INTRODUCTION

Ghrelin, an acylated 28-amino acid polypeptide was primarily isolated from the human and rat stomach (1, 2), and circulating ghrelin is produced predominantly in gastric oxyntic mucosa (2). Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R). GHS-Rs are predominantly expressed in the pituitary and hypothalamus; however their presence has also been shown in other central and peripheral tissues, but at much lower levels (3). Acting on GHS-R, ghrelin strongly and dose dependently stimulates release of growth hormone from the anterior pituitary (1). Beside a release of growth hormone, ghrelin has been found to stimulate a release of adrenocorticotropic hormone, corticosterone, and prolactin (4, 5). Ghrelin stimulates food intake and fat deposition in adult animals (6) and humans (7). Plasma level of ghrelin is increased under negative energy balance conditions, such as fasting, anorexia nervosa or cachexia (2, 8, 9); whereas obesity (8, 10) and food intake (2, 8) decrease plasma ghrelin concentration.

Recent studies have shown that pretreatment with ghrelin exhibits protective effect in the gut. Administration of ghrelin reduces gastric mucosal damage, as well as inhibits the development of experimental pancreatitis. However, this protective effect requires administration of ghrelin before gastric or pancreatic damage and thus has a limited clinical value. The aim of present study was to assess the influence of ghrelin administered after development of acute pancreatitis on the course of this disease. Acute pancreatitis was induced by cerulein. Ghrelin was administered twice a day for 1, 2, 4, 6 or 9 days at the dose of 4, 8 or 16 nmol/kg/dose. The first dose of ghrelin was given 24 hours after last injection of cerulein. The severity of acute pancreatitis was assessed between 0 h and 10 days after cessation of cerulein administration. Administration of caerulein led to the development of acute edematous pancreatitis and maximal severity of this disease was observed 24 hours after induction of pancreatitis. Treatment with ghrelin reduced morphological signs of pancreatic damage such as pancreatic edema, leukocyte infiltration and vacuolization of acinar cells, and led to earlier regeneration of the pancreas. Also biochemical indexes of the severity of acute pancreatitis, serum activity of lipase and amylase were significantly reduced in animals treated with ghrelin. These effects were accompanied by an increase in the pancreatic DNA synthesis and a decrease in serum level of pro-inflammatory interleukin-1β. Administration of ghrelin improved pancreatic blood flow in rats with acute pancreatitis. We conclude that: (1) treatment with ghrelin exhibits therapeutic effect in caerulein-induced experimental acute pancreatitis; (2) this effect is related, at least in part, to the improvement of pancreatic blood flow, reduction in proinflammatory interleukin-1β and stimulation of pancreatic cell proliferation.

Key words: ghrelin, interleukin-1β, amylase, lipase, pancreatic regeneration, pancreatic blood flow
MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 150-170 g. Experimental protocol is in agreement with the “European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purpose” and was approved by the Local Commission of Ethics for the Care and Use of Laboratory Animals. Animals were housed in cages with wire mesh bottoms, with normal room temperature and a 12-hour light-dark cycle.

Acute pancreatitis was induced by cerulein (Sigma-Aldrich, GmbH, Steinheim, Germany) administered intraperitoneally (i.p.) 5 times with 1 hour intervals at a dose of 50 µg/kg/dose. Animals without induction of acute pancreatitis (control) were treated i.p. with saline at the same time as animals treated with cerulein.

Ghrelin was administered i.p. twice a day at the dose of 4, 8, or 16 nmol/kg/dose, the first dose was given 24 hours after last injection of cerulein. The severity of acute pancreatitis was assessed between 0 h and 10 days after cessation of cerulein administration. We used ten rats in each experimental group and each time of observation.

Active N-octanoyl rat ghrelin was synthesized in Yanaihara Institute by a solid phase methodology with Fmoc-strategy using automated peptide synthesizer (Applied Biosystem 9030 Pioneer, Foster, CA, USA) as described previously (16).

Determination of pancreatic blood flow

At the end of studies, rats were anesthetized with ketamine (50 mg/kg i.p., Bioketan, Vetoquinol Biowet, Gorzow Wielkopolski, Poland) and the abdominal cavity was opened. Pancreases were exposed for the measurement of pancreatic blood flow by laser Doppler flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Jarfalla, Sweden), as described previously (17). The pancreatic blood flow was presented as percent change from control value obtained in saline-treated rats without induction of acute pancreatitis.

Biochemical analysis of serum

After the measurement of pancreatic blood flow, arterial blood was taken from the abdominal aorta. Serum lipase and amylase activities were determined with a Kodak Ectachem DT II System analyzer (Eastman Kodak Company, Rochester, NY, USA) using Lipa and Amyl DT Slides (Vitros DT Chemistry System, Johnson & Johnson Clinical Diagnostic, Inc., Rochester, NY, USA).

Serum concentration of interleukin-1β was measured using the BioSource Cytoscreen rat IL-1β kit (BioSource International, Camarillo, California, USA) based on ELISA. Serum concentration of insulin was determined using Insulin (rat) high range enzyme immunoassay (ELISA) (Demeditec Diagnostic GmbH, Kiel, Germany). Serum concentration of glucose was determined with a Chemistry Vitros System analyzer FS 5.1 (Johnson & Johnson Gateway, Somerville, NJ, USA) using enzymatic method GOD-PAP (glucose oxidase-peroxidase-4-aminophenol phenol) (Biolabo, Maizy, France).

Determination of pancreatic DNA synthesis

After the blood withdrawal, the pancreas was carefully dissected out from its attachment to the stomach, duodenum, and spleen. Fat and peripancreatic tissues were trimmed away. The pancreas was weighed and samples of pancreatic tissue were taken for study of DNA synthesis and morphological examination. The rate of DNA synthesis was measured by incubation of minced pancreatic tissue at 37°C for 45 min in 2 ml of medium containing 8 µCi/ml of [3H]thymidine ([6-3H]-thymidine, 20-30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic), as described previously (18). DNA concentration in samples was determined by Giles and Myers procedure (19). DNA synthesis was expressed as [3H]thymidine disintegrations per minute per microgram DNA (dpm/µg DNA).

Histological examination of pancreatic damage

Morphological examination of pancreatic tissue was performed in hematoxilin and cosin stained slides as described previously in detail (20). Slides were examined by two experienced pathologist without knowledge of the treatment given (four slides per animal). The histological grading of edema, leukocytic inflammatory infiltration, vacuolization of acinar cells, hemorrhages and necrosis was made using a scale ranging from 0 (absent) to 3 for maximal alteration. Results of histological examination have been expressed as a predominant histological grading in each experimental group of animals.

Statistical analysis

Results, except histological data, have been expressed as means±S.E.M. Statistical analysis was made by analysis of variance followed by Tukey's multiple comparison test. A difference with a p value of less than 0.05 was considered significant.

RESULTS

Intraperitoneal administration of cerulein caused the development of acute edematous pancreatitis in all rats tested. Immediately, after induction of acute pancreatitis, pancreases were grossly swollen and enlarged with a visible collection of

Fig. 1. Morphological signs of pancreatic damage in acute cerulein-induced pancreatitis seen as pancreatic edema, inflammatory infiltration and vacuolization of acinar cells. In contrast to that, pancreatic islets (PI) exhibit regular intact morphology.
edematous fluid. At light microscopic level, interlobular and moderate intralobular edema was accompanied with moderate perivascular and scarce diffuse inflammatory leukocyte infiltration. Vacuolization was observed in 25-50% of acinar cells (Table 1). Maximal pancreatic edema was observed immediately after cerulein administration; whereas leukocytic inflammatory infiltration and vacuolization of acinar cells were maximally pronounced 24 hours after cerulein administration. Foci of hemorrhage were observed between the 1st and the 2nd day after induction of acute pancreatitis. Necrosis was not found in any time of observation. In contrast to changes observed in the exocrine pancreas, endocrine part of the pancreas was not affected by cerulein-induced pancreatitis. Morphology of pancreatic islets was normal without signs of tissue damage (Fig. 1). Morphological signs of acute pancreatitis were associated with biochemical markers of the severity of acute pancreatitis. Serum lipase (Fig. 2) and amylase (Fig. 3) activities were maximally increased immediately after the cessation of cerulein administration; whereas serum concentration of pro-inflammatory interleukin-1β reached maximal value 24 hours after induction of acute pancreatitis (Fig. 4).

### Table 1. Influence of ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on morphological signs of pancreatic damage in the course of cerulein-induced acute pancreatitis.

<table>
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<tr>
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<th>VACUOLIZATION (0-3)</th>
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Numbers represent the predominant histological grading in each group.
Fig. 2. Influence of ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on serum activity of lipase in the course of cerulein-induced pancreatitis. Mean±S.E.M. N=10 in each group of rats. *P<0.05 compared to control; #P<0.05 compared to cerulein alone at the same time of observation; &P<0.05 compared to cerulein + G4 alone at the same time of observation.

Fig. 3. Influence of ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on serum activity of amylase in the course of cerulein-induced pancreatitis. Mean±S.E.M. N=10 in each group of rats. *P<0.05 compared to control; #P<0.05 compared to cerulein alone at the same time of observation; &P<0.05 compared to cerulein + G4 alone at the same time of observation.

Fig. 4. Influence of ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on serum concentration of pro-inflammatory interleukin-1β in the course of cerulein-induced pancreatitis. Mean±S.E.M. N=10 in each group of rats. *P<0.05 compared to control; #P<0.05 compared to cerulein alone at the same time of observation.
pancreatitis caused the initial decrease in pancreatic blood flow (Fig. 5) and pancreatic DNA synthesis (Fig. 6) followed by a subsequent increase in these parameters during pancreatic regeneration. Maximal reduction in pancreatic DNA synthesis was observed 24 hours after cessation of cerulein administration; whereas pancreatic blood flow reached minimal value immediately after the last dose of cerulein.

Initially, after induction of acute pancreatitis, pancreatic weight was increased by 60% as a result of pancreatic edema (Fig. 7). This effect was followed by subsequent reduction in pancreatic weight. Minimal value of pancreatic weight was observed at the 2nd day of acute pancreatitis.

Pancreatic damage was followed by spontaneous tissue repair and 10 days after induction of pancreatitis, morphological features showed almost normal pancreatic histology, excepting minimal interlobular edema and scarce perivascular leukocytic infiltration in some cases (Table 1). Also serum activities of pancreatic enzymes (Fig. 2 and 3) and pancreatic weight (Fig. 7) reached control values at the 10th day of acute pancreatitis.

Treatment with ghrelin after induction of acute pancreatitis decreased the severity of this disease and accelerated pancreatic regeneration. In histological examination administration of ghrelin reduced pancreatic edema, inflammatory infiltration, vacuolization of acinar cells, and hemorrhages (Table 1). Ghrelin given at the dose of 8 or 16 nmol/kg/dose caused similar beneficial effect on pancreatic morphology, and this effect was stronger than effect of ghrelin given at the dose of 4 nmol/kg/dose. Pancreases of animals treated with ghrelin at the dose of 4 nmol/kg/dose, recovered normal pancreatic morphology after 7 days from induction of acute pancreatitis; whereas pancreases of animals treated with ghrelin given at the dose of 8 or 16 nmol/kg/dose, reached normal morphology after 5 days (Table 1).

Treatment with ghrelin reduced biochemical indexes of the severity of acute pancreatitis. Ghrelin reduced the pancreatitis-evoked increase in plasma activity of lipase (Fig. 2) and amylase (Fig. 3), and these effects were statistically significant for all doses between the 2nd and 3rd day after induction of acute pancreatitis. Ghrelin given at the dose of 8 or 16 nmol/kg/dose

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Fig. 5. Influence of ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on pancreatic blood flow in the course of cerulein-induced pancreatitis. Mean±S.E.M. N=10 in each group of rats. *P<0.05 compared to control; †P<0.05 compared to cerulein alone at the same time of observation.

Fig. 6. Influence of ghrelin given at the dose of 4, 8 or 16 nmol/kg/dose (G4, G8 or G16) on pancreatic DNA synthesis in the course of cerulein-induced pancreatitis. Mean±S.E.M. N=10 in each group of rats. *P<0.05 compared to control; †P<0.05 compared to cerulein alone at the same time of observation.
exhibited significantly stronger effect on serum activity of pancreatic enzymes than ghrelin given at the dose of 4 nmol/kg/dose (Fig. 2 and 3).

Administration of ghrelin reduced the pancreatitis-evoked increase in serum concentration of proinflammatory interleukin-1β (Fig. 4). This effect was significant between the 2nd and 7th day after induction of pancreatitis in animals treated with ghrelin at the dose of 8 or 16 nmol/kg/dose. Influence of ghrelin given at the dose of 4 nmol/kg/dose was weaker and significant effect on serum concentration of interleukin-1β was observed between the 3rd and 5th day after induction of acute pancreatitis.

Ghrelin improved pancreatic blood flow in animals with cerulein induced acute pancreatitis (Fig. 5). For ghrelin, given at the dose of 4 nmol/kg/dose, this effect was statistically significant only after the 3rd day after the induction of pancreatitis. Effect of ghrelin given at the dose of 8 or 16 nmol/kg/dose was more pronounced and statistically significant after the 2nd, 3rd, 7th and 10th day after the induction of acute pancreatitis.

Administration of ghrelin increased pancreatic cell proliferation measured as a rate of pancreatic DNA synthesis (Fig. 6). For ghrelin given at the dose of 8 or 16 nmol/kg/dose, this effect was observed between the 2nd and 10th day after induction of acute pancreatitis. Effect of ghrelin given at the dose of 4 nmol/kg/dose was weaker and apart from the 10th day induction of pancreatitis.

Administration of ghrelin increased pancreatic weight during regeneration of the pancreas, but this effect was statistically significant only at the 3rd day of pancreatitis (Fig. 7). Serum concentration of insulin and glucose in control intact rats was 0.37±0.04 nmol/l and 4.75±0.32 mmol/l, respectively (data not shown on separate figures). Induction of acute pancreatitis by cerulein was without effect on serum concentration of insulin or glucose. Also, administration of ghrelin was without significant effect on these both parameters.

DISCUSSION

The present study has shown, for the first time, that administration of ghrelin exhibits therapeutic effect in the course of acute pancreatitis. It was found as a faster normalization of pancreatic histology and a reduction in biochemical markers of acute pancreatitis.

In morphological features we have found that administration of ghrelin reduces pancreatic edema, vacuolization of acinar cells and inflammatory leukocytic infiltration of pancreatic tissue. Activation of leukocytes and release of proinflammatory cytokines are responsible for local pancreatic damage and development of systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) in the course of acute pancreatitis (21). Proinflammatory cytokines such as interleukin-1β, interleukin-6 and tumor necrosis factor-α (TNF-α) are produced within pancreas and subsequently within distant organs, which develop organ dysfunction during severe pancreatitis (22). Proinflammatory cytokine production is well correlated with disease severity (22) and interleukin-1β plays the essential role in the induction of systemic acute phase response and in the release of other members of pro-inflammatory cytokine cascade (23). Study performed by Norman et al. (24) has shown that administration of interleukin-1 receptor antagonist prevents the rise in serum interleukin-6 and TNF-α, and decreases severity of experimental acute pancreatitis. These data are in agreement with our present observation and probably describe one of the mechanisms involved in therapeutic effect of ghrelin in the course of acute cerulein-induced pancreatitis. We have found that the ghrelin-induced reduction in inflammatory infiltration of pancreatic tissue was associated with a decrease in serum concentration of proinflammatory interleukin-1β, leading to limitation of the severity of acute pancreatitis and acceleration of pancreatic regeneration. This anti-inflammatory effect of ghrelin in the pancreas can be a result of its action on immune cells or pancreatic cells, but most likely ghrelin inhibits inflammatory response in both types of these cells.

The relationship between immune and endocrine system is bidirectional (25). Immune cells secrete cytokines and possess their receptors, but also they are able to secrete various hormones and possess their receptors, which are believed to be specific for endocrine system. On the other hand, endocrine cells are able to produce and secrete cytokines, and their receptors. The presence of GHS-R has been reported in different immune cells, including human leukemic B, T and myeloid cell lines, human peripheral lymphocytes and neutrophils (26). GHS-R mRNA expression
was also shown in immune cells of other species such as in mouse splenic T cells (27, 28) or fish leukocytes (29). Immune cells not only express GHS-R, but also its endogenous ligand, ghrelin. The presence of mRNA for ghrelin has been found among others in leukemic B, T and myeloid human cell lines, and human peripheral lymphocytes and neutrophils (26). Ghrelin mRNA was also detected in the spleen, lymph nodes in humans (3), and in mouse splenic T lymphocytes (27, 28). Biological action of ghrelin, on the immune system, includes attenuation of septic shock (30, 31), promotion of thymopoiesis during aging in mice (28) and inhibition of expression of pro-inflammatory cytokines by human monocytes and T lymphocytes (32). The effect of ghrelin on phagocytic activity of leukocytes is controversial. Administration of ghrelin reduces phagocytic activity of peritoneal macrophages in rats exposed to cold-restraint stress (33). On the other hand, Yada et al. (29) have reported that administration of ghrelin stimulates phagocytosis and superoxide production in fish leukocytes, and this effect may be abolished by pretreatment with GHS-R antagonists. These results indicate that influence of ghrelin on phagocytosis may be different in different species. Ghrelin and GHS-R are also expressed in pancreatic islets (34-36) and pancreatic acinar cells (37). Study performed by Granata et al. (38) has shown that ghrelin prevents cell death and apoptosis of pancreatic ß cells. The influence of ghrelin on the vitality of pancreatic acinar cells on cellular level is unknown, but recent study performed by Kerem et al. (39) has shown that exogenous ghrelin enhances endocrine and exocrine regeneration of the pancreas in partly pancreatectomized rats. Our results are in agreement with these studies. Treatment with ghrelin reduced the severity of acute pancreatitis and accelerated pancreatic regeneration. This latter effect was found in morphological examination and as an increase in pancreatic DNA synthesis. DNA synthesis leads to replication of DNA and this process is necessary for cell division. For this reason rate of DNA synthesis is recognized as an index of cell proliferation. In our present study, morphological features have shown that pancreatic damage in cerulein-induced pancreatitis is limited to the exocrine pancreas. Pancreatic islets have exhibited normal regular morphology. Also serum levels of insulin and glucose were not affected by acute pancreatitis. This last observation is in agreement with clinical data which indicate that hyperglycemia occurs mainly in patients with severe cases of this disease (40). Moreover hyperglycemia in patients with acute pancreatitis is often associated with hyperinsulinemia. For this reason, our results showing the ghrelin-evoked increase in pancreatic DNA synthesis, should be recognized as a marker of stimulation of acinar cells proliferation. This concept is additionally supported by the observation that ß cells have only a very limited potential for regeneration (41), as well as less than 1% of pancreatic cells is localized in pancreatic islets (42). Therapeutic effect of ghrelin in acute pancreatitis seems to be also related to its influence on pancreatic blood flow. Clinical and experimental studies have shown that pancreatic ischemia plays an important role in the initiation of pancreatitis, or the progression to necrotizing pancreatitis (43-45). Microvascular perfusion failure is known to be essential in the development of acute pancreatitis in various clinical settings including cardiac (44) or aortic (46) surgery, hypovolemic shock (47) and transplantation of the pancreas (48). Experimental studies have shown that the severity of acute pancreatitis is closely related to tissue ischemia (49). Moreover, the improvement of pancreatic blood flow (16, 50, 51), as well as anticoagulative treatment (52, 53) inhibit the development of acute pancreatitis and accelerate pancreatic regeneration in the course of this disease. In our present study, induction of acute pancreatitis by cerulein caused initial reduction of pancreatic blood flow followed by subsequent increase in this parameter. Administration of ghrelin significantly enhanced the increase in pancreatic blood flow in rats with acute pancreatitis, leading to faster pancreatic regeneration and reduction in inflammatory response. This observation is in harmony with findings that ghrelin stimulates angiogenesis and deficiency of endogenous ghrelin is involved in ageing-related impairment of angiogenesis (54).

A cause-effect relationship between changes of pancreatic blood flow, an increase in pancreatic DNA synthesis, and healing effects of ghrelin is not clear. Previous studies have shown that an increase in pancreatic blood flow and DNA synthesis may be a cause or/and a result of improvement of pancreatic condition. An increase in pancreatic blood flow reduces the severity of pancreatic damage, but simultaneously a reduction in pancreatic damage improves pancreatic blood flow (50, 51). Another question is whether changes of pancreatic blood flow and DNA synthesis result from the amelioration of pancreatitis or they are caused directly by ghrelin. Our previous study has shown that short-time administration of ghrelin does not significantly affect pancreatic DNA synthesis and pancreatic blood flow in control rats without induction of acute pancreatitis; whereas administration of ghrelin in rats with induction of acute pancreatitis, reduces the pancreatitis-evoked decrease in these parameters (16). This observation suggests that beneficial effect of ghrelin is indirect and is related to reduction of pancreatic damage. This conclusion was additionally supported by data obtained in hypophysectomized rats. Hypophysectomy abolishes the ghrelin-induced increase in serum concentration of growth hormone and IGF-1, as well as eliminates protective effect of ghrelin in acute pancreatitis (16). These observations indicate that ghrelin effect on the pancreas is indirect and mediated, at least in part by growth hormone and IGF-1. Serum activity of lipase and amylase is a well established index of acute pancreatitis severity with high sensitivity and specificity (55). In our present study, treatment with ghrelin has reduced the pancreatitis-evoked increase in serum activity of pancreatic digestive enzymes. This observation is another evidence of therapeutic effect of ghrelin in the course of acute pancreatitis.

Our observation regarding therapeutic effect of ghrelin in acute pancreatitis has raised the question whether this effect has physiological or pharmacological nature. Previous study has shown that induction of acute pancreatitis does not significantly affect serum level of endogenous ghrelin in rat model of this disease (16). Plasma level of ghrelin increases under negative energy balance. In humans, fasting (2), anorexia nervosa (8) or cachexia (9) increase plasma or serum level of ghrelin by about 30, 120 or 33%, respectively; whereas obesity (8, 10) and food intake (2, 8) decrease plasma ghrelin concentration by about 30%. The same effect is observed in rats, fasting increases plasma concentration of ghrelin by about 20% (56) to 30% (57). These data indicate that plasma level of endogenous ghrelin may rose maximally by about 120%. On other hand, previous study has shown that administration of ghrelin at the dose of 4, 8 or 16 nmol/kg/dose increases serum concentration of ghrelin by about 150, 300 and 430%, respectively. These observations indicate that therapeutic effect of ghrelin in the course of cerulein-induced pancreatitis has pharmacological nature. Finally, we conclude that ghrelin exhibits therapeutic effect in caerulein-induced experimental acute pancreatitis and this effect is related, at least in part, to the improvement of pancreatic blood flow, reduction in pro-inflammatory interleukin-1ß and stimulation of pancreatic cell proliferation.

Acknowledgements: This study was partly supported by the Polish Ministry of Science and Higher Education (Project N N401 000635) and grants from Jagiellonian University.

Conflict of interests: None declared.
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Received: November 17, 2009
Accepted: July 15, 2010

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