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Substance P induces tumor necrosis factor-α release from human skin via mitogen-activated protein kinase

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Abstract

Substance P plays an important role in neurogenic inflammation with granulocyte infiltration. To investigate cytokines involved in the substance P-induced inflammation and the mechanism of cell activation, we studied the release of TNF (tumor necrosis factor)-α and histamine from human skin slices in response to substance P and antigen. Substance P induced the release of histamine and TNF-α in a dose-dependent manner at concentrations from 0.8 to 100 μM. PD 098059 (2′-amino-3′-methoxyflavone) selectively inhibited the release of TNF-α, but not the release of histamine induced by either substance P or antigen. SB 203580 ([4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole]) slightly inhibited TNF-α release induced by antigen, but not that induced by substance P, and slightly enhanced histamine release induced by either stimulation. The release of TNF-α in response to either stimulation was inhibited by 1 nM–1 μM dexamethasone, but histamine release was not affected. These results suggest that substance P, in addition to antigen, induced TNF-α release from human skin by a mitogen-activated protein (MAP) kinase, predominantly extracellular signaling-regulated protein kinase (ERK)-dependent, and dexamethasone-sensitive pathway, which is separate from that for histamine release from mast cells.

Keywords: Substance P; TNF-α (tumor necrosis factor-α); Histamine; Dexamethasone; MAP (mitogen-activated protein) kinase; Skin

1. Introduction

Substance P, which is located in cutaneous sensory neurons (Hökfelt et al., 1975), is believed to be a major mediator of neurogenic inflammation (Pernow, 1983). It induces the degranulation of mast cells isolated from human skin (Benyon et al., 1987), releasing chemical mediators such as histamine. Intradermal injection of substance P produces immediate wheal-and-flare responses (Hägermark et al.,1978; Jorizzo et al., 1983; Foreman et al., 1983) and granulocyte infiltration in both human (Smith et al., 1993) and animal skin (Matsuda et al., 1989; Walsh et al., 1995; Yano et al., 1989). It is therefore accepted that substance P can influence late-phase and/or chronic inflammatory responses by inducing granulocyte infiltration in skin. Using experiments with mast cell-deficient mice, Matsuda et al. (1989) and Yano et al. (1989) have demonstrated that this reaction is mast cell-dependent. Iwamoto et al. (1993) reported that the granulocyte infiltration induced by substance P was inhibited by an antagonist of leukotriene B_4, a chemoattractant for granulocytes. We have recently demonstrated the release of leukotriene B_4 in correlation with the release of prostaglandin D_2 and histamine from guinea pig skin in response to substance P (Furutani et al., 1999). On the other hand, Saban et al. (1997) have shown that substance P-induced migration of neutrophils but not of eosinophils was blocked by a leukotriene inhibitor, and suggested the involvement of tumor necrosis factor (TNF)-α, in addition to leukotriene B_4, in the granulocyte infiltration in mouse skin. Various cytokines, including TNF-α, interleukin-4, interleukin-5, interleukin-6, interleukin-8 and interleukin-13, have been reported to be produced by mast cells (Gordon and Galli, 1990; Möller et al., 1993; Okayama et al., 1995; Plaut et al., 1989; Toru et al., 1998). Among these cytokines, TNF-α is particularly important in the development of inflammation with leukocyte infiltration. It up-regulates endothelial adhesion molecules, such as endothelial-leukocyte adhesion molecule (ELAM)-1 and

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vascular cell adhesion molecule (VCAM)-1, which are essential for leukocyte infiltration (Walsh et al., 1991; Wellicome et al., 1990). The lack of TNF-α released from mast cells abolished neutrophil infiltration in mice peritonitis evoked by *Klebsiella pneumoniae* (Malaviya et al., 1996). Moreover, Gordon and Galli (1990) have shown that mast cells are a major source of TNF-α during immunoglobulin (Ig) E-dependent responses. It was also reported that intradermal injection of substance P induced the expression of ELAM-1 on microvascular endothelium (Matis et al., 1990), suggesting the release of TNF-α from skin mast cells in response to substance P. However, it has not been fully investigated whether human skin mast cells release TNF-α in response to substance P. Although Ansel et al. (1993) have shown the expression of messenger RNA (mRNA) and the secretion of TNF-α by murine mast cells in response to substance P, it is widely accepted that characteristics of mast cells vary widely among species and organs (Lawrence et al., 1987; Lowman et al., 1988; Bradding et al., 1995). For instance, glucocorticoids inhibit IgE-mediated histamine release from mouse and rat mast cells (Daëron et al., 1982; Schleimer et al., 1987), but not from human mast cells (Schleimer et al., 1983). Moreover, the mechanism of signal transduction evoked by substance P is not identical with that evoked by antigen. Furthermore, it has been suggested that mast cells lose some of their functions, such as leukotriene B_4 release, when isolated from skin tissues by enzymatic digestion (Levi-Schaffer et al., 1987). In this study, we therefore investigated the release of TNF-α and histamine in response to substance P from human skin slices. To investigate the mechanisms of intracellular signaling for these reactions, we studied the effects of antagonists of mitogen-activated protein (MAP) kinase and dexamethasone on the release of TNF-α and histamine induced by substance P and antigen.

2. Materials and methods

2.1. Chemicals

Substance P was purchased from Sigma Aldrich (Tokyo, Japan). House dust mite antigen was obtained from Torii (Tokyo, Japan). PD 098059 (2′-amino-3′-methoxyflavone) was purchased from Research Biochemicals International (Natick, MA). SB 203580 ([4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole]) was kindly donated by SmithKline Beecham (Essex, UK). All other chemicals used in the present study were purchased from Wako (Osaka, Japan).

2.2. Skin donors

Normal skin tissue was obtained from 13 individuals who were 24- to 82-years-old (six men and seven women) without allergic diseases, when they received skin surgery.

2.3. Sera for sensitization of human skin in vitro

Sera for passive sensitization of human skin were obtained from patients with atopic dermatitis. The levels of the serum specific IgE antibody against house dust mite antigen (Der f-1) measured by the CAP-radioallergosorbent test (CAP-RAST) method (Koro et al., 1999) exceeded 100 UA/ml in all patients.

2.4. Release of histamine and TNF-α in vitro

Human skin slices were prepared as described before (Koro et al., 1999). Briefly, after removal of subcutaneous tissue, a piece of skin was cut into 500-μm-thick slices using a hand microtome. The slices were washed three times in Ca^{2+}- and Mg^{2+}-free Tyrode solution (free Tyrode solution) (Koro et al., 1999) and passively sensitized for 120 min with 20% of a serum containing a high titer of IgE antibody against house dust mite antigen in RPMI-1640. After sensitization and washing three times in free Tyrode solution, 80 mg of the slices (wet weight) was put into each tube and incubated in 300 μl of HEPES-buffered α minimum essential medium (αMEM), supplemented with 10% fetal calf serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin in the presence or the absence of various concentration of substance P or 50 μg/ml of antigen at 37°C for 6 h. The antigen concentration was empirically chosen as that inducing maximal release of histamine from skin tissue in vitro (Koro et al., 1999). The reaction was terminated by cooling the tubes in ice. After centrifugation at $1500 \times g$ at 4°C for 5 min, the supernatants were decanted and stored at −80°C until use. In each experiment, skin slices were incubated in duplicate or triplicate.

2.5. Measurement of histamine

Supernatants of the reaction mixtures were extracted with 2% perchloric acid and histamine in the samples was assayed with an automated fluorometric-high-performance liquid chromatography (HPLC) system (Tosoh, Tokyo Japan) as previously described (Tsuruta et al., 1978). The magnitude of the histamine release was expressed as a percentage of the total histamine content of the skin slices in each tube.

2.6. Measurement of TNF-α

TNF-α in the samples was measured using enzyme-linked immunoassay kits (Biosource International, Camarillo, CA). The measurements were performed according to the manufacturer's instructions. The minimum detectable concentration of human TNF-α was < 0.09 pg/ml. There was no cross-reactivity with any other known cytokine.

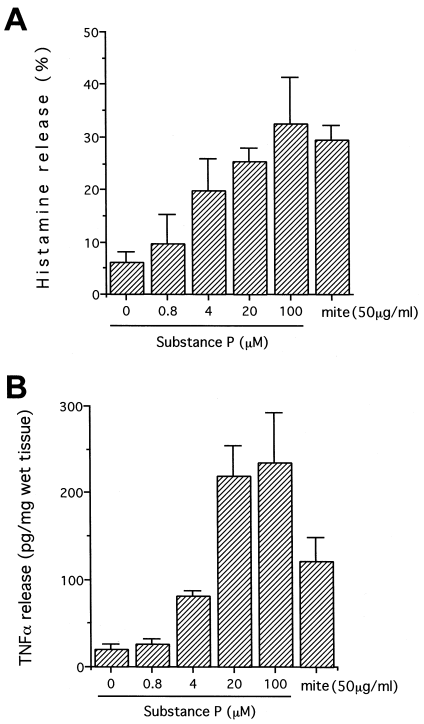

Fig. 1. The release of histamine and TNF-α from human skin tissue in response to substance P or house dust mite antigen.
Human skin slices (80 mg) were passively sensitized with sera containing a high titer of IgE against house dust mite antigen and were incubated in tissue culture medium (300 μl) with various concentrations of substance P or 50 μg/ml house dust mite antigen at 37°C for 6 h. The amounts of histamine and TNF-α released into the supernatants were measured by HPLC (A, histamine) or ELISA (B, TNF-α) as described in the Materials and Methods section. Values are means ± S.E.M. of a number of independent experiments; three to 10 for histamine release and five to six for TNF-α release with triplicate samples.

2.7. Pretreatment of the skin tissues with MAP kinase inhibitors

After passive sensitization, the skin slices were divided into 80 mg wet tissue per tube, incubated in 300 μl of α MEM with PD 098059 (0–50 μM) for 30 min or SB 203580 (0–10 μM) for 15 min and challenged with 100 μM substance P or 50 μg/ml house dust mite antigen as described above. The release of histamine and TNF-α was measured as described above.

2.8. Pretreatment of skin tissues with dexamethasone

The sensitized skin slices were divided among the tubes, incubated in 285 μl of αMEM with or without various concentration of dexamethasone at 37°C for 16 h, and challenged with 20 μM substance P or 50 μg/ml house dust mite antigen as described above. The release of histamine and TNF-α was measured as described above.

2.9. Statistical analysis

The data were analyzed by Student's *t*-test for paired samples. One-way analysis of variance (ANOVA) and Dunn's post-test were used to test the significance of differences among groups.

3. Results

3.1. Release of histamine from human skin

Substance P induced the release of histamine from sensitized human skin tissue in a dose-dependent manner at all concentrations ranging from 0.8 to 100 μM (Fig. 1A). The histamine release induced by 100 μM substance P, $32.4 \pm 8.9\%$ (mean ± S.E.M., $n = 10$) was slightly greater than that induced by antigen, $29.5 \pm 2.0\%$ (mean ± S.E.M., $n = 10$), but the difference was not statistically significant.

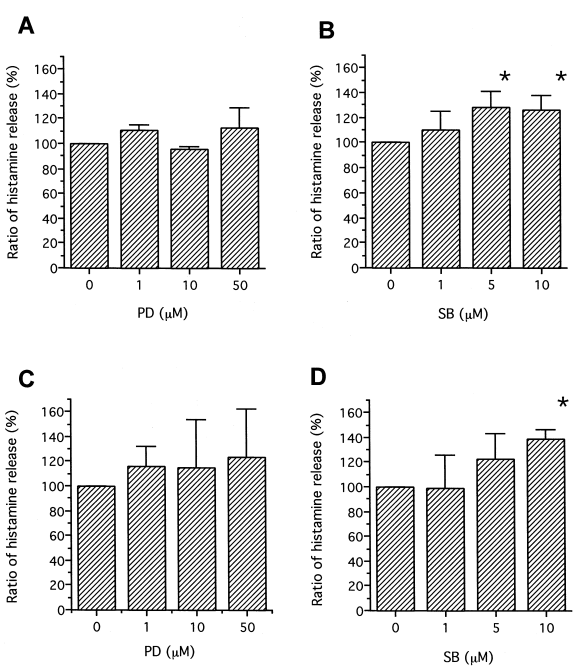

Fig. 2. The effects of MAP kinase inhibitors on histamine release induced by substance P or mite antigen.
The sensitized human skin slices were pre-incubated with indicated concentrations of PD 098059 for 30 min (A, C) or SB 203580 for 15 min (B, D), and stimulated with 100 μM substance P (A, B) or 50 μg/ml mite antigen (C, D) at 37°C for 6 h. The amounts of histamine released in the medium over 6-h incubation periods were assayed by HPLC. The results are expressed as ratios of histamine release under each condition vs. those in the absence of inhibitors. The data are shown as means ± S.D. from three independent experiments. * $P < 0.05$ (ANOVA and Dunn's test).

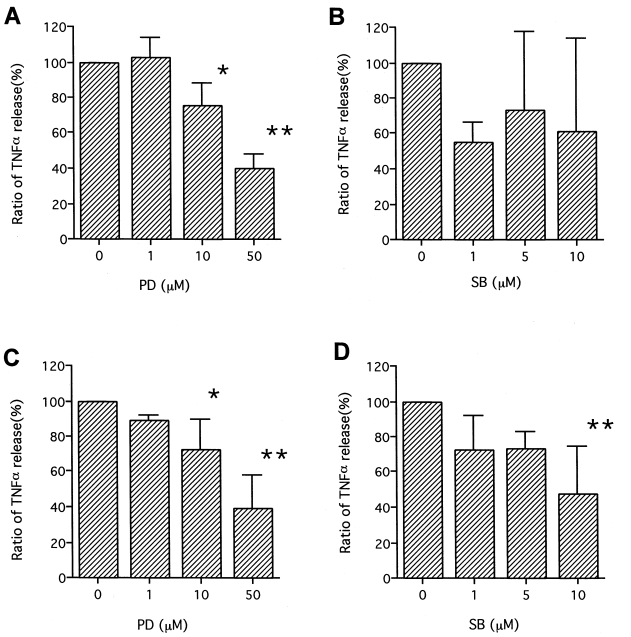

Fig. 3. The effects of MAP kinase inhibitors on TNF-α release induced by substance P or mite antigen.
The sensitized human skin slices were incubated with indicated concentrations of PD 098059 for 30 min (A, C) or SB 203580 for 15 min (B, D) and stimulated with 100 μM substance P (A, B) or 50 μg/ml mite antigen (C, D) at 37°C for 6 h. TNF-α released in the medium was assayed by ELISA. The results are expressed as ratios of TNF-α release under each condition vs. those in the absence of inhibitors. The data are shown as means ± S.D. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (ANOVA and Dunn's test).

3.2. Release of TNF-α from human skin

Substance P also induced the release of TNF-α from human skin tissue in a dose-dependent manner at a concentration from 0.8 to 100 μM (Fig. 1B). The release of TNF-α induced by 100 μM substance P, 234.1 ± 58.8% (mean ± S.E.M., $n = 5$) was significantly higher than that induced by antigen, 120.4 ± 28.1% (mean ± S.E.M., $n = 6$) (*t*-test, $P < 0.01$).

3.3. The effects of MAP kinase inhibitors on the release of histamine and TNF-α from human skin

In order to study the involvement of MAP kinase in the release of histamine and TNF-α, we employed two antagonists against MAP kinase; PD 098059, which inhibits the activation of extracellular signaling-regulated protein kinase (ERK) 2 by preventing phosphorylation of MAP kinase–ERK-kinase (MEK) 1 (Zhang et al., 1997) and SB 203580, a p38 MAP kinase inhibitor. PD 098059 did not affect either the histamine release from sensitized human skin tissue stimulated by 100 μM substance P or that stimulated by house dust mite antigen (Fig. 2A,C). In contrast, SB203580 slightly but significantly enhanced the histamine release from human skin stimulated by either 100 μM substance P or antigen in a dose-dependent manner (Fig. 2B,D).

On the other hand, PD 098059 significantly inhibited the TNF-α release stimulated by substance P and that stimulated by antigen in a dose-dependent manner. The percent inhibition of substance P- and antigen-induced TNF-α release by 50 μM PD 098059 was 60.2 ± 8.2% and 60.7 ± 18.5%, respectively (Fig. 3A,C). SB 203580 also inhibited the release of TNF-α to some extent (Fig. 3B,D). However, the inhibitory effect was not as clear as with PD 098059 and no dose dependence was observed when skin was stimulated by substance P (Fig. 3B).

3.4. Dexamethasone inhibits TNF-α release, but not histamine release from human skin

We next studied the effect of dexamethasone on substance P-induced release of histamine and TNF-α from skin tissue. Pretreatment of skin tissue with dexamethasone inhibited the release of TNF-α induced by substance P in a dose-dependent manner at concentrations from 1 nM–1 μM (Fig. 4B). Statistically significant inhibition was caused by 10 nM (ANOVA, $P < 0.05$, 44.9% inhibition, $n = 3$) or higher concentrations of dexamethasone. The

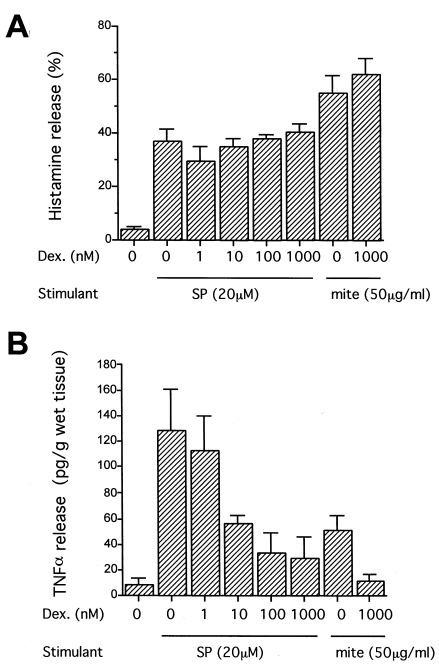

Fig. 4. The effects of dexamethasone on the release of histamine and TNF-α induced by substance P or mite antigen.
The sensitized human skin slices were treated with the indicated concentrations of dexamethasone for 16 h at 37°C and stimulated with 20 μM substance P or 50 μg/ml mite antigen for a further 6 h. Histamine (A) and TNF-α (B) released into the medium was assayed as described in the Materials and Methods section. Value are means ± S.D. from one representative experiment with triplicate samples. Similar results were obtained in four independent sets of experiments.

release of TNF-α induced by antigen was also abolished by 1 μM dexamethasone. On the other hand, none of the dexamethasone concentrations affected histamine release induced from skin tissue by either 20 μM substance P or house dust mite-antigen (Fig. 4A).

4. Discussion

In spite of evidence suggesting roles for substance P and mast cells in the development of cutaneous inflammation with granulocyte infiltration (Matsuda et al., 1989; Smith et al., 1993; Walsh et al., 1995; Yano et al., 1989), the release of cytokines induced from mast cells by substance P and its signal transduction have not been fully investigated. Okayama et al. (1998) have recently reported that substance P induced the release of TNF-α from skin mast cells, which had been enzymatically isolated from three human donors. However, mast cells isolated from skin tissues appear to lose some of their functions during enzymatic digestion (Levi-Schaffer et al., 1987). Moreover, the authors precultured mast cells with a relatively high concentration of stem-cell factor, which significantly augments TNF-α production by high-affinity IgE receptor (FcεRI)-mediated stimuli (Ishizuka et al., 1998). In the present study, we demonstrated the release of TNF-α from freshly isolated normal human skin tissue. All skin tissue obtained from 13 individuals released TNF-α in response to substance P in a dose-dependent manner over concentrations from 0.8 to 100 μM.

The source of TNF-α in this system remains to be identified. Substance P activates macrophages, neutrophils and keratinocytes as well as mast cells in vitro, but the population of neutrophils and macrophages in normal skin is very small. Antagonists against the NK_1 receptor, by which substance P activates macrophages, did not inhibit granulocyte infiltration evoked by substance P in vivo (Walsh et al., 1995). Moreover, Cocchiara et al. (1997) have shown that substance P increased the expression of TNF-α mRNA in peritoneal mast cells, but not that in peritoneal macrophages. Regarding the other cells which constitute skin, Viac et al. (1996) reported the potential of human keratinocytes to produce TNF-α in response to substance P. However, TNF-α was detected only after 48 h of stimulation, whereas we measured TNF-α at 6 h after stimulation. In general, substance P is likely to induce the release of TNF-α from mast cells in the skin.

It is noteworthy that the amount of TNF-α released from human skin stimulated by substance P at 20 and 100μM was significantly greater than that released with 50 μg/ml mite antigen, while the amounts of histamine released at the same concentrations of substance P were similar to those with the antigen. These results suggest that substance P may induce inflammatory cell infiltration by releasing TNF-α as, or more effectively, than antigen, especially at high concentrations.

It was shown that the aggregation of FcεRI on mast cells activates members of MAP kinase families, ERK, p38 MAP kinase, and c-Jun amino-terminal kinase (JNK) (Ishizuka et al., 1997). The roles of each kinase, however, differ among mast cells. Experiments with selective MAP kinase inhibitors have shown that ERK (p42 MAP kinase) critically contributes to the production of TNF-α in a rat mucosal mast cell line (RBL-2H3) (Zhang et al., 1997), whereas the inhibition of ERK MAP kinase had little effect on TNF-α production in MC/9 mast cells. In MC/9 mast cells, selective activation of JNK by enhanced expression of MEK kinase 1 induced strong activation of TNF-α promoter, and the expression of a mutant of JNK2 blunted the activation of TNF-α promoter induced by FcεRI cross-linking, indicating the dominant role of JNK in the production of TNF-α (Ishizuka et al., 1997). In the present study, we demonstrated that both the release of TNF-α from human skin induced by substance P and that induced by antigen were inhibited by PD 098059 (2′-amino-3′-methoxyflavone), an ERK MAP kinase inhibitor. The effect of SB 203580 ([4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole]), a p38 MAP kinase inhibitor, was not as clear as that of PD 098059. SB 203580 partially inhibited the release of antigen-induced TNF-α in a dose-dependent manner ($P < 0.05$), but the effect on substance P-induced release of TNF-α varied among skin preparations. This result may have been due to the heterogeneity of mast cells or of their environments in the skin among donors.

The histamine release induced by antigen and that induced by substance P were both slightly enhanced by SB 203580. Zhang et al. (1997) have reported that antigen-induced release of TNF-α was also enhanced by SB 203580, but inhibited by PD 098059 in rat basophilic leukemia cell (RBL)-2H3 cells. Moreover, Ishizuka, et al. (1999) have recently reported that PD 098059 significantly inhibited the release of serotonin from mouse cultured mast cells. It is plausible that p38 MAP kinase would be involved in the negative regulation of the histamine release from mast cells in human skin.

Glucocorticoids have potent anti-inflammatory activities and are used for treatment of various diseases. However, their mechanisms of action on neurogenic inflammation, especially on human mast cell functions, have not been fully investigated. Both hydrolysis of phosphatidylinositol and exocytosis from rodent mast cells are inhibited by pretreatment with dexamethasone in the range from 10 to 100 nM (Daëron et al., 1982; Schleimer et al., 1987), but dexamethasone has no effect on the release of histamine from human lung mast cells (Schleimer et al., 1983). We have now confirmed that, up to 1 μM, dexamethasone has no effect on the histamine release from human skin challenged with antigen or substance P. However, the release of TNF-α with substance P or with antigen was inhibited by dexamethasone in the range of 1 nM to 1 μM. Recently, Rider et al. (1996) have shown that nanomolar

concentrations of dexamethasone suppressed phosphorylation of Raf-1, MEK-1 and p42 MAP kinase as well as the associated MAP kinase activity. They also showed that such concentrations of dexamethasone did not have any effect on the tyrosine phosphorylation of Lyn, Syk, Vav or subunits of FcεRI, which were required for exocytosis. Together, glucocorticoids may regulate TNF-α production by selectively inhibiting phosphorylation of the regulatory proteins for MAP kinase in human skin mast cells activated by antigen and substance P.

In conclusion, we have demonstrated that substance P induced TNF-α release from human skin, apparently from mast cells. The effects of MAP kinase inhibitors and dexamethasone have revealed the predominant involvement of ERK MAP kinase and a glucocorticoid-sensitive pathway in the release of TNF-α. Further investigation of the mechanisms of mast cell activation by substance P and the development of agents which effectively regulate MAP kinases may result in a new therapy for diseases involving neurogenic inflammation.

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