Key words: ghrelin, blood flow, calcitonin gene related peptide, intestine, protection, oxygen uptake, sensory afferent nerves
INTRODUCTION

Ischemia of the intestine is a serious medical emergency, which in its most severe form presents as an acute total occlusion of the mesenteric trunk, causes over 70% mortality rate in a group of affected patients (1). In cases of subtotal occlusion with marked reduction of the intestinal perfusion due to pathological changes of vasoreactivity, the mesenteric ischemia may present with a severe manifestation including vomiting and diarrhea (2). Pathological changes in the morphology and function of the intestine exposed to the I/R result from an excessive inflammatory response, which may also lead to more general changes, ultimately ending up in an irreversible state of multi organ failure (3). Three stages of acute bowel ischemia could be distinguished from which the first stage resulting mucosal erosions and hemorrhagic ulceraions, could be reversible if stopped with efficient vasodilatatory treatment (4).

Ghrelin is a novel gastrointestinal hormone discovered in 1999 by Kojima et al. (5). Ghrelin is released in a pulsating manner mostly from the ghrelin endocrine cells of the stomach and is involved in the regulation of food intake, particularly the transmission of satiety signals - thus participating in energy homeostasis (6, 7). Recently, this hormone has gained considerable attention because of its divergent and widespread biological activity, which was discovered experimentally in rodents and in humans by means of molecular biology. Ghrelin acts through the GHSR-1a receptor, which is abundantly distributed in the organs of the GI system, the central nervous system (CNS), namely in the midbrain, and adipose tissue (8). Besides the GI tract, the ghrelin receptors were also localized in the heart, lung tissue and kidneys where its physiological role remains still unknown (9-11). There is also evidence of the presence of ghrelin receptors in the medial (muscular) layer of human blood vessels (12). The potent vasodilatatory action of ghrelin was recognized in the splanchnic vasculature (13). Distribution of ghrelin receptors especially among organs of the GI tract suggests that this hormone may act not only in the endocrine system but also in a paracrine and autocrine fashion (14). One of the sites where there is a remarkable concentration of GHSR receptors is within midbrain structures, especially the hypothalamic arcuate nucleus and nucleus tractus solitarii where ghrelin serves as a messenger in the transmission of the satiety signals. The extensive role of vagal afferents in the transmission of the satiety signals has been reported (15, 16) and confirmed in the animals fed with a different diet by showing that intact vagal nerves are essential for the feeding induced by peripherally administered ghrelin. Different effects of ghrelin results from its direct stimulation of the central and peripheral growth hormone secretagogue receptor (GHSR) receptors as both h-ghrelin and m-ghrelin are able to cross the blood-brain barrier at a different rate (17). It was also proposed that adrenergic receptors were involved in the ghrelin-induced transmission of the hunger and satiety signals.

Sibilia et al. (18) and Brzozowski et al. (19) were the first to report the gastroprotective role of centrally and peripherally administered ghrelin against acute lesions induced by ethanol. Furthermore, ghrelin exhibited a potent protective activity against gastric lesions evoked by cold stress and aspirin-induced mucosal impairment. Subsequent analysis of the mechanisms of the protective action of ghrelin conveyed by Brzozowski et al. (20) revealed that the protective action of peripherally administered ghrelin are predominately due to a stimulation of the sensory fibers as their blockade abolished this hormone in afforded protection. CGRP released from the sensory C fibers in response to ghrelin could be responsible for a vasodilatation that promoted vascular perfusion essential for gastric protection (20).

This was confirmed by different authors, who also stated that GI protection depends upon the increased blood flow and excessive oxygen supply to GI organs, both these effects generally attributed to the vasodilatatory action of various gastroprotectors including ghrelin (21-23). Moreover, at least in the stomach, intact vagal innervation was considered as one of the most important prerequisite for ghrelin-induced enhancement of mucosal integrity (20, 24).

Although the mechanisms of the protective actions of ghrelin in the model of gastric mucosal impairment was clinical recognized, there is no evidence so far whether ghrelin could be implicated in the mechanisms of intestinal integrity. Aim of the present study was first to evaluate the importance of peripherally and centrally administered ghrelin on the viability of the exposed intestine to the stress resulting from intestinal ischemia followed by a subsequent reperfusion. Secondly, we wanted to get an insight into the neural mechanism involved in the protective action of ghrelin and accompanying changes in the intestinal blood flow as well as the metabolic activity of the intestine when this hormone was applied peripherally and centrally. Our third goal was to investigate the possible effect of different timing of ghrelin application before and after ischemia along with its ability to exert metabolic effects associated with intestinal protection.

MATERIALS AND METHODS

Experiments were performed on 126 male Wistar weighing 300-350 grams. The animals were housed in cages with wire mesh bottoms, at normal room temperature, and a 12-hour light-dark cycle with no limitation of food and drinking water. Procedures conducted during the investigation conformed the guidelines of the Committee for Research and Animal Ethics of the Jagiellonian University. The animals were fasted for 24 hours before the experiment but were allowed free access to water.

Before the experiment the rats were anesthetized with sodium pentobarbital (10 mg/kg s.c., Vetbutal, Polfa Pulawy, Poland) then placed on a heated table allowing for a body temperature maintained at 37°C as it was monitored by a rectal thermometer (FST TR-100). To avoid oscillations in main blood pressure due to an unsteady breath rate and carbon dioxide-induced oscillations, the animals were artificially ventilated with room air (UGO Basile Rodent Ventilator, Milano, Italy).

Surgical procedures and measurements

A midline neck incision was made, the trachea was exposed and cut, a silicone catheter of a conforming diameter was then inserted. The rats underwent catheterization of the left carotid artery by a cannula filled with saline and connected to the electro manometer (Statham, London, England) for continuous blood pressure recording (expressed in mm Hg), while the heart rate was calculated from phasic blood flow. A small midline incision was made and the anterior mesenteric artery (SMA) was identified and exposed. An ultrasonic probe (RS1) (2 mm of external diameter) was placed on the anterior mesenteric artery trunk and mesenteric blood flow (MBF) was determined with the use of a directional ultrasonic Doppler flowmeter (T206 Transonic systems - Ithaca). Recorded data was expressed in ml/min. Microcirculatory intestinal mucosal blood flow (LDBF) was determined by laser Doppler flowmetry (Periflux 4001 Master, Perimed, Sweden). Spectral Doppler signal inversion in the close pole was detected and processed with the use of fast Fourier analysis. Signal (a 420 nm light beam) was emitted and collected with the use of a fiberoptic probe positioned against the surface of the intestinal mucosa. The probe was secured aside of the animal with the use of a special holder to eliminate...
movement of the tip of the probe against the examined tissue. Microcirculatory blood flow was expressed in arbitrary units (PU). The change in LDBF was calculated in terms of a percentage of control. Intestinal oxygen uptake (VO$_2$) was calculated as an average of the arterio-venous oxygen difference in the mesenteric circulation (A VO$_2$) and MBF. A VO$_2$ ml O$_2$/min was determined from whole blood samples obtained from the aorta and intestinal arcade veins. Oxygen content in blood samples was determined with the use of the AVOximeter (A-VOX 1000 E, Texas, USA), and calculated after a fast analysis based on colorimetry. The results were expressed in O$_2$ ml per 100 ml of the whole blood. The AP, MBF, LDBF and HR were continuously monitored and recorded on a PC computer using the Windows 98 based FlowPress program. After completed measurement, the animals were euthanized by administering an excessive dose of the anesthetics.

**Histological examination and macroscopic determination of intestinal lesions**

The Treitz ligament was identified, and 5 cm distally from this ligament, the intestinal tissue fragments of 10 cm length were collected. The intestinal tissues were processed using standard histological techniques including formalin fixation, dehydration and paraffin embedding, then cut in 4-μm sections and stained with hematoxin and eosin (Hypercenter XP tissue processor Shandon). The specimens were assessed with the use of an optical microscope (100-200 x magnification) by an experienced pathologist that was not provided with information regarding pharmacological agents used. The level of mucosal tissue injury was graded according to the 6-point Chiu scale as follows: 0- normal intestinal mucosa, 1- subepithelial fluid at the tips of the villi, 2- subepithelial fluid at the tips of the villi with partial mucosal desquamation, 3- massive desquamation from the tips of the villi, 4- totally desquamated mucosa with markedly dilated capillaries, 5- derangement of lamina propria, 6- necrosis of full thickness of the mucosal layer. A gross examination of the area of intestinal lesions was conducted by planimetry based on digital photography. Intestinal fragments taken at 5 cm distally from the Treitz ligament were collected and dissected, then photographed at controlled light conditions (artificial lighting). Two-stage deconvolution with spectral frequency analysis was performed then the percentage of ulcerated mucosa was estimated automatically on the basis of color contrast analysis of the injured and healthy mucosa.

**Hormones and drugs used for experimentation and experimental protocol**

Two experimental settings were designed to examine the importance of ghrelin in the mechanism of intestinal protection. In the first series of experiments, the animals were subjected to 30 min of ischemia followed subsequently by 60 min of reperfusion (short I/R), while in the second series, the animals were subjected to 60 min of ischemia subsequently followed by a 120 min reperfusion (long I/R). Ischemia was induced by application of a vascular clamp at the proximal part of the AMA then, after ischemia was completed, the clamp was removed and blood flow was restored. Three basic experimental settings were selected. First, exogenous ghrelin (50 μg/kg i.p.) was applied 30 min prior to the experiment with or without a short and long lasting I/R. Second, ghrelin (5000 ng/kg) was applied i.c.v. 30 min prior to the subsequent animal exposure to the short or long lasting I/R. In the third series of experiments, ghrelin was applied peripherally or centrally and the neural mechanisms of action of this hormone were studied in the animals with capsaicin-induced sensory denervation or those pretreated with selective adrenergic receptor antagonist or sensory system blockade or those who underwent vagotomy.

For this purpose, ghrelin (50 μg/kg i.p.) or (5000 ng/kg i.c.v.) was administered 30 minutes before ischemia with or without nadolol (0.5 mg/kg i.p.), 6-hydroxy dopamine (6-OHDA) (Sigma-Aldrich, Warsaw, Poland) administered i.p. in a single dose of 50 mg/kg or the CGRP receptor antagonist, CGRP$_{8-37}$ (100 μg/kg i.p.) or capsaicin (1% alcohol based solution) administered s.c. according to the protocol described above.

**Animals with vagotomy, sensory denervation and blockade of adrenergic system**

A selected group of animals with vagotomy were prepared. Briefly, the middle laparotomy was performed before ultrasonic and laser Doppler probes were set. The vagal nerves were identified with the use of a magnifying glass (4 x magnification) according to Walker guidelines (25). Bilateral vagotomy was performed by discontinuation of the vagal nerves subdiaphragmatically with micro scissors. Capsaicin-induced sensory denervation was performed a week before the start of the experiment using 1% capsaicin (Sigma) solution that was injected s.c. throughout the period of 3 consecutive days at the increasing doses of 20 mg/kg, 20 mg/kg and 50 mg/kg dissolved in 1 ml of alcohol/saline (20/80%) solution. Injections were performed under ether anesthesia. Sensory deactivation was confirmed by the eye wiping test. Adrenergic deactivation was achieved at 60 minutes before the experiment, using a selective neurotoxin 6-OHDA administered i.p. in the dose of 50 mg/kg.

**Experimental groups**

The animal groups were divided into four experimental series with each group in the series consisting of 7 animals. To study the intestinal circulatory and metabolic effects of the intestinal I/R with or without ghrelin administration applied centrally or peripherally prior and after the short and long ischemic period, the series A and B were established. In series A, the effects of ghrelin administered i.p. at the dose of 50 μg/kg was studied in the following group of rats: 1. ghrelin applied 30 min prior to short ischemic period (30/60 I/R); 2. ghrelin applied 30 min prior to long ischemic period (60/120 I/R); 3. ghrelin applied at the beginning of the reperfusion period in the model of short ischemic period (30/60 I/R), and 4. ghrelin applied at the beginning of reperfusion period, a procedure performed after the long ischemic period (60/120 I/R).

In series B, the effects of ghrelin administered i.c.v. at the dose of 5000 ng/kg was determined in the following groups of rats: 1. ghrelin applied 30 min prior to the short ischemic period (30/60 I/R); 2. ghrelin applied 30 min prior to long ischemic period (60/120 I/R); 3. ghrelin applied at the beginning of reperfusion period, a procedure performed after the short ischemic period (30/60 I/R), and 4. ghrelin applied at the beginning of reperfusion period, a procedure performed after the long ischemic period (60/120 I/R).

Additionally, series C of animals were employed to examine the effect of sensory denervation on the I/R-induced intestinal lesions in rats pretreated with vehicle (saline) or ghrelin (50 μg/kg i.p.) administered 30 minutes before the short I/R (30/60). The following groups of rats with an I/R in series C were used: 1. rats with intact sensory nerves pretreated with vehicle or ghrelin; 2. rats with capsaicin-induced deactivation of sensory nerves pretreated with vehicle and ghrelin; 3. rats with intact sensory nerves pretreated with CGRP$_{8-37}$, the selective CGRP receptor antagonist, co-administered with ghrelin.
Series D was used to determine the effect of vagotomy and suppression of the adrenergic system on the I/R-induced intestinal lesions in rats pretreated with ghrelin (5000 ng/kg i.c.v.) administered 30 minutes before the short I/R (30/60 min) period. The following groups of rats in series D, were used: 1. rats with intact sensory nerves pretreated with vehicle or ghrelin; 2. rats with bilateral vagotomy pretreated with vehicle and ghrelin; 2. rats with the chemical deactivation of sympathetic adrenergic system (chemical sympathectomy) by 6-OHDA pretreated with vehicle or ghrelin; 3. rats with the blockade of peripheral β adrenergic receptors by nadolol (50 mg/kg i.p.) pretreated with vehicle or ghrelin, and 4) rats with bilateral vagotomy and deactivation of sympathetic adrenergic system by 6-OHDA pretreated with vehicle or ghrelin.

**Statistical analysis**

The significance of changes in the measured parameters from control were determined using the Student’s t test for either paired data with a confidence limit of 0<0.05. Percentage differences in specific parameters were compared with control calculated as a mean average ±S.E.M.

**RESULTS**

*Effects of exogenous peripherally administered ghrelin before and after the short lasting I/R (30/60 min) period*

In this set of experiments, ghrelin was administered intraperitoneally 30 min before the onset of ischemia while the measurements of the mesenteric, intestinal blood flow and metabolic parameters (oxygen consumption) were determined at the end of the reperfusion period. Ghrelin applied at a dose of 50 µg/kg caused a significant increase in the mesenteric MBF and the intestinal microcirculatory blood flow (LDBF) by 42±6% and 48±7% when compared to the control group. No significant changes of VO2 were observed (Fig. 1). The intestinal mucosal lesion area was reduced by about 21% when compared to the control (Fig. 3) and reached 3 degree of intestinal impairment due to the histological assessment (Fig. 4).

**Effects of exogenous centrally administered ghrelin before and after the short lasting I/R (30/60 min) period**

Ghrelin was administered intraperitoneally 5 min after the end of ischemic period. A significant increase of the MBF and LDBF were observed by about 25% and 27% respectively, whereas VO2 was significantly increased by about 22% in comparison to the control group. No significant changes of AVO2 were observed (Fig. 1). The intestinal mucosal lesion area was reduced by about 29% in comparison to the control group. No significant changes of AVO2 were observed (Fig. 3). The intestinal mucosal lesion area was reduced by about 25% when compared to the control (Fig. 3) and reached 3 degree of intestinal impairment due to the histological assessment (Fig. 4).

When ghrelin was administered to the third ventricle 30 min before the onset of ischemia a significant increase of MBF and LDBF was observed by about 32% and 35%, respectively (Fig. 2). The value of VO2 and AVO2 were significantly increased by about 25% and 18% when compared to the control values respectively (Fig. 2). The intestinal mucosal lesion area was reduced by about 29% in comparison to the control group (Fig. 3) and reached 3 degree of intestinal impairment due to histological assessment (Fig. 4).

**Effects of exogenous peripherally and centrally administered ghrelin before and after the long lasting I/R (60/120 min) period**

In this series of experiments ghrelin was administered intraperitoneally 30 min before the onset of ischemia. Measurements of the MBF, the LDBF and metabolic parameters (VO2, AVO2) were determined at the end of reperfusion. Ghrelin was significantly increased by about 46±10% and 28±9%, respectively (Fig. 1). The area of the intestinal mucosal lesion was reduced by 38±7% in comparison to the placebo treated control (Fig. 3) and reached 1 degree of intestinal impairment based on histological assessment (Fig. 4).
administered at a dose of 50 µg/kg caused a significant increase of the mesenteric MBF and LDBF by about 28% and 33%, respectively when compared to the control group of placebo treated rats (Fig. 1). Ghrelin applied i.p. evoked a marked increase of VO₂ by about 28%, but AVO₂ remained unchanged. The intestinal mucosa lesion area was reduced by about 23% in comparison to the control placebo treated group (Fig. 3) and reached 2 degree of intestinal impairment observed histologically (Fig. 4).

When ghrelin was administered intraperitoneally 5 min after the beginning of the reperfusion period, a marked increase of MBF and LDBF by about 18% and 23%, respectively was observed when compared to the control group treated with placebo (Fig. 1). An increase of VO₂ by about 17% with no significant changes in the AVO₂ were observed (Fig. 1). The intestinal mucosa lesion area was reduced by about 19% when compared to the vehicle controlled group (Fig. 3), reaching the 4 degree of intestinal impairment under histological assessment (Fig. 4).

When ghrelin was administered i.c.v. to the third ventricle 15 min before or after ischemia, no significant changes in the MBF, LDBF, VO₂, and AVO₂ were observed in ghrelin treated rats when compared those treated with vehicle (Fig. 2). Ghrelin failed to reduce the mucosal lesion area, with no significant improvement of the histological index score of the intestinal mucosa was observed (Figs. 3 and 4).

**Fig. 2.** Effects of intracerebroventricular (i.c.v.) pretreatment with ghrelin (5000 ng/kg) on MBF, LDBF, VO₂, AVO₂. Ghrelin was administered before or after 30 or 60 minutes of intestinal ischemia. The intestinal circulatory and metabolic parameters were determined at the end of short or long lasting reperfusion period. Mean ±S.E.M. values are presented as percentage of change. Asterisk indicates a significant change (p<0.05) as compared to the control. Cross indicated significant difference (p<0.05) between the effects of ghrelin administered before and after ischemic period.

**Fig. 3.** Comparison of the effects of peripherally administered (50 µg/kg i.p.) and centrally administered ghrelin (5000 ng/kg) before and after 30 or 60 minutes duration of intestinal ischemia on I/R induced intestinal mucosa lesion area which was measured immediately after the end of short or long reperfusion period. Results are expressed as mean ±S.E.M. of 6 animals per group and expressed as percentage of change. Asterisk indicates a significant change (p<0.05) as compared to the control. Cross indicated significant difference (p<0.05) between the effects of ghrelin administered before and after ischemic period.
Effect of functional ablation of sensory afferent nerves by capsaicin on ghrelin-induced protection in rats exposed to short lasting I/R

As shown in Fig. 5, the pretreatment with ghrelin (50 µg/kg i.p.) 30 min prior to the short lasting I/R (30/60) period caused a similar changes in MBF, LDBF, VO₂ and AVO₂ as presented in Fig. 1. In contrast, the increase in MBF and LDBF induced by ghrelin were significantly decreased in rats with capsaicin denervation by about 21% and 23%, respectively. The intestinal metabolism as reflected by changes in the VO₂ was decreased by about 22% in capsaicin-denervated rats pretreated with ghrelin when compared to those with intact sensory nerves pretreated with ghrelin alone (Fig. 5). The intestinal lesion area

Fig. 4. Microscopic damage score in the intestinal mucosa of rats exposed to short (30/60) or long (60/120) duration of ischemia/reperfusion (I/R) in control animals and in those pretreated with ghrelin (50 µg/kg i.p.) or ghrelin administered i.c.v. in a dose of 5000 ng/kg. Ghrelin was administered before and after short or long lasting ischemic period. Results are means ±S.E.M. of 5 animals per experimental group compared with the value obtained immediately after control I/R. Cross indicates significant difference (p<0.05) between the effects of ghrelin administered before and after short or the long ischemia.

Fig. 5. Changes of MBF, LDBF, VO₂ and of the area of intestinal mucosal lesions (LA) induced by short duration of the intestinal I/R in rats pretreated with ghrelin (50 µg/kg i.p.) alone or ghrelin applied to animals with sensory denervation induced by chronic pretreatment with capsaicin or those pretreated with CGRP₈₋₃₇ fragment (100 µg/kg i.p.) Results are means ±S.E.M. of 6 animals per group. Asterisk indicates a significant change (p<0.05) as compared to the control. Cross indicates significant difference (p<0.05) between the effects of i.p. ghrelin administration after chronic sensory denervation with capsaicin or after CGRP₈₋₃₇ as compared to ghrelin alone.
(LA) was significantly increased by about 29% in capsaicin-denervated rats comparing to those with intact sensory innervation treated with ghrelin (Fig. 5). Sensory C fibers are known to release neuropeptides (especially CGRP) responsible for the vasodilatation of the mesenteric vessels. Therefore, in order to determine the involvement of sensory nerve endings releasing CGRP in ghrelin-induced intestinal protection, this peptide was administered prior to the short lasting I/R period in rats pretreated with CGRP$_{8-37}$. In rats pretreated with CGRP$_{8-37}$ the results were not significantly different from those obtained in animals with capsaicin-induced sensory denervation. Namely, the values of MBF, LDBF, VO$_2$ were significantly reduced and the intestinal lesion area assessed macroscopically, was significantly increased (Fig. 5). The microscopical assessment revealed that the intestinal damage was significantly increased in rats pretreated with CGRP$_{8-37}$ when compared to the respective values in ghrelin-treated rats without CGRP$_{8-37}$ administration.

**Effects of sympathectomy, β-adrenergic blockade and vagotomy on central ghrelin-induced intestinal protection against lesions induced by short lasting ischemia**

In this series of experiments, the effects of a chemical blockade of the adrenergic peripheral neurons with 6-OHDA (chemical sympathectomy) was employed to determine the effect of the blockade of adrenergic peripheral neurons in the intestinal protection induced by ghrelin. The protective action of ghrelin applied centrally before short lasting ischemia was markedly diminished in rats with sympathectomy when compared to the group of animals without pretreatment with 6-OHDA (Fig. 6). The MBF, LDBF and VO$_2$ were significantly decreased in sympathectomized rats by about 17%, 16% and 18%, respectively, as compared with those recorded in ghrelin treated rats without 6-OHDA pretreatment. To evaluate the role of cholinergic transmission in ghrelin-induced intestinal protection, we employed a separate group of animals with bilateral vagotomy. The values for MBF, LDBF and VO$_2$ values were significantly decreased in sympathectomized animals by about 18%, 16% and 20%, respectively, when compared to those measured with ghrelin alone. A significant increase in the area of intestinal lesion area by about 20% was observed in animals treated with 6-OHDA (Fig. 6).

The importance of β adrenergic receptors in ghrelin-induced intestinal protection was determined by means of their blockade by nadolol. To avoid a blockade of centrally located β receptors and interference with central actions of ghrelin, we have employed nadolol, a non-fat soluble β adrenergic receptor antagonist lacking an ability to cross the brain-blood barrier (BBB). A pretreatment with nadolol in ghrelin-pretreated rats resulted in a significant decrease of vascular relaxation noted previously in the group with centrally administered ghrelin (Fig. 6). Furthermore, there was no increase of resistance of the intestinal tissue to short ischemia on the basis of the area of mucosal lesions observed. An increase of the area of intestinal lesions ulcerations by 25% was observed in comparison to the ghrelin alone group (Fig. 6).

The hypothesis that another functional neural pathway is involved in ghrelin protection could not be excluded, and that is the reason, we had addressed this issue in a separate group of animals with bilateral vagotomy. In vagotomized rats, the MBF, LDBF and VO$_2$ values were decreased in rats treated with ghrelin by about 18%, 16% and 20%, respectively, while a significant increase of the area of gastric lesions by about 20% was observed when compared to the respective values in the ghrelin-treated rats exposed to a short I/R period (Fig. 7). An additional animal group with concomitant vagotomy and adrenergic blockades by 6-OHDA were employed. In this experimental group, the intestinal protective activity of central ghrelin expressed as a lesioned area (LA) was completely lost and this effect was accompanied by the significant inhibition of an increase in the MBF, LDBF and LA observed in ghrelin-treated animals (Fig. 7).

![Intestinal I/R (30/60 min)](image)

Fig. 6. Changes of MBF, LDBF, VO$_2$ and LA induced short duration of the intestinal I/R after pretreatment with ghrelin (i.c.v.) applied alone and in those pretreated with nadolol or 6-OHDA. Results are means ±S.E.M. of 6 rats per group. Asterisk indicates a significant change (p<0.05) as compared to the control. Cross indicates significant difference (p<0.05) as compared with rats pretreated with ghrelin alone.
DISCUSSION

Our study shows for the first time that ghrelin affords the protective activity against intestinal lesions induced by short and long lasting ischemia and subsequent reperfusion. This finding is in consistent with previous research by Sibilia et al. (18) and Brzozowski et al. (19), who described a protective role of intraperitoneally administered ghrelin against the formation of gastric lesions induced by noxious stimuli such as ethanol or gastric ischemia-reperfusion. Moreover, protective properties of ghrelin were described in other organs of the GI tract, namely the pancreas. The results of the studies performed in rats with intestinal subtotal ischemia evoked by acute clamping of SMA revealed the ability of ghrelin to induce protection of the intestinal tissue, which was evidenced after both the central and peripheral administration of this peptide.

We observed that ghrelin-induced protection was accompanied by a marked increase in the mesenteric and intestinal blood flow, which suggests a possible contribution of the intestinal microcirculation to the protective activity of this peptide. Therefore, we propose that ghrelin-induced intestinal protection is due to the ability of the hormone to induce vasodilatation and an increase in the delivery of oxygen and nutrients to the intestinal mucosa. Moreover, since we observed an increase in the oxygen consumption rate after a period of completed reperfusion, we conclude that ghrelin was responsible for the elevation of the metabolic activity of the intestinal tissue, which could correlate with increased resistance of the organ to the I/R-evoked impairment of intestinal mucosa. This remains consistent with our group previous observations that ghrelin-induced protection against gastric lesions caused by stress and ischemia-reperfusion is due to the increased blood flow in the stomach (19, 26). The effect of markedly elevated blood flow both in the intestinal wall capillaries and the mesenteric vessels induced by peripherally administered ghrelin might be at least in part explained by the presence of ghrelin receptors (GHS-1) at the blood vessel wall and ghrelin relaxing action on the smooth muscle of high resistance vessels, namely, arterioles (13, 27).

In the present study, ghrelin was applied peripherally or centrally before an ischemic period and at the beginning of reperfusion. Peripheral administration of ghrelin resulted in a significant difference between the circulatory, metabolic and damaging effects of the hormone when it was administered before or after induction of the ischemia period. A significant increase of mesenteric blood flow, intestinal blood flow and intestinal metabolism were observed in rats administered with ghrelin before the beginning of the ischemic period. This phenomenon was accompanied by an increase of intestinal resistance against I/R-induced intestinal damage as estimated by the decrease in the mucosal lesion area both macroscopically and microscopically. These protective effects of ghrelin administered peripherally were significantly decreased when the hormone was administered after the end of the period of short lasting ischemia or when ghrelin administered before ischemia in groups subjected to long I/R. Similarly, ghrelin significantly increased the mesenteric and intestinal blood flow and counteracted the intestinal mucosal impairment when applied centrally before and after short durations of ischemia.

We believe that central and peripheral ghrelin strongly affects microcirculation, especially in the endothelial layer, which may cause paresis of the smooth muscle layer and finally lessen reactivity to the activating stimuli. Brzozowski et al. (19) and Konturek et al. (28) demonstrated that ghrelin exerts a protective effect on the gastric mucosa against stress-induced lesions, and that the most important mechanism of ghrelin protective activity could be attributed to its local vasodilatory effect and relaxation of the muscular splanchnic arteries, which is in keeping with our hypothesis. Therefore, we think that it is reasonable to propose that the diminished reactivity of ghrelin observed in our model, particularly when hormone was applied after completed ischemia is due to an impairment of the intestinal resistance vessels with subsequent reduction in the
reactivity of circulating ghrelin. Another explanation might be the decrease of metabolism of the based on the decreased oxygen consumption, which reflects the local metabolic rate of the examined organ. This parameter was strongly affected by ischemia alone, which is known to cause internal tissue damage. This failure of intestinal metabolism and blood vessel impairment could attenuate their reactivity to endogenous or exogenous stimuli, such as circulating ghrelin. This peptide acts via GSHR receptors which possess high density in the healthy intestinal tissue (11, 12).

In case of centrally administered ghrelin, a significant vascular and metabolic effect with subsequent protective effects was observed only in the case of short ischemia when the hormone was given prior to the ischemic period. However, these effects were not observed when ghrelin was administered after short ischemic period before the onset of reperfusion. Moreover, we neither observed vascular nor metabolic effects of the hormone given both prior or after long lasting ischemia that finally led to the failure of intestinal viability.

These effects might be explained on the basis of high vulnerability of the neural tissue to toxic substances generated by the ischemic period in the intestinal compartment, which in turn affects the peripheral neurons. It is possible that ischemia-induced decreased conductivity of the mesenteric nerves which was mainly responsible for the lack of reactivity of centrally applied ghrelin (29, 30). We proved that centrally applied ghrelin acts through the sympathetic and parasympathetic visceral innervation suggesting that an impairment of the postganglionic fibers due to ischemia is responsible for the high reactivity of the intestinal tissue in response to centrally applied ghrelin. We assume that intestinal ischemia and hypoxia could impair the brain-gut axis circuit, thus interfering with the central protective effects of this peptide.

This study was designed to evaluate the neural mechanisms involved in the central effect of ghrelin on intestinal lesions because of the abundance of the ghrelin GSHR receptors existing in the midbrain. For instance, the afferent vagal sensory nerves shows an extensive connection with different parts of the CNS, that is crucial for the activity of the autonomic system (31, 32). It has been shown that central and peripheral ghrelin exhibits protection that depends on continuity and function of the vagal nerves. Brzozowski et al. (20) and Wu et al. (33), reported that intact vagal nerves are mandatory for the protective action of ghrelin to occur. Although involvement of the vagal nerves in the ghrelin-mediated protection of the stomach is proven, there is no data confirming its importance in the mediation of the protection of the intestine. Therefore, we attempted to examine the role of the vagal nerve in the protective effects of centrally administered ghrelin against lesions induced by intestinal ischemia and reperfusion. We found that after bilateral subdiaphragmatic ablation of vagal nerves, the ghrelin-induced protection was attenuated when compared to animals with intact innervation and this effect was accompanied by a marked decrease of mesenteric and intestinal blood flow. This finding remains in agreement with previous observation by Brzozowski et al. (20) who employed the same method of interruption of the vagal nerve, although utilizing a different route of ghrelin administration (peripheral vs. central) against gastric lesions. Despite these differences, both studies are in keeping with the notion that indeed vagal nerve activity is necessary to exert the protective effects of ghrelin in the gastrointestinal tract. Ghrelin might activate the cholinergic fibers on the sensory neuropeptide releasing fibers releasing vasoprotective mediators such as CGRP (34, 35). Since we observed significant but not complete abolishment of vascular, metabolic and protective effects of centrally administered ghrelin after vagotomy, an attempt was made to determine the role of the adrenergic system as the other possible descending pathway involved in the ghrelin mediation of signals from the small intestine brain to the gut. There are several pathways which might account for both peripherally and centrally acting ghrelin to activate the hypothalamic adrenergic centers including the NTS-ARC connections, moreover, the presence of ghrelin receptors on the hypothalamic adrenergic centers were documented (36, 37).

Our study shows that the sympathetic neurons could be involved in ghrelin-mediated protection, since after termination of the vagal nerve by vagotomy we failed to observe the complete reversal of the central ghrelin-induced protection against intestinal lesions. Thus, we assume that the "second pathway" in the brain-gut axis with regards to ghrelin-induced signals, might be considered as suggested previously (38). To examine the role of the adrenergic fibers, the chemical sympathectomy was performed by the selective deactivation of adrenergic fibers by the use of neurotoxin 6-hydroxydopamine (6-OHDA). We blocked the adrenergic descending neurons in vivo, both in rats with intact cholinergic innervation and those with vagotomy. In the group with intact vagal nerves a significant but only partial reduction of the ghrelin-induced protection and vascular effects in the intestine was observed. However, in the vagotomized rats the mesenteric circulation and intestinal metabolism as well as the protective activity of this hormone against I/R-evoked lesions were almost completely eliminated. We assumed that adrenergic nerves play a significant role in the transmission of ghrelin derived signals from the central nervous system to the periphery. The hypothesis that other functional neural pathway is involved in the protective mechanism of ghrelin could not be excluded. We conclude that both the vagal nerve and the adrenergic system are involved in ghrelin-induced intestinal protection against lesions caused by a short I/R period. Moreover, we have noticed that ghrelin-induced increase of the oxygen consumption was completely abolished, indicating the possible primary effect of adrenergic system in the metabolic effects of centrally applied ghrelin. Our results differ with the those of Brzozowski et al. (20) who reported no effects of functional ablation on the adrenergic system in the ghrelin-evoked protection of gastric mucosa. Those discrepancies might be simply explained by the divergences in the route of ghrelin administration (peripheral vs. central) and different model, namely gastric but not intestinal lesions, they reported in this particular study. Another difference could be attributed to the adrenergic system in the control of the gastric and intestinal circulation, which after of profound activation of the adrenergic neurons could induce a relaxation of resistance vessels of the intestine via activation of β-adrenergic receptors (39). To test whether β-adrenergic receptors are important in the ghrelin-mediated protection, we have designed an experiment where peripheral β-adrenergic receptors were selectively blocked by a non brain-blood barrier penetrating drug administered before a short I/R period when ghrelin was administered centrally. On the basis of pharmacological studies, it is established that vasodilatation in the mesenteric arteries, increase of metabolic activity, and the oxygen consumption are, at least in part, dependent on β-adrenergic receptors located on the mucosal layer of the mesenteric vessels. The β2 receptor antagonist - nadolol was used in animals pretreated with ghrelin injected i.c.v. to clarify the receptor site of this adrenergic activation by this peptide. According to the literature, it is assumed that ghrelin's central action depends on adrenergic receptors activity and mostly on β2 type of receptors. Under those experimental settings, we observed subsequent marked decrease of the mesenteric blood flow and intestinal blood flow with the concomitant rise of area of the mucosal lesions in comparison with the group without application of antagonist of
β2 receptors. Therefore, we can conclude that the adrenergic system contributes to the ghrelin-induced intestinal protection via an activation of the β2 adrenergic receptors. This contribution of adrenergic β2 receptors to ghrelin-induced intestinal protection might be due to the direct effects of this peptide on the mesenteric vasculature acting through β2 receptors and resulted in vasodilatation. In addition, ghrelin can activate the sensory C fibers via adrenergic β receptors. These C fibers were reported in the splanchnic nervous system as one of the effective mechanisms involved in control of blood flow and secretory functions of the intestine. This hypothesis which specifically addresses the mechanisms of ghrelin-evoked in the adrenergic-sensory interaction requires further studies.

The intestinal wall of rodents presents a dense innervation provided by spinal afferent nerves, which when activated releases different neuropeptides from their synaptic endings (23-35). Evidence has accumulated in the literature indicating that those neuropeptides such as CGRP and substance P (SP) are not only potent vasodilators but also exhibits the protective action against different damaging agents including I/R. Moreover, the sensory fibers are involved in ghrelin-evoked protection of the stomach against stress- and ethanol-induced gastric lesions and of caerulein-induced pancreatitis (20, 40). These observations seem to indicate that the sensory C fibers play an important role in the mediation of ghrelin-induced protection of the stomach and pancreas. In the present study, in animals with capsaicin-induced functional ablation of sensory nerves, the protective and hyperemic effects of peripherally administered ghrelin were eliminated. This supports the notion that ghrelin-induced protection is completely abolished in animals lacking sensory innervation in the different organs of the GI tract including stomach, the intestine and pancreas (19, 20). According to well established knowledge, neuropeptides released from the sensory neurons are capable to relax resistance vessels and therefore capable of increasing both the blood flow and the metabolic rate (41). That was also confirmed by our present observation as we failed to observe an increase in both mesenteric and local intestinal blood flow in animals with sensory denervation. Subsequent lack of the protective effects evoked by ghrelin in those animal groups supports the statement that C fibers play the prominent role in sustained blood flow, and the decreased resistance and increased mucosal viability in the intestine. Since the vasodilatory effects in the gut depends mostly on CGRP (41, 42), we blocked CGRP receptor to which CGRP is known to act as the selective agonist. We found that effects induced by this CGRP antagonist, CGRP38-43 were similar to that evoked by sensory ablation, suggesting CGRP plays a major role in this intestinal protective action of ghrelin. As CGRP is a neuropeptide known for the strong vasodilatory properties, those findings once again underlined the importance of sustaining the level of the flow of oxygen within the blood, and the delivery of nutrients for the survival of the integrity of the intestinal wall in terms of the consequences from I/R-evoked stress. It is not excluded that appetite hormones such as ghrelin can exert synergistic protective effect with glucocorticoids to counteract the deleterious effect of endogenous NADPH/angiotensin II system recently implicated in the pathogenesis of ischemia-reperfusion injury in the stomach (43, 44).

In conclusion, these results of our study revealed that ghrelin plays an important role in the protection of the intestine against short lasting I/R induced intestinal lesions. These protective and hyperemic effects were observed when ghrelin was applied both peripherally and centrally. The protection evoked by ghrelin could be attributed to its ability to increase mean blood flow (MBF) and to cause the local intestinal hyperemia. In terms of long-lasting I/R, the only peripheral but not central administration of ghrelin induced a significant increase of the intestinal mucosa resistance and this is, at least in part, due to the neural component activated by this hormone in the presence of I/R-induced stress. The protective effects of centrally applied ghrelin are mediated both by the vagal nerve and the sympathetic trunks, and the activation of peripherally located β2 adrenergic receptors is responsible for the vasodilatation and the observed intestinal protection. Protective effects of peripheral ghrelin might be due to sensory C fibers activation and CGRP release that seems to play a pivotal role in the protection; this may be due to its strong vasodilatory properties. Our study indicates that ghrelin and its analogs could be considered as a potent pharmacological tool useful both in the prevention and treatment of intestinal ischemia.

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