Interleukin 1β-Induced Increase in Substance P in Rat Myenteric Plexus

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Background: Substance P (SP) is increased in the inflamed intestine of *Trichinella spiralis*-infected rats, but the underlying mechanism is unknown. Interleukin 1β (IL-1β) messenger RNA and protein is expressed in the longitudinal muscle-myenteric plexus (LM-MP) of this model. Thus, the purpose of the study was to examine the ability of human recombinant IL-1β (hrIL-1β) to increase SP in LM-MP preparations from the intestine of noninfected rats. Methods: LM-MP preparations were incubated with hrIL-1β, and immunoreactive SP (IR-SP) was assessed in the tissues by radioimmunoassay or immunohistochemistry. Results: hrIL-1β increased IR-SP in the tissue in a time- and concentration-dependent manner, being maximal after 6 hours at a concentration of 10 ng/mL. The IR-SP could be depleted by scorpion venom, and immunohistochemistry revealed increased staining for SP within nerves of the LM-MP. The action of IL-1β was dependent on protein synthesis, was receptor mediated, and was not due to endotoxin contamination of the cytokine preparation. Conclusions: hrIL-1β stimulates the synthesis of SP in myenteric nerves of rat intestine.

Substance P (SP) is a widely distributed neuropeptide that increases in concentration in inflamed tissues, where it is believed to contribute to the inflammatory process. There are, however, conflicting reports regarding increases in the SP content of the gut in patients with inflammatory bowel disease. Nevertheless, Mantyh et al. showed an up-regulation of SP receptors on arterioles, venules, and lymphoid tissue in the intestinal mucosa of patients with Crohn's disease and speculated that this may have bearing not only on the inflammatory process, but also on the processing of sensory information in the inflamed gut.

We have studied the impact of inflammation on intestinal function using *T. spiralis*-infected rats as a model and have shown changes in cholinergic, adrenergic, and peptidergic nerves in the inflamed jejunum. We found a tetrad increase in immunoreactive SP (IR-SP) in the longitudinal muscle-myenteric plexus (LM-MP) region of the inflamed jejunum; the increased IR-SP could be depleted by scorpion venom treatment of the tissue in vitro, indicating that the neuropeptide was contained in nerves. No increases in IR-SP were observed in the noninfamed ileum of this model, and attenuation of the inflammatory response by corticosteroid treatment prevented the increase in IR-SP. These findings led us to conclude that the increased neuronal IR-SP content was a result of the inflammatory process.

Subsequent studies from this laboratory have addressed putative inflammatory mediators of the above-described changes in myenteric nerves in the *T. spiralis*-infected rat. Recently, we have shown an increase in the expression of messenger RNA (mRNA) for several cytokines in the LM-MP region of the inflamed jejunum of *T. spiralis*-infected rats. In particular, we documented increased expression of interleukin 1β (IL-1β) mRNA and protein in the LM-MP of this model, and it has been shown that IL-1β induces an increase in SP in sympathetic ganglia.

Thus, the purpose of the present study was to investigate the ability of human recombinant IL-1β (hrIL-1β) to increase IR-SP in LM-MP preparations from rat jejunum.

Materials and Methods

Incubation of LM-MP Preparations

Male Sprague-Dawley rats (200-250 g; Charles River Farms, Montreal, Quebec, Canada) were killed by a blow to the head followed by cervical dislocation. The small intestine was removed and placed in Krebs' buffer, containing (in mmol/L) NaCl, 120.9; KCl, 5.9; CaCl₂, 1.2; NaHCO₃, 15.5; NaH₂PO₄, 1.2; and glucose, 11.1; bubbled with 95% oxygen and 5% carbon dioxide. LM-MP segments (20-30 mg) were dissected from the jejunum, as previously

Abbreviations used in this paper: hrIL-1β, human recombinant interleukin 1β; IL-1α, IL-1 receptor antagonist; IR-SP, immunoreactive substance P; LM-MP, longitudinal muscle-myenteric plexus; RIA, radioimmunoassay.

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described.7 The LM-MP was cut into small sections and placed in 24-well tissue culture plates (Becton-Dickinson Labware, Lincoln Park, Nj), together with 1 mL of Roswell Park Memorial Institute (RPMI; Gibco, Grand Island, NY) medium supplemented with 10% fetal calf serum and 10 mmol/L glutamine and the proteinase inhibitor Trasylol (Miles Canada Inc., Etobicoke, Ontario, Canada) (100 kalikrein inactivating units/mL). The LM-MP preparations were incubated for specified times at 37°C in a CO2 incubator (Forma Scientific, Caltech Scientific, Ontario, Canada), then blotted and washed three times with phosphate-buffered saline (PBS). Finally, the tissue was weighed and prepared for measurement of IR-SP or for immunohistochemical analysis (see below).

Measurement of IR-SP

The LM-MP preparations were prepared for IR-SP measurement using the method described by Ferri et al.12 The tissues were placed in 2 mL acetic acid (0.5 mmol/L) on ice and homogenized 3 X 5 seconds at setting 5 using a Kinematica polytron (model CH-6010; Kriens, Luzern, Switzerland). The homogenates were then incubated for 30 minutes in a 37°C water bath, followed by centrifugation for 5 minutes at 11,500g in a bench-top centrifuge (Microfuge B; Beckman, Fullerton, CA). The supernatants were aspirated and stored at -70°C until the measurement of IR-SP. SP was determined using an inhibition-type solid phase radioimmunoassay (RIA) using 125I-SP (sp. act., 200 per Ci mmol/L; Amersham Canada Ltd., Oakville, Ontario, Canada) and a polyclonal rabbit anti-human SP antibody, directed against the C-terminus (Incast Corp, Stillwater, MN) as described previously.13

Identification of IR-SP by Immunohistochemistry

The tissue was pinned flat on cork and submerged in Zamboni’s fixative for 12 hours at 4°C before rinsing with dimethyl sulfoxide for 10 minutes X3, followed by PBS for 10 minutes X3. The tissues were then stored in PBS/0.01% sodium azide at 4°C before immunohistochemical analysis. Detection of SP-like immunoreactivity was performed as previously described by Sharkey et al.14 Briefly, the fixed tissues were incubated with a rabbit anti-SP antibody (1:1000) for 48 hours at 4°C. The antibody was then washed off, and the tissue was further incubated in goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (Dako, Dimension Labs, Ontario, Canada) and a polyclonal rabbit anti-human SP antibody, directed against the C-terminus (Incast Corp, Stillwater, MI) as described previously.13

Protein Synthesis Dependence

Initial experiments investigated the role of protein synthesis by measuring 35S-methionine uptake by the LM-MP preparations. The tissue preparations were preincubated with hrIL-1β together with 10 µCi/mL 35S-methionine (sp. act., 1000 Ci per mmol/L; Amersham) in the presence or absence of cycloheximide (100 µg/mL). After 6 hours, the tissues were washed X3 with PBS and weighed. Finally, they were solubilized with tissue solubilizer (NCS; Amersham), and the radioactivity was counted using a Beckman scintillation counter (model LS-5801, Fullerton, CA). Similar conditions were used to determine the effect of cycloheximide on IR-SP content.

Statistical Analysis

All experiments involved at least four separate experiments, and results are expressed as mean ± 1 SEM. The Students’ paired t test was used to compare two means within each experiment, whereas a one-way analysis of variance was used when comparing more than two means. Statistical significance was inferred when P value < 0.05.
As shown in Figure 2, boiling abolished the increase in IR-SP. The specificity of the hrIL-1β effect was further examined using a selective neutralizing antibody or IL-1ra. As shown in Figure 3, preincubating the cytokine with a specific anti-human IL-1β antibody produced an attenuation of 65%:2155 ± 244 vs. 896 ± 147 pg/mg tissue, hrIL-1β vs. hrIL-1β + anti-IL-1β (P < 0.001). Also as shown in Figure 3, incubation of the tissue with hrIL-1β plus IL-1ra attenuated the cytokine-induced increase in IR-SP by 78%: P < 0.001 (2155 ± 244 vs. 626.5 ± 60.5 pg/mg tissue, hrIL-1β vs. hrIL-1β + IL-1ra). The IR-SP content in LM-MP preparations incubated with either IL-1ra or anti-human IL-1β antibody alone was not altered from control (saline) IR-SP content (see Figure 3).

**Localization of IR-SP**

We used a functional and a morphological approach to determine the source of the increased IR-SP. The origin of the SP was assessed initially using the neural stimulus scorpion venom. As shown in Figure 4, incubation of the tissues with hrIL-1β caused a significant increase in IR-SP content to from 126.9 ± 42.5 pg/mg to 643.9 ± 4 pg/mg tissue (P < 0.001). When the hrIL-1β-treated tissues were subsequently incubated in the presence of 10 µg/mL scorpion venom, there was a 90% decrease in the IR-SP content to 186.5 ± 46.8 pg/mg tissue; the residual IR-SP level in the tissue was similar to that observed in saline-treated controls. When the sodium channel blocker tetrodotoxin (1 µmol/L) was present in the medium, scorpion venom failed to deplete the IL-1β-induced

Figure 1. Concentration-dependence of hrIL-1β–induced changes in SP. LM-MP preparations were incubated for 6 hours with the indicated concentrations of hrIL-1β. Tissues were washed, and IR-SP content was measured by RIA. Results are expressed as IR-SP (picograms per milligram tissue) and are the mean ± SE of 6 experiments (*P < 0.05, **P < 0.001).

**Results**

**Preincubation of LM-MP Preparations With hrIL-1β**

Incubation of LM-MP preparations with hrIL-1β (10 ng/mL) for 4 hours or less produced no significant change in IR-SP content compared with saline-treated controls. However, after 6 hours, there was a significant increase in IR-SP from 285.9 ± 34.7 to 999 ± 178 pg/mg tissue (P < 0.001). Prolonging the incubation to 12 hours produced no increase in IR-SP. In subsequent experiments, a 6-hour incubation period was used.

In control tissues, IR-SP was 159.9 ± 13.3 pg/mg. As shown in Figure 1, preincubating the tissue with hrIL-1β over a concentration range of 0.01–100 ng/mL caused a dose-dependent increase of IR-SP. The lowest hrIL-1β concentration that produced a significant increase in SP was 0.1 ng/mL (252.5 ± 20.9 pg/mg, P < 0.05), and the maximally effective concentration of hrIL-1β was 10 ng/mL, which induced an IR-SP level of 399.2 ± 15.7 pg/mg. Further increase of the hrIL-1β concentration 10-fold produced a submaximal increase in IR-SP content: 301 ± 17.6 pg/mg tissue (P < 0.05).

In subsequent experiments, we explored the mechanism underlying the hrIL-1β–induced increase in the hrIL-1β–stimulated increase in IR-SP using 6-hour incubation periods and 10 ng/mL of hrIL-1β.

**Specificity of hrIL-1β Effect**

To exclude an endotoxin effect of the recombinant cytokine preparation, we boiled hrIL-1β for 20 minutes. As shown in Figure 2, boiling abolished the increase in IR-SP. The specificity of the hrIL-1β effect was further examined using a selective neutralizing antibody or IL-1ra. As shown in Figure 3, preincubating the cytokine with a specific anti-human IL-1β antibody produced an attenuation of 65%:2155 ± 244 vs. 896 ± 147 pg/mg tissue, hrIL-1β vs. hrIL-1β + anti-IL-1β (P < 0.001). Also as shown in Figure 3, incubation of the tissue with hrIL-1β plus IL-1ra attenuated the cytokine-induced increase in IR-SP by 78%: P < 0.001 (2155 ± 244 vs. 626.5 ± 60.5 pg/mg tissue, hrIL-1β vs. hrIL-1β + IL-1ra). The IR-SP content in LM-MP preparations incubated with either IL-1ra or anti-human IL-1β antibody alone was not altered from control (saline) IR-SP content (see Figure 3).
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Figure 3. Specificity of hIL-1β effect. The LM-MP preparations were incubated for 6 hours with saline (□) or with 10 ng/mL hIL-1β (■) in the presence of an IL-1 receptor antagonist (RA) or neutralizing antibody (AB). Tissues were washed, and IR-SP content was measured by RIA. *Different from control, P < 0.001; **Different from IL-1β alone P < 0.01 or a neutralizing IL-1β antibody, P < 0.05). Results are expressed as IR-SP (picograms per milligram tissue) and are the mean ± SE of 5 experiments.

Figure 4. Effect of scorpion venom on residual IR-SP in tissues. The LM-MP preparations were incubated for 6 hours with saline (□) or with 10 ng/mL hIL-1β (■) or with 10 ng/mL hIL-1β for 6 hours followed by stimulation with scorpion venom alone (□□) or scorpion venom plus tetrodotoxin (■■). Tissues were washed and IR-SP content measured by RIA. The results are expressed as IR-SP (picograms per milligram tissue) and are the mean ± SE from 5 experiments. *Increase in IR-SP content compared with saline controls, P < 0.001; **Significantly different from hIL-1β alone, P < 0.001.

Figure 5. Fluorescence micrographs of IR-SP in the LM-MP preparations. (A) IR-SP distribution observed within the intrinsic nerves of saline-treated tissues. IR-SP was predominantly localized in the myenteric plexus and internodal strands. After treatment with hIL-1β (B) the immunoreactive fibers containing SP displayed the same distribution as saline-treated tissues (bar = 100 μm).

Mechanism of Action of IL-1β

As shown in Figure 6, hIL-1β caused a 3.8-fold increase in 35S-methionine uptake by the LM-MP preparations after 6 hours, from 480 ± 100 cpm/mg to 1829 ± 214 cpm/mg tissue (P < 0.01). Coincubation of the hIL-1β with cycloheximide (100 μg/mL) completely inhibited the cytokine-induced increase in 35S-

increase in IR-SP, and the residual IR-SP level was not significantly different from that seen following incubation with hIL-1β alone. Tissues incubated with saline for 6 hours before adding scorpion venom, in the presence or absence of tetrodotoxin, showed no alteration in IR-SP content when compared with saline-treated controls (data not shown).

Immunohistochemical analysis of saline-treated preparations revealed SP-like immunoreactivity localized predominantly in varicose nerve fibers and terminals in the myenteric plexus and internodal strands (Figure 5A). Substance P immunoreactivity was not detected in nerve cell bodies in the plexus. In hIL-1β-exposed tissues, (Figure 5B), the distribution of IR-SP was similar to that seen in saline-treated tissues. However, the major difference between saline- and hIL-1β-treated tissues was the increased intensity of fluorescence within immunoreactive fibers in the myenteric plexus. It is evident from these studies that there is no discernable IR-SP outside of the neural network.
methionine uptake \( (P < 0.001) \). As shown in Figure 7, incubation of the tissues with hrIL-1β in the presence of cycloheximide \( (100 \mu g/mL) \) attenuated the hrIL-1β-induced IR-SP by 88% from 2155 ± 224 pg/mg to 403.8 ± 83 pg/mg tissue \( (P < 0.001) \). There was no change in IR-SP content of tissues incubated either with saline or cycloheximide alone.

**Discussion**

The results of the present study indicate that hrIL-1β caused a time- and concentration-dependent increase in IR-SP in rat LM-MP preparations. The action of hrIL-1β was specific in that it could not be attributed to a heat stable endotoxin contaminant because the effect was abolished by boiling the cytokine and could be blocked using an anti-IL-1β antibody. In addition, the IL-1β effect was receptor mediated, in that it could be blocked by a specific receptor antagonist.15

We have not localized the site of action of IL-1β in the present study. It is possible that the cytokine interacts with receptors on SP-containing nerves in the tissue; neural receptors for IL-1β have been identified, for example, in the brain by autoradiography.16 However, recent studies,11,17 using sympathetic ganglia, show that IL-1β increases the SP content within neurons only in the presence of nonneuronal cells. This raises the possibility that in the present study, IL-1β may be acting on another cell type in the LM-MP preparation to induce the release of an intermediary substance, which in turn increases the SP content in neurons. Enteroendocrine cells or macrophagelike ce,
shown that neutral endopeptidase, which degrades SP, is down-regulated by inflammation in the gut, we feel that reduced degradation of SP cannot explain our data. First, we have been unable to show the ability of IL-1β to down-regulate NEP (unpublished observations). Second, the IR-SP appears to be within nerves and therefore inaccessible to NEP.

In our previous study, capsaicin treatment in vivo attenuated the increase in IR-SP observed in the LM-MP of T. spiralis–infected rats by 80%, suggesting that the increase in SP was located in primary afferents. The capsaicin insensitive increase in IR-SP (20%) in T. spiralis–infected rats likely reflects an increase in the neuropetide within intrinsic nerves of the LM-MP. Because experiments were not performed in capsaicin-treated rats, the proportion of IR-SP in primary afferents detected in the present study cannot be ascertained. However, because the majority of IR-SP in rat LM-MP is in intrinsic enteric nerves, it would constitute the majority of immunoreactivity.

Although the ability of hrIL-1β to stimulate IR-SP in primary afferent nerves remains to be determined, indirect support may be derived from the results of our preliminary experiments using the IL-1α receptor antagonist in T. spiralis–infected rats; IL-1ra treatment of infected rats was associated with a 90% attenuation of the inflammation-induced increase in IR-SP, suggesting that IL-1 may have an effect on capsaicin-sensitive SP-containing nerves, in addition to its effect on intrinsic nerves shown in the present in vitro study.

IL-1β may not be the only factor involved in the increase in IR-SP observed in the LM-MP after 6 days postinfection with T. spiralis. The altered neural environment, as reflected by the marked suppression of acetylcholine and noradrenaline release in the myenteric plexus, may play a role because it is known that SP content of nerves is subject to modulation by the activity of other nerves in the vicinity. In addition, it is probable that other cytokines such as transforming growth factor β and nerve growth factor also contribute to the increased SP in our model, and these possibilities are presently under study in our laboratory.

References


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