ROLE OF HORMONAL AXIS, GROWTH HORMONE - IGF-1, IN THERAPEUTIC EFFECT OF GHRELIN IN THE COURSE OF CERULEIN-INDUCED ACUTE PANCREATITIS

Ghrelin is a ligand for growth hormone secretagogue receptor and stimulates release of growth hormone (GH). Recent studies have shown that treatment with ghrelin exhibits protective and therapeutic effect in the course of experimental pancreatitis. The aim of present study was to examine the role of GH and insulin-like growth factor-1 (IGF-1) in these effects. Acute pancreatitis was induced by cerulein. Study was performed on pituitary-intact hypophysectomized rats. Ghrelin was administered twice a day at the dose of 8 nmol/kg/dose. IGF-1 was given twice a day at the dose of 20 nmol/kg/dose. The severity of acute pancreatitis was assessed 0 h or 1, 2, 3, 5 and 10 days after the last dose of cerulein. Administration of cerulein led to the development of acute edematous pancreatitis. In pituitary-intact rats, treatment with ghrelin reduced biochemical indexes of the severity of acute pancreatitis and morphological signs of pancreatic damage, leading to faster regeneration of the pancreas reduction in serum concentration of pro-inflammatory interleukin-1β and decrease in serum activity of amylase and lipase. These effects were accompanied with an improvement of pancreatic blood flow and an increase in pancreatic DNA synthesis. Hypophysectomy delayed the healing of the pancreas and abolished the therapeutic effect of ghrelin. In hypophysectomized rats with pancreatitis, treatment with IGF-1 exhibits therapeutic effect similar to that observed in ghrelin-treated rats with the intact pituitary. We conclude that therapeutic effect of ghrelin in cerulein-induced pancreatitis is indirect and depends on the release of GH and IGF-1.

Key words: acute pancreatitis, amylase, ghrelin, hypophysectomy, interleukin-1β, lipase, pancreatic regeneration

INTRODUCTION

Ghrelin, an acylated 28-amino acid polypeptide was primary isolated from the human and rat stomach, and the stomach is main source of circulating ghrelin (1, 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 level (3). Acting on GHS-R, ghrelin strongly and dose dependently stimulates release of growth hormone (GH) from the anterior pituitary (1). Beside a release of GH, ghrelin stimulates food intake and fat deposition (4-6), as well as exhibits protective and therapeutic effect in numerous organs, including the gut (7). Treatment with ghrelin protects gastric mucosa against damage evoked by ethanol (8), stress (9) or alendronate (10), as well as accelerates healing of gastric and duodenal ulcers evoked by acetic acid (11), and duodenal ulcers evoked by cysteamine (12). Clinical and animal data indicate that ghrelin reduces inflammation in the colon (13). In the pancreas, pretreatment with ghrelin inhibits the development of cerulein- and ischemia/reperfusion-induced acute pancreatitis (14, 15). Our experimental study has also shown that administration of ghrelin accelerates recovery in the course of cerulein-induced pancreatitis (16). Therapeutic effect of ghrelin in this disease can be a result of direct anti-inflammatory and healing-promoting action of ghrelin or may be indirect effect mediated by GH and insulin-like growth factor-1 (IGF-1).

The concept of direct anti-inflammatory action of ghrelin is supported by findings that GHS-Rs are expressed in pancreatic islets and acinar cells (17-19), as well as in different immune cells involved in the development of inflammation. The presence of GHS-R has been found in human leukemic B, T and myeloid cell lines, human peripheral lymphocytes and neutrophils (20), mouse splenic T cells (21, 22), and fish leukocytes (23). Biological action of ghrelin, on the immune system, includes attenuation of septic shock (24, 25), promotion of thymopoiesis during aging in mice (22) and inhibition of expression of pro-inflammatory cytokines by human monocytes and T lymphocytes (26).

The concept of indirect therapeutic effect of ghrelin in acute pancreatitis is supported by observation that ghrelin strongly
stimulates release of GH and IGF-1 in peripubertal and adult subjects (1, 27). GH is the first step in GH-IGF-1-hepatocyte growth factor (HGF) hormonal axis (28-30). Treatment with GH (31, 32), IGF-1 (15, 33), as well as HGF (34, 35) has been shown to reduce pancreatic damage in experimental acute pancreatitis. Presence of GH receptors has been detected in pancreatic islets and acinar cells (36, 37), as well as in immune cells (38). Also, receptors for IGF-1 are present in the endocrine (39) and exocrine (40) pancreas, and lymphocytes (41, 42). Moreover pancreatic expression of mRNA for IGF-1 and IGF-1 receptor has been found to increase during pancreatic regeneration in acute pancreatitis (43, 44).

The aim of present study was to examine the role of GH and IGF-1 in therapeutic effects of ghrelin in the course of acute pancreatitis. To solve this problem, we have used pituitary-intact rats and hypophysectomized rats. Hypophysectomy allows to remove endogenous source of GH and reduces endogenous level of IGF-1 by 90% (15).

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, at room temperature and with a 12-hour light-dark cycle.

Rats were anesthetized with pentobarbital (30 mg/kg i.p., Vetbutal, Biowet, Pulawy, Poland) and sham-operated or hypophysectomized via the transauricular approach according to a method described previously (45). Two weeks later, 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 8 nmol/kg/dose, the first dose was given 24 hours after last injection of cerulein. 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 (47). DNA concentration in samples was determined by Giles and Myers procedure (48). 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 hematoxlin and eosin stained slides as described previously in detail (49). 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. At the time 0 h at light microscopic level, interlobular and moderate intralobular edema was accompanied with moderate perivascular and scarce diffuse inflammatory leukocyte infiltration. Vacuolization was observed in more than 50% of acinar cells (Table 1). A the time of observation, pancreatic blood flow was presented as percent change from control value obtained in saline-treated rats without induction of acute pancreatitis.
edema, leukocytic inflammatory infiltration and vacuolization of acinar cells were maximally pronounced immediately after cerulein administration. Foci of hemorrhage were observed in some cases at the 2nd day after induction of acute pancreatitis. Necrosis was not found in any time of observation.

Morphological signs of acute pancreatitis were associated with biochemical markers of the severity of acute pancreatitis. Serum amylase (Fig. 1) and lipase (Fig. 2) activities were maximally increased immediately after the cessation of cerulein administration; whereas serum concentration of pro-inflammatory interleukin-1β reached maximal value 2 days after induction of acute pancreatitis (Fig. 3). Cerulein-induced pancreatitis caused the initial decrease in pancreatic blood flow (Fig. 4) and pancreatic DNA synthesis (Fig. 5) followed by a subsequent increase in these parameters during pancreatic regeneration.

Pancreatic damage was followed by spontaneous tissue repair and 10 days after induction of pancreatitis, morphological features showed almost normal pancreatic histology, apart from minimal interlobular edema and scarce perivascular leukocytic infiltration in some cases (Table 1). Also serum activities of pancreatic enzymes (Fig. 1 and 2) and pancreatic DNA synthesis (Fig. 5) 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 pituitary-intact rats. Histological examination has shown that administration of ghrelin reduces pancreatic edema, inflammatory infiltration, vacuolization of acinar cells, and hemorrhages (Table 1). Treatment with ghrelin reduced the pancreatitis-evoked increase in plasma activity of amylase (Fig. 1) and lipase (Fig. 2), and these effects were statistically significant between the 2nd and 3rd day after induction of acute pancreatitis. Administration of ghrelin reduced the pancreatitis-evoked increase in serum concentration of pro-inflammatory interleukin-1β (Fig. 3). This effect was significant between the

| Numbers represent the predominant histological grading in each group. |

| Table 1. Influence of ghrelin (G), hypophysectomy (HP) and IGF-1 administration on morphological signs of pancreatic damage in the course of cerulein-induced acute pancreatitis. |
|-----------------------------------------------|-------------|-------------|-------------|-------------|-------------|
| CONTROL                                  | 0           | 0           | 0           | 0           | 0           |
| HP                                       | 0-1         | 0           | 0           | 0           | 0           |
| CERULEIN th                               | 2           | 2           | 3           | 0           | 0           |
| CERULEIN 2 days                          | 1           | 2           | 1           | 0           | 0           |
| CERULEIN 2 days+G                        | 0-1         | 2           | 0-1         | 0           | 0           |
| CERULEIN 2 days+IGF-1                    | 0-1         | 2           | 1           | 0           | 0           |
| CERULEIN 3 days                          | 1           | 1           | 0           | 0           | 0           |
| CERULEIN 3 days+G                        | 0           | 0           | 0           | 0           | 0           |
| CERULEIN 5 days                          | 1           | 2           | 0           | 0           | 0           |
| CERULEIN 5 days+G                        | 0           | 0           | 0           | 0           | 0           |
| CERULEIN 10 days                         | 1           | 1           | 0           | 0           | 0           |
| CERULEIN 10 days+G                       | 0           | 0           | 0           | 0           | 0           |
| CERULEIN 10 days+IGF-1                   | 0           | 0           | 0           | 0           | 0           |
2nd and 5th day after induction of pancreatitis in pituitary-intact animals. Ghrelin improved pancreatic blood flow in animals with cerulein induced acute pancreatitis (Fig. 4). This was statistically significant at the 2nd, 3rd and 10th day after the induction of acute pancreatitis. In pituitary-intact rats, administration of ghrelin increased pancreatic cell proliferation measured as a rate of pancreatic DNA synthesis (Fig. 5). This effect was statistical significant between the 2nd and 10th day of acute pancreatitis.

Hypophysectomy alone was without effect on parameter tested (Figs 1-5) apart from mild interlobular edema of the pancreas observed in some cases (Table 1).

Cerulein-induced acute pancreatitis was more severe in hypophysectomized rats than in pituitary-intact rats. Pancreatic edema, inflammatory infiltration and vacuolization of acinar cells was more pronounced at the 2nd, 3rd and 10th day of acute pancreatitis (Table 1). These changes were associated with a significant increase in serum activity of pancreatic enzymes (Fig. 1 and 2) and serum concentration of interleukin-1β (Fig. 3). Pancreatic blood flow (Fig. 4) and pancreatic DNA synthesis (Fig. 5) were lower than in pituitary-intact rats with acute pancreatitis.

Administration of ghrelin was without significant effect on the pancreatic damage evoked by cerulein-induced pancreatitis in hypophysectomized rats. Pancreatic edema, inflammatory infiltration and vacuolization of pancreatic acinar cells reached the same grade as in treated with saline hypophysectomized rats with acute pancreatitis (Table 1). Also, biochemical parameters of acute pancreatitis (Figs 1-3), pancreatic blood flow (Fig. 4) and pancreatic DNA synthesis (Fig. 5) were similar.

In hypophysectomized rats with cerulein-induced pancreatitis, administration of IGF-1 significantly reduced biochemical indexes of the severity of acute pancreatitis such as serum activity of amylase (Fig. 1) and lipase (Fig. 2), and serum concentration of pro-inflammatory IL-1β (Fig. 3). Moreover, treatment with IGF-1 significantly reversed the pancreatitis-evoked fall of pancreatic blood flow (Fig. 4) and pancreatic DNA synthesis (Fig. 5). These parameters (Figs 1-5) reached values similar to that as in pituitary intact rats treated with ghrelin after induction of acute pancreatitis. Moreover, morphological features demonstrated a reduction in pancreatic edema, inflammatory infiltration and vacuolization of pancreatic...
Fig. 3. Influence of ghrelin (G), hypophysectomy (HP) and IGF-1 administration on serum concentration of interleukin-1β in the course of cerulein-induced pancreatitis (CIP). Mean±S.E.M. N=10 in each group of rats. aP<0.05 compared to control; bP<0.05 compared to CIP alone in pituitary-intact rats at the same time of observation; cP<0.05 compared to CIP alone in hypophysectomized rats at the same time of observation.

Fig. 4. Influence of ghrelin (G), hypophysectomy (HP) and IGF-1 administration on pancreatic blood flow in the course of cerulein-induced pancreatitis (CIP). Mean±S.E.M. N=10 in each group of rats. aP<0.05 compared to control; bP<0.05 compared to CIP alone in pituitary-intact rats at the same time of observation; cP<0.05 compared to CIP alone in hypophysectomized rats at the same time of observation.

Fig. 5. Influence of ghrelin (G), hypophysectomy (HP) and IGF-1 administration on pancreatic DNA synthesis in the course of cerulein-induced pancreatitis (CIP). Mean±S.E.M. N=10 in each group of rats. aP<0.05 compared to control; bP<0.05 compared to CIP alone in pituitary-intact rats at the same time of observation; cP<0.05 compared to CIP alone in hypophysectomized rats at the same time of observation.
acinar cells in hypophysectomized rats treated with IGF-1 after induction of acute pancreatitis (Table 1).

**DISCUSSION**

Previous studies have shown that administration of ghrelin exhibits protective (14, 15) and therapeutic (16) effect in acute pancreatitis in pituitary-intact rats. Our present observation is in agreement with these data. Ghrelin administration caused a faster normalization of pancreatic histology, as well as a reduction in biochemical markers of acute pancreatitis. Morphological examination has shown that treatment with ghrelin reduces pancreatic edema, vacuolization of acinar cells and inflammatory leukocytic infiltration of pancreatic tissue. Reduction in inflammatory infiltration was associated with reduction in serum concentration of pro-inflammatory interleukin-1β and serum activity of pancreatic enzymes, amylase and lipase. In acute pancreatitis, pro-inflammatory cytokines such as interleukin-1β, interleukin-6 and tumor necrosis factor-α (TNF-α) are produced within pancreas and subsequently within distant organs, and severity of acute pancreatitis is well correlated with the level of these cytokines (50).

Serum activity of lipase and amylase is a well established index of acute pancreatitis severity with high sensitivity and specificity (51). The ghrelin-evoked reduction in serum activity of pancreatic digestive enzymes in pituitary-intact rats with acute pancreatitis is another evidence of therapeutic effect of ghrelin in the course of acute pancreatitis. Clinical and experimental studies have shown that pancreatic ischemia plays an important role in the initiation of acute pancreatitis, or the progression of this disease to necrotizing pancreatitis (52-54). Severity of acute pancreatitis is closely related to tissue ischemia (55) and an improvement of pancreatic blood flow (16, 56, 57), as well as anticoagulative treatment (58, 59, 60) inhibit the development of acute pancreatitis and accelerate pancreatic recovery. 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 (16, 34, 56, 59), as well as our present study have shown that induction of acute pancreatitis by cerulein leads to initial inhibition of pancreatic DNA synthesis followed by subsequent increase in this parameter. In our present study, administration of ghrelin has increased pancreatic DNA synthesis in pituitary-intact rats with acute pancreatitis. This observation indicates that therapeutic effect of ghrelin in acute pancreatitis depends at least in part, on growth promoting effect of this peptide.

The most important finding of our present study is the observation that therapeutic effect of ghrelin in acute cerulein-induced pancreatitis is indirect and depends on the release of growth hormone and IGF-1. This conclusion is supported by results obtained in pituitary-intact and hypophysectomized rats. In pituitary-intact rats, our study has shown that treatment with ghrelin exhibits therapeutic effect in acute pancreatitis. On the other hand, it is known that administration of ghrelin at the dose of 8 nmol/kg/dose in rats with intact hormonal axis, ghrelin-growth hormone-IGF-I, leads to the increase in serum concentration of growth hormone and IGF-1 by about 70% and 150%, respectively (15). Moreover, treatment with growth hormone (31, 32) and IGF-1 (15, 33) has been shown to reduce pancreatic damage in experimental acute pancreatitis. These data suggest involvement of growth hormone and IGF-1 in therapeutic effect of ghrelin in cerulein-induced acute pancreatitis.

Hard evidences that healing effect of ghrelin in acute pancreatitis is related to growth hormone and IGF-1 release, have been obtained in experiments with hypophysectomized rats. First of all, previous studies have shown that hypophysectomy increases serum concentration of endogenous ghrelin by about 60% (11); however our present study has shown that hypophysectomy inhibits pancreatic regeneration and increases the severity of acute pancreatitis. It has been manifested as an increase in morphological signs of pancreatic damage, serum activity of pancreatic digestive enzymes and serum concentration of pro-inflammatory interleukin-1β. These findings were associated with reduction in pancreatic blood flow and pancreatic DNA synthesis.

Next support for the conclusion that healing effect of ghrelin in the course of acute pancreatitis depends on the release of IGF-1 brings the observation that administration of ghrelin in hypophysectomized rats failed to exhibit any therapeutic effect in acute pancreatitis. Morphological features of pancreatic tissue, serum level of amylase, lipase and interleukin-1β, as well as pancreatic blood flow and pancreatic DNA synthesis were the same as in in hypophysectomized rats without treatment with ghrelin. On the other hand, it must be pointed out that hypophysectomy induces not only a lack growth hormone and IGF-1 but also insufficiency of other pituitary hormones, which may affect the course of acute pancreatitis. To solve this problem, we have used hypophysectomized rats treated with IGF-1 at the dose of 20 nmol/kg/dose. This dose of IGF-1 increases serum concentration of this peptide to the level observed in pituitary-intact rats treated with ghrelin at the dose 8 nmol/kg/dose (11). Our present study has shown that administration of IGF-1 in hypophysectomized rats reduces morphological signs of pancreatic damage and biochemical indexes of the severity of acute pancreatitis, as well as accelerates recovery in this disease. This observation brings additional evidence that healing effect of ghrelin in acute pancreatitis depends on growth hormone and IGF-1 release. Similar, IGF-1-related therapeutic effect of ghrelin has been observed by us in the stomach and duodenum (11).

Our findings are in opposition to reports that ghrelin acting on GHS-Rs may directly inhibit expression of pro-inflammatory cytokine and reduce pro-inflammatory response and nuclear-kB activation (26, 61). These discrepancies suggest that direct anti-inflammatory effect of ghrelin is insufficient to reduce inflammatory response in serious inflammation such as acute pancreatitis, as well as that the hypophysectomy-induced reduction in serum level of IGF-1 totally abolishes direct therapeutic effect of ghrelin in the course of acute pancreatitis. Finally, we conclude that treatment with ghrelin accelerates recovery in the course of acute pancreatitis and this effect is indirect and depends on the release of growth hormone and IGF-1.

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