Heat shock proteins (HSPs) have been reported to protect the pancreatic cells from the acute damage produced by caerulein overstimulation. However, the effects of caerulein, melatonin or hyperthermia preconditioning on mRNA signal for HSP60 in the pancreatic acinar cells has not been examined yet. The aims of this study were: 1. To investigate the gene expression for HSP60 in the pancreatic AR42J cells stimulated by melatonin, caerulein or combination of both these substances. 2. To compare the above changes with mRNA signal for HSP60 in pancreatic AR42J cells subjected to hyperthermia preconditioning. AR42J cells were incubated in standard medium at 37°C for: 0, 1, 3, 5, 12 or 24 h, under basal conditions. Above cells were then subjected to heat shock (42°C) for 0, 1 or 3 h. In the next part of the study AR42J cells were incubated in presence of caerulein (10⁻¹¹, 10⁻⁹ or 10⁻⁷ M), melatonin (10⁻⁸ or 10⁻⁶ M), or combination of above under basal conditions or following heat shock pretreatment. Gene expression for HSP60 was determined by RT-PCR. The mRNA signal for HSP60 has been observed in AR42J cells under basal conditions, and this signal was markedly and time-dependently increased in these cells subjected to hyperthermia preconditioning. Incubation of AR42J cells in presence of melatonin (10⁻⁸ or 10⁻⁶ M) resulted in the significant and dose-dependent increase of gene expression for HSP60 in both groups of AR42J cells: preconditioned and in those, which were not subjected to hyperthermia. Caerulein stimulation reduced mRNA signal for HSP60. The strongest signal has been observed after the exposition of AR42J cells to hyperthermia preconditioning, combined with melatonin and caerulein. We conclude that: 1. Gene expression for HSP60 has been detected in pancreatic AR42J cells under basal conditions. 2. Hyperthermia preconditioning resulted in a significant and time-dependent increase of HSP60 signal in pancreatic AR42J cells. 3. HSP60 gene expression was significantly increased in pancreatic AR42J cells stimulated by melatonin whereas caerulein reduced this signal. 4. The strongest gene expression for HSP60 has been found in the cells subjected to the combination of hyperthermia preconditioning, caerulein and melatonin.

**Key words:** Melatonin, caerulein, HSP60, AR42J cells, RT-PCR.
INTRODUCTION

Melatonin, is known as a main product of the pineal gland. Subsequent studies have revealed that this indoleamine could be produced in the eye (retina, ciliary body) as well as in the gastrointestinal tract (stomach and intestine), where it is synthesized in the enterochromaffine cells (1-4).

In the digestive tract melatonin has been found to protect the gastric and duodenal mucosa against peptic ulcers. This beneficial effect of melatonin has been related to its ability to scavenge the oxygen radical species, to improve the gastric blood flow and to stimulate prostaglandin (PG) generation in the gastric mucosa (5, 6). Specific melatonin receptors have been detected in the pancreas (7). Melatonin through its antioxidant and immunomodulative properties have been reported to protect the pancreas against its acute damage (8, 9). Recently melatonin has been found to stimulate pancreatic enzyme secretion in vivo and to increase plasma CCK level (10, 11). Despite this findings the physiological and pathological role of melatonin in the pancreas has not been fully explained.

It has been shown that hyperthermia preconditioning stimulates heat shock protein (HSP) synthesis in the pancreatic tissue. This protein have been demonstrated to protect the pancreas in various models of acute pancreatitis (AP). Above effect has been related to the inhibition of intrapancreatic activity of trypsynogen caused by HSP (12, 13). So far, little information is available concerning HSP gene expression changes in the pancreatic cells in AP and the influence of the melatonin on the mRNA signal for HSP was not studied.

The aims of this study were: 1. To investigate the effect of hyperthermia preconditioning on HSP60 mRNA signal in AR42J cells, 2. To determine changes of mRNA signal for HSP60 in the AR42J cells following stimulation of these cells by caerulein or melatonin, and 3. To asses the HSP60 gene expression in AR42J cells subjected to melatonin and caerulein combined with hyperthermia preconditioning.

MATERIAL AND METHODS

Cells and drugs

The study was performed on AR42J cells (rat pancreatic acinar cell line) (American Type Culture Collection, Rockville, MD, USA). Cell culture was conducted in the medium, containing RPMI 1640 Medium with Glutamax-I (Gibco BRL, Gaithersburg, MD, USA) and 10% fetal bovine (FBS, head-inactivated; Gibco BRL) in the ratio 1:1 and with addition of 100 U/ml Penicyline and 100 µg/ml Streptomycine (Sigma, St. Louis, MO, USA) in the standard condition (37°C and 5% CO2). Plateled cells were collected by the TRizol REAGENT (GIBCO BRL).

Caerulein (Takus) from Pharmacia GmbH, Erlangen, Germany and melatonin from Sigma Co (St. Louis, MO, USA) were used for the experiments.
Experimental protocol:

Part A
The study on the effects of caerulein and melatonin on HSP60 gene expression in AR42J cells.

In this part of the study cells were stimulated with the increasing concentrations of caerulein (10^{-11}, 10^{-9} or 10^{-7}M), whereas the others were given increasing doses of melatonin (10^{-8} or 10^{-6}M). Cells of both groups were incubated with tested substances for: 0, 1, 3, 5, 12 or 24 h. Subsequently, the most effective concentrations of caerulein, that was 10^{-11}M, or melatonin (10^{-8}M), were selected for further experiments. Time-course experiments (data not shown) showed that the most effective incubation time was the 3 h and this time was used in further studies.

The separate group of cells was stimulated by combination of caerulein (10^{-11}M) and melatonin (10^{-8}M) during 3 h.

Part B
The study on of the effects of caerulein and melatonin on the AR42J cells preconditioned by hyperthermia.

In this part of the study cells underwent hyperthermia preconditioning by exposition to the temperature of 42 °C, during: 0, 1 and 3 h. Then hyperthermically preconditioned AR42J cells were incubated in the presence of caerulein (10^{-11}M), melatonin (10^{-8}M) or combination of above during 3 h.

Determination of gene expression for HSP60 in the AR42J cells.

AR42J cells were taken (and immediately frozen in liquid nitrogen) from the control and from all experimental groups. Total RNA was extracted by a guanidinum isothiocyanate-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 producer standard procedure (Promega Corporation, USA).

Primers for HSP60 were synthesized by Gibco BRL/Life Technologies (Eggenstein, Germany). The HSP60 sense primer was 5’ TAT GGC TAT CGC TAC TGG TG , while the HSP60 antisense primer was 5’ AAC CTT CAA CAC GGC TAC TC. Primers were designed using programme Primer Premier 4 (Sigma, St. Louis USA) The expected length of this PCR product was 300 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 MgCl2, 0.5 mM dNTPs in a volume of 50 µl. To maximize the amplification specificity, hot-start PCR was performed for 29 cycles (94°C for 5 min, 94°C for 1 min, 58°C for 1 min and 72°C for 2 min). Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gels containing ethidium bromide. Then visualization under UV light was performed. To compare the level of expression of HSP60 mRNA against the reference gene (β - actin) mRNA data, the image analysis was employed. PCR products were analyzed using program Gel-Pro analyzer (Media Cybernetics, Silver Spring, Mass, USA).
RESULTS

Part A
The study of the effects of caerulein and melatonin on HSP60 gene expression in AR42J cells.

HSP60 mRNA signal in AR42J cells was detected in all samples examined (Fig. 1-3). This signal was present in the control cells as well as in the those stimulated by various concentrations of caerulein (10⁻¹¹, 10⁻⁹ or 10⁻⁷ M). Incubation of the cells with this secretagogue caused a small but significant decrease of HSP60 signal, as compared to the control value (Fig. 1).

Gene expression of HSP60 was detected in the AR42J cells following the addition of melatonin (10⁻⁸ or 10⁻⁶M) to the medium. The ratio of HSP60/β-actin mRNA signal in the control cells was 0.27 ± 0.02 after 3 hours of incubation. Application of melatonin (10⁻⁸ or 10⁻⁶M), to AR42J cells resulted in the marked increases of this ratio to 1.25 ± 0.03 and 0.97 ± 0.04 respectively (Fig. 2).

Fig. 1. Gene expression for HSP60 measured by RT-PCR in AR42J cells incubated under basal conditions (lane 1), stimulated by caerulein at concentrations of: 10⁻¹¹ M (lane 2), 10⁻⁹ M (lane 3) and 10⁻⁷ M (lane 4). M- molecular weight marker: HSP60 - 300bp and β-actin 764bp. NC- negative control.
The strongest HSP60 mRNA signal was detected in AR42J cells treated with combination of melatonin (10^{-8}M) and caerulein (10^{-11}M). In these cells the ratio of HSP60/β-actin reached 1.8 ± 0.03 (Fig. 3).

Part B
The study of the effects of caerulein and melatonin on gene expression of HSP60 in the AR42J cells subjected to hyperthermia preconditioning.

Hyperthermia (42° C for 1 or 3h) resulted in the time-dependent increase of HSP60 mRNA signal in all examined cell samples. The ratio of HSP60/β-actin mRNA increased gradually at time intervals 0, 1 and 3 hours, to the levels of: 0.4 ± 0.03, 0.78 ± 0.04 and 1.1 ± 0.04 respectively (Fig. 4 A).

Addition of caerulein (10^{-11}M) to AR42J cells subjected to hyperthermia preconditioning decreased mRNA signal for HSP60, as compared to the preconditioned group without secretagogue administration. The ratio of

*Fig. 2. Changes of HSP60 mRNA signal detected by RT-PCR in AR42J cells incubated under basal conditions (lane 1), or treated with melatonin at concentrations of 10^{-8}M (lane 2) or 10^{-6}M (lane 3). M-molecular weight marker: HSP60 - 300bp and β-actin 764bp. NC - negative control.*
HSP60/β-actin was at the level of 0.2 ± 0.03, 0.48 ± 0.04 and 0.8 ± 0.04 at time points: 0, 1 and 3 h, respectively (Fig. 4 B).

To the contrary, addition of melatonin (10^-8M) to the medium containing hyperthermically preconditioned cells, produced statistically significant increase of HSP60 gene expression, as compared to the cells subjected to hyperthermia alone. The ratio of HSP60/β-actin increased in parallel to the time of hyperthermia preconditioning and reached the levels of 0.4 ± 0.03, 1.08 ± 0.04 and 1.42 ± 0.04 after: 0, 1 and 3 hours of incubation respectively (Fig. 4 C).

The strongest mRNA signal for HSP60 was detected in hyperthermically preconditioned cells stimulated by the combination of melatonin (10^-8M), and caerulein (10^-11M). The ratio of HSP60/β-actin, was proportional to the time of hyperthermia preconditioning and reached the values of 0.41 ± 0.03, 1.3 ± 0.04 and 1.65 ± 0.03 at time points: 0, 1 and 3 hours respectively (Fig. 4 D).
DISCUSSION

The presence of heat shock proteins (HSPs) have been previously detected in the pancreas (14). Previous data from the rat study have demonstrated that in caerulein-induced pancreatitis the HSP60 level in the pancreas was markedly reduced (15). In spite of this observation the effect of caerulein on gene expression for HSP in pancreatic acinar cells has not been examined yet.

Fig. 4. The ratio of HSP60/β-actin detected by RT-PCR in AR42J cells incubated under basal conditions (lane 1) or in the AR42J cells subjected to hyperthermia preconditioning at time intervals 0 h (lane 2), 1 h (lane 3), or 3 h (lane 4) without [A] or with addition of caerulein (10^{-11}M) [B], melatonin (10^{-8}M) [C], or combination of above [D]. M-molecular weight marker: HSP60 - 300bp and β-actin 764bp. NC - negative control.
Our present data show that incubation of AR42J cells with caerulein leads to the significant decrease of HSP60 mRNA signal in these cells. Caerulein overstimulation is known to produce acute pancreatitis, and it is likely, that under these conditions the HSP production in the pancreatic acinar cells is markedly reduced leading to these cells damage as reported previously (15).

Our results revealed for the first time that melatonin is able to increase the gene expression for HSP60 in AR42J cells and the highest level of HSP60 signal has been observed following the application of melatonin together with caerulein to the medium of these cells.

Melatonin is known to protect the pancreas against the acute damage caused by caerulein overstimulation, by improving the antioxidant status of pancreatic tissue, by modulating the immune response (increase of pro- and decrease of anti-inflammatory cytokines), and by improving the pancreatic blood flow (8, 9, 16).

Our results suggest that melatonin promoted protection against caerulein-induced pancreatitis (16) may be mediated by upregulation of HSP in acinar cells. Although caerulein alone diminished HSP60 signal in AR42J cells, its combination with melatonin augmented the HSP60 expression in these cells. These discrepant effects of caerulein on HSP60 in pancreatic cells requires further studies and explanation. It is very likely that melatonin contributes to the activation of HSP and that this melatonin effect could take a part in the protection of pancreatic tissue from the damage caused by acute inflammation. Above notion is supported by the previous observation showing that luzindole (melatonin antagonist of MT2 receptor) aggravated the pancreatic damage caused by acute caerulein-induced pancreatitis (16).

The protective effect of melatonin have been also demonstrated in the stomach. This indole acts as a free radical scavenger and an antioxidant. It has been also reported to improve the gastric blood flow, to increase iNOS gene expression and NO generation, to stimulate PG synthesis in the gastric mucosa, as well as to regulate CGRP release from sensory nerve endings (5, 6, 17, 18).

Previous data have shown that HSP60 and HSP70 exhibit immunomodulative properties leading to the suppression of NF-kappaB generation and to the decrease of the pro-inflammatory cytokines production (19). The production of anti-inflammatory cytokines have been stimulated by above HSPs in acute pancreatitis (20, 21). In the stomach subjected to acute ethanol injury the increase of HSP70 gene expression induced by NO-ASA has been reported. Under these conditions the expression of the antioxidative enzymes was markedly increased (22).

Our data have shown that the addition of melatonin to the medium containing hyperthermically preconditioned AR42J cells markedly increased HSP60 mRNA signal in these cells. It has been demonstrated that hyperthermia preconditioning stimulates expression of HSP in the pancreatic tissue. Hyperthermia-provoked protection against caerulein-induced pancreatitis may be mediated through the increase of HSP content in pancreatic acinar cells (23, 24). The mechanism of protective action of HSP in acute pancreatitis could be related to the prevention
from the intracellular activation of trypsinogen and to the stabilization of the cellular cytoskeleton by HSP (12, 13). HSP60 and HSP70 take a part in the formation of tridimensional structure of proteins and in the elimination of damaged or irregularly synthesized proteins from the cells.

Previous data have shown that water immersion and restraint stress caused the increase of HSP60 expression but hyperthermia activates HSP72. It has been reported that the increase of HSP60 gene expression in the pancreas of rats exposed to water immersion and restraint stress was correlated with attenuation of pancreatic damage produced by acute caerulein-induced pancreatitis. It is also believed, that hyperthermia, in contrast to the caerulein stimulation, leads to the increase of TGF-β generation and to the activation of HSP70 production in the pancreas. This observation suggests, that TGF-β₁ takes a part in the protection of pancreas against acute pancreatitis, and in the stimulation of pancreatic regeneration (25, 26). In the early stage of CIP, the short increase of TGF-β₁ in the pancreas was observed, which is likely to be the result of neutrophil and platelet infiltration (25, 26).

It has been reported in the experimental study that the administration of HSP coinducer drug-candidate, BRX-220 to the rats with advanced caerulein-induced pancreatitis, leads to the stimulation of HSP generation in the pancreatic cells resulting in the pancreatic protection against acute pancreatitis (27, 28). These results should be particularly important for the understanding the protective role of HSP in the pancreas subjected to caerulein overstimulation (27).

Our present in vitro findings indicate that melatonin is able mRNA signal for HSP60 in pancreatic AR42J cells. It is very likely that melatonin could stimulate HSP generation in the pancreatic acinar cells, and that this mechanism could be involved in the protective effect of indoleamine on the pancreas. To verify this hypothesis further studies are needed.

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