

Nociceptin Modulates Bronchoconstriction Induced by Sensory Nerve Activation in Mouse Lung

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Nociceptin/orphanin FQ (N/OFQ), the endogenous ligand for the N/OFQ peptide receptor (NOP), inhibits tachykinin release in the airway of several animal models. The aim of this study was to investigate the role of the N/OFQ–NOP receptor system in bronchoconstriction induced by sensory nerve activation in the isolated mouse lung. We used C57BL/6J NOP^{+/+}, NOP^{-/-}, and Balb/C mice sensitized (or not) to ovalbumin. Bronchopulmonary function coupled with measurements of endogenous N/OFQ levels before and after capsaicin-induced bronchoconstriction in the presence or absence of NOP-selective agonists/antagonists are presented. N/OFQ significantly inhibited capsaicin-induced bronchoconstriction in both naive and sensitized mice, these latter animals displaying airway hyperresponsiveness to capsaicin. The inhibitory effect of N/OFQ were not observed in NOP^{-/-} mice, and were mimicked/abolished by the selective NOP agonist/antagonist University of Ferrara Peptide (UFP)-112/UFP-101 in NOP^{+/+} mice. UFP-101 alone potentiated the effect of capsaicin in naive mice, but not in sensitized mice. Endogenous N/OFQ levels significantly decreased in sensitized mice relative to naive mice. We have demonstrated that a reduction in endogenous N/OFQ, or the lack of its receptor, causes an increase in capsaicin-induced bronchoconstriction, implying a role for the N/OFQ–NOP receptor system in the modulation of capsaicin effects. Moreover, for the first time, we document differential airway responsiveness to capsaicin between naive and sensitized mice due, at least in part, to decreased endogenous N/OFQ levels in sensitized mice.

Keywords: endogenous nociceptin/orphanin FQ; airway responsiveness; allergic asthma; sensory nerves; nociceptin/orphanin FQ peptide receptor

Nociceptin/orphanin FQ (N/OFQ) is a heptadecapeptide derived from a larger precursor protein, prepro-N/OFQ (1). This peptide is the endogenous ligand of an opioid-like G protein-coupled receptor recently named the N/OFQ peptide (NOP) receptor (2). This receptor has an overall 60% homology with the classical MOP, DOP, and KOP (μ , δ , κ) opioid receptors (3). Recent studies indicate that N/OFQ has a broad spectrum of physiologic functions and pharmacological effects in both the central and peripheral nervous systems, as well as in some nonneuronal tissues (2). In the central nervous system, NOP receptor activation by N/OFQ inhibits the release of several neurotransmitters, including noradrenaline and glutamate (4),

whereas, in the periphery, this system is able to inhibit excitatory, nonadrenergic, noncholinergic responses in isolated bronchi (5). Several studies have reported the role of N/OFQ–NOP receptor system in the airway, showing its ability to inhibit acetylcholine release in the trachea (6), and the contractions of the guinea pig isolated bronchus induced by electrical field stimulation (7). Moreover, N/OFQ inhibits capsaicin-induced bronchoconstriction in the isolated guinea pig lung. This is via an inhibition of tachykinin release from nonmyelinated C fibers of afferent sensory terminal nerves that innervate all compartments of the pulmonary wall, from trachea to bronchiole (5, 8). Pharmacological studies have demonstrated that many processes involved in asthma, such as bronchoconstriction, mucus hypersecretion, and plasma extravasation, are mimicked by the release of sensory neuropeptides, such as calcitonin gene-related peptide, substance P (SP), and neurokinin (NK) A (9). Fischer and colleagues (7) have shown that airway afferent nerve cell bodies expressed mRNA for the NOP receptor, and that airway N/OFQ-immunoreactive fibers were distinct from airway SP-immunoreactive fibers. Moreover, the same authors have documented that an induction of sensory neuropeptides in nodose ganglion neurons is crucially involved in the increase of airway hyperresponsiveness (AHR) in the late response to ovalbumin (OVA) challenge (10).

Active sensitization with OVA is a commonly used model for allergic airway diseases. Although this model may not entirely reflect the situation in human allergic asthma, many similarities are observed, including histologic features, allergen-induced eosinophilia, and early and late-phase airway obstruction after allergen challenge (10). The aims of the current study were: (1) to evaluate the role of the N/OFQ–NOP receptor system in bronchoconstriction induced by sensory nerve activation using the novel and selective NOP receptor agonist University of Ferrara Peptide (UFP)-112 (11, 12) and antagonist UFP-101 (13, 14), and knockout mice for the NOP receptor (NOP^{-/-}); and (2), using actively sensitized and OVA-challenged mice, to investigate the role of the N/OFQ–NOP receptor system in allergic airway disease.

MATERIALS AND METHODS

Animals

We used 9- to 12-week-old C57BL/6J (NOP^{+/+} and NOP^{-/-}) and Balb/C mice sensitized (or not) to OVA. The NOP^{-/-} mice were obtained by backcrossing hybrid C57BL/6J-129 (15) with CD-1 mice for nine generations (16). The animals were housed in a controlled environment, maintained on a 12-hour light/dark cycle, and allowed food and water *ad libitum*. NOP^{+/+} and NOP^{-/-} animals were all genotyped by PCR (16). A group of Balb/c mice were sensitized to OVA as described by Roviezzo and colleagues (17). All experimental procedures were in accordance with Italian D.Lgs. 116/92.

Bronchopulmonary Function Measurements

We used an isolated, perfused mouse lung model prepared essentially as described previously (17). After preparation, the lungs were perfused

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and ventilated for 45 minutes without treatment to obtain a steady baseline. Subsequently, generation of a capsaicin repetitive dose-response curve (0.5–5 nM) was performed with or without NOP receptor agonist/antagonist treatment.

Drugs were administered intravenously. Capsaicin-induced increased lung resistance (R_L) was referred to percent increase over basal value.

In sensitized and naive mice, N/OFQ (0.01–0.1 and 1 μ M) and the selective NOP receptor agonist, UFP-112 (1 nM, 0.01 and 0.1 μ M), were given 15 minutes before capsaicin-induced bronchoconstriction. Moreover, in NOP^{+/+}, naive, and sensitized mice, the effects of the selective NOP receptor antagonist, UFP-101 (1 μ M), in the presence or absence of N/OFQ (1 μ M), on capsaicin-induced bronchoconstriction was also assessed. In NOP^{-/-} mice, N/OFQ (1 μ M) and UFP-112 (0.1 μ M) were administered 15 minutes before capsaicin treatment. In a separate set of experiments, lung tissues were recovered from naive and sensitized mice (not receiving N/OFQ) at baseline or at the end of capsaicin dose-response curve studies, and immediately frozen until radioimmunoassay (RIA) analysis.

Extraction of Samples

Tissue samples (lungs) were sonicated in 10 volumes (vol/wt) of boiling 1 M acetic acid, and maintained at 90°C for 10 minutes. After centrifugation (12,000 rpm for 20 min at 4°C), the supernatants were separated and subjected to chromatographic extraction as previously described (18).

RIA

Immunoreactive N/OFQ (ir-N/OFQ) present in tissue extracts was measured by a specific RIA according to a validated procedure (19).

Lyophilized extracts were reconstituted with 50 μ l of methanol/0.1% HCl (1/1) and assayed in duplicate. Aliquots of 25 μ l were mixed with 100 μ l of 125I-N/OFQ (Bachem, St. Helens, Merseyside, UK) and 100 μ l of antiserum (96:2+; kindly supplied by Prof. SC I. Nylander, Uppsala University, Sweden). The antiserum was used at the appropriate dilution, as previously described (19). The antiserum, raised against N/OFQ, shows 0.5% cross-reactivity with the nociceptin fragment N/OFQ (1–13), and less than 0.1% cross-reactivity with nocistatin and the following opioid peptides: dynorphin A (DYN A), dynorphin B (DYN B), their truncated or elongated forms DYN A (1–6), DYN A (1–32), DYN B (1–29), or with Met-enkephalin, Met-enkephalinArg⁶Phe⁷, Leu-enkephalin, and β -endorphin.

RIA tubes were incubated at 4°C for 24 hours. A charcoal slurry (1 ml/tube) was used to separate free and antibody-bound peptide (15% horse serum, 3% charcoal, 0.3% dextran in RIA buffer). Bound peptide was separated by centrifugation (5,000 $\times$ g at 4°C), and 1 ml aliquots of the supernatants were counted for 1 minute on a Beckman 5,500 γ counter (Beckman, Fullerton, CA). The detection limit of the RIA assay was 1–2 fmol/tube.

RIA curves and data were analyzed using the GraphPad Prism 3.03 software for Windows (GraphPad Software, San Diego, CA).

Drugs

Capsaicin and OVA were obtained from Sigma-Aldrich (Milan, Italy). Peptides (N/OFQ, UFP-112, and UFP-101) used in this study were prepared and purified in house (University of Ferrara), as previously described (14).

Statistical Analysis

All data are expressed as means ($\pm$ SEM). Statistical evaluation was performed by ANOVA, followed by the Student–Newman–Keuls post test. The threshold of statistical significance was set at a *P* value of less than 0.05.

RESULTS

N/OFQ–NOP Receptor System in NOP^{+/+} Mice

Capsaicin (0.5–5 nM) produced a bronchoconstriction in lungs obtained from NOP^{+/+} mice, with maximal effect at 5 nM (R_L from 0.38 $\pm$ 0.02 to 0.80 $\pm$ 0.03 cm H₂O/L/s); higher con-

centrations induced lung collapse. The maximum increase in resistance obtained with capsaicin represents approximately 40% of 1 mM acetylcholine-induced bronchoconstriction (data not shown).

N/OFQ treatment (0.01–0.1–1 μ M) produced a statistically significant decrease in capsaicin-induced increases in R_L , with maximal inhibition at 1 μ M (R_L from 0.35 $\pm$ 0.02 to 0.39 $\pm$ 0.03 cm H₂O/L/s) (Figure 1A). UFP-112 (1 nM or 0.01–0.1 μ M) mimicked the inhibitory effect of N/OFQ (R_L from 0.36 $\pm$ 0.02 to 0.41 $\pm$ 0.01 cm H₂O/L/s) (Figure 1B).

Pretreatment with UFP-101 (1 μ M) abolished the inhibitory effects of N/OFQ on capsaicin-induced bronchoconstriction (R_L from 0.37 $\pm$ 0.01 to 0.78 $\pm$ 0.03 cm H₂O/L/s). When UFP-101 was used alone, 15 minutes before capsaicin, it significantly increased capsaicin-induced bronchoconstriction (R_L from 0.34 $\pm$ 0.02 to 0.88 $\pm$ 0.02 cm H₂O/L/s) (Figure 2).

The effects of N/OFQ, UFP-112, and UFP-101 seen with capsaicin-induced bronchoconstriction were not observed with acetylcholine-induced bronchoconstriction (Figure 3).

N/OFQ–NOP Receptor System in NOP^{-/-} Mice

In NOP^{-/-} mice, capsaicin produced a bronchoconstriction, with maximal effect at 5 nM (R_L from 0.37 $\pm$ 0.02 to 1.5 $\pm$ 0.012 cm H₂O/L/s) (Figure 4). Both NOP receptor agonists, N/OFQ (1 μ M; R_L from 0.38 $\pm$ 0.01 to 1.56 $\pm$ 0.014 cm H₂O/L/s) and UFP-112 (0.1 μ M; R_L from 0.42 $\pm$ 0.03 to 1.34 $\pm$ 0.018 cm H₂O/L/s), and the antagonist, UFP-101 (R_L from 0.42 $\pm$ 0.03 to

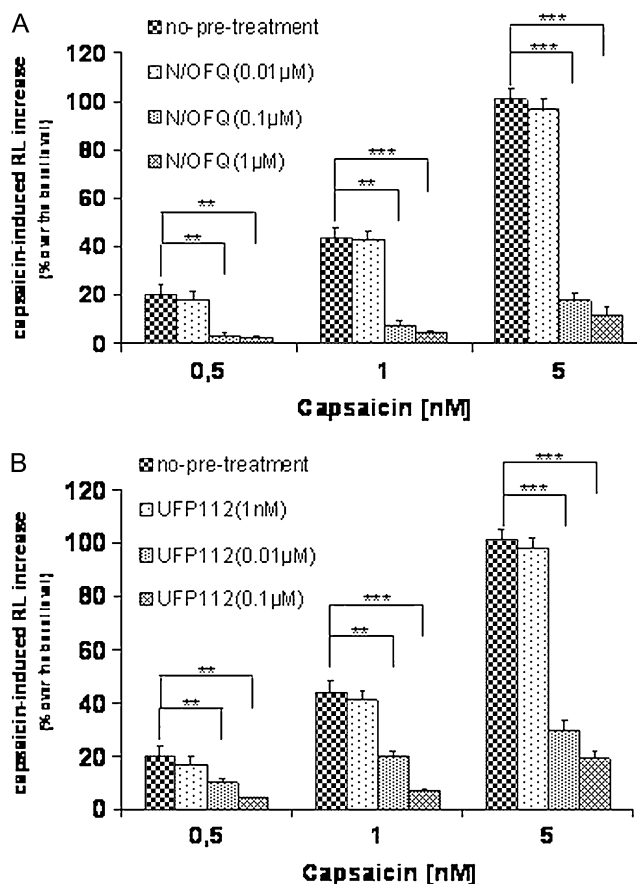


Figure 1. Effects of nociceptin/orphanin FQ (N/OFQ) (0.01, 0.1, and 1 μ M) (A) and University of Ferrara Peptide (UFP)-112 (1 nM, 0.01 μ M, and 0.1 μ M) (B) on capsaicin-induced bronchoconstriction in N/OFQ peptide (NOP)^{+/+} mouse lungs. All values are expressed as means ($\pm$ SEM); *n* = 5 per group. ***P* < 0.01; ****P* < 0.001.

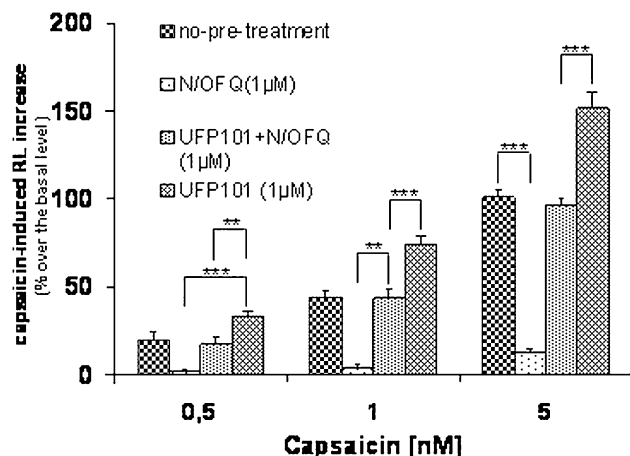


Figure 2. Effects of N/OFQ (1 μ M) and UFP-101 (1 μ M) alone and their coapplication on capsaicin-induced bronchoconstriction in NOP^{+/+} mice lungs. All values are expressed as means ($\pm$ SEM); $n = 5$ per group. ** $P < 0.01$; *** $P < 0.001$.

1.34 $\pm$ 0.018 cm H₂O/L/s), did not modify capsaicin-induced bronchoconstriction at doses effective in NOP^{+/+} mice (Figure 4).

N/OFQ–NOP Receptor System in Sensitized Mice

In sensitized mice, capsaicin-induced bronchoconstriction was increased (0.5–1.5 nM) (R_L from 0.38 $\pm$ 0.03 to 1.52 $\pm$ 0.015 cm H₂O/L/s) with respect to naive mice (R_L from 0.36 $\pm$ 0.02 to 0.71 $\pm$ 0.03 cm H₂O/L/s). The maximum increase in resistance obtained with capsaicin represents approximately 80% of 1 mM acetylcholine-induced bronchoconstriction (data not shown).

Despite the increased responsiveness to capsaicin, both NOP receptor agonists, N/OFQ (1 μ M; R_L from 0.39 $\pm$ 0.03 to 0.47 $\pm$ 0.02 cm H₂O/L/s) (Figure 5A) and UFP-112 (0.1 μ M; R_L from 0.40 $\pm$ 0.03 to 0.51 $\pm$ 0.02 cm H₂O/L/s) (Figure 5B), inhibited capsaicin-induced bronchoconstriction in a statistically significant manner. This inhibitory effect was abolished by UFP-101 (1 μ M; R_L from 0.40 $\pm$ 0.02 to 1.57 $\pm$ 0.016 cm H₂O/L/s) (Figure 6). When the antagonist was used alone before capsaicin administration, it did not modify capsaicin airway responsiveness (R_L from 0.37 $\pm$ 0.02 to 1.50 $\pm$ 0.011 cm H₂O/L/s) (Figure 6).

N/OFQ RIA

Basal levels of ir-N/OFQ in the lung were significantly higher in tissues obtained from naive compared with sensitized mice. Moreover, after capsaicin administration, lung ir-N/OFQ levels were significantly decreased in naive, but not in sensitized mice (Figure 7A). ir-N/OFQ in lung perfusates of sensitized mice was increased after capsaicin administration, confirming the lung tissue ir-N/OFQ data (Figure 7B).

DISCUSSION

The present study describes the effect of NOP receptor activation on capsaicin responses in the isolated mouse lung. Our data demonstrate that N/OFQ is a potent and effective inhibitor of increased R_L evoked by capsaicin. In NOP^{+/+} mice, we have observed that bronchoconstriction induced by capsaicin was significantly decreased by N/OFQ treatment. Because N/OFQ failed to modulate the bronchoconstriction induced by a direct contractile agonist, such as acetylcholine, its effects against capsaicin may likely derive from a prejunctional action (i.e., inhibition of neurotransmitter release from sensory nerves). This observation is consistent with the findings of others, who

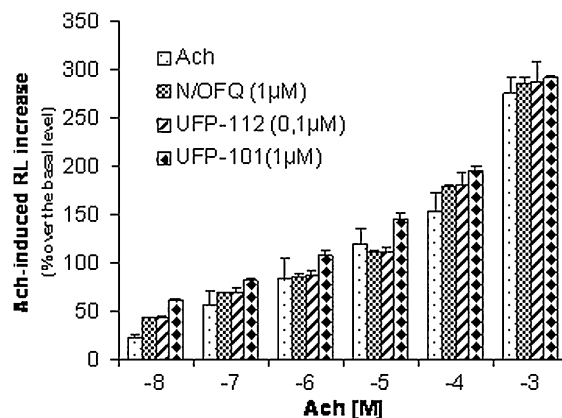


Figure 3. Effects of N/OFQ (1 μ M), UFP-112 (0.1 μ M), and UFP-101 (1 μ M) alone and their coapplication on acetylcholine (10 nM–1 mM)-induced bronchoconstriction. All values are expressed as means ($\pm$ SEM); $n = 5$ per group.

showed that N/OFQ is able to inhibit bronchoconstriction and cough induced by capsaicin in the guinea pig (9, 20).

These results were confirmed with selective NOP ligands—namely, the full agonist, UFP-112 (12, 21), and the pure antagonist, UFP-101 (5, 22, 23), which, respectively, mimicked and blocked the effects of N/OFQ.

An important aspect of our results is related to the ability of UFP-101 to significantly increase capsaicin-induced bronchoconstriction when given alone. Contrary to the present findings, Corboz and colleagues (24) have shown that the nonpeptide NOP receptor antagonist J-113397, given alone, did not affect capsaicin-induced bronchoconstriction in the guinea pig. However, this disagreement could possibly be explained by differences in the models used. Indeed, we used *in vitro* isolated and perfused mouse lung preparation compared with *in vivo* guinea pig. The direct involvement of the NOP receptor in both the inhibitory effect of N/OFQ and UFP-112, and in the potentiating action of UFP-101 on capsaicin-evoked bronchoconstriction, was demonstrated using NOP^{−/−} mice. However, probably more important is the observation that, in NOP^{−/−} mice, UFP-101 alone did not modify airway responsiveness to capsaicin, and

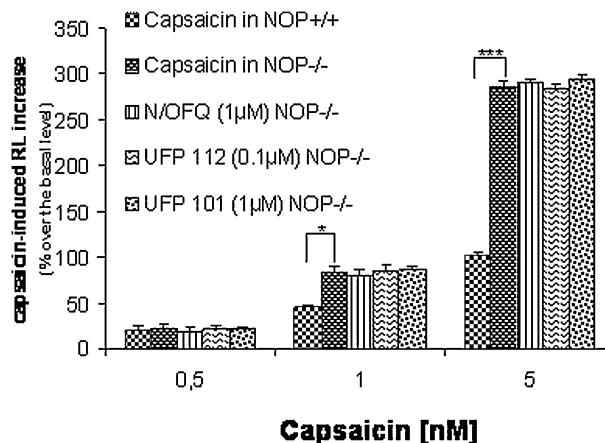


Figure 4. Capsaicin-induced bronchoconstriction in NOP^{+/+} and NOP^{−/−} mouse lungs and the effects of UFP-112 (0.1 μ M), UFP-101 (1 μ M), or N/OFQ (1 μ M) on the response to this constrictor. All values are expressed as means ($\pm$ SEM); $n = 5$ per group. * $P < 0.05$; *** $P < 0.001$.

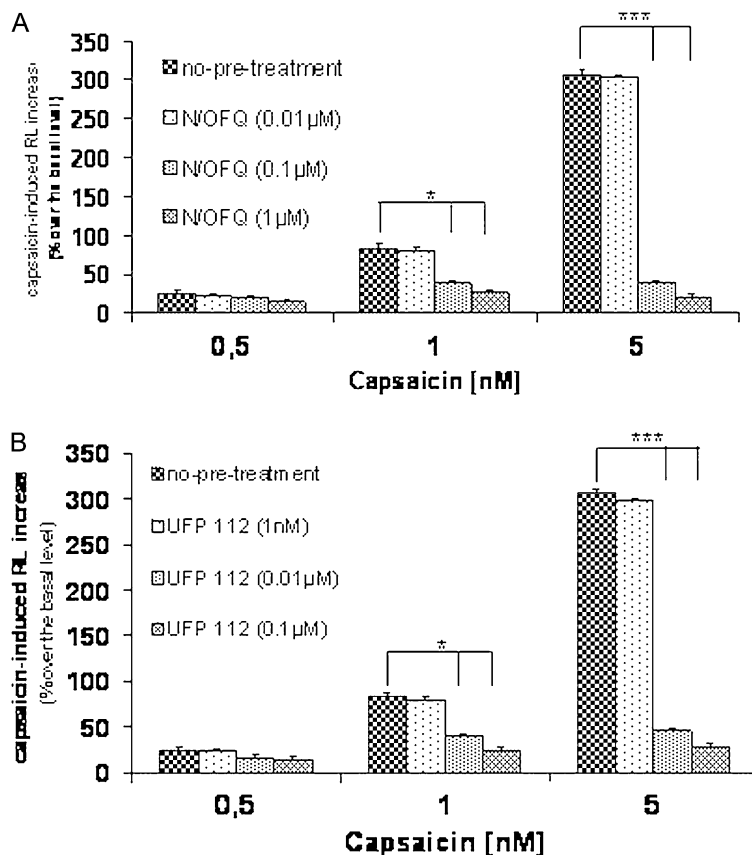


Figure 5. Effects of N/OFQ (0.01, 0.1, and 1 μ M) (A) and UFP-112 (1 nM, 0.01 and 0.1 μ M) (B) on capsaicin-induced bronchoconstriction in sensitized mouse lungs. All values are expressed as means ($\pm$ SEM); $n = 5$ per group. * $P < 0.05$; *** $P < 0.001$.

the responsiveness to capsaicin was similar to that observed in response to UFP-101 in NOP^{+/+} mice.

For the first time, these data suggest an involvement of endogenous N/OFQergic signaling in the modulation of capsaicin-induced bronchoconstriction.

As reported in the literature, N/OFQ-mediated inhibition of capsaicin-induced bronchoconstriction may be related to an effect on capsaicin-induced release of NKs from capsaicin-sensitive sensory nerve endings (25). In the respiratory tract of several animal models of asthma, tachykinergic sensory afferent nerves play an important role in the regulation of airway smooth muscle tone and neurogenic inflammation through storage and release of SP and NKA (25). To verify that the N/OFQ-NOP receptor system was also involved in a model of allergic asthma, we evaluated the role of this system in a group of animals sensitized and challenged with OVA. This model has been well characterized, and shows many similarities to asthma of human airways (26). Although our sensitized mice displayed an AHR to capsaicin, as reported by Fischer and colleagues (10), N/OFQ and UFP-112 inhibitory effects on capsaicin-induced bronchoconstriction were preserved. Again, it is important to note that, even if UFP-101 preserved its inhibitory action on N/OFQ effects, when used alone before capsaicin, it did not modify airway responsiveness to capsaicin in sensitized mice. It is evident from our results that the N/OFQ-NOP receptor system differentially modulates airway reactivity to capsaicin in naive and sensitized mice, and that endogenous N/OFQ could have an underlying role in these differences. Our hypothesis was supported by endogenous N/OFQ contents in the lung and perfusate before and after capsaicin administration. Indeed basal levels of ir-N/OFQ were significantly higher in lung obtained from naive compared with sensitized mice. Moreover, ir-N/OFQ levels significantly decreased after capsaicin administration in naive mice only. In addition, ir-N/OFQ levels in the

perfusate confirmed these results, allowing us to propose that, after capsaicin-induced bronchoconstriction, a large amount of endogenous N/OFQ was released in naive mice, but not in sensitized mice. Therefore, considering that an induction of sensory neuropeptides has been documented in allergen-sensitized animals (10), we can also postulate, in our animal model, a reduction of endogenous N/OFQ.

In conclusion we have demonstrated that a reduction in endogenous N/OFQ or the lack of its receptor causes an increase in capsaicin-induced bronchoconstriction, implying a role for the N/OFQ-NOP receptor system in the modulation of capsaicin effects.

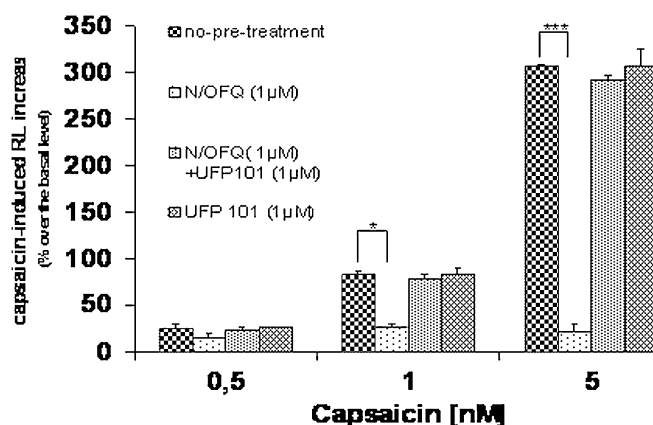


Figure 6. Effects of N/OFQ (1 μ M) and UFP-101 (1 μ M) alone and their coapplication on capsaicin-induced bronchoconstriction in sensitized mouse lungs. All values are expressed as means ($\pm$ SEM); $n = 5$ per group. * $P < 0.05$; *** $P < 0.001$.

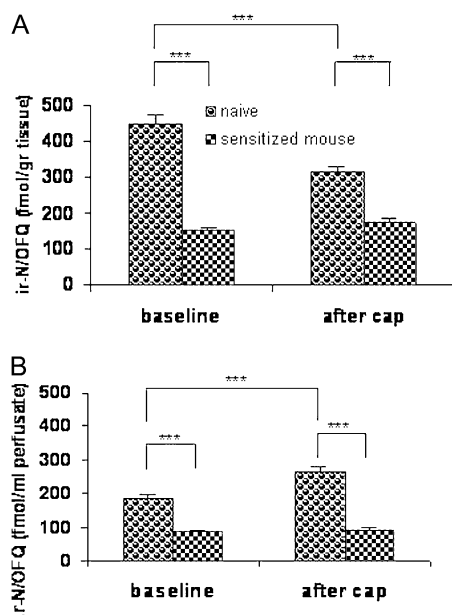


Figure 7. N/OFQ levels in pulmonary tissue (A) and perfusate (B) of naive and sensitized mice at baseline and after capsaicin (0.5, 1, and 5 nM). All values are expressed as means ($\pm$ SEM); $n = 5$ per group. *** $P < 0.001$.

Moreover, for the first time, we have documented differential airway responsiveness to capsaicin between naive and sensitized mice due, at least in part, to decreased endogenous N/OFQ levels in sensitized mice.

Because AHR is a crucial pathological process in asthma, this study could add the N/OFQ–NOP receptor system to the list of potential sites for the investigation of mechanisms involved in asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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