-pretreated rats were isolated and stimulated in vitro, and levels of $\text{TNF-}\alpha$, nitrite, and O_2^- were evaluated in the AM supernatants. The $\text{TNF-}\alpha$ production in response to LPS (3 $\mu\text{g}/\text{ml}$) was markedly increased in vehicle and capsaicin groups, but

TABLE 3

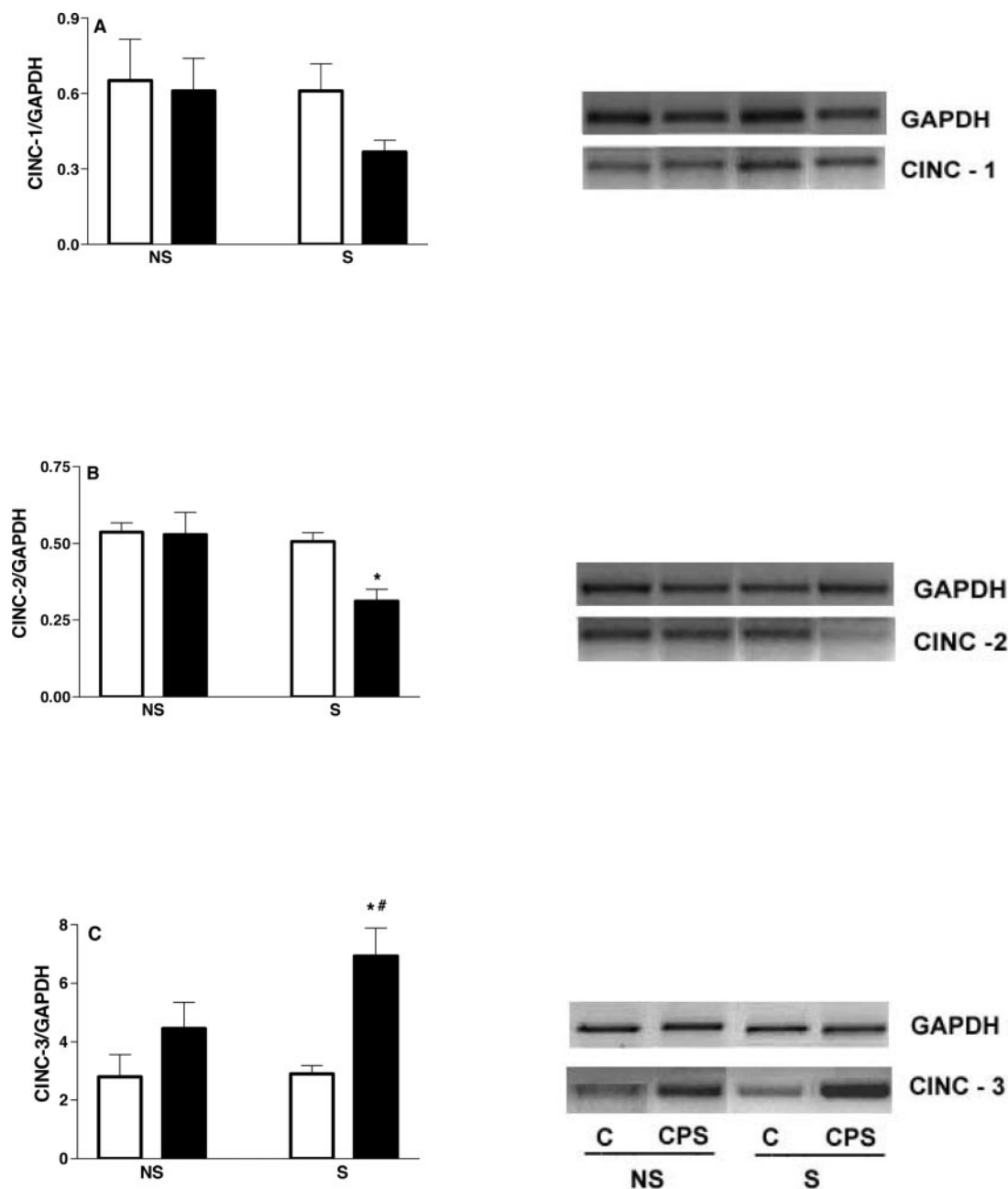
In vitro chemotaxis of neutrophils in response to fMLP (10 μM), PAF (0.1 μM), and substance P (50 μM)

Peripheral neutrophils were isolated from vehicle- or CPS-pretreated rats. Neutrophils from vehicle or capsaicin-pretreated rats were incubated with each chemotactic agent for 2 h (37°C , 5% CO_2). Results are expressed as mean of total cell number values $\pm$ S.E.M. of three independent experiments of three replicates in each.

	Neutrophils ($\times 10^5/\text{ml}$)			
	Nonsensitized		OVA sensitized	
	Vehicle	CPS	Vehicle	CPS
MEM	4.2 $\pm$ 0.3	3.3 $\pm$ 0.2	2.8 $\pm$ 0.3	2.5 $\pm$ 0.3
fMLP	13.1 $\pm$ 2.7*	13.9 $\pm$ 1.0*	9.9 $\pm$ 1.2*	11.6 $\pm$ 1.6*
PAF	18.6 $\pm$ 1.8*	17.5 $\pm$ 4.3*	11.3 $\pm$ 1.9*	12.3 $\pm$ 4.1*
Substance P	17.5 $\pm$ 1.2*	18.90 $\pm$ 1.1*	ND	ND

ND, not determined.

* $P < 0.05$ compared with untreated cells (MEM).



no significant differences among both of these groups could be detected (Table 4). The production of O_2^- in PMA (10 nM)- and zymosan (100 particles/cell)-stimulated AM was markedly increased in both vehicle and capsaicin groups, but no differences among them could be observed (Table 4). In addition, the elevated levels of nitrite in AM stimulated with LPS (3 μ g/ml) did not significantly differ between vehicle and capsaicin groups (Table 4).

Discussion

In our present study, the OVA challenge in immunized rats treated with capsaicin as neonates promoted a higher neutrophil migration at 6 h in comparison with vehicle-pretreated animals, and that was accompanied by larger release of $TNF-\alpha$, IL-6, IL-10, and LTB_4 in BAL fluid, as well as by higher CINC-3 expression in the lung tissues. This suggests

that in normal conditions, capsaicin-sensitive sensory C-fibers act to attenuate allergic neutrophil infiltration; consequently, permanent loss of C-fibers by capsaicin neonatal treatment represents a state that primes the lung tissue, which in turn evokes large neutrophil recruitment upon antigen challenge.

There is evidence that priming of neutrophils can result in exaggerated neutrophil functions and inflammatory mediator release, leading to abnormal homing and activation of inflammatory cells in remote tissues (Wagner and Roth, 2000). Priming of neutrophils is, therefore, one of the pivotal processes that regulate their functional responses. To explore the possibility that capsaicin neonatal treatment primed peripheral neutrophils that could explain their massive lung accumulation after antigen challenge, we first evaluated the ability of neutrophils to adhere in fibronectin-coated plates

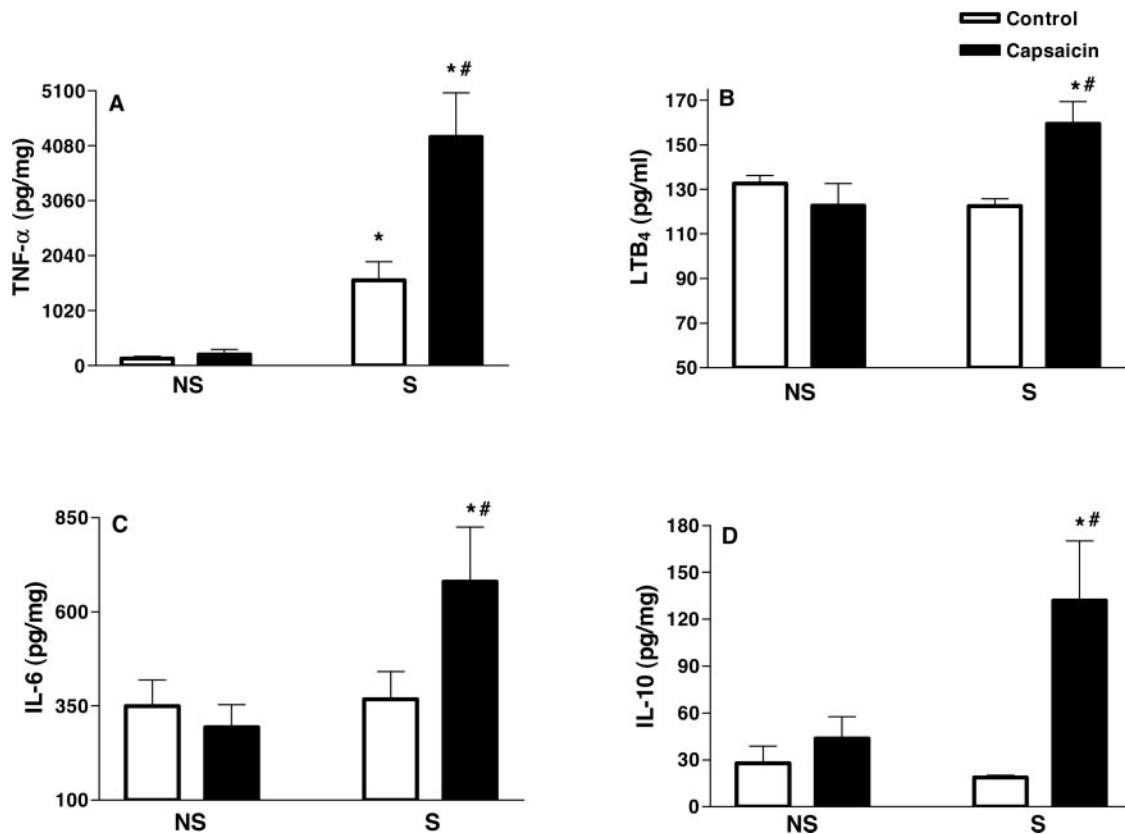


Fig. 3. Levels of TNF- α (A), LTB₄ (B), IL-6 (C), and IL-10 (D) after OVA challenge in BAL fluid of vehicle- and capsaicin-pretreated rats. At adult ages, nonsensitized (NS) and sensitized (S) rats were intratracheally injected with OVA, and BAL fluid was collected 6 h post-OVA challenge. Results are mean values $\pm$ S.E.M. from six animals. *, $P < 0.05$ compared with nonsensitized rats. #, $P < 0.05$ compared with sensitized rats of vehicle group.

upon stimulation with fMLP and PMA. The β_2 integrins (Mac-1 and LFA-1) are accepted to be the major integrin mediators of neutrophil adhesion to endothelial cells during migration, but a β_2 -independent mechanism via the integrin VLA-4 has also been shown to influence the infiltration of neutrophils into lungs (Burns et al., 2001). Chemotactically stimulated human neutrophils express VLA-4 when allowed to transmigrate across endothelial layers or when treated with fMLP, PAF, or IL-8 (Kubes et al., 1995). In fibronectin-coated plates, both Mac-1 and VLA-4 have been shown to mediate the adhesion of human neutrophils (Conran et al., 2003), thus mimicking the in vivo studies. In our study, stimulation of peripheral neutrophils with fMLP or PMA increased the cell adhesion to fibronectin-coated plates, but no differences in neutrophil adhesion between the vehicle and capsaicin groups could be detected. Furthermore, the anti-VLA-4 or anti-Mac monoclonal antibodies prevented the increased adhesion of stimulated neutrophils irrespective if neutrophils were obtained from vehicle- or capsaicin-pretreated rats, strongly suggesting that modifications in cell adhesion capacity and/or adhesion molecule expression do not explain the higher in vivo accumulation of lung neutrophils in the capsaicin group. Next, we examined the capacity of neutrophils to migrate in vitro in response to the chemoattractants fMLP, PAF, and substance P. These agents directly or indirectly influence neutrophil migration across endothelium and pore filters via G protein activation (Volpi et al., 1985; Carolan and Casale, 1993). However, the enhanced chemotaxis responses to these chemoattractants did not differ among vehicle or capsaicin groups. Taken together, data

with cell chemotaxis and adhesion are clear to suggest that peripheral neutrophils of capsaicin-pretreated rats are not found primed while in the circulating blood.

The IL-8 family of chemokines plays a major role in neutrophil infiltration into inflamed tissues. In the rat, CINC-3 have been considered the functional homologs of IL-8 and comprise the isomers CINC-1, CINC-2 α , CINC-2 β , and CINC-3 (Watanabe et al., 1991). These cytokines are potent chemotactic factors for rat neutrophils both in vitro and in vivo and can be induced by LPS, IL-1, and TNF- α (Watanabe et al., 1991). Furthermore, CINC-3 have similar ability to induce chemotaxis and to activate rat neutrophils, suggesting that their contribution to neutrophil infiltration depends on the amount of each present in a certain inflammatory site (Nakagawa et al., 1998). Thus, our data that only CINC-3 mRNA expression is increased in lung tissue from capsaicin-pretreated rats upon antigen exposure indicate a pivotal role for this cytokine in the airways neutrophil infiltration of the capsaicin neonatal model. Likewise, increased mRNA rat expression for CINC-3 has been detected in the lung injury induced by IgG immune complex (Shanley et al., 1997). The reduction of CINC-2 expression in the lung homogenates of capsaicin-pretreated rats is unclear, and additional studies are required to elucidate this aspect. Of interest, CINC-3 is able to inhibit neutrophil chemotaxis and calcium mobilization induced by CINC-1 and CINC-2 (Shibata et al., 2000). Whether elevation of CINC-3 levels down-regulate expression of the other homologues remains to be elucidated.

The exposure of capsaicin-pretreated rats to OVA significantly increased the levels of TNF- α , IL-6, and IL-10 in BAL

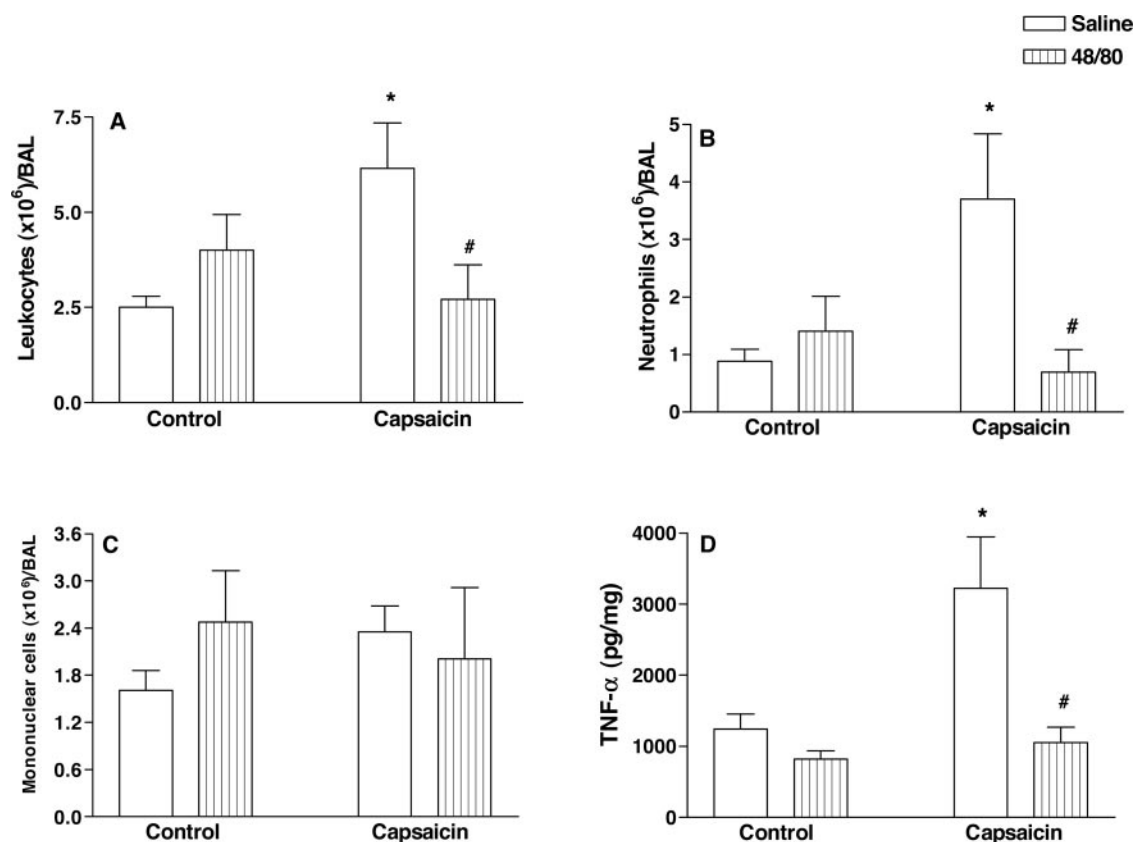


Fig. 4. Effect of chronic treatment with compound 48/80 in neutrophils counts (top panel) and TNF- α levels (bottom panel) in BAL fluid of vehicle- and capsaicin-pretreated rats. The BAL fluids were collected 6 h post-OVA challenge. Results are mean values $\pm$ S.E.M. from six animals. *, $P < 0.05$ compared with vehicle animals. #, $P < 0.05$ compared with saline of capsaicin groups.

TABLE 4

TNF- α , O $_2^-$, and nitrite production in supernatant of alveolar macrophages obtained from vehicle- and capsaicin (CPS)-pretreated rats and stimulated in vitro with PMA (10 nM), zymosan (100 particles/cells), and/or LPS (3 μ g/ml, 6 h)

Results are mean values $\pm$ S.E.M. from six animals.

	Vehicle	CPS
TNF- α (ng/ml)		
Basal	3.4 $\pm$ 0.32	3.1 $\pm$ 0.6
LPS	4.4 $\pm$ 0.24*	4.5 $\pm$ 0.3*
O $_2^-$ (nmol/10 ⁶ cells)		
Basal	0.5 $\pm$ 0.2	0.4 $\pm$ 0.2
PMA	7.0 $\pm$ 1.1*	7.5 $\pm$ 0.7*
Zymosan	2.1 $\pm$ 0.3*	3.0 $\pm$ 0.4*
Nitrite (μ M)		
Basal	6.6 $\pm$ 1.0	7.0 $\pm$ 1.8
LPS	21.5 $\pm$ 1.6*	19.6 $\pm$ 0.6*

* $P < 0.05$ compared with basal levels.

fluid compared with vehicle-pretreated rats. Although the role of IL-6 in the lung pathophysiology is still controversial since it may present pro- and anti-inflammatory effects, the functions of IL-10 offering clinical protection and reduction of lung pathology is well established (Morrison et al., 2000). Interleukin-10 reduces the levels and expression of TNF- α by mononuclear cells and mast cells (Weber et al., 1995). In our study, in BAL fluid of vehicle-treated rats (OVA challenged), a moderate increase in TNF- α with no significant changes in the levels of IL-6 and IL-10 was detected. However, OVA challenge in capsaicin-pretreated rats caused marked elevations of TNF- α and IL-6 in BAL fluid accompanied by concomitant elevations in the IL-10 levels, thus suggesting that

lung injury by OVA challenge in capsaicin-pretreated rats undergoes down-regulation by this cytokine. Furthermore, in immune inflammation in mice, TNF- α induces in vivo peritoneal neutrophil migration via mechanisms involving release of LTB $_4$ (Canetti et al., 2001), a finding that may explain the moderate (but significant) increase in LTB $_4$ levels in BAL fluid of capsaicin-pretreated rats.

Alveolar macrophages are richly present in alveoli, distal airspaces, and conducting airways, and produce both Th1 (TNF- α) and Th2 (IL-6 and IL-10) cytokines, along with nitric oxide and lipoxygenase products (Hamid et al., 2003). Therefore, the enhanced production of cytokines and lipid mediators in lungs of capsaicin-pretreated rats points to this cell type as a potential source involved in the generation and release of these inflammatory mediators. However, in our study, the enhanced production of TNF- α , nitrite, and O $_2^-$ in stimulated alveolar macrophages did not differ among vehicle and capsaicin groups, suggesting that the higher neutrophil influx of capsaicin-pretreated rats cannot be explained by an up-regulation of alveolar macrophages. The failure of capsaicin-pretreated rats to increase the expressions of iNOS and COX-2 in the lung tissues exposed to antigen challenge reinforces this suggestion since macrophages are recognized as major cells in producing NO and arachidonic acid metabolites in lung disease states (Hamid et al., 2003).

C-fibers are found beneath and within the epithelium, around blood vessels, and within the bronchial smooth muscle layer. Mast cells are also located close to blood vessels, nerves, epithelia, and bronchial smooth muscle. Functional

and morphological relationship exists between mast cells and capsaicin-sensitive sensory nerves in the airways mucosa (Alving et al., 1991). The sensitized mast cells store cytokines such as TNF- α within its granules and release them upon antigenic presentation, making it a pivotal cell in the allergic asthmatic response to allergens. Furthermore, TNF- α produced by mast cells exerts critical roles in neutrophil influx under different pathological conditions such as the delayed-type hypersensitivity reactions (Biedermann et al., 2000) and bacterial infections (Tessier et al., 1998). Activated mast cells also produce LTB₄ and a variety of other cytokines, including IL-6, IL-10, and CINC-3 (Weber et al., 1995; Shanley et al., 1997). Thus, connective tissue and/or mucosal mast cells in the lung can be considered as target cells in connection with C-fibers implicated in the enhanced neutrophil recruitment into the airways of capsaicin-pretreated rats. To investigate this possibility, control and capsaicin-pretreated rats were treated chronically with the mast cell activator compound 48/80, and both neutrophil counts and TNF- α levels in BAL fluid were examined in the OVA-challenged rats. Chronic treatment of rats with compound 48/80 is a classical model used to deplete the histamine/5-HT stores in connective tissue mast cells (Di Rosa et al., 1971) leading to a virtual disappearance of connective tissue mast cells in airway tissues (Tam et al., 1988; Damazo et al., 2001). Additionally, both mucosal and connective tissue mast cells are able to release TNF- α in response to OVA challenge (Trezena et al., 2003) and compound 48/80 (Gibbs et al., 2001). We found it reasonable to speculate that pulmonary mast cells undergo a large reduction of TNF- α levels in conditions of chronic treatment with this compound. Our results showed that chronic treatment with compound 48/80 restored the TNF- α levels to control values and prevented the neutrophil infiltration in BAL fluid seen in capsaicin-pretreated rats. Taken together, these data are indicative that C-fiber degeneration at neonatal stages causes an up-regulation of airways mast cells and enhancement of TNF- α production, which upon OVA challenge, lead to degranulation and large release of inflammatory mediators. Interestingly, neonatal treatment with capsaicin in rats (Ahlstedt et al., 1986) and lambs (Ramirez-Romero et al., 2000) has been shown to increase mast cell population around blood vessels, pleura, and the entire respiratory tract. Additional studies are required to establish the mechanisms by which C-fibers act to attenuate allergic neutrophil infiltration in the airways. Substance P activates pertussis toxin-sensitive GTP-binding proteins in mast cells, causing the release of TNF- α via mitogen-activated protein kinase pathways (Azzolina et al., 2003). Hence, the substance P-induced TNF- α release would be exacerbated in conditions of permanent loss of C-fibers where airways mast cells are found up-regulated. Another possibility to explain our findings deals with a potential deficiency of somatostatin in the airways of capsaicin-pretreated rats. Somatostatin, released from activated sensory nerve terminals, reduces mast cell activity and decreases protease secretion (Saavedra and Vergara, 2003). Consequently, the negative feedback normally exerted by somatostatin in mast cells would be lost in conditions of C-fiber degeneration, making such cells more susceptible to allergen exposure.

In conclusion, our findings show that capsaicin treatment at neonatal stages of the rat and hence chronic neuropeptide depletion primes pulmonary mast cells that, upon activation

by antigen at adult ages, produce large amounts of cytokines such as TNF- α and CINC-3, thus triggering the massive neutrophil airways infiltration.

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