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J. JAWOREK[*], J. BONIOR[***], A. LEJA-SZPAK[***], K. NAWROT[***], M. KOT[***], R. TOMASZEWSKA[**], J.STACHURA[**], W.W. PAWLIK[*], S.J. KONTUREK[*]

SENSORY NERVES IN CENTRAL AND PERIPHERAL CONTROL OF PANCREATIC INTEGRITY BY LEPTIN AND MELATONIN

[*] Department of Physiology
[**] Department of Pathology
[***] Faculty of Health Care
University School of Medicine Jagiellonian University Kraków Poland

Central nervous system affects pancreatic secretion of enzymes however, the neural modulation of acute pancreatitis has not been investigated. Leptin and melatonin have been recently reported to affect the inflammatory response of various tissues. The identification of specific receptors for both peptides in the pancreas suggests that leptin and melatonin could contribute to the pancreatic protection against inflammation. The aim of this study was: 1/ to compare the effect of intracerebroventricular (i.c.v.) or intraperitoneal (i.p.) administration of leptin or melatonin on the course of caerulein-induced pancreatitis (CIP) in the rat, 2/ to examine the involvement of sensory nerves (SN) and calcitonin gene-related peptide (CGRP) in pancreatic protection afforded by leptin or melatonin, 3/ to assess the effect of tested peptides on lipid peroxidation products (MDA + 4-HNE) in the pancreas of CIP rats, 4/ to investigate the influence of leptin or melatonin on nitric oxide (NO) release from isolated pancreatic acini and 5/ to determine the effects of caerulein and leptin on leptin receptor gene expression in these acini by RT-PCR. CIP was induced by subcutaneous (s.c.) infusion of caerulein (25μg/kg) to the conscious rats, confirmed by the significant increases of pancreatic weight and plasma amylase and by histological examination. This was accompanied in marked reduction of pancreatic blood flow and significant rise of MDA + 4-HNE in the pancreas. Leptin or melatonin were administered i.p. or i.c.v. 30 min prior to the start of CIP. Deactivation of SN was produced by s.c. capsaicin (100 mg/kg). An antagonist of CGRP, $CGRP_{8-37}$ (100 μg/kg i.p.), was given together with leptin or melatonin to the CIP rats. MDA + 4-HNE was measured using LPO commercial kit. NO was determined using the Griess reaction. Pretreatment of CIP rats with i.p. leptin (2 or 10 μg/kg) or melatonin (10 or 50 mg/kg) significantly attenuated the severity of CIP. Similar protective effects were observed following i.c.v. application of leptin (0.4 or 2 μg/rat) but not melatonin (10 or 40 μg/rat) to the CIP rats. Capsaicin deactivation of SN or administration of $CGRP_{8-37}$ abolished above beneficial effects of leptin on CIP, whereas melatonin-induced protection of pancreas was unaffected. Pretreatment with i.p. melatonin (10 or 50 mg/kg), but not leptin, significantly reduced MDA + 4-HNE in the pancreas of CIP rats. Leptin (10^{-9} - 10^{-6} M) but not melatonin (10^{-8} - 10^{-5} M) significantly stimulated NO release from isolated pancreatic acini. Leptin receptor gene expression in these acini was significantly increased by caerulein and leptin. We conclude that 1/ central or peripheral pretreatment with leptin protects the pancreas against its damage induced by CIP, whereas melatonin exerts its protective effect only when given i.p., but not following its i.c.v. adminstration, 2/ activation of leptin receptor in the

pancreatic acini appears to be involved in the beneficial effects of leptin on acute pancreatitis, 3/ the protective effects of leptin involve sensory nerves, CGRP and increased generation of NO whereas melatonin-induced protection of the pancreas depends mainly on the antioxidant local effect of this indole, and scavenging of the radical oxygen species in the pancreatic tissue.

K e y w o r d s: *leptin, melatonin, sensory nerves, lipid peroxidation, nitric oxide, leptin receptor gene expression*

INTRODUCTION

Previous reports have shown that central nervous system (CNS) plays and important role in the regulation of exocrine pancreatic functions. It has been demonstrated that administration of CGRP or dopamine directly into the brain is able to inhibit pancreatic enzyme secretion, whereas secretin or TRH given to the central nervous system produced stimulation of this secretion (1 – 4).

So far no study was undertaken to determine the effects of intracerebroventricular (i.c.v.) administration of hormones on the course of acute pancreatitis and on the pancreatic integrity.

Recently two hormones leptin and melatonin received particular attention due to their ability to modulate the immune response of the organism (5 - 7).

Melatonin has been discovered first in the pineal gland, but following reports revealed that the gastrointestinal tract contains a considerable amount of this hormone (8). As a pineal hormone melatonin contribute to the regulation of circadian rhytms, but its unique property is the protection of gastric mucosa against the damage caused by reactive oxygen species (ROS) (9 - 10). In acute pancreatitis the excessive production of ROS is responsible for the lipid membrane peroxidation, alteration of cytoskeleton, to the premature activation of digestive enzymes in the pancreatic tissue and to the DNA and protein damage (11 - 13).

Leptin, the product of *ob* gene is released by adipocytes, but recent finding have shown that the gastric mucosa may be also an important source of this peptide (14). Leptin is known to regulate food intake and body weight, as well as to influence the activity of immune cells and the cytokine production (7, 15 - 17).

Both hormones, leptin and melatonin, have been found to protect the various tissue from the injury, including the gastric mucosa, or nervous tissue (18 - 21). Our recent study have shown that leptin is able to protect the pancreas against the damage produced by overstmulation with caerulein, and that above pancreatoprotective effect of leptin involves modulation of cytokine production (22). Receptors for melatonin have been characterized in the pancreatic tissue and in the CNS, but the physiological significance of melatonin receptors in the pancreas remains unknown (23, 24). Melatonin has been recently reported to diminish acute pancreatic damage, however the mechanism of this effect have not been fully explained (25).

In acute pancreatitis the degree of tissue damage depends on the balance between noxious inflammatory factors and pancreatic defense mechanisms. Sensory nerves are of great importance in the prevention of pancreatic integrity from the damage

produced by acute inflammation (26, 27). Activation of these nerves releases a variety of neurotransmitters such as: CGRP, tachykinins or nitric oxide (NO), leading to the pancreatic hyperemia and increasing pancreatic resistance against acute inflammation (26–28). Deactivation of afferent nerves often leads to the disturbances of pancreatic microcirculation (27, 28). The decrease of pancreatic blood flow produces hypoxia and pancreatic cell damage, followed by the activation of secretory enzymes, autodigestion and aggravation of pancreatitis (29).

The aim of this study was: 1/ to compare the effects of central (i.c.v.) or peripheral (i.p.) administration of melatonin or leptin on the course of caerulein-induced pancreatitis (CIP), 2/ to investigate the involvement of sensory nerves, and CGRP in the effects of leptin or melatonin on course of CIP, 3/ to assess whether the generation of ROS in the pancreas of CIP rats could be affected by these hormones, 4/ to determine the effects of leptin or melatonin on the release of nitric oxide (NO) from isolated acini obtained from the pancreas of intact rats, 5/ to examine the expression of leptin receptor gene in the isolated pancreatic acini exposed to leptin and caerulein stimulation.

MATERIAL AND METHODS

Following items were purchased: caerulein (Takus) from Pharmacia GmbH, Erlangen, Germany, leptin (murine recombinant), melatonin, capsaicin and $CGRP_{8-37}$, an CGRP receptor antagonist, were from Sigma Co (St. Louis, MO, USA), essential and nonessential amino acid mixture from Serva Feinbiochemica (GmbH, Heidelberg, Germany) and purified collagenase from Worthigton Biochemica Co.(Freehold, N.J., USA). NO assay commercial kit was from Cayman Chemical Co. (Ann Arbor, Mich, USA). The BIOXYTECH LPO-586 kit was purchased from Oxis International Inc. (Portland, OR, USA).

Studies were performed on male Wistar rats weighing 150 - 200 g and fasted for 24 h before the experiment, while drinking water was available *ad libitum.* Animals were housed in cages under standard conditions, on commercial pellet chow, at room temperature with a 12-h light and dark cycle.

During the experiments the rats were placed in individual Bollman cages. Acute caerulein-induced pancreatitis (CIP) was produced by s.c. infusion of caerulein at a total standard dose of 25 μg/kg (5 μg/kg-h for 5 h). Caerulein was diluted in the saline and infused at a rate 1 ml/h. For the first part of the study various doses of leptin (2 or 10 μg/kg.), or melatonin (10 or 50 mg/kg) were dissolved in 0.5 ml of saline and administered i.p. as a bolus injection 30 min prior to the start of caerulein or saline (control experiments) infusion. In the second part of the study leptin (0.4 or 2 μg/rat), or melatonin (10 or 40 μg/rat), dissolved in 20 μl of vehicle saline was given i.c.v. 30 min prior to the start of caerulein or saline infusion. In some experiments the rats were injected first with an antagonist of CGRP receptors; $CGRP_{8-37}$ (100 μg/kg i.p.) and then a standard dose of leptin (10 μg/kg i.p. or 2 μg/rat i.c.v.) or melatonin (50 mg/kg i.p. or 40μg/rat i.c.v) was applied. This was followed 30 min later by caerulein or saline (control tests) infusion to the rats.

The involvement of sensory nerves in the pancreatic protection afforded by leptin or melatonin given i.p. or i.c.v. to the rats was studied in the animals with sensory nerves deactivated by pretreatment with large dose of capsaicin. Capsaicin was given to the rats at total dose of 100 mg/kg 10 days before the tests, as described previously (30).

Experimental Protocol

The study consists of four parts (A, B, C, D). In part A leptin or melatonin was given to the rats i.p. as a bolus injection prior to the start of caerulein infusion to induce CIP. In this part of the study we used rats with intact sensory nerves and group of animals with sensory nerves deactivated with capsaicin.

For part B of study the rats with intact and capsaicin-deactivated sensory nerves were also used. To assess the central effects of leptin or melatonin above substances were administered i.c.v. prior to the start of CIP.

To investigate the involvement of CGRP the effects of leptin or melatonin on the CIP, an antagonist of CGRP; $CGRP_{8-37}$ was used in intact rats in parts A and B of this study.

Part C was concerned with the effects of leptin or melatonin on the generation of NO in isolated pancreatic acini obtained from the normal rat pancreas.

In part D we investigated leptin receptor gene expression (*db*) in the normal rat pancreatic acini and the effect of caerulein and leptin on this expression.

Each part of the study consists of several experimental groups of rats, 6-8 fasted rats in each single group.

The experimental protocol has been approved by the Jagielloninan University Ethical Committee for Animal Experimentation.

PART A
The study on the involvement of sensory nerves or CGRP in the effects of peripheral (i.p.) administration of leptin or melatonin on CIP

The following study groups, each consisting of 6-8 animals with intact sensory nerves and 6-8 rats with sensory nerves deactivated with capsaicin, were employed including: 1/Control (vehicle saline s.c.), 2/ Leptin 10 μg/kg i.p.), dissolved in 0.5 ml of saline, followed by s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h, 3/ Melatonin (10 or 50 mg/kg i.p.), dissolved in 0.5 ml of saline, followed by s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h, 4/ Vehicle (0.5 ml) injected i.p. followed 30 min later by s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h to induce CIP.

To test the involvement of sensory nerves neurotransmitter CGRP in the effects of leptin or melatonin on CIP, rats with intact sensory nerves were injected i.p. with CGRP antagonist; $CGRP_{8-37}$ (100 μg /kg) followed 15 min later by administration of leptin (10 μg/kg i.p) or melatonin (50 mg/kg i.p) in separate tests. Subsequently s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h was performed to produce CIP..

The effects of leptin alone (2 or 10 μg /kg i.p.), melatonin alone (10 or 50 mg/kg i.p.), $CGRP_{8-37}$ alone (100 μg /kg i.p) or combined with leptin or melatonin were also tested in rats receiving vehicle saline instead of caerulein infusion.

Part B
The study on the effects of central (i.c.v.) administration of leptin or melatonin on CIP and on the involvement of sensory nerves or CGRP in these effects

For this part of the study leptin or melatonin was dissolved in 20 μl of saline and administered into right lateral cerebral ventricle (i.c.v.) of the rats as described previously (18, 22). Briefly, under light ether anesthesia, an incision was made along the midline of the skull, the skull bones were cleaned of connective tissue and the intersection between the sagittal and coronary sutures was visualized. A point at a distance of 2.5 mm from both sutures was found and at this point a

small hole was made in the skull using a needle with a sharp end. The hole was made with a rotary movement of the needle and the head wound was closed by a clip. The effectiveness of i.c.v. administration was verified by injecting 20 μl of 0.1% of toluidine blue.

For this part of the study several experimental groups consisting of 6-8 animals with intact sensory nerves and 6-8 rats with sensory nerves deactivated with capsaicin were used, including: 1/ Control; 20 μl of vehicle saline given i.c.v. followed 30 min later by s.c. infusion of vehicle saline to the rats, 2/ Vehicle (20 μl) injected i.c.v. followed 30 min later by s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h to induce CIP, 3/ Leptin (0.4 or 2 μg /rat i.c.v.), dissolved in 20 μl of saline, followed by s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h, 4/ Melatonin (10 or 40 μg/rat i.c.v.), dissolved in 20 μl of saline, followed by s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h.

To test the involvement of sensory nerves neurotransmitter; CGRP in the effects of leptin or melatonin on CIP, rats with intact sensory nerves were used and injected i.p. with CGRP antagonist; $CGRP_{8-37}$ (100 μg /kg) followed 15 min later by administration of leptin (2 μg/rat i.c.v.) or melatonin (40 μg/rat i.c.v.) with subsequent s.c. infusion of caerulein at a dose of 5 μg/kg-h during 5 h.

The effects of leptin alone (0.4 or 2 μg/rat i.c.v.), melatonin alone (10 or 40 μg/rat i.c.v), $CGRP_{8-37}$ alone (100 μg /kg i.p) or combined with leptin or melatonin, given i.c.v., were also tested in rats receiving vehicle saline instead of caerulein infusion.

Examination of pancreatic blood flow (PBF) and plasma amylase

Following 5 hours injection of caerulein or vehicle saline (in control tests) the animals were shortly anesthetized with Vetbutal (0.5 ml/rat), weighted and the abdominal cavity was opened. The pancreas was exposed for measurement of the blood flow by a laser Doppler flowmeter (LDF) using a Laserflo, model BPM Blood Perfusion Monitor (Vasamedics Inc., St Paul, MN, USA) as described previously (30). Pancreatic blood flow (PBF) was measured in five different regions of the pancreas and was expressed as percent change from control value obtained from the rats injected with saline.

Immediately after measurement of PBF, the abdominal aorta was exposed and blood was withdrawn into EDTA containing tubes for determination of plasma amylase, that was determined using an enzymatic method (Amylase reagent, Dialab Diagnostic Ges. MBH, Wien, Austria) as described previously (30).

Pancreatic weight and histological examination

The pancreas was carefully dissected from its attachment to the stomach, duodenum and the spleen, rinsed and weighted. Pieces of the pancreas were excised from the body portion, fixed in 10% formaline and stained with haematoxylin and eosin (H&E). Pancreatic samples were examined by professional histologist without the knowledge of the treatment given. The histological grading of edema, leukocyte infiltration and vacuolization was made using a scale ranging from 0 to 3 as described previously (30).

Determination of lipid peroxidation products (MDA + 4HNE) in the pancreatic tissue

The samples of fresh pancreatic tissue were taken for measurement of lipid peroxidation products: malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), using LPO-586 commercial kit, according to the manufacturer's protocol. Briefly, samples of pancreatic tissue weighing about 300 mg were homogenized in the phosphate buffer, (20 mM pH 7.4). Than, 10 μl of 0.5 M butylated hydroxytoluene in acetonitrile was added to each sample to prevent tissue oxidation. Samples were centrifuged and the pellets were immediately frozen at -70^{o} C until assay. MDA + 4-HNE was measured in duplicate and expressed as nM/g of tissue .

PART C

The effect of leptin or melatonin on NO^-_2 /NO^-_3 release from isolated pancreatic acini

Pancreatic acini were isolated from the pancreas of intact rats by collagenase digestion and suspended in KRH medium (pH 7.4), as described previously (31). Acinar suspensions were incubated in shaking bath at 37 ºC for 30 min in presence of various concentrations of leptin (10^{-9} – 10^{-6} M), or melatonin (10^{-8} – 10^{-5} M). Spontaneous NO release by the pancreatic acini was measured by incubating the acini without the addition of leptin or melatonin. The supernatant was then separated from the pellet by centrifugation at 1000 rpm for 5 min. The acini were dissolved by lysing buffer containing Triton X-100. NO release by pancreatic acini was quantified as nitrite (NO^-_2) and nitrate (NO^-_3) levels in the supernatant and was determined according to the method that is based on the Griess reaction (32) using a commercially available kit (Cayman Chemicals Assay Kit; Cayman Chemicals Co.).

PART D

Studies of gene expression for leptin receptor in the isolated pancreatic acini and on the effect of caerulein and leptin on this expression by RT-PCR

Pancreatic acini were isolated from the pancreas of intact rats by collagenase digestion and suspended in KRH medium (pH 7.4), as described previously (33). Acinar suspensions were incubated in the medium for 3 h in the presence of leptin (10^{-6} M), supramaximal concentration of caerulein (10^{-8} M), or combination of above. Control samples were incubated without the addition of tested substances. Immediately after incubation the supernatant was separated from the acini by centrifugation at 1000 rpm for 5 min. Then pancreatic acini were immediately frozen in liquid nitrogen. Total RNA was extracted by a guanidinum isothiocyanat-e/phenol chlorophorm single step extraction kit from Stratagene. DNA synthesis was performed from 1 μg total cellular RNA using Promega Reverse Transcriptase System according to produced standatd procedure (Promega Corporation, USA).

Primers for leptin receptor were synthetized by GIBCO BRL/Life Technologies (Eggenstein, Germany). The nucleotide sequences of the rat leptin receptor was based on the published cDNA sequences encoding rat *db* receptor (32). The leptin receptor sense primer was 5' AGT CAC TCA GTG CTT ATC C, while the leptin receptor antisense primer was 5' AGT CCT TGT GCCCAG GAA C. The expected lenght of this PCR product was 436 bp. Concomitantly amplification of control rat β-actin (ClonTech, Palo Alto, CA, USA) was performed on the same sample to assess the RNA integrity. Reaction mixtures for PCR contained cDNA templates, 50 pmol of each primer, and 2.5 U of Taq DNA polymerase (Promega Co, USA) in 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs in a volume of 50 μl. To maximize the amplification specificity, hot-start PCR was performed for 30 cycles (94º C for 1 min, 55º C for 45 s and 72º C for 2 min). Polymerase chain reaction products were detected by electophoresis on a 1.5% agarose gels containing ethidium bromide. Then visualization under UV light was performed. To compare the level of expression of leptin receptor mRNA against the reference gene (β-actin) mRNA data, the image analysis was employed. PCR products were analyzed using program Gel-Pro Analyzer (Fotodyne Incorporated, USA).

Statistical analysis

Comparison of the differences between the mean values of various groups of experiments were made by analysis of variance and the Student's t test for unpaired data. A difference with a p. value of < 0.05 was considered statistically significant. Results are expressed as means ± SEM.

RESULTS

PART A

The study on the involvement of sensory nerves or CGRP on the effects of peripheral (i.p.) administration of leptin or melatonin on CIP

Pancreatic blood flow (PBF), plasma amylase activity and pancreatic weight

Subcutaneous infusion of caerulein (5μg/kg–h during 5 h) to the rats with intact sensory nerves produced CIP in all animals tested. CIP was manifested by a 40% reduction in PBF, accompanied by significant increase of pancreatic weight and plasma amylase activity (by 200% and 500%, respectively). Above changes of PBF, pancreatic weight and plasma amylase observed in the rats with intact sensory nerves were not significantly different from these produced by infusion of caerulein (5μg/kg–h during 5 h) to the rats with capsaicin-deactivated afferent nerves *(Figs 1 - 5)*.

In the CIP rats with intact sensory nerves pretreatment with leptin (2 or 10 μg/kg i.p.), resulted in the significant and dose-dependent attenuation of all changes produced by pancreatic overstimulation with caerulein. Dose of 10 μg/kg of leptin given i.p. to the rats 30 min prior to the start of CIP resulted in the normalization of PBF, accompanied by 50% reduction in pancreatic weight and plasma amylase activity, as compared to the values obtained in the CIP rats, without leptin pretreatment *(Fig. 1)*.

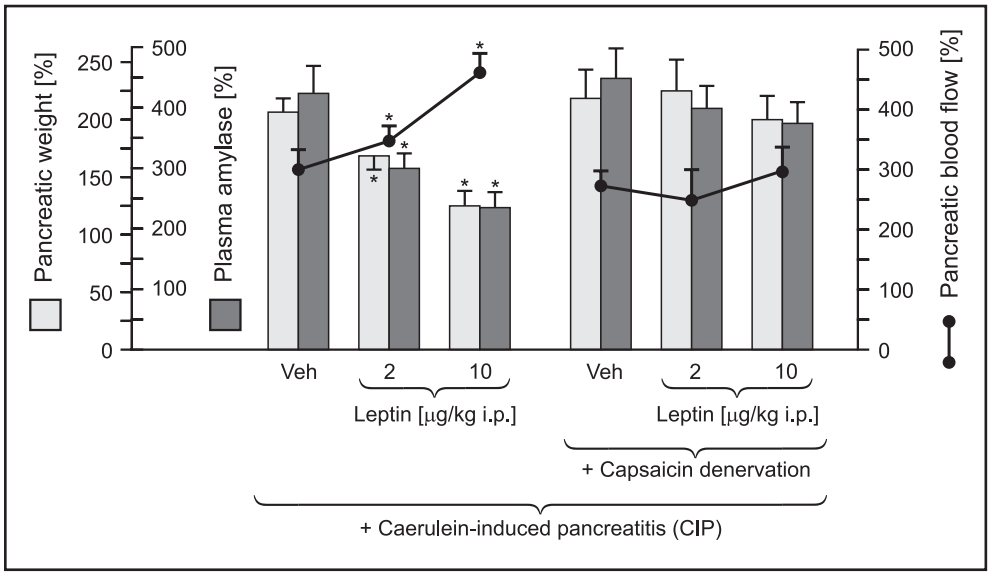

Fig. 1. Pancreatic weight, pancreatic blood flow and plasma amylase activity in the rats with intact or capsaicin-deactivated sensory nerves subjected to caerulein-induced pancreatitis (CIP), pretreated with vehicle saline (Veh.), or with leptin (2 or 10 μ/kg i.p.). The results are expressed as percent of value obtained from control rats, infused with vehicle saline alone. Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP without leptin pretreatment. Means ± SEM of 6-8 rats in each experimental group.

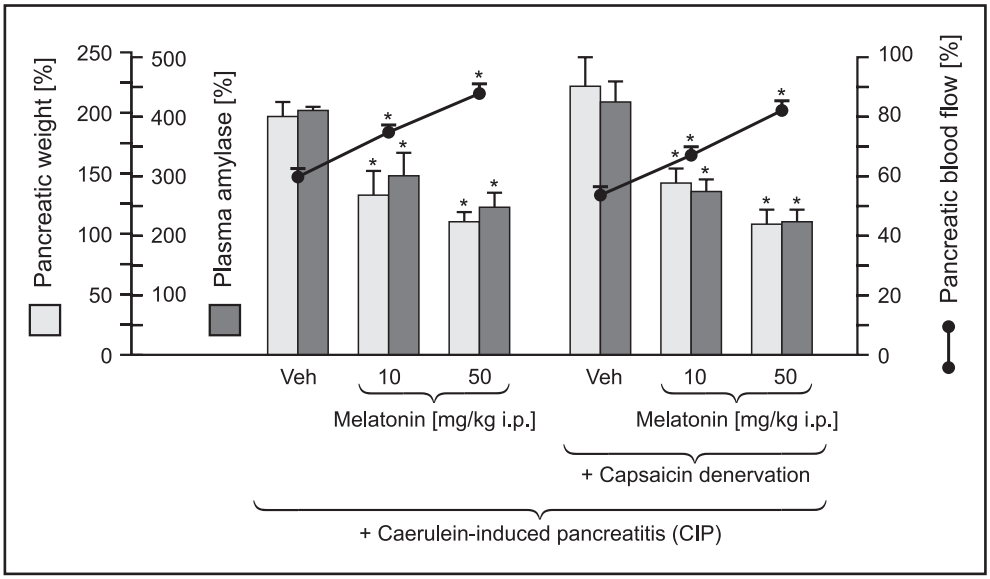

Fig. 2. Pancreatic weight, pancreatic blood flow and plasma amylase activity in the rats with intact or capsaicin-deactivated sensory nerves subjected to caerulein-induced pancreatitis (CIP), pretreated with vehicle saline (Veh.), or with melatonin (10 or 50 mg/kg i.p.). The results are expressed as percent of value obtained from control rats, infused with vehicle saline alone. Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP without melatonin pretreatment. Means ± SEM of 6-8 rats in each experimental group.

Pretreatment of the intact rats with melatonin (10 or 50 mg/kg i.p.) given 30 min prior to the start of CIP partially reversed all harmful effects of CIP on the pancreas; PBF was significantly and dose-dependently improved, pancreatic weight and plasma amylase activity was significantly reduced, as compared to the values observed in the rats with CIP alone *(Fig.2)*.

Deactivation of afferent nerves with capsaicin completely abolished all protective effects of leptin (2 or 10 μg/kg i.p.) on the pancreas of CIP rats *(Fig. 1)*. To the contrary, functional ablation of sensory nerves with capsaicin failed to affect the protection afforded by melatonin (10 or 50 mg/kg i.p.) on CIP *(Fig.2)*.

In the CIP rats pretreated with leptin (10 μg/kg i.p) administration of CGRP antagonist; $CGRP_{8-37}$ (100 μg/kg i.p.) prior to the injection of leptin, resulted in the partial reversion of pancreatoprotective effects of leptin, whereas melatonin-induced protection of the pancreas was not significantly altered by this CGRP antagonist *(Fig. 3)*. $CGRP_{8-37}$, given alone (100 μg/kg i.p.) to the control rats, as well as to the rats subjected to CIP, failed to influence significantly pancreatic weight, PBF and plasma amylase.

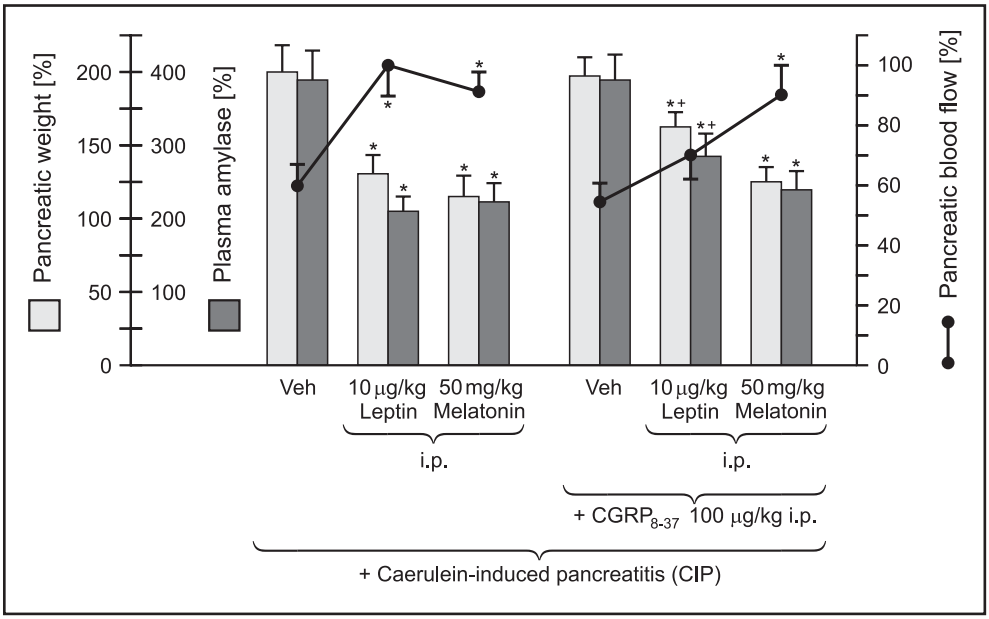

Fig. 3. Pancreatic weight, pancreatic blood flow and plasma amylase activity in the rats subjected to caerulein-induced pancreatitis (CIP), pretreated with vehicle saline (Veh), leptin (10 μg/kg i.p.) or melatonin (50 mg/kg i.p.) with or without the addition of $CGRP_{8-37}$ (100 μ/kg i.p.). The results are expressed as percent of value obtained from control rats, infused with vehicle saline alone. Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP without leptin or melatonin pretreatment. Cross indicates significant ($p < 0.05$) change, as compared to the value obtained from the CIP rats without $CGRP_{8-37}$ pretreatment. Means ± SEM of 6-8 rats in each experimental group.

Injection of leptin, melatonin or $CGRP_{8-37}$ alone to the control rats receiving infusion of vehicle saline instead of caerulein did not affect significantly any of parameters tested in both groups of animals; intact or capsaicin pretreated rats. These results were omitted for the sake of clarity.

Histological examination

Infusion of caerulein (5μg/kg-h x 5 h) produced typical pancreatic lesions in all tested rats *(Fig. 4, Tables 1-4)*. The pancreas was grossly swollen and enlarged. Peritoneal fluid was present in all animals. Edema was accompanied by perivascular infiltration of leukocytes, and the vacuolization in acinar cells. In rats infused with caerulein and pretreated with leptin (2 or 10 μg/kg i.p.) all these changes were significantly less pronounced, edema was markedly diminished, infiltration was reduced and vacuolization was significantly decreased. Ablation of sensory nerves completely reversed above beneficial

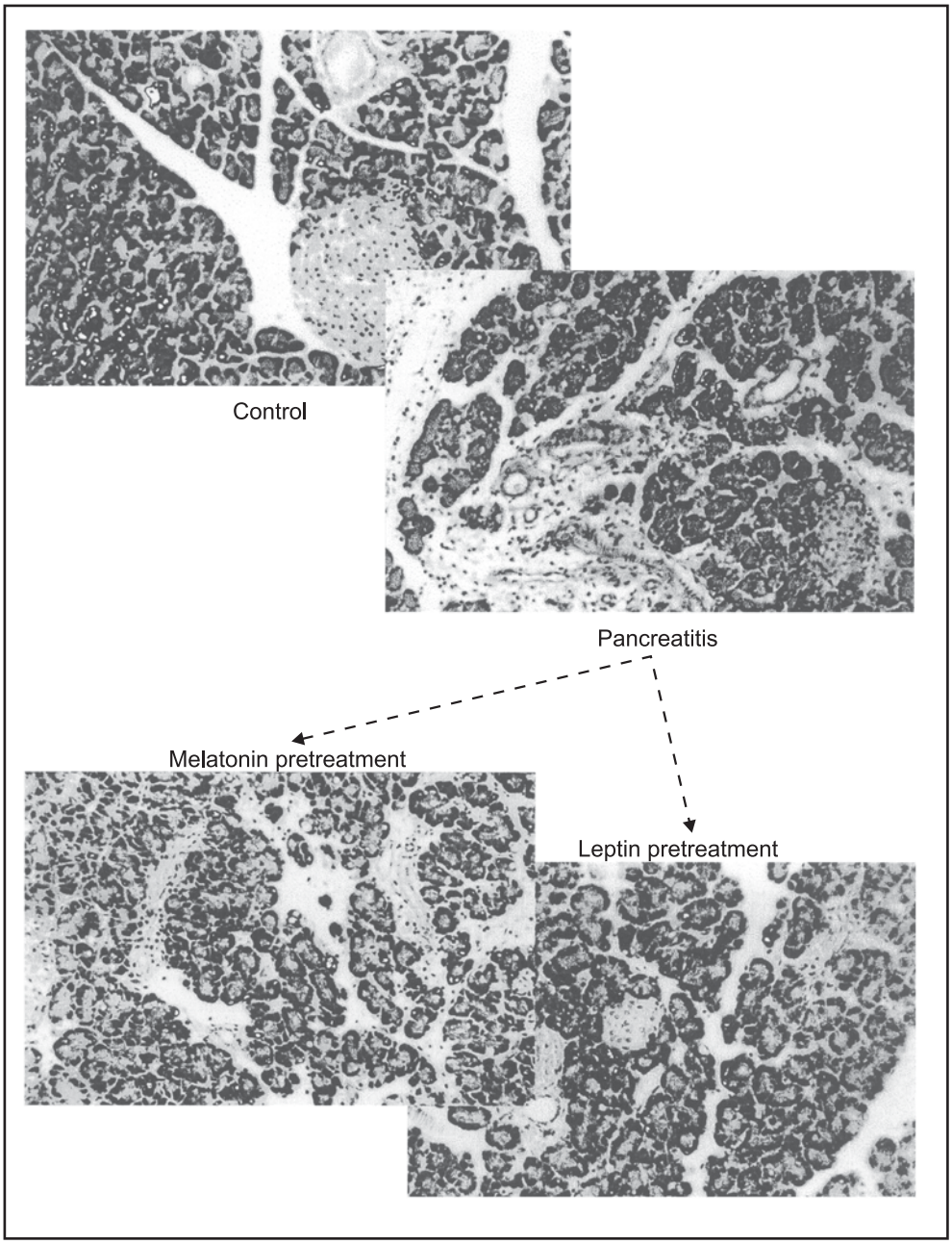

Fig. 4. Histological section of pancreas from intact rats (control), from those subjected to CIP alone (pancreatitis), from the animals with CIP pretreated with protective dose of leptin 10 μg/kg i.p. (Leptin pretreatment) and from the animals with CIP pretreated with protective dose of melatonin 50 mg/kg i.p. (Melatonin pretreatment). Hematoxylin/eosin stain. Magnification × 165.

Table 1. Histological changes induced by s.c. infusion of caerulein alone (CIP) (5 μg/kg-h x 5 h), leptin alone administered i.p. at doses of 2 or 10 μg/kg, or combination of above agents without or with addition of $CGRP_{8-37}$ to the rats with intact sensory nerves (top panel) and to the animals with sensory nerves deactivated with capsaicin (bottom panel). Asterisk indicates significant change as compared to the value obtained with infusion of caerulein alone. Cross indicates significant increase above the values obtained from the CIP animals pretreated with leptin (10 μg/kg i.p.) without $CGRP_{8-37}$.

Rats with intact sensory nerves			
	Edema (0-3)	Infiltration (0-3)	Vacuolization (0-3)
Control	0	0	0
CIP alone	2.5 ± 0.1	2.3 ± 0.2	2.8 ± 0.1
CIP+ leptin 2 μg/kg i.p..	1.6 ± 0.1*	1.2 ± 0.2*	1.3 ± 0.1
CIP + leptin 10 μg/kg i.p.	1.0 ± 0.1*	0.66 ± 0.2*	1.1 ± 0.1*
CIP + leptin 10 μg/kg i.p. + $CGRP_{8-37}$ 100 μg/kg i.p.	1.8 ± 0.2*+	1.5 ± 0.3*+	2.0 ± 0.1*+

Rats with sensory nerves deactivated with capsaicin			
	Edema (0-3)	Infiltration (0-3)	Vacuolization (0-3)
Control	0	0	0
CIP alone	2.7 ± 0.1	2.5 ± 0.2	2.7 ± 0.3
CIP+ leptin 2 μg/kg i.p.	2.5 ± 0.1	1.8 ± 0.4	2.8 ± 0.1
CIP + leptin 10 μg/kg i.p.	2.8 ± 0.1	2.6 ± 0.4	2.5 ± 0.1

effects of leptin (2 or 10 μg/kg i.p) on the pancreas of CIP rats, whereas administration of $CGRP_{8-37}$ (100 μg/kg i.p.) to the rats with CIP pretreated with leptin (10 μg/kg i.p.) resulted in the partial reversal of pancreatoprotective effect of leptin *(Table 1)*.

Pretreatment of the CIP rat with melatonin (10 or 50 mg/kg i.p.) resulted in significant improvement of pancreatic integrity and attenuation of all morphological changes produced by caerulein overstimulation in the pancreas. The beneficial effects of melatonin on the pancreas of CIP rats were not significantly affected by previous deactivation of sensory nerves with capsaicin or by the pretreatment with $CGRP_{8-37}$ (100 μg/kg i.p.) *(Fig. 4, Table 2)*.

In the control rats from both groups; intact or capsaicin-deactivated and infused with vehicle saline instead of caerulein, administration of leptin (2 or 10 μg/kg i.p.), melatonin (10 or 50 mg/kg i.p), or $CGRP_{8-37}$ (100 μg/kg i.p.) did not affect significantly pancreatic morphology. These results were omitted for the sake of clarity.

Table 2. Histological changes induced by s.c. infusion of caerulein alone (CIP) (5 μg/kg-h x 5 h), melatonin alone administered i.p. at doses of 10 or 50 mg/kg, or combination of above agents without or with addition of $CGRP_{8-37}$ in the rats with intact sensory nerves (top panel) and to the capsaicin-pretreated animals (bottom panel). Asterisk indicates significant change as compared to the value obtained with infusion of caerulein alone.

Rats with intact sensory nerves			
	Edema (0 - 3)	Infiltration (0 - 3)	Vacuolization (0 - 3)
Control	0	0	0
CIP alone	2.5 ± 0.1	2.3 ± 0.3	2.8 ± 0.1
CIP+ melatonin i.p.10 mg/kg	1.8 ± 0.2*	2.1 ± 0.6	2.0 ± 0.3
CIP+ melatonin i.p.50 mg/kg	1.7 ± 0.3*	1.8 ± 0.1	2.1 ± 0.3*
CIP+ melatonin i.p.50 mg/kg + $CGRP_{8-37}$ 100 μg/kg i.p.	1.8 ± 0.1*	1.9 ± 0.0	2.0 ± 0.2*

Rats with sensory nerves deactivated with capsaicin			
	Edema (0 - 3)	Infiltration (0 - 3)	Vacuolization (0 - 3)
Control	0	0	0
CIP alone	2.7 ± 0.1	2.5 ± 0.2	2.7 ± 0.3
CIP+ melatonin i.p.10 mg/kg	2.0 ± 0.2*	1.9 ± 0.6	2.5 ± 0.3
CIP+ melatonin i.p.50 mg/kg	1.5 ± 0.3*	1.7 ± 0.1*	2.3 ± 0.3

Effect of i.p. administration of leptin or melatonin on lipid peroxidation products (MDA + 4HNE) in the pancreatic tissue

The content of lipid peroxidation products (MDA + 4-HNE) examined in the pancreatic tissue obtained from vehicle-treated, intact rats was very low. Deactivation of sensory nerves with capsaicin did not affect significantly the level of MDA + 4-HNE in the pancreas of vehicle treated rats *(Fig. 5)*.

In both groups of CIP rats, in those with intact sensory nerves and in the animals with capsaicin deactivated afferent nerves, the caerulein overstimulation produced similar dramatic increase in the pancreatic generation of MDA + 4-HNE *(Fig. 5)*. In the pancreas of CIP rats administration of melatonin (10 or 50 mg/kg i.p.), given 30 min prior to the start of caerulein infusion, resulted in a significant reduction of lipid peroxidation. Deactivation of sensory nerves with capsaicin failed to affect significantly above reduction of MDA + 4-HNE content produced by melatonin in the pancreas of CIP rats *(Fig. 5)*.

Pretreatment with leptin (2 or 10 μg/kg i.p.), did not changed significantly the generation of MDA + 4-HNE in the pancreatic tissue of both groups of rats, with intact sensory nerves and with capsaicin deactivation *(Fig. 5)*.

Pretreatment of the CIP rats with $CGRP_{8-37}$ (100 μg/kg i.p.) had no significant influence on the effects of melatonin (50 mg/kg i.p.) on lipid peroxidation in the

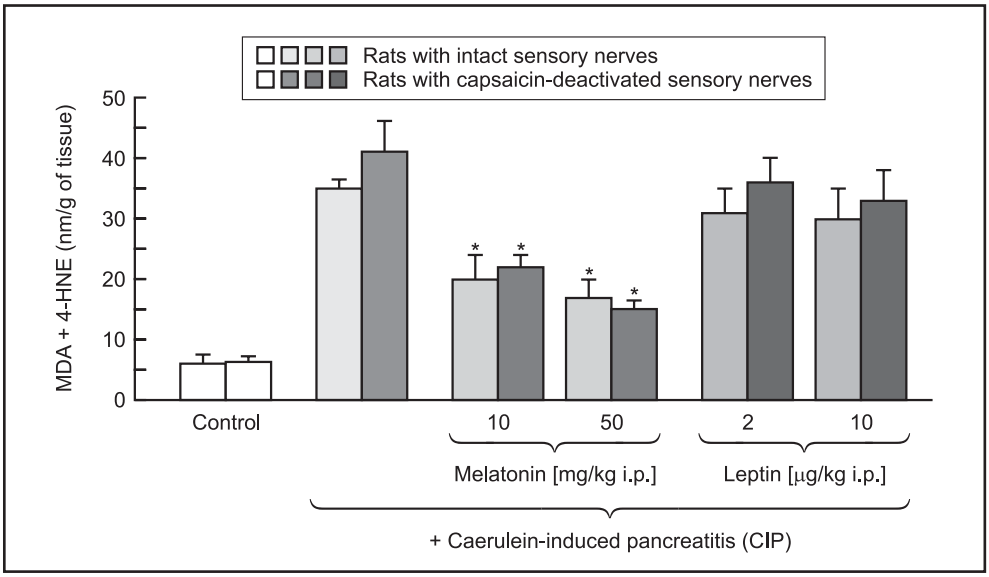

Fig. 5. Lipid peroxidation products (MDA + 4-HNE) in the pancreatic tissue obtained from the rats with intact or capsaicin-deactivated sensory nerves, subjected to caerulein-induced pancreatitis (CIP) with or without pretreatment with melatonin (10 or 50 mg/kg i.p.) or with leptin (2 or 10 μg/kg i.p.). Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP alone. Control = value obtained from the rats pretreated with vehicle saline alone. Means ± SEM of 6-8 rats in each experimental group.

pancreas of CIP rats. $CGRP_{8-37}$ (100 μg/kg i.p.) given to the CIP rats with or without pretreatment with leptin failed to affect significantly MDA + 4-HNE level in the pancreatic tissue of the rats with CIP, and these results were omitted for the sake of clarity.

In the vehicle treated, control rats administration of leptin or melatonin failed to affect significantly the generation of MDA + 4-HNE in the pancreatic tissue and ablation of sensory nerves was without the effect on this content of lipid peroxidation products under basal conditions. Above results were omitted for the sake of clarity.

PART B

The study on the effects of central (i.c.v.) administration of leptin or melatonin on CIP and on the involvement of sensory nerves or CGRP in above effects

Pancreatic blood flow (PBF), plasma amylase and pancreatic weight

In all rats pretreated with vehicle saline administered i.c.v. prior to infusion of caerulein (5μg/kg-h x 5 h) acute pancreatitis was observed that was similar to that found in intact rats *(Figs 6 and 7)*.

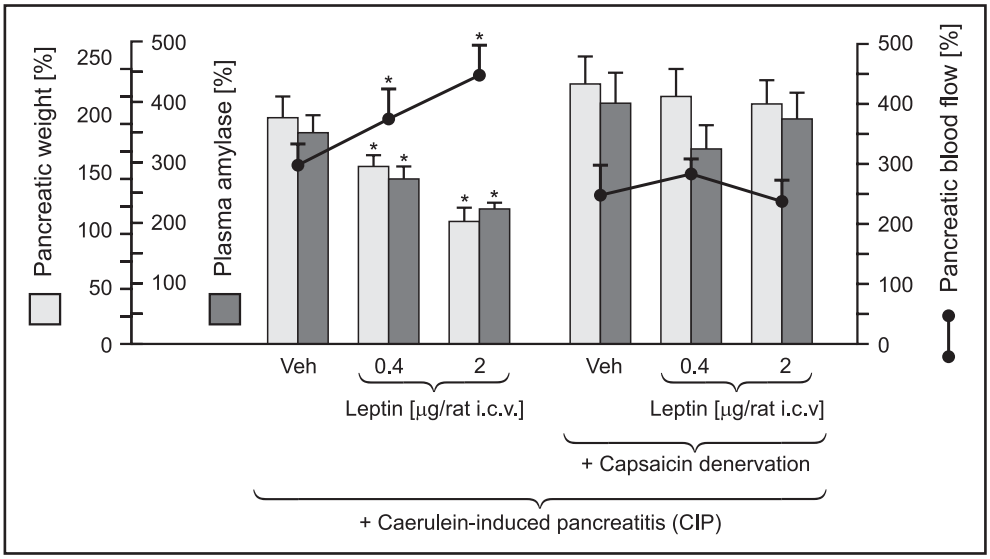

Fig. 6. Pancreatic weight, pancreatic blood flow and plasma amylase activity in the rats with intact or capsaicin-deactivated sensory nerves, subjected to caerulein-induced pancreatitis (CIP), pretreated with vehicle saline (Veh.), or with leptin (0.4 or 2 μg/rat i.c.v.). The results are expressed as percent of value obtained from control rats, infused with vehicle saline alone. Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP without leptin pretreatment. Means ± SEM of 6-8 rats in each experimental group.

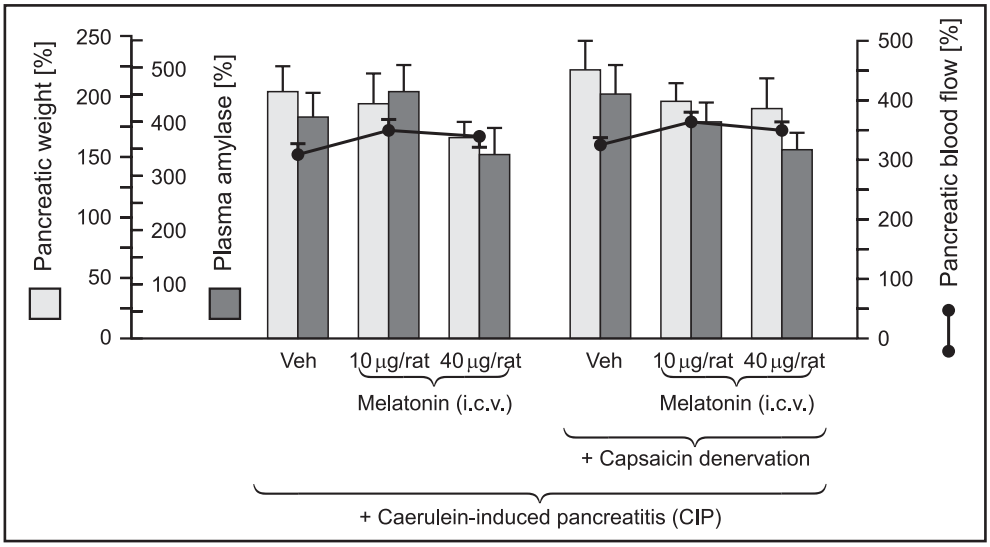

Fig. 7. Pancreatic weight, pancreatic blood flow and plasma amylase activity in the rats with intact or capsaicin-deactivated sensory nerves subjected to caerulein-induced pancreatitis (CIP), pretreated with vehicle saline (Veh.), or with melatonin (10 or 40 μg/rat i.c.v.). The results are expressed as percent of value obtained from control rats, infused with vehicle saline alone. Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP without melatonin pretreatment. Means ± SEM of 6-8 rats in each experimental group.

As shown on *Fig. 6* central administration of leptin (0.4 or 2 μg/rat i.c.v.) to the rats prior to the start of CIP resulted in the attenuation of the pancreatic edema, plasma amylase activity and improvement of PBF similar to that observed in CIP rats pretreated with leptin (2 or 10 μg/kg) given i.p. *(Fig.1)*. In rats with capsaicin deactivated sensory nerves pretreatment with leptin (2 or 10 μg/kg) given i.p. or i.c.v. completely reversed above beneficial effects of leptin on CIP *(Fig. 5)*.

Central administration of melatonin (10 or 40 μg/rat i.c.v.*)* prior to the induction of CIP failed to affect significantly pancreatic weight, plasma amylase or PBF in the rats with intact or capsaicin deactivated sensory nerves *(Fig 7)*.

Administration of CGRP antagonist, $CGRP_{8-37}$ (100 μg/kg i.p.), to the CIP rats pretreated with leptin (2 μg/rat i.c.v.) resulted in a significant attenuation of protective effects of leptin on the pancreas similar to that observed following i.p. leptin pretreatment *(Fig. 8)*. $CGRP_{8-37}$ (100 μg/kg i.p.) given to the CIP rats pretreated with melatonin (40 μg/rat i.c.v.) did not affect the inflammatory changes produced in the pancreas by caerulein overstimulation *(Fig. 8)*. $CGRP_{8-37}$, given alone (100 μg/kg i.p.)

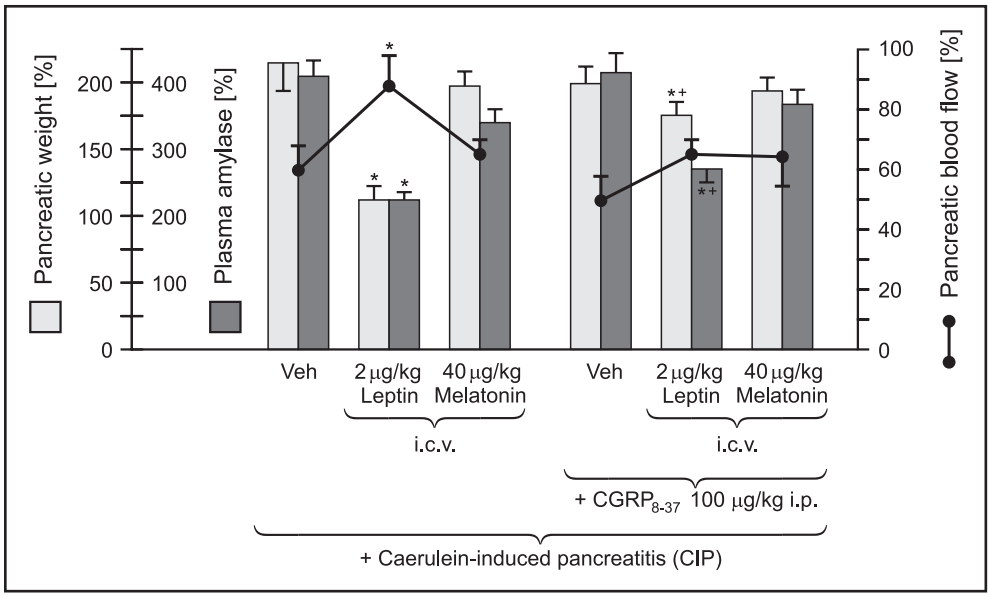

Fig. 8. Pancreatic weight, pancreatic blood flow and plasma amylase activity in the rats subjected to caerulein-induced pancreatitis (CIP), pretreated with vehicle saline (Veh.), leptin (2 μg/rat i.c.v.) or melatonin (40 μ/rat i.c.v.) with or without the pretreatment with $CGRP_{8-37}$ (100 μg/kg i.p.). The results are expressed as percent of value obtained from control rats, infused with vehicle saline alone. Asterisk indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats subjected to CIP without leptin or melatonin pretreatment. Cross indicates significant ($p < 0.05$) change, as compared to the value obtained from the rats CIP without $CGRP_{8-37}$ pretreatment. Means ± SEM of 6-8 rats in each experimental group.

Table 3. Histological changes induced by s.c. infusion of caerulein alone (CIP) (5 μg/kg-h x 5 h), leptin alone administered i.c.v. at doses of 0.4 or 2 μg/rat, or combination of above agents without or with addition of $CGRP_{8-37}$. Asterisk indicates significant change as compared to the value obtained with infusion of caerulein alone. Cross indicates significant increase above the values obtained from the CIP animals pretreated with leptin (2 μg/rat i.c.v.) without $CGRP_{8-37}$.

	Edema (0-3)	Infiltration (0-3)	Vacuolization (0-3)
Control	0	0	0
CIP alone	2.2 ± 0.1	2.1 ± 0.3	2.2 ± 0.1
CIP+ leptin 0.4 μg/rat i.c.v.	1.6 ± 0.5	1.7 ± 0.2	1.7 ± 0.0
CIP + leptin 2 μg/rat i.c.v.	1.2 ± 0.2*	1.0 ± 0.2*	0.8 ± 0.3*
CIP + leptin 2 μg/rat i.c.v. + $CGRP_{8-37}$ 100 μg/kg i.p.	1.7 ± 0.2*+	1.9 ± 0.1	1.5 ± 0.3*+

Rats with sensory nerves deactivated with capsaicin			
	Edema (0-3)	Infiltration (0-3)	Vacuolization (0-3)
Control	0	0	0
CIP alone	2.5 ± 0.2	2.3 ± 0.1	2.7 ± 0.4
CIP + leptin 0.4 μg/rat i.c.v.	2.0 ± 0.3	1.9 ± 0.2	2.2 ± 0.2
CIP + leptin 2 μg/rat i.c.v.	2.2 ± 0.4	2.0 ± 0.2	2.6 ± 0.4

Table 4. Histological changes induced by s.c. infusion of caerulein alone (CIP) (5 μg/kg-h x 5 h), melatonin alone administered i.c.v. at doses of 10 or 40 μg/rat, or combination of above without or with addition of $CGRP_{8-37}$ in rats with intact sensory nerves (upper panel) and with capsaicin deactivated nerves (bottom panel).

	Edema (0 - 3)	Infiltration (0 - 3)	Vacuolization (0 - 3)
Control	0	0	0
CIP	2.5 ± 0.1	2.3 ± 0.3	2.2 ± 0.1
CIP + melatonin i.c.v. 10 μg/rat	2.2 ± 0.5	2.0 ± 0.2	2.0 ± 0.0
CIP + melatonin i.c.v. 40 μg/rat	2.5 ± 0.2	1.9 ± 0.2	1.8 ± 0.4
Melatonin i.c.v. 40 μg/rat + $CGRP_{8-37}$ 100 μg/kg i.p.	2.3 ± 0.1	2.1 ± 0.1	2.2 ± 0.1

Rats with sensory nerves deactivated with capsaicin			
	Edema (0 - 3)	Infiltration (0 - 3)	Vacuolization (0 - 3)
Control	0	0	0
CIP alone	2.5 ± 0.2	2.3 ± 0.1	2.7 ± 0.4
CIP + melatonin i.c.v. 10 μg/rat	2.6 ± 0.1	1.9 ± 0.3	2.2 ± 0.3
CIP + melatonin i.c.v. 40 μg/rat	2.1 ± 0.2	1.9 ± 0.4	2.3 ± 0.2

to the control rats, receiving i.c.v. injection of physiological saline instead of leptin or melatonin, as well as to the rats subjected to CIP, failed to influence significantly pancreatic weight, PBF and plasma amylase in these animals.

In the control rats, subjected to infusion of vehicle saline instead of caerulein, central administration of leptin (0.4 or 2 μg/rat i.c.v.) or melatonin (10 or 40 μg/rat i.c.v.*)* was without the effect on above pancreatic parameters tested and these results were omitted for the sake of clarity.

Histological examination

In the control rats receiving vehicle saline given i.c.v., instead of tested substances, overstimulation with caerulein produced typical pancreatic lesions *(Table 3 and 4)*.

Pretreatment of the CIP rats with leptin (0.4 or 2 μg/rat i.c.v.) produced significant and dose-dependent reduction in pancreatic lesion and above beneficial effects of i.c.v. leptin administration on CIP were almost completely abolished following $CGRP_{8-37}$ (100 μg/kg i.p.) pretreatment or the ablation of sensory nerves with capsaicin *(Table 3)*.

Intracerebroventricular pretreatment with melatonin (10 or 40 μg/rat i.c.v.), of the CIP rats failed to influence cell edema, leukocyte infiltration and vacuolization of the acinar cells produced by caerulein overstimulation in the rats with intact sensory nerves and in those with sensory nerves deactivated with capsaicin. Administration of $CGRP_{8-37}$ (100 μg/kg i.p.) to the CIP rats with intact sensory nerves pretreated with melatonin (40 μg/kg i.p.) did not produced any significant change of pancreatic inflammation produced by CIP *(Table 4)*.

Central administration of leptin (0.4 or 2 μg/rat i.c.v.) or melatonin (10 or 40 μg/rat i.c.v.) to the control rats infused with vehicle saline, instead of caerulein, failed to influence significantly pancreatic morphology in animals with intact sensory nerves and in those subjected to deactivation of afferent nerves with capsaicin. Above results were omitted for the sake of clarity.

Effect of i.c.v. administration of leptin or melatonin on lipid peroxidation products (MDA + 4HNE) in the pancreatic tissue

Central pretreatment with leptin (0.4 or 2 μg/rat i.c.v.) or with melatonin (10 or 40 μg/rat i.c.v.), failed to affect the generation of MDA + 4-HNE in the pancreas of CIP rats. In the control rats, infused with vehicle saline instead of caerulein, i.c.v. administration of leptin or melatonin alone did not influence significantly the lipid peroxidation in the pancreatic tissue. Above results were omitted for the sake of clarity.

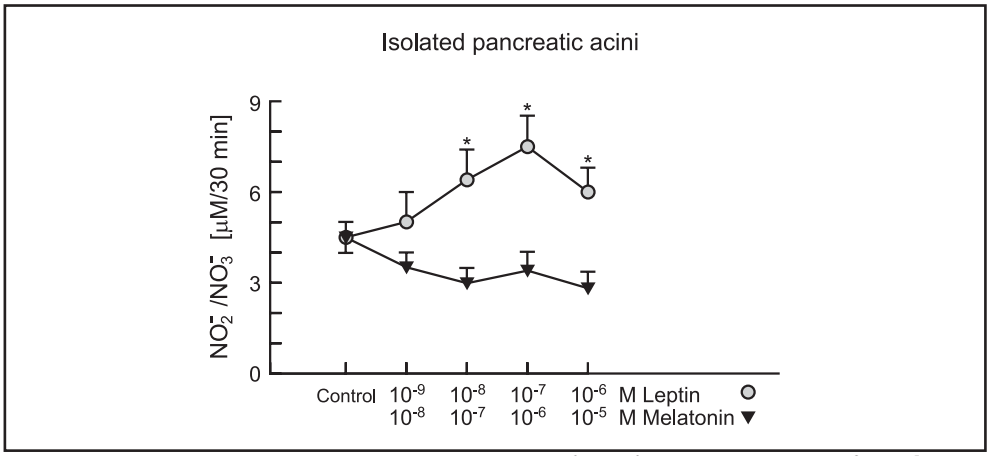

Fig. 9. Effects of increasing concentrations of leptin (10^{-9} - 10^{-6} M) or melatonin (10^{-8} - 10^{-5} M) on NO_2/NO_3 release from the isolated pancreatic acini obtained from the intact rats. Control = unstimulated NO_2/NO_3 release. Asterisk indicates significant ($p < 0.05$) change, as compared to the control. Means ± SEM of 6 separate experiments.

PART C

The effect of leptin or melatonin on NO^-_2/NO^-_3 release from isolated pancreatic acini

Incubation of pancreatic acini, isolated from normal rat pancreas, in presence of increasing concentrations of leptin (10^{-9} – 10^{-6} M) resulted in the dose-dependent increase of NO release from these acini, achieving the highest level at 10^{-7} M of leptin, whereas melatonin (10^{-8} – 10^{-5} M) failed to affect significantly the NO^-_2/NO^-_3 release by the isolated pancreatic acini *(Fig 9)*.

PART D

Studies of gene expression for leptin receptor in the isolated pancreatic acini and on the effect of caerulein and leptin on this expression, by RT-PCR

The signal for leptin receptor gene expression was detectable in the pancreatic acini under basal conditions as well as in those treated with caerulein alone (10^{-8} M), with leptin alone (10^{-6} M) or with their combination *(Fig 10)*. Stimulation of the acini with leptin, caerulein or combination of leptin + caerulein, resulted in the increase of mRNA for leptin receptor, over that observed in the unstimulated acini. The strongest signal was observed in the pancreatic acinar cells exposed to combination of both tested substances. The ratio of leptin receptor mRNA over β-actin mRNA confirmed that leptin receptor gene expression was significantly elevated in the acini incubated in presence of caerulein (10^{-8} M) together with leptin (10^{-6} M) *(Fig.10)*.

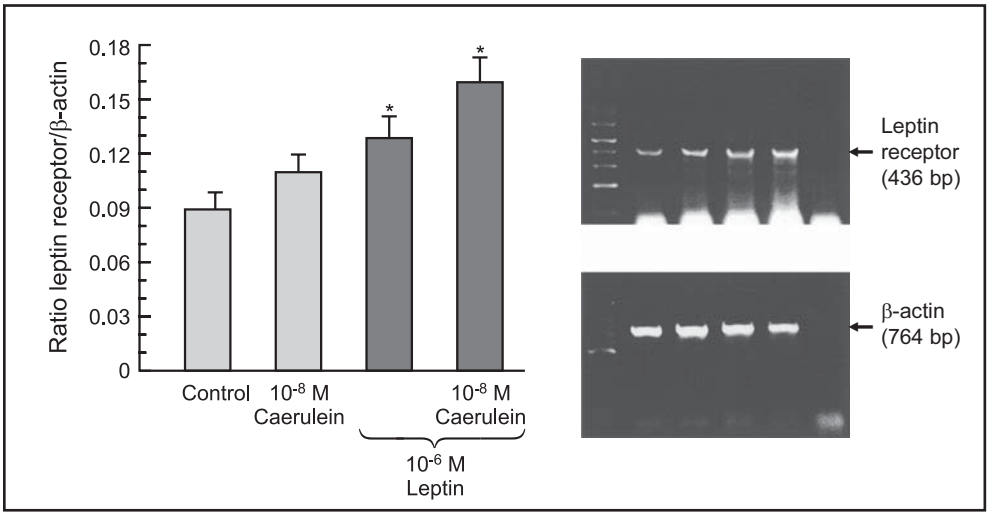

Fig. 10. The ratio of leptin receptor over beta actin mRNA in the isolated pancreatic acini and expression of leptin receptor mRNA by RT-PCR in the acini incubated under basal conditions (lane 1), from the acini incubated in presence of caerulein at concentration of 10^{-8} M (lane 2), leptin at concentration of 10^{-6} M (lane 3), or combination of above (lane 4). M – molecular weight marker (436 bp). Asterisk indicates significant ($p < 0.05$) change, as compared to the unstimulated control. Means ± SEM of 3 separate experiments.

DISCUSSION

This study demonstrates for the first time that sensory nerves and CGRP are involved in the protective effects of leptin on the pancreas subjected to caerulein overstimulation, whereas melatonin-induced protection of the pancreas does not depend on these nerves.

Herein we confirm our previous observations that leptin given peripherally (i.p.) or into the central nervous system (i.c.v.) is capable of reducing pancreatic acute damage (22).

Moreover, our previous study have demonstrated, that pancreas is able to produce endogenous leptin and that this "pancreatic" leptin, could limit the extend of pancreatic damage. These protective effects of leptin could be attributed to the modulation of cytokine production; decrease of pro-inflammatory TNFα and the increase of anti-inflammatory IL-4 by leptin (22, 34). The mechanism of leptin-induced protection of the pancreas could also involve the reduced secretion of digestive enzymes, because leptin has been reported to directly inhibit amylase release from isolated pancreatic acini (35).

The most important observation of our present study is the finding that leptin receptor gene expression is present in the pancreatic acini isolated from the intact rats. The presence of specific leptin receptors has been recently shown in the pancreatic acinar cell line AR42J (33). Herein we demonstrate that normal pancreatic acini exhibit

the gene expression for leptin receptor and that leptin as well as caerulein can enhance this signal for leptin receptor. It is very likely that protective action of leptin on the pancreas depends, at least in part, on the activation of leptin receptors in the pancreatic acinar cells.

Specific leptin receptors have been also detected in the CNS, mainly in the hypothalamus (17). It is not clear whether leptin exerts its beneficial effect on the pancreas though its specific receptors in the pancreas itself or in the brain. Both ways of action may be probable. It is also not excluded, that leptin administered i.c.v. could increase the release of leptin and could affect its peripheral receptors sites in the pancreas. This notion is supported by our previous study showing that central application of leptin leads to the increase of leptin in plasma (22).

In the inflammed pancreas exogenous leptin improved the PBF, reduced leukocyte infiltration and limitated of TNFα production (22). Sensory nerves are particularly important for microcirculatory response and stimulation of these nerves could produce pancreatic hyperemia. (27, 28). Our results clearly demonstrate that capsaicin-induced deactivation of sensory nerves completely reversed the protective effect of leptin on the pancreas and leads to the significant reduction in pancreatic microcirculation. Since the integrity of sensory nerves is essential for the protective effect of leptin on the pancreas it is very likely that endogenous leptin could exert its protective effect on the gland though the activation of specific receptors on pancreatic sensory nerves. This notion is supported by the observation that leptin is able to affect the synaptic transmission in the pancreatic neurons *via* its specific receptor (36, 37). However, it is not excluded that activation of central leptin receptors in the hypothalamus could modify the activity of primary sensory nerves though descending neuronal pathways.

Activation of pancreatic sensory nerves releases several neurotransmitters and one of them is CGRP (28). This peptide is known to produce gastrointestinal hyperemia, which was reversed by antagonizing the CGRP receptor with $CGRP_{8-37}$ (18). The CGRP receptors have been identified on pancreatic acini and CGRP is believed to participate in the pancreatic protection against CIP (27, 37). In this study CGRP receptor antagonist, $CGRP_{8-37}$, reversed the beneficial effects of peripheral as well as central leptin administration on the pancreas of CIP rats. Pretreatment with $CGRP_{8-37}$ given together with leptin reduced hyperemia produced by leptin in the pancreas, leading to the increase of pancreatic edema and plasma amylase in the rats with CIP. Thus, the major finding of the present study is the observation that sensory nerves and CGRP, released from their endings, contribute to the protective effect of leptin on the pancreas.

Previous studies have demonstrated that leptin as well as CGRP are able to release nitric oxide from vascular endothelium and that NO is involved in the gastroprotective and metabolic effects of leptin (18, 39, 40).

In the pancreas NO could be released from sensory nerves, vascular epithelium or directly from the pancreatic acini (30, 41, 42). It has been shown that NO produced in the pancreas is involved in the protection of pancreatic acinar cells against the damage caused by their overstimulation (41, 42). Our recent study provided an evidence that

leptin is able to affect NO synthesis in the pancreas (34). The mRNA expression for inducible form of NO synthase (iNOS) was inhibited by leptin, whereas the signal for constitutive isoenzyme (cNOS) increased in the pancreas of CIP rats pretreated with leptin (34). The results of present study demonstrate that leptin, could stimulate directly the release of NO from isolated pancreatic acini, and we assume that this NO released in the pancreatic gland could also participate in the protection of pancreas against the damage induced by CIP.

Our results are in agreement with the previous observation of Wenbo et al, who reported that melatonin is effective in the protection of the pancreas from the acute damage (25). We found that this protective effect of i.p. melatonin was accompanied by a significant rise of PBF and marked reduction in the generation of ROS in the pancreatic tissue. It is of interest that the beneficial effects of melatonin have been only observed following peripheral administration of this substance, whereas its central application of melatonin failed to affect significantly pancreatic damage induced by CIP. Deactivation of sensory nerves by capsaicin did not influence significantly the protection of the pancreas afforded by i.p. melatonin pretreatment. Also administration of $CGRP_{8-37}$, the antagonist of CGRP, which is released from the sensory nerve endings, was ineffective in the pancreatic protection afforded by melatonin. Above observations indicate that sensory nerves and its neurotransmitter CGRP are not involved in the melatonin-induced protection of the pancreas against CIP. Also NO seems unlikely to contribute to pancreatic protection obtained with peripheral application of melatonin. In contrast to the stimulatory effect of leptin on NO release from pancreatic acini, exposition of these acini to melatonin failed to affect the generation of NO and its release from the acinar cells.

Previous studies have shown that melatonin prevent from acute or chronic gastric lesions and protect the neural tissue from the damage induced by ROS (20, 21, 25). Melatonin received particular attention as pancreatoprotector due to its antioxidative properties. ROS, are responsible for the damage of cell membranes during an oxidative stress (10). Melatonin reduced oxidative stress by several ways. It is an effective scavenger of hydroxyl radical produced by reduction of oxygen and of the peroxyl radicals generated during the peroxidation of lipids, thus melatonin could prevent lipid membrane peroxidation (20, 21). Additionally, melatonin stimulate antioxidative enzymes such as; superoxide dysmutase or catalase (44). Melatonin was found to inhibit oxidative damage in many tissues (10, 21, 22). Oxidative stress in acute pancreatitis results from the disturbances of pancreatic microcirculation leading to the aggravation of pancreatic damage (13). The ability of melatonin to cross morpho-physiological barriers and to enter subcellular compartments is essential for its protective antioxidative effects (10).

We observed that melatonin diminished pancreatic damage by improving pancreatic blood flow and by causing the significant reduction of the lipid peroxidation, that may be responsible for the prevention of cell membrane from destruction. The earliest morphological events during overstimulation of the pancreas with caerulein involve

the degradation of acinar cell cytosceleton followed by premature activation of pancreatic enzymes and autodigestion of the gland (45). ROS produced during inflammmation are mostly responsible for the disassembly of pancreatic cell cytoskeleton (12–14). It is very likely that melatonin is able to limit the dearangement of digestive enzymes segregation inside the pancreatic acinar cell and the disturbances of enzyme exocytosis by preventing the lipid membranes from peroxidation during CIP induction.

It is of interest, that we have not found any significant effect of leptin on lipid peroxidation in the intact pancreatic gland, despite of spectacular pancreatoprotective action of this peptide. It could be explained that leptin influences the pancreas though the activation of sensory nerves, CGRP release from their endings and *via* the increased NO generation. In contrast to melatonin, leptin does not seem to affect directly the antioxidative mechanisms in the pancreas.

We conclude that melatonin, exerts its pancreatoprotective effect following the peripheral, not central, administration of this substance. We confirm our previous observation that leptin was effective in the pancreatic protection when was given intraperitoneally as well as intracerebroventricularly to the rats with CIP. The detection of leptin receptor in the pancreatic acniar cells suggests that these receptors may be involved in above beneficial effects of leptin on pancreatitis. The protective effects of leptin involve sensory nerves and increased generation of NO, whereas melatonin-induced protection of the pancreas depends, in major extend, on the local effect produced by this indole, particularly by its scavenging of the ROS in the pancreatic tissue.

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Author's address: Dr hab. Jolanta Jaworek, Chair of Physiology, University School of Medicine Jagiellonian University, ul. Grzegórzecka 16, Kraków, Poland