Gastrin-releasing peptide receptor (GRPR) mediates chemotaxis in neutrophils

Rafael Sanguinetti Czepielewski,a,b 1, Bárbara Nery Porto,a,b 1, Lucas Bortolotto Rizzo,a Rafael Roeslerd,e,f, Ana Lúcia Abujamra,c,d,e, Larissa García Pinto,a Gilberto Schwartzmannd,e,g, Fernando de Queiroz Cunha,a and Cristina Bonorinoa,b 2

aLaboratory of Cellular and Molecular Immunology, Biomedical Research Institute (IPB), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), 90610-900, Porto Alegre, RS, Brazil; bDepartment of Cellular and Molecular Biology (FABIO), PUCRS, 90619-900, Porto Alegre, RS, Brazil; cLaboratory of Neuropharmacology and Neural Tumor Biology, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, 90050-170, Porto Alegre, RS, Brazil; dCancer Research Laboratory, University Hospital Research Center (CPE-HCPA), Federal University of Rio Grande do Sul, 90035-003, Porto Alegre, RS, Brazil; eNational Institute for Translational Medicine, Porto Alegre, RS, Brazil; fDepartment of Internal Medicine, School of Medicine, Federal University of Rio Grande do Sul, 90035-003, Porto Alegre, Brazil; and gDepartment of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, 14049-900, Ribeirão Preto, São Paulo, Brazil

Edited* by Philippa Marrack, Howard Hughes Medical Institute, National Jewish Health, Denver, CO, and approved November 30, 2011 (received for review July 13, 2011)

Neutrophil migration to inflamed sites is crucial for both the initiation of inflammation and resolution of infection, yet these cells are involved in perpetuation of different chronic inflammatory diseases. Gastrin-releasing peptide (GRP) is a neuropeptide that acts through G protein-coupled receptors (GPRCs) involved in signal transmission in both central and peripheral nervous systems. Its receptor, gastrin-releasing peptide receptor (GRPR), is expressed by various cell types, and it is overexpressed in cancer cells. RC-3095 is a selective GRPR antagonist, recently found to have antiinflammatory properties in arthritis and sepsis models. Here we demonstrate that i.p. injection of GRP attracts neutrophils in 4 h, and attraction is blocked by RC-3095. Macrophage depletion or neutralization of TNFα abrogates GRP-induced neutrophil recruitment to the peritoneum. In vitro, GRP-induced neutrophil migration was dependent on PLC-β2, PI3K, ERK, p38 and independent of Gαi protein, and neutrophil migration toward synovial fluid of arthritis patients was inhibited by treatment with RC-3095. We propose that GRP is an alternative chemotactic receptor that may play a role in the pathogenesis of inflammatory disorders.

Neuropeptides are used by neurons as signaling molecules to regulate synaptic transmission and plasticity (1). Nonetheless, these molecules can be versatile, also acting as chemical messengers outside the nervous system. Recent reports showed that neuropeptides are produced as a result of immune pathologies (2), whereas others appear to induce cytokine production by immune cells (3).

Gastrin-releasing peptide (GRP) is a neuropeptide that induces gastrin secretion in the gastric tract (4). It acts by binding to the gastrin-releasing peptide receptor (GRPR or BB2), a member of the G protein-coupled receptor (GPCR) superfamily expressed in the gastric, respiratory, and nervous systems, as well as endocrine glands and muscle (5). GRPR mediates gastrointestinal motility and hormone and neurotransmitter release in the gut, intestine, colon, and other organs (6). It has roles in the nervous system, controlling the circadian cycle, anxiety, fear, stress, and modulation of memory (7). It is overexpressed in cancer cells, and the production of GRP together with GRPR overexpression results in autocrine growth stimulation (6). Selective GRPR antagonists were thus comparable between both methods. Collectively, these results indicate that GRP-induced neutrophil migration occurs specifically through activation of GRPR. The chemotactant effect of GRP in vivo was restricted to neutrophils, because no recruitment of macrophages or B or T cells was observed in any of the time points, from 1.5 to 72 h (Fig. 1 C and D), as assessed by both Diff-Quick staining and flow cytometry. In our hands, a peritoneal lavage of an uninfluenced mouse yielded between 5 and 10 million cells, and injection of GRP did not significantly alter total numbers of cells. The absolute numbers of neutrophils migrating to the peritoneum after GRP i.p. were thus comparable between both methods. Collectively, these results indicate that GRP has a selective effect over neutrophils in vivo, inducing their migration through activation of GRPR.

Results

GRP Induces Neutrophil Migration in Vivo. It has been previously shown that GRP antagonist RC-3095 has antiinflammatory activity in animal models of inflammation (9, 10, 16). We hypothesized that GRP could have proinflammatory potential, so we tested whether GRP would have a dose-dependent effect on neutrophil recruitment in vivo. We performed a kinetic analysis, looking at different time points after GRP injection. I.p. injection of human GRP induced neutrophil recruitment after 4 h in a dose-dependent fashion, the highest numbers being obtained with 0.6 μg per cavity (Fig. 1A). Pretreatment of mice with RC-3095 immediately before injection of GRP abolished neutrophil accumulation in the peritoneum (Fig. 1B), indicating that GRP-induced neutrophil migration occurs specifically through activation of GRPR. The chemotactant effect of GRP in vivo was restricted to neutrophils, because no recruitment of macrophages or B or T cells was observed in any of the time points, from 1.5 to 72 h (Fig. 1 C and D), as assessed by both Diff-Quick staining and flow cytometry. In our hands, a peritoneal lavage of an uninfluenced mouse yielded between 5 and 10 million cells, and injection of GRP did not significantly alter total numbers of cells. The absolute numbers of neutrophils migrating to the peritoneum after GRP i.p. were thus comparable between both methods. Collectively, these results indicate that GRP has a selective effect over neutrophils in vivo, inducing their migration through activation of GRPR.

We report that GRP can be an endogenous inflammatory mediator, acting as a chemotactant through GRPR. In addition, it activates specific signaling pathways that promote neutrophil migration. We propose that GRP triggers neutrophil recruitment both indirectly, through macrophages, as well as directly, binding to GRPR in these cells.

GRP-Induced Neutrophil Recruitment in Vivo Depends on Macrophages and TNF-α Production. Neutrophil migration to sites of inflammation in vivo is mediated by the release of cytokines and chemokines by resident cells. We decided to investigate the role of macrophages on neutrophil migration induced by GRP in vivo. We performed macrophage depletion by i.p. injection of


The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

1R.S.C. and B.N.P. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: cbonorino@pucrs.br.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109961109/-/DCSupplemental.
GRP induces neutrophil recruitment to the peritoneal cavity of mice. Mice were injected i.p. with different doses of GRP (0.06–6 μg/cavity) and recovered cells were counted after 4 h. **p < 0.01 compared with saline-treated group; (B) GRP (0.6 μg/cavity), RC-3095 (6 μg/cavity), or RC-3095 (6 μg/cavity) + GRP (0.6 μg/cavity) and control group, saline, ***p < 0.001 compared with GRP-injected group; and (C) GRP (0.6 μg/cavity). After 1.5, 4, 8, 16, 24, 48, and 72 h, animals were killed, cells were cytospin-fuged, stained with Diff-Quick, and counted. Filled circles, neutrophils (Ne); in saline-groups; open circles, GRP-treated groups. Filled squares, mononuclear cells (MN) in controls; open squares, mononuclear cells counted in GRP-treated groups. ***p < 0.001 compared with saline-injected group; (D) GRP (0.6 μg/cavity). After 1.5, 4, 8, 16, 24, 48, and 72 h, cells in the peritoneal fluid were analyzed by FACS. Gates 1 to 4 were determined on the basis of FSC × SSC distribution and staining with anti-CD14, CD11c, CD4, and B220. G1 = lymphocytes; G2 = larger lymphocytes and DCs; G3 = macrophages; G4 = neutrophils. Filled forms, saline groups; open forms, GRP-treated groups. Data representative of four independent experiments (n = 4 for each group of treatment) and expressed as the mean ± SE of the percentage or number of cells.

GRP Has a Direct Chemotactic Effect on Neutrophils. It has recently been demonstrated that neutrophils express GRPR (12). Chemokines (17) and leukotrienes (21) and molecules released by damaged tissues (22, 23) act as chemotactants, acting directly on neutrophils to induce migration. We investigated whether GRP, a neuropeptide, would induce neutrophils to migrate up a gradient of GRP in vitro, in a Transwell system. Nanomolar amounts of GRP induce neutrophil migration in a dose-dependent manner (Fig. 3A) in a bell-shaped curve. These results reflect previous studies on macrophages (19), lymphocytes (20), and mast cells (24), showing that GRP modulates immune cell function in a nanomolar range. To investigate the effects exerted by RC-3095 over migration, we performed a dose–response curve of this inhibitor using the optimal dose of GRP determined previously. We observed that RC-3095 does not inhibit GRP-induced migration while in equivalent nanomolar amounts (Fig. 3B). However, a 10-fold increase of the inhibitor blocks the effect of GRP on neutrophil migration, and a 20-fold increase completely abolishes it.

To exclude the possibility that GRP was simply inducing chemokinesis, and not chemotaxis, we performed a checkerboard analysis. For this, the optimal concentration of GRP (1 nM) was placed in the upper wells, lower wells, or both upper and lower wells of the Transwell chamber (25). GRP was only able to induce neutrophil chemotaxis, and a 20-fold increase completely abolishes it.

Neutrophil Migration Induced by GRP Is Independent of Gai Proteins, but Dependent on PLC-β, PI3K, and MAPK. The classical pathway of neutrophil migration involves triggering GPCR receptors, which release Gαi subunits from Gai proteins, activating specific signalizing pathways (26). However, GRPR preferentially couples to Gq proteins (27), which have not been identified with neutrophil migration. Given the complexity of GPCR biology, we hypothesized that GRPR could associate with Gai protein to induce migration. To investigate this, we pretreated neutrophils with pertussis toxin (PTx), which irreversibly inhibits Gai proteins (28) and allowed...
cells to migrate toward GRP or leukotriene B$_4$ (LTB$_4$). Pretreating neutrophils with PTx abrogated their migration to LTB$_4$, but did not affect migration toward GRP (Fig. 4A), suggesting that Gpr proteins are not involved in neutrophil chemotaxis induced by GRP.

Because GRPR is coupled to Gq protein, and triggering Gq-coupled receptors leads to activation of phospholipase C-β (PLC-β) (29), we investigated its involvement on GRP-induced migration. The pretreatment of cells with a selective inhibitor of PLC-β, U-73122, abolished neutrophil migration toward GRP (Fig. 4B), indicating that GRP activates PLC-β to induce chemotaxis.

Neutrophil migration induced by GPCR ligation is known to be dependent on PI3K and MAPK signaling routes (30). To determine downstream signaling pathways activated in GRP-induced neutrophil migration, we pretreated the cells with selective inhibitors of PI3K (LY29004), as well as of ERK 1/2 (PD98059) and p38 MAPK (SB203580). Blocking these pathways completely inhibited recruitment toward GRP (Fig. 4C), suggesting that GRP activates PI3K and MAPK, similarly to other chemotactic molecules (31). We also investigated whether treatment of neutrophils with GRP would activate these signaling pathways, analyzing phosphorylation of MAPKs and AKT (as a reading of PI3K activation) (32). Results showed in Fig. 4D support the findings that GRP rapidly signals through GRPR, activating these pathways, which promote neutrophil chemotaxis.

**Neutrophil Migration Toward Synovial Fluid (SF) of Rheumatoid Arthritis (RA) Patients Can Be Blocked by RC-3095.** Previous studies revealed an antiinflammatory effect of RC-3095 in murine arthritis (9) and sepsis models (10). This effect was attributed to the inhibition of IL-1β and TNF-α production. Other studies reported an increase of GRP concentrations in SF of arthritis patients, correlating with severity of disease (33, 34).

We hypothesized that at least part of the effect of RC-3095 in arthritis could be explained by an inhibition of neutrophil recruitment to the arthritic joint. We analyzed neutrophil migration toward SF from RA patients. The concentrations of GRP in samples of SF from patients varied between 0.8 and 1.8 ng/mL in our samples ($n = 6$) (Fig. 5A). Analysis in the Transwell system indicated that SF induced neutrophil migration, comparably to GRP alone (Fig. 5B). This migration was abrogated by RC-3095, indicating that GRP in the SF can activate GRPR and trigger neutrophil chemotaxis. Interestingly, the inhibition of neutrophil migration by RC-3095 was comparable to that observed with pretreatment of neutrophils with a CXCR2 antagonist (SB225002). The concentration of IL-8 in SF samples of RA was greater than 300 pg/mL (311.12 ± 3.45 pg/mL). To exclude the possibility that GRP in SF could be binding directly to CXCR2, we allowed neutrophils pretreated with SB225002 to migrate toward GRP. This pretreatment did not inhibit GRP-induced neutrophil migration (Fig. 5C), suggesting the effect of GRP was restricted to GRPR binding.

**Discussion**

Neutrophil migration is governed by gradients of chemoattractants, which include chemokines, complement-derived peptides (26), bacterial peptides (35), and lipid mediators (21). Despite the different nature of the ligands, all of them bind to and activate GPCR (26). In this study, we describe a unique function for the neuropeptide GRP and its receptor, GRPR, in the chemotaxis of neutrophils, showing that GRP can directly induce the GRPR-mediated neutrophil migration.

In vivo, macrophages play a role in the recruitment of neutrophils to the peritoneum by GRP. When we stimulated murine peritoneal macrophages or human monocytes with GRP in vitro...
and analyzed TNF-α and chemokine secretion by these cells, we observed induction of TNF-α in murine macrophages and MCP-1 in human monocytes 2 h after GRP stimulation. These results suggest that an early induction of such mediators in macrophages by GRP may occur in vivo, contributing to neutrophil recruitment, and this is consistent with neutrophil peaking at 4 h after GRP injection. Our results show that at 24 h, these mediators are no longer induced, and consistently, no neutrophils migrate to the peritoneum at this time point. Different stimuli induce in-vitro neutrophil migration toward the other molecule. Alternatively, GRPR agonists are capable of inducing neutrophil chemotaxis. These are pathways known to be downstream ofGPCR, and different neutrophil chemotactants, namely fMLP, IL-8, LTB4, and C5a, and ERK and p38 to stimulate neutrophil migration (31, 42), after triggering either classical or alternative chemokine receptors.

Several neuropeptide receptors have seven-spanned structure and are associated with G-proteins (2). It is possible that the phenomenon characterized in this study is actually a general mechanism mediating the interaction of endogenous neuropeptides and immune cells. Some neuropeptides, produced by immune cells have potent antiinflammatory actions and were found to have important roles in the maintenance of tolerance in different immunological disorders (2). However, here we demonstrate the proinflammatory activity of the neuropeptide GRP. Our results also provide a unique candidate mechanism for the antiinflammatory effect of the GRPR antagonist RC-3095 in models of arthritis (9) and sepsis (10). We can also extend our model to explain the proinflammatory effect of GRP, because a GRP blocking agent effectively prevented asthma exacerbation in animal models (12). The effective role of GRP in inflammation has not been characterized. The present study demonstrates that injection of GRP can induce an inflammatory response.

An unexpected finding was the suggestion of an interaction between GRPR and CXCR2, implicating some level of hierarchy or cooperation between the two receptors. Neutrophils prioritize between different chemotactic cues, favoring p38-controlled migration toward bacterial end targets over P2X5-controlled migration toward endogenous chemotactants (42). Prioritization is regulated by the phosphatase and tensin homolog (PTEN) (43), which inhibits chemotactant-derived P2X5 signaling, allowing unidirectional migration to an end-target chemotactant. It is possible that GRP and IL-8 are viewed by neutrophils as equivalent chemocytactants, and the blockage of either receptor in the presence of the other’s agonist inhibits the P2X5 pathway, inhibiting migration toward the other molecule. Alternatively, GRPR...
could be interacting with CXCR2 directly. A recent study demonstrated that GRPR can heteromerize with an isoform of the μ-opioid receptor MOR1D (44). This interaction explains the usual itch that accompanies morphine treatments, which could now be inhibited by preventing the interaction between the two GPCRs. In that study, heteromerization was followed by internalization of the complexed receptors. It is possible that in our system, GRP and CXCR2 form a complex and this complex is internalized upon treatment with each of the antagonists. These two intriguing possibilities deserve further investigation and will probably reveal new properties of chemoattractant receptors, including GRPR.

Finally, the migration of neutrophils to arthritic joints has been linked to disease severity (45). The presence of GRP in inflammatory scenarios, independently of infection, could directly recruit neutrophils via a nonclassical pathway, as well as induce macrophages to produce other neutrophil-recruiting molecules (Fig. S1), and both are blocked by RC-3095. The fact that GRP is a neuropeptide poses the intriguing possibility that neutrophil recruitment, and consequent disease exacerbation, could ensue in situations of psychological stress, when GRP can be released by neurons (46). Disease relapse in response to stress is a common aspect of autoimmune diseases (47), as well as asthma (48). Future studies on the in vivo mechanisms mediating cell migration induced by GRP will test this hypothesis.

Methods

Further information can be found in SI Methods.

Mice and Patients. C57BL/6 females, 6–8 wk old, were purchased from FEPPS and housed in accordance with National Institutes of Health Guidelines. Synovial fluid from RA patients were provided by the Rheumatology Service of São Lucas Hospital (PUCRS). All patients signed an informed consent term approved under Ethics protocol no. 858/05-CEP. This study was approved by the Ethics committee at PUCRS under protocol no. CEUA 10/00186.

Human Neutrophil Isolation and Chemotaxis Assay. Neutrophils were isolated from heparin-treated venous blood of healthy human volunteers using Histopaque-1077 (Sigma-Aldrich). Neutrophil chemotaxis was assayed in a Transwell system (Corning). Stimuli was added to the bottom wells, and neutrophils were added to top wells and incubated for 2 h at 37 °C. For checkerboard assay, stimuli were added to top wells, and incubated for 2 h at 37 °C. For checkerboard assay, stimuli were added to top wells, and incubated for 2 h at 37 °C. For checkerboard assay, stimuli were added to top wells, and incubated for 2 h at 37 °C.
**Murine Macrophage and Human Monocyte Purification.** Peritoneal macrophages were obtained by peritoneal and adherent cells stimulated with GRP (10 nM) for 2 or 24 h. Human monocytes were isolated from peripheral blood mononuclear cells obtained by density gradient centrifugation with Histopaque-1077, and adherent cells were stimulated with GRP for 2 or 24 h. Supernatants were collected and frozen until cytokine/chemokine analysis.

**Flow Cytometry.** Peritoneal cells were stained with anti-CD14 PE, CD11c PE-Cy7, CD45 FITC, and B220 PE-Cy5.5. Murine macrophages and human monocytes were stained with GRP for 24 h and culture supernatants analyzed for cytokines by Th1/Th2 Cytometric Bead Array (BD Biosciences), according to the manufacturer’s instructions.

**ELISA.** GRP (Phoenix Pharmaceuticals), TNF, and chemokines were determined according to the supplier’s instructions.

**Statistical Analysis.** Data are presented as mean ± SE. Results were analyzed using GraphPad Prism 5. Statistical differences among the experimental groups were evaluated by ANOVA with Tukey or Bonferroni correction or with Student’s t test. The level of significance was set at P < 0.05.

**Acknowledgments.** The authors thank Dr. José C. Alves-Filho for the PTx gift, Drs. Maria M. Campos and Rafael Zanin for Phosphoryl reagents, and Dr. Marcelo Bozza for the U-73122 gift. The authors acknowledge grant support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Grant 474697/2008-8. R.R. is the recipient of a CNPq fellowship and B.N.P., a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Programa Nacional de Pós-Doutorado (CAPES-PNPD) fellowship. R.R. and G.S. are supported by CNPq Grant 303703/2009-1 (to R.R.), the National Institute for Translational Medicine, and the South American Foundation for Anticancer Drug Development.