Suppression of Experimental Autoimmune Encephalomyelitis by Ghrelin

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Michael-Mark Theil,*† Sachiko Miyake,2* Miho Mizuno,* Chiharu Tomi,* J. Ludovic Croxford,* Hiroshi Hosoda,‡ Julia Theil,‡† Stephan von Hörsten,‡ Hiroaki Yokote,* Asako Chiba,* Youwei Lin,§ Shinji Oki,* Takashi Akamizu,‖ Kenji Kangawa,‖ and Takashi Yamamura2*

Ghrelin is a recently identified gastric hormone that displays strong growth hormone-releasing activity mediated by the growth hormone secretagogue receptor. While this unique endogenous peptide participates in the regulation of energy homeostasis, increases food intake, and decreases energy expenditure, its ability to inhibit the production of proinflammatory cytokines in vitro indicates its role in the regulation of inflammatory process in vivo. Here we examine the effect of exogenous ghrelin on the development of experimental autoimmune encephalomyelitis (EAE), a representative model of multiple sclerosis. In the C57BL/6 mouse model of EAE induced by sensitization to myelin oligodendrocyte glycoprotein 35–55 peptide, we found that alternate-day s.c. injections of ghrelin (5 μg/kg/day) from day 1 to 35 significantly reduced the clinical severity of EAE. The suppression of EAE was accompanied by reduced mRNA levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the spinal cord cellular infiltrates and microglia from ghrelin-treated mice at the peak of disease, suggesting the role of ghrelin as an antiinflammatory hormone. Consistently, ghrelin significantly suppressed the production of proinflammatory cytokines in LPS-stimulated microglia in vitro. These results shed light on the new role of ghrelin in the regulation of inflammation with possible implications for management of human diseases. *The Journal of Immunology, 2009, 183: 0000–0000.

Ghrelin does not only stimulate GH release, but it also increases food intake, regulates energy homeostasis, and decreases energy expenditure by lowering the catabolism of fat (4, 8, 9). Because of its orexigenic and adipogenic character, ghrelin may be potentially useful for the treatment of anorexia and cachexia (10, 11). Although the precise mechanisms remain to be clarified, the orexigenic activities of ghrelin may be mediated by another feeding regulatory hormone neuropeptide Y (NPY) via stimulation of Y1 and Y5 receptors (12). Furthermore, the antagonistic effect of ghrelin on leptin-induced decrease of food intake seems to be mediated by ghrelin-induced release of NPY and subsequent stimulation of the Y1 receptor (13).

Ghrelin has been shown to exhibit antiinflammatory functions against T cells and macrophages in vitro (14–16). The potential activity of ghrelin as an antiinflammatory reagent in vivo was shown in several animal models, including bowel disease (17), arthritis (16, 18), sepsis, and endotoxemia (16, 19, 20). Here we report that s.c. injections of ghrelin could significantly attenuate the clinical severity of the representative model of experimental autoimmune encephalomyelitis (EAE) induced in C57BL/6 (B6) mice by sensitization against myelin oligodendrocyte glycoprotein (MOG)35–55 peptide. Furthermore, we demonstrate that in vivo treatment with ghrelin significantly suppressed the mRNA levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in microglia and infiltrating T cells derived from the spinal cords of ghrelin-treated mice. Finally, we confirm that LPS-stimulated microglia and monocytes produced lower amounts of proinflammatory cytokines when they were pretreated with ghrelin in vitro. In conclusion, the present study indicates the potential use of ghrelin as an antiinflammatory drug to control human CNS pathology.

Materials and Methods

Mice and reagents

We used female B6 mice (CLEA Japan) between 6 and 10 wk of age in specific pathogen-free conditions. Animal care and use were in accordance
with institutional guidelines. Animal experiments were approved by our institutional review committee. Rat MOG35–55 (amino acid sequence MEVGWYRSPFSRVVHLYRNGK) was synthesized at Toray Research Center (Tokyo, Japan). Ghrelin and des-acyl ghrelin (Table I) were synthesized as previously described (4, 7).

**Table I. Amino acid sequence of mouse ghrelin and des-acyl ghrelin**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequencea</th>
<th>Ser7 acylation</th>
<th>Reference</th>
</tr>
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<tr>
<td>Ghrelin</td>
<td>GSSFLSPRHKQAKQREKSPKPKPLQPR</td>
<td>n-Octanoic acid (4)</td>
<td>(7)</td>
</tr>
<tr>
<td>Des-acyl ghrelin</td>
<td>GSSFLSPRHKQAKQREKSPKPKPLQPR</td>
<td>n-Octanoic acid (4)</td>
<td>(7)</td>
</tr>
</tbody>
</table>

*The underlined letter S represents the third serine (Ser7).

**Immunization and clinical assessment of EAE**

We immunized mice (n = 5–15 per group) s.c. in the tail base with 100 μg of MOG35–55–peptide dissolved in 0.1 ml of PBS and 0.1 ml of CFA containing 1 mg of *M. tuberculosis* H37Ra (Difco). Shortly after immunization and 48 h later, the mice were injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories). Clinical scores of EAE were daily assigned as follows: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or moribund; and 6, death. The cumulative scores were calculated for individual mice by summing up the daily scores.

**Administration of ghrelin and des-acyl ghrelin**

For EAE treatment, we s.c. injected ghrelin and des-acyl ghrelin diluted in 0.9% saline. In the first series of experiments, mice were injected with ghrelin or des-acyl ghrelin at doses of 0.5, 5, or 50 μg/kg every other day for 35 days. Sham-treated animals were injected with 0.9% saline (standard protocol). In the next experiment, we injected the mice with 5 μg/kg ghrelin every day from day 1 to 10 (induction phase treatment) or from day 11 to 20 (effector phase treatment) and in-between with 0.9% saline. The controls were injected every day from day 1 to 20 with 0.9% saline (alternative protocol).

**Assessment of histological EAE**

To evaluate the histological manifestations of EAE, we treated mice with 5 μg/kg ghrelin or 0.9% saline following the standard protocol and sacrificed them on day 17 postimmunization. The spinal cords were removed and stained with H&E and Luxol fast blue for histopathological analysis.

**Flow cytometry and isolation of mononuclear cells from the CNS**

B6 mice were challenged for EAE, treated following the standard protocol with 5 μg/kg ghrelin or 0.9% saline and sacrificed on day 17 postimmunization. We removed spleen, lymph nodes (LN), and thymus as well as spinal cord from the ghrelin- and saline-treated mice for flow cytometer analysis. Single-cell suspensions were prepared according to standard methods. The spinal cord cell suspensions were centrifuged at 200 × g for 10 min and resuspended in 4 ml of 70% isotonic Percoll (Amersham Biosciences)/PBS and overlaid by equal volumes of 37%–70% isotonic Percoll. The gradient was centrifuged at 500 × g for 15 min and the mononuclear cells were harvested from the 37%–70% interface, washed, and counted. The cells were stained for 5 min with anti-FcRγ III/II mAb (BD Pharmingen), washed, and labeled with the following mAbs for surface phenotype analysis: FITC-CD4 mAb, FITC-CD19 mAb, PE-CD8a mAb, PE-NK1.1 mAb, PE-CD25 mAb, allophycocyanin-Alexa Fluor 700-CD8α mAb, PE-CD11b mAb, and PE-CD19 mAb (BD Pharmingen) and FITC-F4/80 mAb (Dainihon Seiyaku). The cytometric analysis was performed using a FACS Calibur operated by CellQuest software (BD Biosciences).

**Cytokine and cell proliferation assay**

MOG35–55-immunized B6 mice were treated s.c. with 5 μg/kg/day of ghrelin or 0.9% saline every day from day 1 to 10. The LN cells were collected on day 11 after immunization and suspended in our standard lymphocyte culture medium (RPMI 1640 supplemented with 5 × 10−5 M 2-ME, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin) added with 1% syngeneic mouse serum. The cells were cultured in 96-well round-bottom plates at 1 × 10^5/well for 72 h in the presence of 100 μg/ml MOG35–55. Levels of IFN-γ, IL-17, and IL-4 in the supernatant were determined by using a sandwich ELISA. Proliferative responses were measured using a Beta-2I25 counter (Pharmacia) to detect the incorporation of [3H]thymidine (1 μCi/well) for the final 16 h of culture.

**Evaluation of encephalitogenic T cell induction in B6 mice treated with ghrelin**

To evaluate whether in vivo ghrelin treatment may affect the induction of encephalitogenic T cells after immunization with MOG35–55, we evaluated the ability of the lymphoid cells from ghrelin- or saline-treated mice to passively transfer EAE into naive recipients. Donor B6 mice were immunized with MOG35–55 and treated every day from day 1 to 10 with 5 μg/kg/day of ghrelin or 0.9% saline. We removed spleens and LN from the donor mice on day 11 and prepared lymphoid cell suspensions. The lymphoid cells were stimulated with MOG35–55 (5 μg/ml) in the standard medium added with FCS (10%) for 96 h and then we isolated the CD4+ T cells for cell transfer by depletion of CD8+ and NK1.1+ cells. In brief, the MOG35–55-stimulated total lymphoid cells were labeled with PE-CD8a mAb, PE-NK1.1 mAb, and PE-CD19 mAb (BD Pharmingen) for 30 min, washed, and incubated with anti-PE microbeads (Miltenyi Biotec) for 15 min. Using autoMACS (Miltenyi Biotec), we isolated CD4+ T cells (CD8+CD19+ and NK1.1− fraction) as a pass-through and suspended the cells in PBS. We injected 1.0 × 10^7 of the cells into the peritoneal cavity of syngeneic recipient mice that had been X-irradiated (550 rad) shortly before. We also injected 200 ng of pertussis toxin i.p. on the same day and 48 h later.

**Reverse transcription and real-time PCR**

To analyze the mechanism of ghrelin effects in vivo, we extracted total RNA from spinal cord, spleen, thymus, and LN samples using the RNeasy Mini Kit (Qiagen). The RNA was subjected to reverse transcription with the Advantage RT-for-PCR kit (BD Biosciences). Real-time PCR was conducted in the LightCycler quantitative PCR system (Roche Molecular Biochemicals) by using the LightCycler-FastStart DNA Master SYBR Green 1 kit (Roche Molecular Biochemicals). We followed the manufacturer’s specification using 4 mM MgCl2, and 1 μM primers. The primers used are as follows: TNF-α, CTGGAAGGGAATGGTTT (sense) and GGTACATGTGGCAGATCTTT (antisense); IL-1β, CACCGCTTTCAGGACAC (antisense); TGF-β, TGCAGCTGCAGAAGTATTAAA (sense) and GCTGAATGAAAGGCCTGTA (antisense); and HPRT, GGTGGATCACAAGGCCTTGC (sense) and GAGCGGAGGAGGAGTGT (antisense). Values are presented as the relative amount of transcript of each sample normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

**In vitro effect of ghrelin on RAW 264.7 monocytes treated with LPS**

To examine the effect of ghrelin on monocytes, RAW 264.7 monocytes (American Type Culture Collection) were suspended in the standard culture medium supplemented with 10% FCS and cultured in 96-well flat bottom plates at 1 × 10^5/well overnight. Various concentrations of ghrelin (10−6, 10−8, 10−10 M) were added to the culture and 1 h later the cells were stimulated with LPS (Sigma-Aldrich) at various doses (0.1, 1, 10 μg/ml). After 2 h of incubation at 37°C, supernatants were collected and the levels of TNF-α and IL-6 were detected by using a sandwich ELISA.

**Isolation of microglial cells from the CNS**

The spinal cords were incubated with 35 mg/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals) in RPMI 1640 medium at 37°C for 30 min. Mononuclear cells were harvested from the 37%–70% interface, washed, and counted. The cells were stained with 5 min with anti-FcRγ III/II mAb (BD Pharmingen), washed, and labeled with the following mAbs for surface phenotype analysis: FITC-CD4 mAb, FITC-CD19 mAb, PE-CD8a mAb, PE-NK1.1 mAb, PE-CD25 mAb, allophycocyanin-Alexa Fluor 700-CD8α mAb, PE-CD11b mAb, and PE-CD19 mAb (BD Pharmingen) and FITC-F4/80 mAb (Dainihon Seiyaku). The cytometric analysis was performed using a FACS Calibur operated by CellQuest software (BD Biosciences).
Fisher post hoc test was applied. Probability values of were subjected to two-way ANOVA. In case of significant differences, a
ments, we injected 0.5, 5, or 50
complete recovery, this EAE model shows persistent paralysis
administrated with the vehicle, 0.9% saline, alone. B. The mice were injected from day 1 every other day with 5 μg/kg des-acyl ghrelin, whereas controls
were subjected to 0.9% saline injections. C. Following an alternative protocol, mice were treated from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9% saline, while controls were treated every day with 0.9% saline injections. Data represent mean ± SEM. *, Significant differences between the groups (p < 0.05; Mann-Whitney U test).

FACSria (BD Biosciences). The total RNA was extracted from the isolated cells and was subjected to reverse transcription and real-time PCR.

In vitro effect of ghrelin on microglia cells treated with LPS
Mononuclear cells were prepared from brains of untreated non-EAE mice incubated with Liberase Blendzyme 3 and DNase I as described above and were isolated on 40%–80% discontinuous Percoll gradients. Isolated cells were suspended in DMEM supplemented with 10% FCS and cultured in 96-well flat bottom plates at 2 × 10⁶/well in the presence of ghrelin (10⁻⁶ M) overnight and later stimulated with LPS at different doses (0.01, 0.1 μg/ml). After 5 h of incubation at 37°C, supernatants were collected and the levels of TNF-α were detected by using a sandwich ELISA.

Statistical analysis
The differences in the clinical score between ghrelin-, des-acyl ghrelin-, and sham-treated groups were analyzed by the nonparametric Mann-Whitney U test. FACS analysis, real-time PCR, ELISA, and proliferation data were subjected to two-way ANOVA. In case of significant differences, a Fisher post hoc test was applied. Probability values of <0.05 were considered as statistically significant.

Results
Ghrelin inhibits EAE
To explore the modulatory effects of ghrelin on inflammatory de-myelinating diseases, we employed a model of EAE actively induced in B6 mice with MOG35–55. Although classical forms of EAE are typically characterized by acute paralysis followed by complete recovery, this EAE model shows persistent paralysis with partial recovery as a reflection of persistent inflammatory demyelination in the CNS (21, 22). In the first series of experiments, we injected 0.5, 5, or 50 μg/kg ghrelin to the mice every other day from day 1 to 35 postimmunization, while the control mice were injected with 0.9% saline. The results showed that the continuous injections of 5 μg/kg ghrelin suppressed most efficiently the clinical signs of EAE (Fig. 1A), whereas a lower (0.5 μg/kg) or a higher dose (50 μg/kg) showed only a marginal effect (data not shown). The treatment with 5 μg/kg ghrelin did not significantly alter either the onset or peak score of EAE. However, significant differences were noted in mean clinical score after day 25 postimmunization between the ghrelin-treated and the control mice (Fig. 1A).

Moreover, the effect of ghrelin on EAE was specific as des-acyl ghrelin, an acyl-modified ghrelin, which lacks the n-octanoic acid on the third serine, and consequently its binding ability to GHS-R (7) (Table I) had no modulatory effect on EAE at any concentration examined (Fig. 1B and Table II). Thus, the discrepant results obtained with ghrelin and des-acyl ghrelin indicate that ghrelin treat-
ment would ameliorate the clinical course of EAE via activation of the GHS-R.

To further characterize the effects of ghrelin on EAE, we next examined if treatment lasting for a shorter duration may also be immunomodulatory in vivo. We injected 5 μg/kg ghrelin every day from day 1 to 10 postimmunization (roughly corresponding to the induction phase) or from day 11 to 20 (roughly corresponding to the effector phase). As shown in Fig. 1C, both protocols showed similar levels of disease suppression, although it was less notable than the continuous treatment from day 1 to 35 (Table II).

Ghrelin does not influence cellular infiltration into CNS
In the previous results on prophylactic or therapeutic treatment of EAE, clinical suppression of EAE was generally associated with a significant reduction of cellular infiltration in the CNS (23). To clarify if histological manifestation of EAE is also suppressed by ghrelin treatment, we treated MOG35–55-immunized B6 mice with 5 μg/kg ghrelin or 0.9% saline every other day and prepared sections of spinal cords at the peak of disease (day 17 after immunization) (Fig. 2). Clinical signs were milder in the ghrelin-treated mice compared with saline-treated ones. However, histology of the spinal cord sections with H&E staining revealed equivalent levels of cellular infiltration in ghrelin- and saline-treated mice. To confirm this, we isolated mononuclear cells from spinal cords of the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Mean Day of Onset ± SEM</th>
<th>Mean Maximal Score ± SEM</th>
<th>Mean Cumulative Score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>8/8</td>
<td>16.38 ± 1.13</td>
<td>3.75 ± 0.33</td>
<td>55.44 ± 7.14</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>7/8</td>
<td>17.86 ± 1.30</td>
<td>3.29 ± 0.33</td>
<td>36.71 ± 9.99</td>
</tr>
<tr>
<td>Des-acyl ghrelin</td>
<td>6/8</td>
<td>18.83 ± 2.55</td>
<td>3.67 ± 0.40</td>
<td>49.33 ± 12.99</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6/8</td>
<td>18.00 ± 0.71</td>
<td>3.80 ± 0.44</td>
<td>49.05 ± 8.09</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>7/8</td>
<td>15.14 ± 0.51</td>
<td>4.43 ± 0.07</td>
<td>50.43 ± 3.10</td>
</tr>
<tr>
<td>(1–10)</td>
<td>6/8</td>
<td>16.00 ± 0.73</td>
<td>3.17 ± 0.53</td>
<td>34.00 ± 7.25</td>
</tr>
<tr>
<td>(11–20)</td>
<td>7/8</td>
<td>16.29 ± 1.25</td>
<td>3.50 ± 0.45</td>
<td>38.72 ± 8.79</td>
</tr>
</tbody>
</table>

The table shows the results of three separate experiments (n = 8 mice in each group of the three experiments).

The table shows the results of three separate experiments (n = 8 mice in each group of the three experiments).

After induction of EAE with MOG35–55, mice were treated in two different experiments following the standard protocol of every other day s.c. treatment with 5 μg/kg ghrelin or 5 μg/kg des-acyl ghrelin. The controls were injected with 0.9% saline (vehicle).

Following an alternative protocol, we treated the mice from days 1–10 (induction phase treatment) or from days 11–20 (effector phase treatment) with 5 μg/kg ghrelin and in-between with 0.9% saline, while controls were injected every day with 0.9% saline only. Data represent mean ± SEM.
GHRELIN INHIBITS EAE

Table III. Cytokine production and proliferation of MOG35–55-specific T cells after ghrelin treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CPM ± SEM</th>
<th>INF-γ ± SEM</th>
<th>IL-17 ± SEM</th>
<th>IL-4 ± SEM</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>47,590 ± 10,988</td>
<td>2,087 ± 487</td>
<td>820 ± 211</td>
<td>ND</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>36,663 ± 9,058</td>
<td>2,883 ± 615</td>
<td>674 ± 148</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mice were immunized with MOG35–55 and treated with 5 μg/kg ghrelin or 0.9% saline everyday from day 1 to 10 (n = 3/group). Popliteal and inguinal LN cells were harvested on day 11 after immunization and stimulated with 10 μg/ml MOG35–55. CPM marks the proliferative response to MOG35–55. The cytokines were measured in the supernatant by sandwich ELISA after 72 h of stimulation. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. ND, Not detectable.

Ghrelin does not inhibit the induction of MOG35–55-reactive T cells

To elucidate the immunomodulatory mechanism of ghrelin, we examined the cytokine production and proliferative response of draining LN cells to MOG35–55 that were obtained from MOG35–55-sensitized mice treated for 10 days every day with ghrelin or saline. The LN cells were collected on day 11 after immunization and stimulated with MOG35–55 in vitro. Accordingly, we harvested the supernatant and measured the levels of INF-γ, IL-17, and IL-4 by using ELISA. Although the IL-4 concentration was under the detection level, INF-γ and IL-17 could be detected in the MOG35–55-stimulated culture supernatant (Table III). There was no significant difference in the level of INF-γ and IL-17 when we compared ghrelin-treated and saline-treated groups. Furthermore, ghrelin-treated mice did not differ from saline-treated mice in the proliferative response of the draining LN cells to MOG35–55. We also examined the frequency of CD4+CD25+FOX3+ regulatory T cells in the lymph nodes and spleens using flow cytometry and did not find significant differences between ghrelin-treated and saline-treated mice (data not shown). These results indicate that in vivo ghrelin treatment did not inhibit the induction of MOG35–55-reactive T cells.

Ghrelin does not affect induction of pathogenic autoimmune T cells

To further confirm that MOG35–55-reactive T cells are normally induced in ghrelin-treated mice, we evaluated if the ability of the MOG35–55-sensitized lymphoid cells, obtained from MOG35–55-immunized mice, to transfer EAE into naïve mice could be affected by in vivo ghrelin treatment. To this aim, we immunized donor mice with MOG35–55 and treated them every day with ghrelin or saline from immunization up to day 10. Next day, we pooled lymphocytes from spleen and LN and cultured them in the presence of MOG35–55. Three days later, CD4+ T cells were purified and injected into recipient mice as described in Materials and Methods. It was theoretically possible that in vivo ghrelin treatment does not
inhibit induction of MOG$_{35-55}$-reactive T cells, but would prohibit the ability to cause EAE in vivo. In postulating that this could happen, CD4$^+$ T cells from ghrelin-treated donors should be less encephalitogenic than those from saline-treated mice. The results showed that transfer of activated CD4$^+$ T cells either derived from saline- or ghrelin-treated donors induced passive EAE in the recipients, showing approximately the same clinical course and severity (Fig. 4). Thus, it can be concluded that ghrelin treatment does not affect the induction of encephalitogenic MOG$_{35-55}$-reactive CD4$^+$ T cells.

**Ghrelin decreases mRNA levels of proinflammatory cytokines in the CNS**

After demonstrating that ghrelin does not suppress the infiltration of inflammatory cells in the spinal cord, we wondered whether the cytokine milieu in the ghrelin-treated mice could be significantly altered. To answer the question, we analyzed the mRNA levels of pro- and antiinflammatory cytokines (IFN-$\gamma$, TNF-$\alpha$, IL-1$\beta$, IL-6, IL-4, IL-10, and TGF-$\beta$) in the spinal cord, spleen, LN, and thymus of ghrelin- and saline-treated mice at the peak of disease (day 17) by using quantitative PCR. Although ghrelin treatment had no effect on the mRNA levels of IL-4, IL-10, and IFN-$\gamma$ in the spinal cord, spleen, LN, and thymus (data not shown), we found significantly reduced levels of TNF-$\alpha$ ($p < 0.0015$), IL-1$\beta$ ($p < 0.025$), and IL-6 ($p < 0.025$) in the spinal cord of ghrelin-treated mice, compared with saline-treated ones (Fig. 5A). In contrast, the level of TGF-$\beta$ showed a trend for slight elevation in the spinal cord. We also found a diminished level of TNF-$\alpha$ mRNA ($p < 0.0001$) in the spleen of ghrelin-treated mice (Fig. 5B), whereas we saw no significant change in any of the cytokines that we measured in LN or thymus of ghrelin-treated mice (Fig. 5, C and D). Because TNF-$\alpha$, IL-1$\beta$, and IL-6 mRNAs were selectively down-regulated in the spinal cord, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. This idea was consistent with the fact that ghrelin treatment did not inhibit the induction of MOG$_{35-55}$-reactive T cells.

**Ghrelin suppresses the proinflammatory cytokine production of LPS-stimulated monocytes**

To verify the postulate that in vivo treatment with ghrelin may ameliorate EAE by targeting monocytes, we examined in vitro effects of ghrelin on the monocytic cell line RAW 264.7 that robustly produce proinflammatory cytokines when stimulated with LPS. The RAW 264.7 line cells were first exposed to various doses of ghrelin for 1 h and then stimulated with LPS. We harvested the supernatant 2 h later and measured the levels of TNF-$\alpha$ and IL-6 by ELISA. The results revealed that prior exposure to ghrelin would significantly suppress the production of TNF-$\alpha$ ($p < 0.02$) and IL-6 ($p < 0.05$) by LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 6). The inhibitory effect of ghrelin was very potent, as in addition to the effects on LPS-stimulated monocytes, even the basal production of TNF-$\alpha$ ($p < 0.008$) and IL-6 ($p < 0.03$) was significantly reduced by in vitro ghrelin treatment. Given that in vivo treatment with ghrelin could suppress the
development of EAE without altering histological EAE or T cell-derived cytokine balance, the ghrelin-mediated suppression of monocyte-produced TNF-α and IL-6 would strongly support the postulate that monocytes are the main target cells in ghrelin-mediated suppression of EAE.

**Ghrelin inhibits the expression of proinflammatory cytokines in microglia**

The proinflammatory cytokines are known to be produced not only by CNS-infiltrating macrophages but also by T cells and microglia in the course of EAE. To investigate which cells are important in the ghrelin-mediated suppression of EAE, we first examined the expression of proinflammatory cytokines in macrophages. Unexpectedly, the mRNA of IL-1β, IL-6, and TNF-α did not alter in CNS-infiltrating macrophages of ghrelin-treated mice compared with the control mice (Fig. 7A). We next examined the expression of these cytokines in other cell types also known as a source of inflammatory cytokines and found reduced expression of these cytokines in microglia (Fig. 7B). Additionally, the expression of inflammatory cytokines was decreased in CNS-infiltrating T cells (Fig. 7C). Hence, these results suggest that microglia might play a crucial role in ghrelin-mediated inhibition of EAE.

**Ghrelin inhibits the proinflammatory cytokine production of LPS-stimulated microglia**

We next examined the effect of ghrelin on microglia. To test whether ghrelin directly affects microglia, we isolated mononuclear cells from the brains of untreated mice. In untreated non-EAE mice, most (≈77%) of the brain mononuclear cells were CD11b⁺ cells, and the majority of CD11b⁺ cells (≈95%) were considered as CD45low microglia cells. Among these mononuclear cells, CD19⁺ B cells were <0.1% and CD3⁺CD45⁺ T cells were 1–1.5%. We cultured the isolated mononuclear cells in the presence of ghrelin overnight and stimulated them with LPS in different doses for 5 h. The TNF-α levels in the culture supernatant were measured by using ELISA. In the presence of ghrelin, the TNF-α levels were significantly reduced (Fig. 8). These results suggest that ghrelin directly affects microglia by reducing the production of inflammatory cytokines.

**Discussion**

Starvation is known to have immunosuppressive effects (24–26). Although little was known about the mechanistic link between starvation and immunity, recent studies have shed light on the immunomodulatory potency of a range of feeding regulatory hormones such as leptin and NPY. For example, serum leptin is decreased after acute starvation in parallel with immunosuppression or Th2 bias, whereas exogenous leptin would correct the altered Th1/Th2 balance toward Th1 (27, 28). In contrast, NPY is increased after starvation. Exogenous NPY would shift the Th1/Th2 balance toward Th2 and can ameliorate the severity of EAE (29). Interestingly, both peptide hormones are linked to ghrelin in an endocrine feedback system (30). Ghrelin itself is increased after

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**FIGURE 6.** Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated monocytes. The monocytes were treated with various concentrations of ghrelin (10⁻⁶ M, 10⁻⁸ M, 10⁻¹⁰ M) 1 h before stimulation with 0.1, 1.0, and 10 µg/ml LPS. The (A) TNF-α and (B) IL-6 production was measured 2 h after LPS stimulation by sandwich ELISA. Data represent mean ± SEM of duplicate samples from one out of three independent experiments. Significant differences at 10⁻⁶, 10⁻⁸, and 10⁻¹⁰ M (p < 0.05; two-way ANOVA) are depicted as *, **, and *** respectively.

**FIGURE 7.** Effect of ghrelin on proinflammatory cytokine mRNA expression in infiltrating cells and microglia. Total mRNA was extracted from (A) macrophages, (B) microglia, and (C) T cells obtained on day 20 postimmunization from the spinal cords of MOG₆₅-₇₅-immunized mice treated with ghrelin or saline. The IL-1β, IL-6, and TNF-α mRNA expression levels were measured by real-time PCR. Data are presented as relative amount of transcript normalized to the housekeeping gene GAPDH.

**FIGURE 8.** Effect of ghrelin on the proinflammatory cytokine production of LPS-stimulated microglia. The microglia cells were treated with ghrelin (10⁻⁶ M) overnight and later stimulated with 0.01 and 0.1 µg/ml LPS. Five hours after stimulation, the TNF-α production was measured using ELISA. Data represent mean ± SEM of duplicate samples from one out of two independent experiments. *, Significant differences between the groups (p < 0.05; two-way ANOVA).
starvation, and it can potently stimulate the release of NPY in the CNS (12). Moreover, ghrelin shows antagonistic effects against leptin (31). Although the available data on the action of ghrelin on leptin or NPY may not be extrapolated to speculate about its role in the immune system, we decided to explore whether ghrelin may exhibit beneficial effects in the modulation of EAE. Furthermore, ghrelin was reported to have protective effects on endotox shock in rats (32). Additionally, the wide range of GHS-R expression within the immune cells strongly suggested the immunomodulatory potential of ghrelin (6). Considering its endocrine interactions, ghrelin becomes an interesting candidate for the in vivo modulation of EAE.

To evaluate the effects of ghrelin on the immune system in vivo, we used the representative EAE model induced with MOG 35–55 in B6 mice. Subcutaneous injections of ghrelin significantly suppressed EAE severity, especially after the peak of disease, while the EAE onset occurred almost similarly in both ghrelin- and sham-treated mice. Priming phase treatment (days 1–10) as well as effector phase treatment (days 11–20) also showed disease-suppressing effects, suggesting a modulatory role of ghrelin during all phases of disease. The unacylated ghrelin form, des-acyl ghrelin, failed to suppress EAE, demonstrating that the disease suppression was mediated by the GHS-R.

The histological findings at day 17 were similar in all animals regardless of the applied treatment. The inflammatory cell infiltration and demyelination occurred in both groups, suggesting a ghrelin effect independent of cell trafficking at the peak of disease. Moreover, we found by FACS analysis that the number of mononuclear cells isolated from the spinal cord and their composition did not significantly alter among ghrelin- and sham-treated mice at the same time point. Our data showed no statistically significant changes in the examined cell subsets, which supported the histological findings of unaffected immune cell traffic to the CNS. This discrepancy between analogous inflammatory status in the spinal cord on the one hand and less severe disease on the other hand in ghrelin-treated mice was remarkable, suggesting cytokine regulation as the possible mechanism of EAE suppression.

Leptin and NPY both influence the Th1/Th2 balance in opposing directions (27–29). Since ghrelin is the most potent NPY-releasing hormone and NPY suppresses EAE by a Th2 bias (29), we examined whether ghrelin affects the Th1/Th2 balance similar to NPY and if its potential mechanism of EAE suppression is primarily mediated on immune cells or secondarily through NPY release. To investigate the effect of ghrelin on the cytokine balance, we measured the cytokine responses of MOG 35–55-primed T cells from mice treated with ghrelin or saline. The evaluated IFN-γ, IL-17, and IL-4 levels as well as the proliferative response did not significantly alter between ghrelin- and sham-treated mice. Underlying these observations, we conclude that the suppression of EAE mediated by ghrelin does not affect the T cell-derived cytokine balance. To further address whether ghrelin acts via the NPY pathway, we determined the encephalitogenic potential of CD4+ T cells from ghrelin-treated mice to cause passive EAE in syngeneic recipients. We treated donor animals with ghrelin or saline for 10 days after priming with MOG 35–55, and lymphoid cells from the mice were stimulated with MOG 35–55. Three days later, CD4+ T cell blasts were isolated and transferred to naïve mice. The CD4+ T cells from ghrelin-treated mice did not differ from those from saline-treated mice in the ability to mediate passive EAE, indicating that ghrelin does not primarily affect induction of encephalitogenic CD4+ T cells in vivo. While NPY attenuates EAE by a Th2 bias of encephalitogenic CD4+ T cells (29), our findings likely suggest that ghrelin interacts independently of NPY in the amelioration of EAE.

To further clarify the mechanism of ghrelin-mediated EAE suppression, we examined the mRNA levels of several cytokines of ghrelin- and sham-treated mice at the peak of disease. Our data demonstrate significantly reduced levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the spinal cord and lower levels of TNF-α in the spleen of ghrelin-treated mice. In contrast, the level of TGF-β showed a trend for slight elevation in the spinal cord. The importance of TNF-α for initiating and sustaining inflammation is well described, as well as its essential role in the development of acute EAE (33, 34). The proinflammatory role of IL-1β and IL-6 in the immunopathology of EAE is also generally accepted (35–38). Thus, the inhibition of TNF-α, IL-1β, and IL-6 must be considered as an important mechanism in the ghrelin-mediated EAE suppression.

Given the selective down-modulation of the proinflammatory cytokines, we suspected that monocytes could be potential target cells in the ghrelin-mediated EAE suppression. However, the analysis of infiltrating cells and residual microglia revealed that the suppression of proinflammatory cytokines was prominently led by microglia. A decreased expression of these cytokines was also observed in infiltrating T cells. Considering that the transfer of T cells obtained from ghrelin-treated mice induced a similar disease course compared with control mice, the reduction of proinflammatory cytokines in microglia might be important in the ghrelin-mediated suppression of EAE.

In conclusion, the present study demonstrates for the first time to our knowledge that the gastric hormone ghrelin suppresses actively induced EAE by inhibiting production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 with microglia as the main target cells. These findings support an antiinflammatory property of ghrelin, shedding light on its role in immune-endocrine interactions. Consequently, we speculate that ghrelin may serve as an antiinflammatory drug to control human CNS pathology involving the production of proinflammatory cytokines.

Disclosures
The authors have no financial conflicts of interest.

References


