ROLE OF ENDOGENOUS MELATONIN AND ITS MT, RECEPTOR IN THE MODULATION OF CAERULEIN-INDUCED PANCREATITIS IN THE RAT.

The present study investigated the involvement of endogenous melatonin in the prevention of pancreatic damage provoked by caerulein-induced pancreatitis (CIP) by using the luzindole, the antagonist of melatonin MT$_2$ receptors. CIP was produced by subcutaneous infusion of caerulein to conscious rats (25 µg/kg). Luzindole (1, 2 or 4 mg/kg) was given as an intraperitoneal bolus injection 30 min prior to the start of CIP. Lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured in the pancreas by LPO-584 commercial kit. CIP was confirmed by histological examination and manifested by significant increases of plasma activities of amylase, lipase and tumor necrosis factor α (TNFα) (by 500%, 1000% and 600%, respectively) comparing to the control values. This was accompanied by a 40% limitation in pancreatic blood flow (PBF) and by 200% increase of MDA+4-HNE in the pancreas of CIP rats. Administration of luzindole to the CIP rats reduced PBF, aggravated the histological manifestations of pancreatitis, resulted in the significant augmentation of pancreatic MDA + 4-HNE content, and produced the marked increases of plasma levels of lipase, amylase and TNFα, comparing to the values observes in the rats with CIP alone. These results suggest that endogenous melatonin through its receptor MT$_2$ plays an important role in the attenuation of pancreatic damage produced by overstimulation with caerulein.

Key words: luzindole, caerulein-induced pancreatitis, lipid peroxidation, pancreatic blood flow, tumor necrosis factor alpha.
INTRODUCTION

Melatonin (5-methoxy-N-acetyltryptamine), is a pineal product, whose physiological significance is obscure (1, 2). Beside its involvement in the regulation of circadian and seasonal rhythms, melatonin as efficient scavenger may protect various tissues against the damage by radical oxygen species (ROS) (3 - 5). This protective effect of melatonin has been related to both its scavenging of ROS and activating of the antioxidative enzymes such as superoxide dismutase (SOD) or glutathione peroxidase (GSH-PX) (6 - 8). This hormone received attention due to its immunomodulatory properties and it has been demonstrated to influence the cytokine production in inflammatory and cancer diseases (9 - 11). Melatonin exerts its biological effects though its specific membrane receptors classified into three subtypes, two of which; MT1 and MT2, were found in mammals (12, 13). Melatonin binding sites have been widely distributed in the brain and in others tissues such as retina, gastrointestinal mucosa, smooth muscles or melanophores (12-15). Pharmacological antagonists of melatonin receptors were useful tool to investigate the physiological role of this indoleamine in the organism. Luzindole (2-benzyl-N-acetyl-tryptamine), a competitive MT2 receptor antagonist, has been shown originally to inhibit dopamine release in rabbit retina, (16). Luzindole is the best known melatonin analogue and it has been used in numerous studies to assess the significance of the melatonin receptors in central nervous system and in the peripheral tissues (17 - 20).

Previous reports have demonstrated that a considerable amount of melatonin is produced in the gastrointestinal tract, but the physiological role of this indole in the gut is unclear (2, 14, 21). Exogenous melatonin and that, produced endogenously from L-tryptophan, have been recently shown to protect the gastric mucosa against acute lesions and to reduce tissue damage induced by acute pancreatitis (22 - 25). However, it is not know whether above protective activity of melatonin represents pharmacological effect of this hormone, or whether endogenous melatonin could also exert a detectable influence on gastrointestinal tissues.

Our recent study has shown that melatonin is able to prevent pancreatic lesions induced by overstimulation with caerulein and that above favorable effect of melatonin is related to the decreased lipid peroxidation and independent from the activation of sensory nerves and generation of nitric oxide in the pancreas (24). This observation prompted us to determine if endogenous melatonin could contribute to the natural defense mechanism against tissue damage caused by acute pancreatitis. To solve this problem we investigated the effects of luzindole; an antagonist of MT2 receptors in the rats with caerulein-induced pancreatitis.
MATERIAL AND METHODS

Animals and drugs

Studies were performed on male Wistar rats weighing 150 - 200 g. Animals were housed in cages under standard conditions, on commercial pellet chow, at room temperature with a 12-h light and dark cycle. Rats were deprived of food 17 h prior to the start of experiment, while drinking water was available ad libitum.

Following items were purchased: caerulein (Takus) from Pharmacia GmbH, Erlangen, Germany, melatonin from Sigma Co, (St Louis, MO, USA), TNFα solid phase enzyme linked immunosorbent assay (ELISA) kits was from BioSource International, Inc. (Camarillo, CA, USA). The BIOXYTECH LPO-586 kit was purchased from Oxis International Inc. (Portland, OR, USA).

Experimental protocol.

All experiments were carried out at the same time at the morning. 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 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. Luzindole was dissolved in 0.5 ml of DMSO (99.8%) and then in 0.9% saline, and given to the rats as 1.0 ml bolus intraperitoneal (i.p.) injection 30 min prior to the start of CIP. In control tests 1.0 ml of vehicle was administered instead of tested substance.

The following study groups, were employed including: 1/Control (1.0 ml of vehicle i.p.), followed 30 min later by s.c. infusion of 0.9% saline for 5 h. 2/Vehicle (1.0 ml) injected i.p. followed 30 min later by s.c. infusion of caerulein at a total dose of 25 µmg/kg (5 µg/kg-h for 5 h) to induce CIP, 3/ Luzindole (1, 2 or 4 mg/kg i.p.), dissolved in 1.0 ml of vehicle, followed by s.c. infusion of caerulein at a total dose of 25 µg/kg for 5 h, 4/ Luzindole (1, 2 or 4 mg/kg i.p.), dissolved in 1.0 ml of vehicle followed by s.c. infusion of 0.9% saline.

The study protocol was approved by the Ethics Committee on Animal Experiments of the Jagiellonian University.

Examination of pancreatic blood flow (PBF), and on plasma activities of amylase, lipase and TNFα

Following 5-h infusion of caerulein, or saline, the animals were anesthetized with Vetbutal (0.5 ml/kg i.p.), and then the abdominal cavity was opened. Pancreatic blood flow was measured by a laser Doppler flowmeter using a Laserflo, model BPM 403 A (Blood Perfusion Monitor, Vasdamedics Inc. St Paul, Mn, USA) as previously described (24, 25). Blood flow was measured in five different pancreatic regions in each rat and expressed as the percent change of the control value. Immediately afterwards blood was drawn from the inferior vena cava for plasma measurement of amylase, lipase, and tumor necrosis factor alpha (TNFα). Plasma amylase was measured with the modified sacharogenic method using Alpha Diagnostics kit as described elsewhere (24, 25). Lipase was measured using an automatic analyser Kodak Ektachem chemistry slides (Lipa). Plasma TNFα was measured with the ELISA method (Enzyme Linked Immuno-Sorbent Assay) using the kit of Bio-Source International, Camarillo, California, USA.

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 (24, 25).

**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). Then, 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°C until assay. MDA + 4HNE was measured in duplicate and expressed as nM/g of tissue.

**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**

**Effects of luzindole on pancreatic blood flow (PBF), pancreatic weight and on plasma amylase, lipase and TNFα activities**

Single dose injection of luzindole (1, 2 or 4 mg/kg i.p.) to the control rats, receiving infusion of vehicle saline instead of caerulein, failed to affect significantly pancreatic weight, plasma levels of amylase, lipase or TNFα (Figs

![Fig. 1. Pancreatic weight in rats subjected to caerulein-induced pancreatitis (CIP), without or with pretreatment with various doses of luzindole. Asterisk indicates significant (p < 0.05) change, as compared to the value obtained from the rats subjected to CIP alone. Cross indicates significant (p < 0.05) increase above the control value. Control = value obtained from the rats pretreated with vehicle saline alone. Means ± SEM of 6 -8 rats in each experimental group.](image-url)
In the control rats administration of luzindole (1, 2 or 4 mg/kg i.p.) resulted in slight, and insignificant reduction of PBF (Fig 5).

Subcutaneous infusion of caerulein (5µg/kg-h during 5 h) to the rats produced CIP in all animals tested. CIP was manifested by a 40% reduction in PBF, accompanied by significant increase of pancreatic weight, plasma amylase, lipase and TNFα activities (by 200%, 500%, 1000% and 600%, respectively) (Figs 1 - 5).

Intraperitoneal administration of luzindole at dose of 4 mg/kg given to the rats 30 min prior to the start of CIP produced significant increase of pancreatic weight, plasma amylase, lipase, and TNFα activities over the values obtained from the rats subjected to CIP alone (Figs 1 - 5). Luzindole given to the rats with
CIP at doses of 2 or 4 mg/kg i.p. resulted in the significant limitation of PBF as compared to the values observed in the rats with CIP without luzindole pretreatment (Fig. 5). Dose of 2 mg/kg i.p. of luzindole produced marked increase of pancreatic weight and plasma lipase level, but did not affect significantly plasma activities of amylase and TNFα, comparing to the values obtained in rats with CIP alone (Figs 1 - 4). Pretreatment of the CIP rats with luzindole given at dose of 1 mg/kg failed to affect significantly any of above pancreatic parameters tested (Figs 1-5).

Fig. 4. Pancreatic blood flow in rats subjected to caerulein-induced pancreatitis (CIP), without or with pretreatment with various doses of luzindole. Asterisk indicates significant (p < 0.05) change, as compared to the value obtained from the rats subjected to CIP alone. Cross indicates significant (p < 0.05) decrease below the control value Control = value obtained from the rats pretreated with vehicle saline alone. Means ± SEM of 6 -8 rats in each experimental group.

Fig. 5. TNFα plasma level in rats subjected to caerulein-induced pancreatitis (CIP), without or with pretreatment with various doses of luzindole. Asterisk indicates significant (p < 0.05) change, as compared to the value obtained from the rats subjected to CIP alone. Cross indicates significant (p < 0.05) increase above the control value Control = value obtained from the rats pretreated with vehicle saline alone. Means ± SEM of 6 -8 rats in each experimental group.
Effects of luzindole on lipid peroxidation products (MDA + 4-HNE) in the pancreatic tissue

Administration of increasing doses of luzindole (1, 2 or 4 mg/kg i.p.) failed to affect significantly MDA + 4-HNE in the pancreatic tissue of control rats, infused with vehicle saline, instead of caerulein (Fig 6).

Following caerulein infusion to produce CIP, the level of lipid peroxidation products in the pancreas significantly increased by about 500%. Pretreatment of CIP rats with luzindole given at dose of 4 mg/kg significantly increased MDA + 4-HNE content in the pancreas, as compared to the value obtained in the rats with CIP alone (Fig. 6). Luzindole given at lower doses (1 or 2 mg/kg i.p.) to the rats with CIP failed to affect significantly lipid peroxidation in the pancreas of CIP rats (Fig 6).

Histological examination

In the control rats, infused with vehicle saline instead of caerulein, administration of luzindole (1, 2 or 4 mg/kg i.p.) did not change significantly pancreatic morphology (Fig 7, Table 1).

Infusion of caerulein (5µg/kg-h x 5 h) produced typical pancreatic lesions in all tested rats (Fig 7, Table 1). 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. Following the pretreatment of CIP rats with luzindole at doses of 2 or 4 mg/kg i.p. the significant aggravation of pancreatic edema and neutrophil infiltration of pancreatic tissue over the values obtained from the rats with CIP alone was observed (Fig 7, Table 1). Luzindole at doses of 1, 2, or 4 mg/kg i.p failed to affect significantly acinar cell vacuolization produced by CIP. Dose of 1 mg/kg
i.p. of luzindole did not produced any significant change of pancreatic morphology in the rats with CIP (Table 1).

DISCUSSION

The results of present study provides the evidence that administration of luzindole, melatonin MT₂ receptor antagonist, aggravates pancreatic damage.
induced by caerulein overstimulation. This observation suggests that endogenous melatonin may be involved in the modulation of inflammatory response in acute pancreatitis.

Previous reports have shown that in addition to pineal gland, the gastrointestinal tract is a major source of circulating melatonin (2, 14, 21). Despite of the demonstration of melatonin binding sites in the gut and in the pancreas the physiological significance of this hormone is largely unknown (14). Exogenous melatonin is able to reduce gastric mucosal lesions caused by stress or ischemia/reperfusion and could effectively protect the liver against the damage produced by toxic oxidative reaction (6, 22, 27). Recent reports have demonstrated that melatonin given in pharmacological doses also attenuates pancreatic damage induced by caerulein-induced acute pancreatitis in the rats (23- 25). However, the evidence concerning the physiological role of melatonin in the gastrointestinal tract gut was poorly documented.

Melatonin received particular attention due to its ability to protect the various tissues against the damage caused by oxidative stress (5, 6, 22, 25, 28 -31). Pharmacological doses of melatonin prevent from the organ injury associated with septic shock, inflammation or ischemia/reperfusion (5, 6, 22, 25, 32). Administration of melatonin has been found to reduce lipid peroxidation and to improve the clinical outcome in septic newborns (33). This indole is also effective in reducing neuronal damage and thought to prevent the brain from the

**Table 1** Effect of increasing doses of luzindole (1, 2 or 4 mg/kg i.p.) on pancreatic morphology in the rats without or with caerulein-induced pancreatitis (CIP).

Asterisk indicates significant (p < 0.05) increase over the value obtained with CIP alone. CONTROL = values observed in the rats pretreated with saline instead of caerulein. Means ± SEM of 6 rats in each experimental group.

<table>
<thead>
<tr>
<th></th>
<th>EDEMA (0-3)</th>
<th>INFILTRATION (0-3)</th>
<th>VACUOLISATION (0-3)</th>
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<tr>
<td>CONTROL</td>
<td>0.2 ± 0.1</td>
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<td>0.0</td>
</tr>
<tr>
<td>LUZINDOLE 1 mg/kg + NaCl</td>
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<td>0.2 0.0</td>
<td>0.0</td>
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<tr>
<td>LUZINDOLE 2 mg/kg + NaCl</td>
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<td>0.0</td>
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<td>CIP alone</td>
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<td>2.0 ± 0.1</td>
<td>2.2 ± 0.0</td>
</tr>
<tr>
<td>LUZINDOLE 1 mg/kg + CIP</td>
<td>2.2 ± 0.9</td>
<td>2.0 ± 0.0</td>
<td>2.3 ± 0.5</td>
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<tr>
<td>LUZINDOLE 2 mg/kg + CIP</td>
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<td>2.5 ± 0.1*</td>
<td>2.3 ± 0.7</td>
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<tr>
<td>LUZINDOLE 4 mg/kg + CIP</td>
<td>3.0 ± 0.0*</td>
<td>2.8 ± 0.2*</td>
<td>2.25 ± 0.4</td>
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neurodegenerative diseases, such as Parkinson or Alzheimer (34, 35). Because of its protective effects exerted on neurons melatonin has been suggested as an anti-aging hormone (36). It has been also attempted as therapeutical agent in the cancer, affective disorders, anxiety and endotoxic shock (11, 29, 37). In addition melatonin has been demonstrated to reduce the thirst and fever caused by bacterial lipopolysaccharide (17).

All above effects of melatonin results from its chemical properties. Numerous studies have demonstrated that this indoleamine acts as a direct scavenger of ROS and stimulates the antioxidant enzymes such as glutathione peroxidase (GSH-PX) or catalase (CAT) (33, 38). Melatonin decreases lipid peroxidation, acts to stabilize cell membranes, and enhances their resistance to oxidative stress (31, 39, 40).

Acute pancreatitis is a serious disease whose pathogenesis is intensively studied. A large body of evidence suggests that ROS could play an essential role in pancreatic tissue damage caused by acute inflammation (41 - 43). In acute pancreatitis the decrease of pancreatic blood flow leads to the accumulation of activates macrophages and increased concentration of ROS in the vicinity of pancreatic acinar cells (43). Peroxidation of lipid membranes, disintegration of cytoskeleton and intracellular compartments by ROS might lead to the disturbances of digestive and lizosomal enzymes transport within the acinar cell leading to this cell destruction (41, 44). Enzymes and ROS produced by pancreatic acinar cells and released into the interstitium reaches the capillaries and leads to their injury. The increase of the capillary permeability initiates tissue edema and propagation of the destructive processes (45).

In acute pancreatitis the degree of tissue damage depends upon the balance between the inflammatory factors promoting the cytotoxicity (ROS, endotoxins, pro-inflammatory cytokines) and, on the other hand, on the activation of the natural defense mechanism (endogenous nitric oxide, prostaglandins), which increases the pancreatic resistance against the damage (41-43, 46-47). It is very likely that endogenous melatonin is involved in these anti-inflammatory processes in acute pancreatitis and could be one of the beneficial factors limiting the propagation of the cell destruction.

Previous reports has demonstrated that exogenous melatonin decreases lipid peroxidation in acute experimental pancreatitis (22, 24, 25). Our present study shows that pretreatment of the CIP rats with melatonin MT\_2 receptor antagonist; luzindole leads to the increase of lipid peroxidation products (MDA + 4-HNE), this providing indirect evidence of ROS generation in the pancreas.

The major finding of our study the observation that luzindole produced the decrease of pancreatic blood flow in rats under basal conditions and following the overstimulation of the pancreas with caerulein. Melatonin, by itself, has been reported to affect the vascular myocytes contractivity, however the results of the studies concerning above vasoactive action of melatonin are controversial. Melatonin, has been found to release vascular smooth muscles and this
vasorelaxant effect was induced by the activation of MT₂ melatonin receptors (48-50). However some studies have reported that melatonin could also potentiate the contraction of the vessels of tail artery stimulated by phenylephrine and that this effect is dependent upon the activation of MT₁ melatonin receptor (19, 48, 51). The results of present study showing that luzindole, MT₂ receptor antagonist, produced the decrease of pancreatic blood flow, support and reinforced the hypothesis that melatonin MT₂ receptors are implicated in the regulation of vascular muscle tone by producing the relaxation of blood vessels.

Another, we believe important finding of our study, is the observation that luzindole could influence the production of TNFα in acute pancreatitis. Previous publications, including our reports, have demonstrated that serum level of this cytokine increases in acute pancreatitis (52, 53). TNFα, released from macrophages, play an important role in the initiation of pathological processes in pancreatic inflammation (54, 55). Herein we demonstrate, for the first time, that administration of luzindole resulted in a significant increase of this pro-inflammatory cytokine blood level. Melatonin has been reported to influence the inflammatory cells and to affect the production of cytokines (9). This substance is able to reduce the production of TNFα induced by endotoxins (32, 49). Our study presents the indirect evidence supporting the notion that endogenous melatonin is involved in the regulation of cytokine production.

In summary, this study shows that the blockade of melatonin MT₂ receptors by luzindole, aggravates pancreatic inflammation, thus providing evidence that endogenous melatonin acts through its MT₂ receptor to improve the resistance of pancreatic tissue against the damage caused by overstimulation with caerulein.

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