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Short Paper

Effects of co-administration of ghrelin agonist (GHRP-2) and GH on TNF-α, IL-6 and iNOS gene expression induced by LPS in the mouse brain

Zendehdel, M.[1*]; Allahdini, P.[2]; Safarpour, E.[3]; Abrehdari, Z.[2]; Pourrahimi, M.[1] and Mazaheri Nezhad Fard, R.[4]

[1]Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [2]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran; [3]Department of Biology, Qom Branch, Islamic Azad University, Qom, Iran; [4]Rastegar Reference Laboratory, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

[*]**Correspondence:** M. Zendehdel, Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. E-mail: zendedel@ut.ac.ir

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Summary

The aim of this study was to examine the anti-inflammatory effects of co-administration of growth hormone-releasing peptide-2 (GHRP-2) and growth hormone (GH) on tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) gene expression induced by LPS in the mouse brain. Thirty-five male NMRI mice (25±5 g) were injected through the mouse tail vein with saline, GHRP-2 (100 μg/kg), GH (25 μg/kg) or GHRP-2 + GH, 30 min before the intraperitoneal injection of LPS (5 mg/kg). Then, inflammation was induced by the intraperitoneal injection of LPS. The control animals received sterile saline in the first and second injections. Changes in the expression level of TNF-α, IL-6 and iNOS genes were studied in the mouse brain by a semi quantitative RT-PCR method. The results of this study showed that GHRP-2 or GH significantly decreased the expression of TNF-α and IL-6 genes in brain 2 h after the injection of LPS. Co-administration of GHRP-2 and GH markedly reduced the expression of TNF-α and IL-6 genes. LPS had no effect on the expression of iNOS gene in brain. The data suggest that co-administration of GHRP-2 and GH has a protective effect in brain inflammation induced by LPS through inhibition of TNF-α and IL-6.

Key words: LPS, GHRP-2, GH, Brain, Mouse

Introduction

Inflammation and microglial activation are two common phenomena of the pathogenesis in multiple neurodegenerative diseases, including Alzheimer, Parkinsons and Multiple Sclerosis (Qin *et al*., 2007). Microglia, the resident innate immune cell in brain, actively monitors its environment and can become overactive in response to diverse cues to produce cytotoxic factors, such as nitric oxide and tumor necrosis factor-α (TNF-α) (Li *et al*., 2004; Nagaya *et al*., 2004). To have a better understanding of the etiology and pathology of neurodegenerative diseases, identification of the mechanisms responsible for the progressive activation of microglias and related neural damages is necessary (Qin *et al*., 2007). Lipopolysaccharide (LPS) is a substance found in the cell wall of gram-negative bacteria that reliably produces anorexia in experimental animals because of its ability to produce acute inflammation (Garcion *et al*., 1998). Ghrelin, a 28-amino acid acylated peptide produced and secreted principally by the X/A-like enteroendocrine cells of stomach, was discovered in 1999 as a natural ligand for growth hormone secretagogue receptor (GHSR-1) (Wu and Kral, 2004). Ghrelin has been reported to significantly downregulate the circulating levels of cytokines in endotoxemia (Li *et al*., 2004). Administration of GHRP-2, a

synthetic ghrelin receptor agonist, reduces interleukin-6 (IL-6) production by macrophages in response to LPS (Colton and Gilbert, 1987; Granado *et al*., 2005). Evidence shows that the suppression of growth hormone (GH) action has clinical consequences when the inflammation is severe or chronic (Bergad *et al*., 2000). To determine if GH potentiates GHRP-2 anti-inflammatory effects, the current study was designed for the first time to examine the effects of co-administration of GHRP-2 and GH on the expression of TNF-α, IL-6 and iNOS genes induced by LPS in the mouse brain.

Materials and Methods

Thirty-five male NMRI mice (25±5 g), purchased from Pasteur Institute of Iran, were used in this study. Animals were housed under constant conditions in a temperature controlled room (22 ± 2°C) at 12:12 h light-dark cycles. All the experiments were performed according to international and current guidelines for the care of the laboratory animals.

After one week of acclimatization, mice were randomly divided into five groups of seven animals each. Group I mice, served as control, were injected with saline (0.5 ml, IV) in the first injection and saline (0.5 ml, IP) in the second injections. Group II mice were injected with saline (0.5 ml, IV) in the first injection and LPS (5 mg/kg, IP) (bacterial strain 0111: B4; Sigma Co., USA) in the second injection (Wu *et al*., 2009). Groups III, IV and V were conducted similar to the group II except that the mice received GHRP-2 (100 µg/kg, IV), GH (25 µg/kg, IV) (Techno Gene, Switzerland) and GHRP-2 (100 µg/kg, IV) + GH (25 µg/kg, IV) instead of saline in the first injection, respectively (Granado *et al*., 2005; Wu *et al*., 2009). The first injection was administered 30 min prior to the second injection (Wu *et al*., 2009). Mice were sacrificed by decapitation 2 h after the second injection. The brains of mice were removed and snap frozen in liquid nitrogen and then stored at -70°C. Lateral tail vein was used for the IV injection.

Isolation of total RNA

The total RNA for the reverse-transcriptase polymerase chain reaction (RT-PCR) was extracted from the brain samples treated with saline, LPS, GHRP-2, GH, and a combination of GHRP-2 and GH. Total RNA was isolated using RNX^{TM} Plus solution (Cat No.: RN7713C) based on the manufacturer's instruction (CinnaGen, Iran). Concentration of RNA was measured using a biophotometer at 260 and 280 nm wavelengths. Furthermore, the quality of RNA was approved by electrophoresis on agarose-formaldehyde gels. The extracted RNA was treated with DNase (Ambion, USA) to eliminate contaminant DNA. RNA samples were stored at -70°C until use. Equivalent amounts of total RNA were used to synthesize complimentary DNA (cDNA). cDNA synthesis was carried out using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, USA; Cat No. K1622) according to the manufacturer's instruction.

Quantitative RT-PCR

The expression levels of TNF-α, IL-6 and iNOS genes were assessed using a quantitative RT-PCR method. The primer sequences used in this study are listed in Table 1. To normalize signals from various samples of RNA, GAPDH mRNA (mouse housekeeping gene) were coamplified as internal standard. The ratios of the intensities of iNOS, TNF-α and IL-6 to GAPDH bands were assessed using

Table 1: Primer sequences for RT-PCR

Primer	Sequence (5′ to 3′)	Amplicon (bp)
iNOS Forward	GGCCACCTTGTTCAGCTAC	147
iNOS Reverse	TTCAGAGTCTGCCCATTGCT	
IL-6 Forward	GCCTTCCCTACTTCACAAGT	146
IL-6 Reverse	CCATTGCACAACTCTTTTCTC	
TNF-α Forward	GCCTCCCTCTCATCAGTTCTA	103
TNF-α Reverse	ACTTGGTGGTTTGCTACGAC	
GAPDH Forward	GTCGGTGTGAACGGATTTGGC	156
GAPDH Reverse	GTTGAATTTGCCGTGAGTGGAGTC	

LabWorks™ software version 6.5 and normalized with the intensity of GAPDH bands.

Statistical analysis

Data were expressed as means±SEM from seven mice per group. Statistical significance of difference was determined using analysis of variance (One-Way ANOVA). The ANOVA analysis was followed by Bonferroni test and Student's t-test. Differences in values were considered significant if $P<0.05$.

Results

TNF-α and IL-6 significantly increased 2 h after the injection of LPS as compared to control animals ($P<0.05$). As shown in Figs. 1A, B, 2A and B, administration of GHRP-2 or GH alone significantly decreased the expression of TNF-α and IL-6 genes 2 h after the LPS injection. In contrast, co-administration of GHRP-2 and GH markedly reduced TNF-α and IL-6 gene expression as compared with the reduction caused by GHRP-2 or GH alone ($P<0.05$). GHRP-2 decreased the expression of TNF-α and IL-6 genes, which had been induced by LPS in brain by 19 and 37%, respectively ($P<0.05$; Figs. 1A, B, 2A and B). GH decreased the expression of TNF-α and IL-6 genes, which had been induced by LPS in brain by 29 and 45%, respectively ($P<0.05$; Figs. 1A, B, 2A and B). Co-administration of GHRP-2 and GH markedly reduced the expression of TNF-α and IL-6 genes induced by LPS in brain by 59 and 61%, respectively ($P<0.05$; Figs. 1A, B, 2A and B); suggesting that GHRP-2 and GH have synergistic effects on TNF-α and IL-6 genes induced by LPS in brain. However, LPS had no effect on the expression of iNOS gene in brain.

Discussion

In the current study, it was demonstrated that GHRP-2 significantly decreased the expression of TNF-α and IL-6 genes induced by LPS in brains of mice. The beneficial role of ghrelin in downregulating cytokines has been reported previously (Li *et al*., 2004). When mice were treated with LPS alone, a

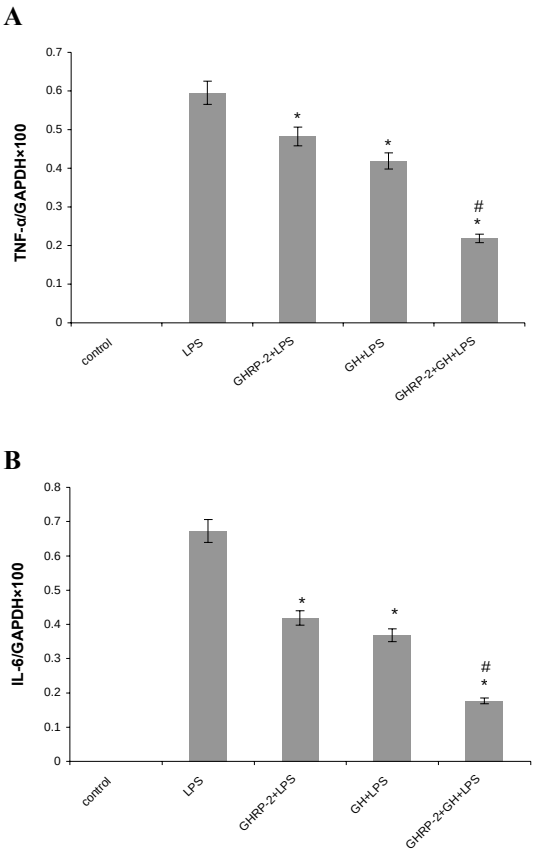

Fig. 1: Effect of GH (25 µg/kg, IV) and GHRP-2 (100 µg/kg, IV) on the expression of TNF-α (A) and IL-6 (B) induced by LPS (5 mg/kg, IP) in mice brains. Data are presented as means±SE (n=7). * P<0.05, compared to the corresponding LPS group. # P<0.05, compared to the corresponding GHRP-2 or GH alone

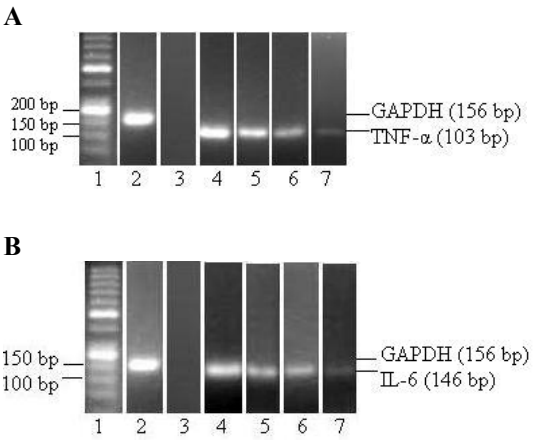

Fig. 2: Effect of GH (25 µg/kg, IV) and GHRP-2 (100 µg/kg, IV) on the expression of TNF-α (A) and IL-6 (B) induced by LPS (5 mg/kg, IP) in mice brains. 1: Molecular marker (50 bp); 2: GAPDH (156 bp); 3: Control; 4: LPS; 5: LPS + GHRP-2; 6: LPS + GH; Column 7: LPS + GHRP-2 + GH

dramatic increase in TNF-α and IL-6 levels was observed as expected. The ghrelin beneficial effect in downregulating TNF-α and IL-6 in sepsis is mediated by the ghrelin receptors on macrophages (Wu *et al*., 2007). Studies demonstrate the presence of GHSR in afferent neurons of no-dose ganglia, suggesting that ghrelin signals are transmitted to the brain by vagal afferent nerves and ghrelin can stimulate the vagus nerve via the central ghrelin receptors (Date *et al*., 2002). It can be pointed out that anti-inflammatory properties of ghrelin are mediated through the stimulation of vagus nerves or the inhibition of sympathetic nerve activation (Lin *et al*., 2004; Wu *et al*., 2007). Interestingly, the results of the current study demonstrate that the co-administration of GHRP-2 and GH significantly decreased the inflammatory responses in a synergistic mode in mice, suggesting that GH potentiates the GHRP-2 responsiveness. Our findings indicate that the treatment of mice with LPS did not induce the iNOS gene expression in mice brains, as proved by RT-PCR. iNOS immunoreactivity is strongly observed in macrophages, 5 h after the LPS hippocampal injection (Garcion *et al*., 1998). In this study, iNOS was not normally expressed in brain of mice 2 h after LPS injection. Therefore, LPS possibly induce iNOS in the brain 2 h after the LPS injection.

In conclusion, simultaneous administration of GHRP-2 and GH reduced TNF-α and IL-6 genes expression induced by LPS in a mouse model of brain inflammation. This report demonstrates that anti-inflammatory effects of GHRP-2 and GH may provide a potential neuroprotective benefit in various neurodegenerative disorders. The administration of a combination of GHRP-2 and GH may be a good treatment in neurodegenerative diseases. Further understanding of the mechanism of GHRP-2 action will provide important insights into potential therapeutic interventions for the inflammation-related neurodegenerative diseases.

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