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Central effects of glucagon-like peptide-1 on cold-restraint stress-induced gastric mucosal lesions

Soğuk-kısıtlama stresi ile oluşturulan gastrik mukozal hasar üzerine "glucagon-like peptide-1" in santral etkisi

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Background/aims: *Intracerebroventricular (i.c.v.) glucagon-like peptide-1 (GLP-1) has been shown to prevent gastric mucosal lesions induced by reserpine and ethanol. Here, we aimed to investigate the effects of i.c.v. GLP-1 on stress-induced gastric mucosal lesions and the mechanisms which may mediate these effects.* ***Methods:*** *Rats were equipped with intravenous and i.c.v. cannulas under ether anesthesia. To induce cold-restraint stress, rats were kept individually in wire cages, specifically prepared according to their sizes, at 7-9°C for 5 hours. They were then decapitated, and their stomachs were removed and scored for mucosal damage. GLP-1 (1, 10, 100, 1000 ng/10 µl; i.c.v.) was injected 5 min before cold-restraint stress induction. Rats were pretreated with exendin-(9-39) (2.5 ng/10 µl; i.c.v. and 250 ng/kg; intraperitoneal [i.p.]), calcitonin gene-related peptide (CGRP)-(8-37) (10 µg/kg; i.p.), N^G-nitro-L-arginine methyl ester (L-NAME) (3 mg/kg; i.v.), indomethacin (5 mg/kg; i.p.) and atropine (1 mg/kg; i.p.) to investigate mechanisms which may mediate the gastroprotective effect of GLP-1.* ***Results:*** *GLP-1 (1000 ng/10 µl; i.c.v.) significantly prevented gastric mucosal lesions induced by cold-restraint stress ($p<0.01$). Intracerebroventricular (i.c.v.), but not i.p., injection of exendin-(9-39) significantly blocked the gastroprotective effect of the peptide ($p<0.05$). Pretreatment with CGRP-(8-37), L-NAME and indomethacin also prevented the gastroprotective effect of i.c.v. GLP-1 ($p<0.05$, $p<0.05$ and $p<0.01$, respectively), while pretreatment with atropine did not prevent the gastroprotective effect of the peptide.* ***Conclusions:*** *We conclude that i.c.v GLP-1 inhibits the gastric mucosal damage induced by cold-restraint stress via the activation of its specific receptors, and CGRP, nitric oxide and prostaglandins, but not cholinergic muscarinic receptors, mediate this effect.*

Key words: Glucagon-like peptide-1, intracerebroventricular, gastroprotection, exendin-(9-39), CGRP-(8-37), L-NAME, indomethacin, atropine

Amaç: *İntraserebroventriküler (i.c.v.) "glucagon-like peptide-1" (GLP-1)'in reserpin ve etanol ile oluşturulan gastrik mukozal lezyonları önlediği gösterilmiştir. Bu çalışmada, stres ile oluşturulan gastrik mukozal lezyonlarda i.c.v. GLP-1'in etkisini ve bu etkide aracılığı olabilecek mekanizmaların araştırılması amaçlandı.* ***Yöntem:*** *Sıçanlara eter anestezisi altında intravenöz ve intraserebroventriküler kanül yerleştirildi. Soğuk-kısıtlama stresi uygulamak için, sıçanlar kendi büyüklüklerine göre hazırlanan tel kafesler içinde 7-9°C'de 5 saat bekletildi. Dekapite edildikten sonra mideleri çıkartıldı ve mukozal lezyonlar skorlandırıldı. GLP-1 (1, 10, 100, 1000 ng/10 µl; i.c.v.) soğuk-kısıtlama stresi uygulanmadan 5 dakika önce enjekte edildi. GLP-1'in etkisinde aracılığı olan mekanizmaları araştımak amacıyla sıçanlara exendin-(9-39) (2,5 ng/10 µl; i.c.v. and 250 ng/kg; i.p.), CGRP-(8-37) (10 µg/kg; i.p.), (L-NAME) (3 mg/kg; i.v.), indometazin (5 mg/kg; i.p.) ve atropin (1 mg/kg; i.p.) ön tedavisi uygulandı.* ***Bulgular:*** *GLP-1 (1000 ng/10 µl; i.c.v.) soğuk-kısıtlama stresi ile oluşturulan gastrik mukozal lezyonları anlamlı olarak önledi ($p<0.01$). Exendin-(9-39)'in i.c.v. enjeksiyonu peptidin gastroprotektif etkisini bloke etti ($p<0.05$), ancak i.p. enksiyonu etki göstermedi. CGRP-(8-37), L-NAME ve indometazin ön tedavileri de i.c.v. GLP-1'in gastroprotektif etkisini bloke ederken (sırayla $p<0.05$, $p<0.05$ and $p<0.01$) atropin bu etkiyi önlemedi.* ***Sonuç:*** *I.c.v. GLP-1'in soğuk-kısıtlama stresi ile oluşturulan gastrik mukozal hasarı spesifik reseptörlerinin aktivasyonu yoluyla önlediği, CGRP, NO ve prostaglandinlerin bu etkiye aracılık ettiği ancak periferik kolinerjik muskarinik reseptörlerin bu etkide rol oynamadığı düşünülmektedir.*

Anahtar kelimeler: "Glucagon-like peptide-1", intraserebroventriküler, gastroproteksiyon, exendin-(9-39), CGRP-(8-37), L-NAME, indometazin, atropin

INTRODUCTION

Stress-induced gastric mucosal damage is a result of the breakdown of mucosal defense mechanisms under both clinical and experimental conditions (1). Mucus and epithelial cell barrier constitute the structural elements of gastric mucosal defense (2). Mucine production, bicarbonate secretion and mucosal microcirculation are the physiological preventive mechanisms during acute gastric

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damage development (3). It has been reported that acid hypersecretion is not the main factor but rather only plays a permissive role in the development of stress-induced gastric mucosal damage (3), and that the damage is mainly due to the mucosal ischemia (4). Locally-secreted prostaglandins (PGs), sensory neuropeptides and nitric oxide (NO) contribute to the regulation of gastric blood flow and maintenance of mucosal integrity (5-7).

Research in the last two decades has revealed a strong relationship between stress ulcer and the brain-gut peptides. It has been suggested that the brain-gut peptides, which decrease gastric motility and acid secretion and increase mucus secretion and gastric blood flow, may be important for the prevention of gastric mucosal damage development (8-10). Among these peptides, glucagon-like peptide-1 (GLP-1) seems to be a strong candidate for this function. GLP-1 is formed by the post-translational processing of the preproglucagon gene in the small intestine and central nervous system (11, 12). It has been shown that both peripheral and central effects of GLP-1 are mediated by its specific receptors, and these effects are antagonized by the specific GLP-1 receptor antagonist exendin-(9-39) (13, 14). GLP-1 receptors and GLP-1 immunoreactive fibers are widely distributed in the brain (15-17). Both GLP-1 and its receptors have been found in significant amounts in the nucleus tractus solitarius (15, 18, 19). A high density of GLP-1 binding sites and GLP-1 gene expression have been demonstrated in the hypophysis, area postrema and paraventricular, supraoptic and arcuate nuclei of the hypothalamus (18, 20). Coexpression of GLP-1 receptors, vasopressin and oxytocin mRNAs in neurons of the rat hypothalamic supraoptic and paraventricular nuclei may lead to regulation of the secretion of these peptides by GLP-1 (21). Intracerebroventricularly (i.c.v.)-injected GLP-1 stimulates the secretion of luteinizing hormone (LH), thyroid stimulating hormone (TSH), corticosterone and vasopressin (16, 17, 22, 23), induces anorexia and adipsia, produces taste aversions, induces pica behavior and inhibits gastric emptying (16, 24-27). Thus, it has been suggested that GLP may act as a central regulator for several neuroendocrine and autonomic functions (16, 28).

Centrally-injected GLP-1 has been shown to prevent gastric mucosal lesions induced by several ulcerogenic agents, such as ethanol and reserpine (29), but its gastroprotective role against stress, which is a natural ulcerogen, has not yet been investigated. Here, we aimed to investigate the effects of i.c.v.-injected GLP-1 on stress-induced gastric mucosal lesions, and the mechanisms which may mediate these effects. For this purpose, we planned to investigate the roles of the specific central and/or peripheral receptors of the peptide, calcitonin gene-related peptide (CGRP), NO synthase-NO (NOS-NO) system, cyclooxygenase-prostaglandin (COX -PG) system, and the cholinergic pathway in the possible gastroprotective effect of GLP-1.

MATERIALS AND METHODS

Animals

Male Wistar rats (n=113) (Experimental Animals Breeding and Research Center, Uludağ University Medical Faculty, Bursa, Turkey), weighing 250-300 g were used in this study. Rats were housed 4-6 in a cage under constant environmental conditions (20-24°C; 12-h light-dark cycle). The animals were fasted for 24 h before the experiments with free access to tap water. The surgical and experimental protocols used were approved by the Animals Care and Use Committee of Uludağ University.

Surgical Procedures

Ether anesthesia was used during surgery. For i.v. injections, rats were implanted through the right femoral vein with a PE 10 tubing filled with heparinized saline. For i.c.v. injections, a burr hole was drilled through the skull 1.5 mm lateral to the midline and 1-1.5 mm posterior to the bregma on the right side. Through this hole, a 10 mm length of 20 gauge stainless steel hypodermic tubing was directed toward the right lateral ventricle. The cannula was lowered 4.2-4.5 mm below the surface of the skull perpendicularly and was fixed to the skull with acrylic cement. Animals were housed individually and allowed to recover for 5 days. At the end of the experiments, 5 µl of a methylene blue solution was injected into the cerebral ventricle through the cannula, and the placement of the inner end of the cannula was verified for each rat. After decapitation, the brains were removed and sections were observed macroscopically to ascertain whether the cannula had been correctly placed into the lateral cerebral ventricle.

Induction and Evaluation of Gastric Mucosal Lesions

To induce cold-restraint stress, rats were kept indi-

vidually in wire cages, specifically prepared according to their sizes, at 7-9°C for 5 hours. This regimen of cold-restraint stress has been reported previously to produce gastric ulcers reliably in food-deprived rats (30, 31). At the end of this period, the animals were decapitated, and their stomachs were removed and opened along the greater curvature. The number and severity of gastric lesions were evaluated according to the following rating scale:

0: no lesion

1: mucosal edema and petechiae

2: 1-5 small lesions (1-2 mm)

3: more than 5 small lesions or 1 intermediate lesion (3-4 mm)

4: 2 or more intermediate lesions or 1 gross lesion (greater than 4 mm)

5: perforated ulcers

To observe the effect of i.c.v. cannulation on gastric mucosa, five rats were implanted with i.c.v. cannulas. They were decapitated 5 days later, and their stomachs were removed and compared with those of naive rats (n=5).

Experimental Protocols

Effect of i.c.v. GLP-1 on stress-induced gastric lesions

GLP-1 (1, 10, 100, 1000 ng/10 µl) or saline (10 µl) was injected i.c.v. 5 mins before cold-restraint stress induction. Five hours later, rats were decapitated and gastric lesions were evaluated.

Role of its specific receptors in the gastroprotective effect of i.c.v. GLP-1

GLP-1 receptor antagonist exendin-(9-39) was injected i.c.v. (2.5 ng/10 µl) or intraperitoneally (i.p.) (250 ng/kg), 10 mins before saline (10 µl; i.c.v.) or GLP-1 (1000 ng/10 µl; i.c.v.) injection.

Role of CGRP in the gastroprotective effect of i.c.v. GLP-1

Rats received CGRP-(8-37), a CGRP receptor antagonist (10 µg/kg; i.p.), 15 mins before GLP-1 (1000 ng/10 µl; i.c.v.) or saline (10 µl; i.c.v.) injection.

Involvement of the NOS-NO system in the gastroprotective effect of i.c.v. GLP-1

Rats received N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor (3 mg/kg; i.v.), 1 min before GLP-1 (1000 ng/10 µl; i.c.v.) or saline (10 µl; i.c.v.) injection.

Involvement of the COX-PG system in the gastroprotective effect of i.c.v. GLP-1

Rats were pretreated with a COX inhibitor indomethacin (5 mg/kg; i.p.), 60 mins before GLP-1 (1000 ng/10 µl; i.c.v.) or saline (10 µl; i.c.v.) injection.

Role of the cholinergic system in the gastroprotective effect of i.c.v. GLP-1

Rats were pretreated with a muscarinic cholinergic receptor antagonist, atropine (1 mg/kg; i.p.), 10 mins before GLP-1 (1000 ng/10 µl; i.c.v.) or saline (10 µl; i.c.v.) injection.

Drugs

All drugs were injected to conscious, freely moving rats. GLP-1 (7-36), atropine sulphate, L-NAME, indomethacin and CGRP-(8-37) were purchased from Sigma (Sigma Chemical Co., MO, USA) and dissolved in saline. The doses of the drugs used were selected either according to the dose-response studies performed (for exendin-(9-39)) or according to the literature [9,25,30]. Intracerebroventricular injections were performed using a Hamilton microsyringe.

Statistical Analysis

Data are presented as means ± S.E. Non-parametric Kruskal-Wallis test was used to determine statistical significance. Differences were considered to be significant at $p<0.05$.

RESULTS

Effect of i.c.v. GLP-1 on stress-induced gastric lesions

Following 5 hours of cold-restraint stress, the average ulcer scores were 3.8±0.1. Only the highest dose of GLP-1 (1000 ng/10 µl; i.c.v.) significantly prevented the gastric mucosal lesions induced by cold-restraint stress (average ulcer score: 2.1±0.3; $p<0.01$) (Figure 1), and this dose was used throughout the experiments.

Role of its specific receptors in the gastroprotective effect of i.c.v. GLP-1

Intraperitoneal injection of GLP-1 receptor antagonist exendin-(9-39) did not affect the gastroprotective activity of i.c.v. GLP-1, while i.c.v. injection of exendin-(9-39) significantly blocked the gastroprotective effect of the peptide ($p<0.05$) (Figure 2). The average ulcer scores were 1.5±0.2 and 3.7±0.1, respectively.

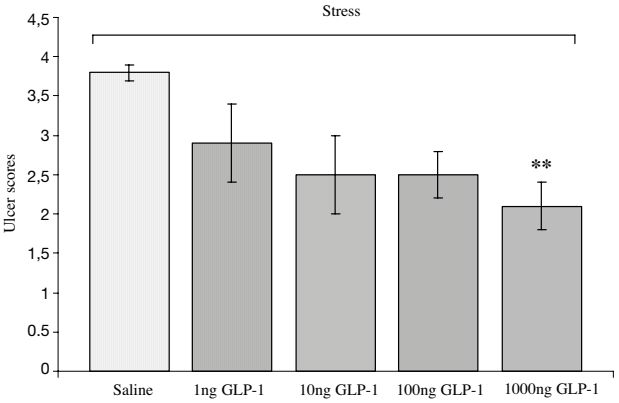

Figure 1. Effects of various doses of intracerebroventricular (i.c.v.) GLP-1 on gastric mucosal damage induced by cold-restraint stress. GLP-1 (1, 10, 100, 1000 ng/10 µl) or saline (10 µl) was injected i.c.v. 5 mins before cold-restraint stress induction. Gastric lesions were evaluated 5 hours later. Results were presented as means ± SE. Each group consisted of 6-7 rats

**p<0.01 with respect to the saline group

Role of CGRP in the gastroprotective effect of i.c.v. GLP-1

Injection of CGRP-(8-37), a CGRP receptor antagonist (10 µg/kg; i.p.), significantly inhibited the gastroprotective effect of i.c.v. GLP-1 (1000 ng/10 µl) ($p<0.05$) (Figure 3). The average ulcer score in this group was 3.6±0.2.

Figure 2. Involvement of central and peripheral GLP-1 receptors in the gastroprotective effect of intracerebroventricular (i.c.v.) GLP-1. GLP-1 receptor antagonist exendin-(9-39) was injected both i.c.v. (2.5 ng/10 µl) and intraperitoneally (i.p.) (250 ng/kg), 10 mins before saline (10 µl; i.c.v.) or GLP-1 (1000 ng/10 µl; i.c.v.) injection. Cold-restraint stress was induced 5 mins later and gastric lesions were evaluated following the 5-hour cold-restraint stress period. Results were presented as means ± SE. Each group consisted of 6-7 rats

*p<0.05 with respect to the saline group, #p<0.05 with respect to the GLP-1 group

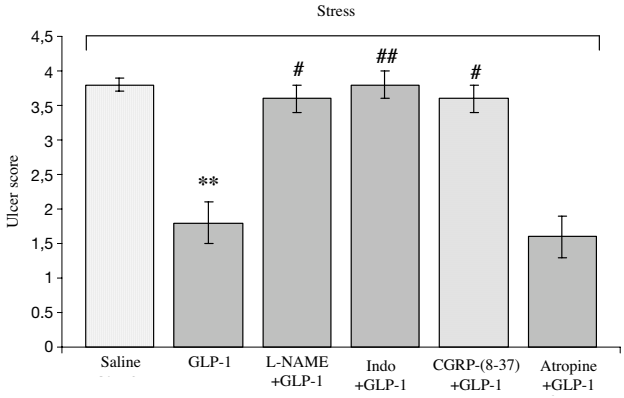

Figure 3. Role of calcitonin gene-related peptide (CGRP)-(8-37), NG-nitro-L-arginine methyl ester (L-NAME), indomethacin and atropine in the gastroprotective effect of intracerebroventricular (i.c.v.) GLP-1. Rats received CGRP-(8-37) (10 µg/kg; i.p.), 15 mins before, L-NAME (3 mg/kg; i.v.), 1 min before, indomethacin (5 mg/kg; i.p.), 60 mins before or atropine (1 mg/kg; i.p.), 10 min before GLP-1 (1000 ng/10 µl; i.c.v.) or saline (10 µl; i.c.v.) injection. Cold-restraint stress was induced 5 mins later and gastric lesions were evaluated following the 5-hour cold-restraint stress period. Results were presented as means ± SE. Each group consisted of 6-7 rats

Indo: Indomethacin, **p<0.01 with respect to the saline group, #p<0.05 with respect to the GLP-1 group, ##p<0.01 with respect to the GLP-1 group

Involvement of the NOS-NO system in the gastroprotective effect of i.c.v. GLP-1

Injection of L-NAME (3 mg/kg; i.v.) 1 min before GLP-1 (1000 ng/10 µl; i.c.v.) significantly prevented the gastroprotective effect of GLP-1 ($p<0.05$) (Figure 3). The average ulcer score in this group was 3.6±0.2.

Involvement of the COX-PG system in the gastroprotective effect of i.c.v. GLP-1

COX inhibitor indomethacin (5 mg/kg; i.p.) also blocked the gastroprotective effect of 1000 ng/10 µl i.c.v. GLP-1, when applied 60 mins before the injection of the peptide ($p<0.01$) (Figure 3). The average ulcer score in this group was 3.8±0.2.

Role of the cholinergic system in the gastroprotective effect of i.c.v. GLP-1

The average ulcer score of the rats pretreated with a muscarinic cholinergic receptor antagonist, atropine (1 mg/kg; i.p.), 10 mins before GLP-1 (1000 ng/10 µl; i.c.v.) injection was 1.6±0.2. This score was not significantly different from that of the saline-GLP-1 group.

None of the antagonists alone had any effects on gastric mucosal lesions (Table 1). The average ulcer scores of the naive rats and those implanted with i.c.v. cannulas only were both 0±0.

Table 1. Effects of antagonists on gastric mucosal damage induced by cold-restraint stress, when injected alone

Groups	n	Ulcer score
Saline	7	3.8 ± 1
Exendin-(9-39) (i.p.)	7	3.4 ± 1
Exendin-(9-39) (i.c.v.)	7	3.2 ± 1
L-NAME	7	3.9 ± 2
Indomethacin	7	3.8 ± 1
CGRP-(8-37)	7	3.7 ± 1
Atropine	7	2.8 ± 1

L-NAME: N^G-nitro-L-arginine methyl ester, CGRP: Calcitonin gene related peptide, i.p.: Intraperitoneal, i.c.v.: Intracerebroventricular

DISCUSSION

We have previously shown that centrally-injected GLP-1 prevents gastric mucosal damage induced by various models (29,32). However, the gastroprotective effect of GLP-1 against stress, a natural ulcerogen, has not yet been investigated. This study has revealed that i.c.v. GLP-1 prevents the gastric mucosal lesions induced by cold-restraint stress in rats. Stress-induced acute gastric mucosal lesions are due to stimulation of specific cerebral pathways that regulate autonomic functions, decrease mucosal blood flow, and increase gastric motility, degranulation of mast cells and leukocyte activation (33). Centrally-injected GLP-1 may prevent cold-restraint stress-induced gastric mucosal damage by affecting one or more of these factors. It has been reported that GLP-1-containing neurons are activated in several experimental stress models (34, 35), and that centrally-injected GLP-1 increases plasma corticosterone levels by stimulating corticotropin-releasing hormone (CRH) neurons and also increases arginine vasopressin (AVP) secretion (21, 36, 37). Therefore, it has been suggested that central GLP-1 may be involved, at least partially, in the hypothalamic stress response (34-37). Central injections of both CRH and AVP produce protective effects on stress-induced ulcers (31, 38). Similar to these two stress hormones, GLP-1 may exert a protective effect on stress-induced gastric mucosal damage by stimulating the sympathetic nervous system. In fact, the protective effect of the sympatho-adrenergic system in stress-induced ulcers has been previously reported by several authors (39, 40).

GLP-1 receptors are widely distributed both centrally and peripherally (13-17). GLP-1 receptors are also present on afferent fibers and may produce various effects, including the activation of vagal afferents (25,41). It has been shown that the discovery of both GLP-1 and its specific receptors, which are synthesized in the same brain regions, led to better understanding of the effects of the peptide in the central nervous system. The effects of GLP-1 are antagonized by exendin-(9-39) (13, 14). Exendins are a group of peptides that are isolated from helodermatid venom and have both structural and functional similarities to GLP-1. Exendin-4 acts as an agonist and exendin-(9-39) acts as an antagonist for the truncated form of GLP-1 (42).

To investigate whether the gastroprotective effects of centrally-injected GLP-1 on stress-induced gastric mucosal lesions are mediated by its specific receptors, and to decide if these effects are mediated centrally or peripherally, the specific GLP-1 receptor antagonist exendin-(9-39) was injected both i.c.v. and i.p. Centrally, but not peripherally-injected exendin-(9-39) inhibited the gastroprotective effect of i.c.v. GLP-1, which suggests that central mechanisms are involved in this effect.

The mechanisms that contribute to the gastrointestinal effects of GLP-1 are not well-defined. Some authors have suggested that GLP-1 acts directly by stimulating the gastric receptors (43), while others have suggested an indirect effect through neural pathways. It has been reported that GLP-1 inhibits the gastrointestinal vagal inputs via its receptors in the nucleus tractus solitarius and dorsal motor nucleus of the vagus nerve (44), and exerts its inhibitor effects through the sympathetic nervous system (45).

Capsaicin-sensitive sensory neurons and CGRP, which is released from these fibers, seem to be involved in the inhibitor effects of GLP-1, since some of these effects are abolished following afferent fiber denervation by capsaicin (25). Capsaicin-sensitive sensory neurons are nociceptive neurons, which are activated by various noxious stimuli (46). These neurons may play an important role in gastroprotection, by releasing CGRP and consequently increasing the gastric mucosal blood flow (47). We found that CGRP receptor antagonist CGRP-(8-37) prevented the gastroprotective effect of i.c.v. GLP-1, suggesting the involvement of CGRP receptors in this effect. Similarly, injection of L-NAME inhibited the gastroprotective effect of the peptide, showing that the NOS-NO system also contributes to the mechanism. Another finding is the inhibition of the gastroprotective effect of GLP-1 by a non-selective COX inhibitor, indomethacin. Hence, it has been shown that the gastric

PGE_2 content significantly decreases following the cold-restraint stress (48). Harada et al. (49) reported that capsaicin-sensitive sensory neurons are involved in the gastric release of both PGI_2 and PGE_2. Both PGs are vasodilators and they might exert strong gastroprotective effects through vasodilation and/or other cytoprotective mechanisms.

Release of CGRP due to the activation of capsaicin-sensitive sensory neurons leads to an increase in the secretion of endothelial NO, PGI_2 and PGE_2, and consequently, an increase in the gastric mucosal blood flow. As a result, CGRP, NO, PGI_2 and PGE_2 maintain the integrity of the gastric mucosa by increasing the gastric mucosal blood flow (2, 47, 49, 50). Since we have found that CGRP, NO and PGs are involved in the gastroprotective effect of i.c.v. GLP-1, the peptide seems to exert this effect by increasing the gastric mucosal blood flow and thus contributing to the maintenance of mucosal integrity. It has been widely accepted that mucosal ischemia is the most important factor in the development of stress-induced gastric mucosal damage (3, 4). It has been reported that gastric mucosal blood flow is a protective factor against mucosal damage and vasodilator mediators such as locally-released PGs, neuropeptides and NO help to provide the mucosal resistance (5-7, 47-50).

Stress increases the motility of the gastrointestinal tract and this may be an important factor responsible for the gastric mucosal damage induced by stress (8-10). Thus, the gastroprotective effect of i.c.v. GLP-1 may also be partially due to its inhibitor effect on gastric motility, since it has been reported that i.c.v. GLP-1 inhibits gastric emptying via vagal afferents, which has been shown to be mediated by the specific receptors of the peptide and sensory afferent denervation abolishes this effect (25).

We found that peripherally-injected atropine did not change the effect of i.c.v. GLP-1, suggesting that peripheral muscarinic receptors are not involved in the central gastroprotective effect of the peptide. We have previously reported that central, but not peripheral, muscarinic cholinergic receptors mediate the gastroprotective effect of centrally-injected GLP-1 on ethanol-induced gastric mucosal lesions. It has also been shown that the positive chronotropic effect of the peptide is not related to the inhibition of the vagal tone (51, 52). These data suggest that peripheral muscarinic receptors or vagal efferents do not mediate the peripheral effects of centrally-injected GLP-1.

We thus conclude that i.c.v GLP-1 inhibits the gastric mucosal damage induced by cold-restraint stress via the activation of its specific receptors, and CGRP, NO and PGs, but not peripheral cholinergic muscarinic receptors, mediate this effect.

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