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Cutting Edge: Nociceptin Stimulates Neutrophil Chemotaxis and Recruitment: Inhibition by Aspirin-Triggered-15-Epi-Lipoxin A_4[1]

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The nociceptin receptor (Noci-R) is a G protein-coupled receptor present in neural tissues and its activation by nociceptin is involved in the processing of pain signals. Here, we report that Noci-R is present and functional on peripheral blood polymorphonuclear leukocytes (PMN). Human PMN express mRNA for Noci-R, its nucleotide sequence determined, and specific binding with [^{125}I]-labeled nociceptin gave an apparent K_d ~1.5 nM for this PMN opioid receptor. Nociceptin evoked PMN chemotaxis with maximal activity at 100 pM, without intracellular Ca^{2+} mobilization. When injected in murine air pouches, nociceptin elicited leukocyte infiltration in a concentration-dependent fashion. Nociceptin-stimulated PMN infiltration was inhibited by treating mice with a synthetic analog of the aspirin-triggered lipid mediator 15-epi-lipoxin A_4. The present results identify nociceptin as a potent chemoattractant and provide a novel link between the neural and immune systems that are blocked by aspirin-triggered lipid mediators and may be relevant in neurogenic inflammation. ***The Journal of Immunology,*** **2001, 166: 3650–3654.**

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Pain often occurs as a consequence of inflamed and damaged tissues; conversely, it is now recognized that the activation of nociceptors may cause inflammation (1). Primary afferent nerve fibers control cutaneous blood flow and vascular permeability by releasing vasoactive peptides and neuropeptides, that in turn can evoke inflammation by stimulating production of inflammatory mediators (2). These vascular reactions and the additional recruitment and activation of leukocytes are the hallmarks of neurogenic inflammation (3).

The neuropeptide orphanin FQ/nociceptin is an endogenous ligand of the human opioid receptor-like, ORL1, (hereafter termed nociceptin receptor (Noci-R)[5]) (4), a member of the opioid receptors family (5). Nociceptin and its receptor are present in brain and are involved in the processing of pain signals (6, 7). Nociceptin is also present in the periphery at primary afferent nerve fibers (8) and may thus act on tissues such as the skin, visceral organs, and blood vessels, suggesting that additional physiological functions of this neuropeptide have yet to be appreciated.

In the present report, we demonstrate that Noci-R is also present and functional in human leukocytes, and questioned whether nociceptin acts on these cells. Because polymorphonuclear leukocyte (PMN) migration is a critical component of inflammation and is held to play a role in neuropathic pain, it was of interest to characterize the potential relationship between these key cells of host defense and the neural network. Aspirin (ASA) is well appreciated for its analgesic and antipyretic actions (9). ASA therapeutic mechanism of action includes inhibition of prostaglandin formation, thought to be critical in its both analgesic and anti-inflammatory properties. Cyclooxygenase (COX)-2, an isoform of COX that generates prostanoids, is constitutively expressed in neuronal systems (9). Recently, we have found that, when acetylated by ASA, the ability of COX-2 to generate prostanoids is blocked yet stimulates the biosynthesis of ASA-triggered-15-epi-lipoxin A_4 (ATL). These novel lipid mediators are the carbon 15 epimers of the native lipoxins (10). ATL can contribute to the beneficial action of ASA in that they down-regulate key events in acute inflammation (10). The present findings indicate that peripheral blood PMNs express Noci-R (K_d ~1.5 nM), and that its high affinity ligand nociceptin stimulates chemotaxis and recruitment in

[5] Abbreviations used in this paper: Noci-R, nociceptin receptor; ASA, aspirin; ATL, ASA-triggered-15-epi-lipoxin A_4; ATL-1, 15-epi-16-(*para*-fluoro)-phenoxy-lipoxin A_4; $[Ca^{2+}]_i$, intracellular Ca^{2+}; LTB_4, leukotriene B_4; PMN, polymorphonuclear leukocyte; COX, cyclooxygenase; $DPBS^{2+}$, Dulbecco's PBS with $CaCl_2$ and $MgCl_2$.

vivo. Moreover, a fluorinated ATL stable analog, namely 15-epi-16-(*para*-fluoro)-phenoxy-lipoxin A_4 (ATL-1) is a potent inhibitor of nociceptin-stimulated leukocyte recruitment in vivo. Therefore, nociceptin may constitute a novel mediator of neurogenic inflammation that can be regulated by ATL.

Materials and Methods

Isolation and incubation of human leukocytes

PMNs from healthy volunteers were obtained as originally described (11), with modifications (12). Monocytes were isolated according to (13). PMN chemotaxis was performed using a microchamber technique as in Falk et al. (14), with modifications (15). The cells migrating across the filter (Neuroprobe, Gaithersburg, MD) onto the lower surface were counted in four high power fields under light microscopy in triplicate. Results are presented as the chemotactic index calculated by the following ratio: cells migrating to chemoattractants/cells migrating to vehicle. Nociceptin was purchased from Phoenix Pharmaceuticals (Belmont, CA) and fMLP was obtained from Sigma (St. Louis, MO); both were used throughout. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels were monitored as in (16).

RT-PCR and sequencing

Total RNA from PMNs or monocytes was extracted with TRIzol (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. Total RNA (1 μg) was used in each reaction using Titan One tube RT-PCR (Roche Molecular Biochemicals, Indianapolis, IN). RT-PCR was performed according to the following profile: 50°C for 30 min for reverse transcription, then 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, repeated 35 times for PCR, followed by a final extension at 72°C for 10 min. Internal primers for human Noci-R were 5′-GAT GAA GAG ATC GAG TGC CTG GTG-3′ (P1) and 5′-GGC CTT GAA GTT CTC ATC CAG GAA-3′ (P2); with an expected PCR product of 402 bp in length. These primers were designed in view of the originally reported human nucleotide sequence Noci-R (5) to involve two exons (separated by an intron of 118 bp), which allowed discrimination between genomic DNA and expressed mRNA. Primers used to amplify the full coding sequence of Noci-R were 5′-ACC TGT CGT CGA CTG CCA GCC GGC-3′ (P3) and 5′-CAC CAG GAG GGC AGC TTT AGT CTG-3′ (P4), with an expected PCR product of 1471 bp in length according to the cDNA sequence. The latter primers were derived from the consensus sequence resulting from reported human sequences (GenBank and EMBL accession nos. X77130, U30185, NM__000913, and L40949).[6] Primers were used in paired combinations to obtain overlapping fragments of 782 bp (P1-P4) and 1090 bp (P3-P2) in length, which together with the 402 bp fragment, span the entire coding region of the receptor. RT-PCR products were chromatographed in 1% agarose gel containing ethidium bromide and photographs of the gels were taken under ultraviolet illumination. Bands extracted from the gel (Qiaex II gel extraction kit; Qiagen, Valencia, CA) were analyzed by fluorescent dideoxy terminator-automated sequencing (BWH Automatic Sequencing and Genotyping Core Facility, Boston, MA).

Nociceptin binding with human PMN

[^{125}I]-Labeled nociceptin binding was performed with freshly isolated human PMN. Cells were suspended in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$ ($DPBS^{2+}$). Aliquots (10×10^6/0.1 ml PMN) were incubated with 0.25 nM of [^{125}I]-labeled nociceptin (specific activity ~2000 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) in the absence or presence of increasing concentrations of unlabeled nociceptin (Phoenix Pharmaceuticals) for 1 h at 14°C. Incubations were terminated by adding 1 ml ice-cold $DPBS^{2+}$. The bound and unbound radioligands were separated by brief centrifugation. Cell pellets were washed with 1 ml of ice-cold $DPBS^{2+}$. The radioactivity associated with the cell pellets was determined using a scintillation counter (Beckman Coulter, Fullerton, CA). Nonspecific binding was determined in the presence of 100 nM of unlabeled nociceptin.

Murine air pouches

Six-day murine air pouches were raised on the dorsal skin of 6-wk-old BALB/c mice and experiments were performed essentially as in Ref. 17. Briefly, 1.5 h after local injection of nociceptin into the air pouch cavity, mice were sacrificed and individual air pouches were lavaged twice with 2 ml sterile PBS. The lavage exudates were centrifuged and the cell pellets were suspended in PBS and enumerated. The synthetic analog of 15-epi-lipoxin A_4, ATL-1 (18), was a generous gift from D. Perez, W. Guilford, and J. Parkinson (Berlex Biosciences, Richmond, CA) and its integrity was quantitated by liquid chromatography-tandem mass spectrometry as in Ref. 18. The ATL-1 analog (10 μg), diluted in sterile PBS, was delivered as a bolus injection either into the tail vein or locally into the 6-day air pouch immediately before nociceptin injection.

Statistical analyses

Statistical analyses were performed using the ANOVA test, and significance was considered to be attained at $p < 0.05$.

Results and Discussion

Expression and coding sequence of the Noci-R mRNA in peripheral blood leukocytes

Total RNA was extracted from freshly isolated human PMNs and monocytes for RT-PCR was performed using specific primers for the human Noci-R (see *Materials and Methods*). Both PMNs and monocytes gave a band with the expected length, ~400 bp (Fig. 1), supporting the notion that both cell types express this opioid-like receptor. To verify its identity, we sequenced the Noci-R-like cDNA from isolated PMN. Using a combination of primers (see *Materials and Methods*) with PMN cDNA permitted sequencing of a continuous segment of this receptor that spans the entire coding region (5) of the putative PMN Noci-R (not shown). Sequence alignment of the PMN-derived cDNA revealed identity with the reported sequence obtained from brainstem tissues (5). Because both monocytes and PMN possess mRNA for Noci-R that was cell ratio- (PMN/monocyte) dependent ($n = 3$; not shown), we conducted direct specific binding experiments with ^{125}I-labeled nociceptin and PMN. Human PMN displayed specific [^{125}I]-labeled nociceptin binding with apparent K_d ~1.5 nM. Fig. 2 shows representative results from three separate donors. Nonspecific binding accounted for ~48% of the total binding for PMN with the commercially isolated [^{125}I]-labeled nociceptin. These results indicated that circulating PMNs express Noci-R mRNA and displayed high affinity recognition sites, raising the question of whether these receptors are functional on PMN.

Nociceptin is a potent chemoattractant for PMNs

Next, we determined whether synthetic nociceptin evoked PMN chemotaxis, using a microchamber migration assay with isolated human PMNs. Nociceptin-induced chemotaxis was evident within a wide concentration range (10^{-14}–10^{-6} M) giving a steep rise and maximum effect at 10^{-10} M (Fig. 3). At the optimal concentration of 10^{-10} M, the chemotactic index was similar to that obtained with 10^{-8} M of fMLP, a surrogate of microbial-derived *N*-formylated proteins and a well-appreciated chemoattractant for

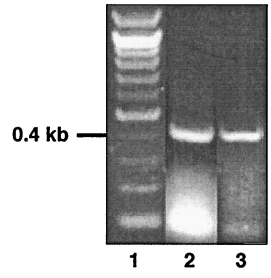

FIGURE 1. Human leukocytes express Noci-R. RT-PCR of leukocyte RNA was performed as described in *Materials and Methods*. Positives were obtained with both PMNs (*lane 2*) and monocytes (*lane 3*) with a PCR product of the expected length, ~400 bp. *Lane 1*, DNA markers.

[6] The sequence presented in this paper has been deposited in the EMBL Nucleotide Sequence Database (accession no. AF348323).

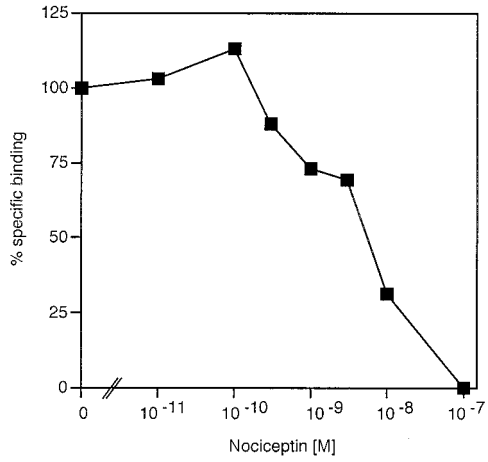

FIGURE 2. Competitive [^{125}I]-labeled nociceptin binding with human PMN. Human PMN (10×10^6) were incubated with [^{125}I]-labeled nociceptin for 1 h at 14°C in the presence of increasing concentrations of unlabeled nociceptin (■). Bound and unbound radioligands were separated by centrifugation and specific binding was determined. Results are representative of one experiment of three separate donors.

PMNs (19) that was used here as a reference agonist for purpose of direct comparison. These results are in line with the involvement of other neuropeptides such as neurotransmitters in leukocyte chemotaxis, as recently reported for norepinephrine (20), which stimulates chemotaxis of monocytes, at an optimal concentration of 10^{-10} M. At these potential physiologically relevant concentrations (10^{-14}–10^{-6} M), nociceptin is apparently not recognized by other opioid receptors nor does the Noci-R bind other opioid ligands (4, 21). Specific antagonists for the Noci-R that are devoid of agonist activity are not currently available (22). Although it is possible that other receptors present in PMNs might mediate nociceptin's direct chemotactic activity, the present results suggest that this neuropeptide acts via engagement of its own recognized receptor Noci-R (4, 6) present on PMN (Figs. 1 and 2). Also, the proposed antagonist [Phel-Psi(CH_2)-NH)Gly(2)]nociceptin-(1–13)NH_2 (22) did compete at 14°C for specific binding in the 10^{-8}–10^{-5} M range with human PMN (data not shown).

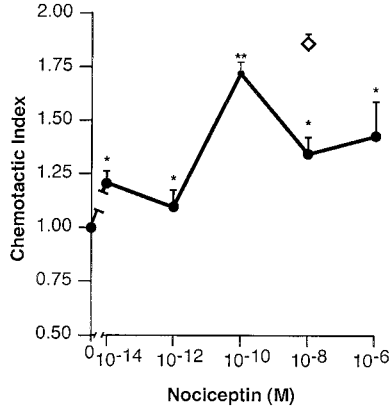

FIGURE 3. Nociceptin is a potent chemoattractant for human PMNs. Freshly isolated PMNs were incubated in the presence of indicated concentrations of nociceptin and PMN migration was quantitated as described in *Materials and Methods*. Results are expressed as the mean ± SEM from at least three different donors. For purpose of direct comparison, ◇ denotes fMLP at 10^{-8} M. *, Significantly different ($p < 0.05$) compared with PMN exposed to vehicle alone (control). **, Significant differences of $p < 0.01$.

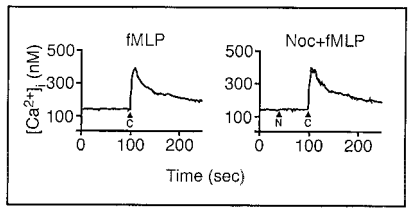

FIGURE 4. Nociceptin-stimulated chemotaxis in human PMNs is Ca^{2+}-independent. $[Ca^{2+}]_i$ mobilization was monitored with human PMN incubated at 37°C with chemoattractants. PMNs were exposed to 100 nM fMLP, 100 nM LTB_4, or 10 nM IL-8 (see Table I) alone or following a 60-s incubation with 100 nM nociceptin. The arrowheads indicate addition of nociceptin (N; at 40 s), and that of chemoattractant (C; at 100 s). Traces are representative of experiments with fMLP from four independent experiments, each performed with the three different chemoattractants.

Nociceptin responses are very potent in that it is ~10,000-fold the potency of substance P (23), and 1,000-fold that of bradykinin (24) in a flexor-reflex pain model. In the present experiments, nociceptin's action on PMN chemotaxis showed a peak in activity at a concentration of 100 pM, which is 100- to 1,000-fold more potent than that observed for the synthetic chemoattractant peptide fMLP (19). Thus, our results affirm that nociceptin is a highly potent biopeptide and indicate that its actions extend to the immune system.

Nociceptin acts on PMNs independently of $[Ca^{2+}]_i$ mobilization

PMN chemoattractants typically stimulate production of superoxide anions and induce $[Ca^{2+}]_i$ mobilization (25). No detectable superoxide anion production in PMNs was observed following exposure of PMN to nociceptin ($n = 3$; data not shown). Also, when PMNs were exposed to a broad range of concentrations of nociceptin (10^{-14}–10^{-6} M) it did not stimulate a rise in $[Ca^{2+}]_i$. Although nociceptin did not alter the $[Ca^{2+}]_i$ responses to either the chemoattractants fMLP (Fig. 4) or IL-8 (not shown), this neuropeptide did potentiate in a very modest but statistically significant manner $[Ca^{2+}]_i$ mobilization in response to leukotriene B_4 (LTB_4) (Table I). These results, together with ^{125}I-labeled nociceptin specific binding, suggest that nociceptin may represent a new class of neural-derived chemoattractant molecules, possessing a distinct profile of leukocyte activation than classical chemoattractants (e.g., soluble PMN agonists) such as fMLP, LTB_4, or IL-8 that each evoke the rapid mobilization of Ca^{2+} (25).

Nociceptin stimulates leukocyte trafficking in vivo

To establish whether this neuropeptide can act as a chemoattractant in vivo, we examined nociceptin in the murine dorsal

Table I. *Nociceptin and $[Ca^{2+}]_i$ mobilization by chemoattractants*[a]

	Chemoattractant		
	fMLP (100 nM)	IL-8 (10 nM)	LTB_4 (100 nM)
Percent of control value	99 ± 3	98 ± 3	113 ± 2[b]

[a] Rise in $[Ca^{2+}]_i$ mobilization from PMNs incubated with chemoattractants (as in Fig. 4). The results, in percentage, show the response of cells preincubated with nociceptin compared to that of cells incubated with other agonists alone. Results presented are the mean ± SEM, from data obtained in four separate experiments.

[b] Significant differences ($p < 0.05$) from LTB_4 alone. Rises in $[Ca^{2+}]_i$ in the absence of nociceptin were 316 ± 35 nM, 214 ± 19 nM, and 433 ± 91 nM, for fMLP, IL-8, and LTB_4, respectively (mean ± SEM; $n = 4$).

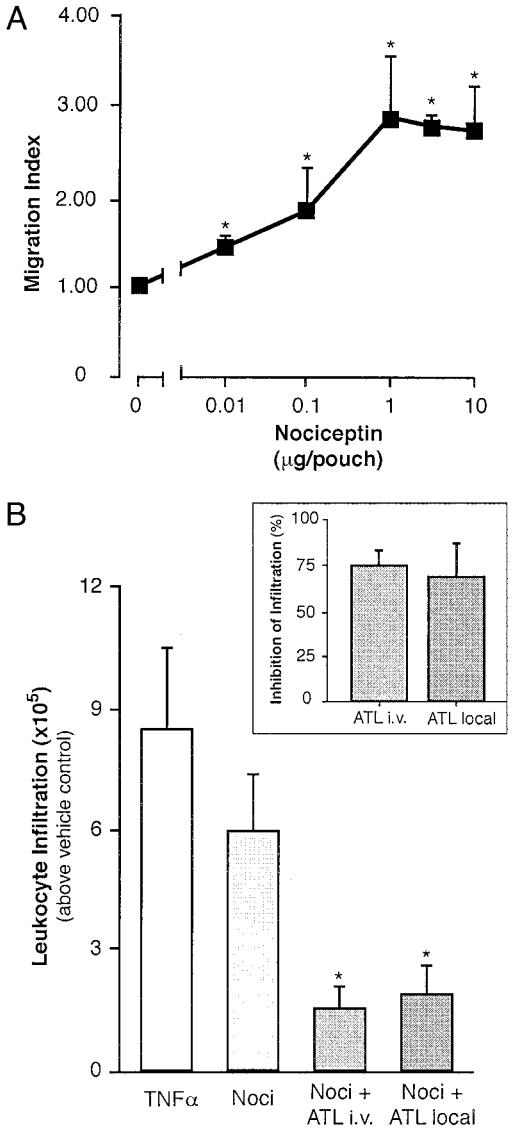

FIGURE 5. Nociceptin elicits leukocyte recruitment in vivo. Inhibition by ASA-triggered 15-epi-LXA_4. *A,* Sterile PBS, alone, or containing indicated amounts of nociceptin, was injected into the 6-day murine air pouches. After 1.5 h, the exudates were collected and the total number of leukocytes was enumerated. *, Denotes statistically significant difference ($p < 0.05$) from air pouches injected with PBS alone. *B,* ATL analog (10 μg) was injected locally into the air pouch or i.v. before nociceptin (Noci; 1 μg/pouch), 20 ng TNF-α is shown as a reference. Results are expressed as mean ± SEM ($n = 4$). *, Statistically different ($p < 0.05$) from Nociceptin alone.

air pouch, an established animal model for evaluating leukocyte infiltration (26, 27). When injected locally into the air pouch, nociceptin elicited leukocyte infiltration in a concentration-dependent manner (Fig. 5*A*). Statistically significant leukocyte migration into the air pouch was observed with doses as low as 10 ng nociceptin and the number of cells within the exudates increased with higher amounts of this peptide, up to 1 μg, above which there was no additional increase in PMN numbers. Leukocyte recruitment values obtained with 1 μg nociceptin were within the same range (Fig. 5*B*) of those induced by local injection of recombinant murine TNF-α, a well appreciated stimulus for PMN recruitment in this model (27). These findings, together with results of direct specific binding with human PMN, suggest that nociceptin, when present in peripheral tissues, can stimulate diapedesis and leukocyte accumulation. Because ATL are bioactive anti-inflammatory lipids that may mediate some of ASA beneficial actions (10), we tested whether an ATL stable analog could regulate nociceptin-stimulated leukocyte recruitment. Local delivery of 10 μg ATL-1 into the air pouch or by i.v. injection potently inhibited nociceptin-driven leukocyte infiltration (Fig. 5*B*). To determine whether 1–100 nM ATL could directly inhibit chemotaxis of human PMN, isolated cells were exposed to ATL followed by nociceptin, and chemotaxis was assessed as in Fig. 3. In these experiments, previous exposure to ATL did not block nociceptin-stimulated PMN chemotaxis in vitro ($n = 3$; not shown). LXA_4, ATL, and their analogs inhibit isolated PMN degranulation in response to immune complexes (28) and inhibit PMN responses to certain soluble agonists directly as well as indirectly by, for example, inhibiting TNF-α-stimulated chemokine production in vivo (17). Nociceptin directly stimulated PMN chemotaxis with isolated cells (Fig. 3) and stimulated recruitment of leukocytes in vivo that could involve direct nociceptin-stimulated PMN chemotaxis as well as nociceptin-stimulated generation of other endogenous chemoattractants in vivo (Fig. 5), the formation of which appears to be blocked by ATL in vivo. Together, these results identify a novel link between the nervous and the immune system, namely the stimulation of leukocyte trafficking by nociceptin.

To our knowledge, the present results are the first to show a role for nociceptin and its receptor in leukocyte recruitment. Leukocyte recruitment to inflammatory sites is essential for host defense and the generation of appropriate immune responses. During this process, PMNs also contribute to the onset of nociception and hyperalgesia, namely via their ability to release neuropeptides such as substance P (29) and generate lipid mediators, including LTB_4 and prostaglandin E_2 (12). PMNs are also considered a significant source of proinflammatory cytokines such as TNF-α, which can evoke leukocyte infiltration (27). In view of the present results it is possible that nociceptin, which can be released from afferent nerve fibers, contributes to leukocyte trafficking, particularly PMN, toward a site of nociception, where it may serve as a signal to amplify, both directly and/or indirectly via potential release of additional endogenous chemoattractants, the recruitment of PMNs to the site of inflammation that in turn evoke pain.

In summary, the present results further elucidate the complex dialogue that can occur between immune and neural systems (30). Along these lines, it is of interest that recent data indicate that hematopoietic and neural lineages may be more closely associated than previously recognized (31). Hence, these results provide evidence that nociceptin evokes PMN recruitment that is likely to be relevant in the genesis of neurogenic inflammation, and may provide a basis for new therapeutic approaches to manage pain and inflammation, based on ATL structures.

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