

**Effect of natural products on the pathogenesis of acute
experimental pancreatitis**

PhD thesis

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INTRODUCTION

Acute pancreatitis

Acute pancreatitis is an inflammatory disease caused by the premature activation of pancreatic exocrine enzymes which leads to the injury of the gland and other organs. Approximately 3000 patients develop acute pancreatitis annually in Hungary. Alcohol and gallstones are the etiological factors in almost 80% of the cases, but several other factors are believed to be important in the initiation of the inflammatory cascade.

The clinical classification into mild and severe forms has prognostic relevance. Mild edematous pancreatitis is considered to be a self-limiting disease with low complication rate and it can be treated in most patients by fasting. In contrast to this, the mortality of the severe necro-hemorrhagic form (10-20%) is still high and except biliary pancreatitis it does not have a specific therapy.

Pathogenesis of acute pancreatitis

The molecular background of the pathogenesis of acute pancreatitis is still not fully understood. Intracellular activation of the exocrine enzymes is believed to be the key step in the development of the disease. The best established theory pronounces the central role of the fusion of zymogen granules and lysosomes as the result of a secretory blockade caused by cholecystokinin (CCK) hyperstimulation. In the formed autophag vacuole the lysosomal enzyme cathepsin B can activate the pancreatic trypsinogen. Auto-activation of trypsinogen which is facilitated by an acidic pH and high calcium concentration is another possible mechanism.

As activated enzymes can enter the systemic circulation and injure the tissue of the lung and other organs, local pancreatic changes can be accompanied by systemic alternations.

Trypsin can activate the complement system and thereby lead to the production of chemotactic complement factors (C3a, C5a). These factors attract macrophages and neutrophils to the tissue which produce further cytokines. The released tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL), IL-6, nitric oxide and platelet activating factor can amplify the inflammatory response. Pancreatic acinar cells and infiltrating leucocytes can produce oxygen and nitrogen derived free radicals (FR) which can damage the lipid membranes of the cell. Lipid peroxidation is leading to the increased permeability of the membranes and finally to cell death. In the early phase of pancreatitis FRs are responsible for edema formation. Tissue edema and also systemic stress results in the slow down of pancreatic microcirculation and as a consequence of this it leads to formation of thrombi which causes the hypoxia and necrosis of the tissue. This is probably the reason for the conversion of mild edematous pancreatitis to necrohemorrhagic pancreatitis.

In the investigations of inflammatory processes, attention has recently turned to the activation of the transcription factor nuclear factor kappa B (NF- κ B). NF- κ B binding elements have been found in the promoter region of many genes encoding TNF-alpha, IL-1 and 6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and many other cytokines and enzymes. In the pancreas, NF- κ B exists as a homo or heterodimer of two subunits called p65 and p50. Under physiological conditions, NF- κ B is located in the cytosol of the cell and is bound to the inhibitory protein I-kappaB (I- κ B). During the inflammatory processes, I- κ B is phosphorylated by I- κ B kinase and becomes the target of degradation, and the unbound NF- κ B can translocate to the nuclei. NF- κ B is believed to be a key factor in the development of acute pancreatitis. Inhibition of NF- κ B activation has been shown to exert anti-inflammatory effects in acute pancreatitis.

Experimental pancreatitis

The pathomechanism and especially the early events of acute pancreatitis can not be sufficiently studied in clinical settings; therefore several experimental models were developed for the induction of pancreatitis in different species and with varying severity. Mild edematous pancreatitis can be induced in rodents by the administration of secretagogues like CCK-octapeptide (CCK-8) or cerulein or by ligation of the pancreatic duct. Acute necrotizing pancreatitis can be evoked by the retrograde injection of taurocholic acid to the biliopancreatic duct, by intraperitoneal injections of L-arginine or in mice by a choline-deficient ethionine-supplemented diet. The lethality of these models is high. The CCK-8 and the L-arginine-induced pancreatitis models were used in our experiments to evoke edematous or necrotizing pancreatitis respectively. These models are simple, highly reproducible and the severity of the illness is dose dependent.

Antioxidant or anti-inflammatory drugs in the treatment of experimental pancreatitis.

Several antioxidant drugs were applied in the therapy of acute experimental pancreatitis in the past. These substances were able to influence some parameters of acute pancreatitis but clinically they were not efficient enough to reduce the severity of human pancreatitis. Therefore our attention was turned to substances which can target a broader spectrum of pathological mechanisms.

Melatonin

The pineal product melatonin is known to play a role in the regulation of the circadian rhythm. The antioxidant activity of melatonin has recently received significant attention. The protective effects of melatonin have been documented in experimental models of numerous diseases like stroke, Alzheimer, Parkinson and Huntington diseases, fetal brain

injury, hypoxia/reperfusion-induced heart, liver, retina and gut injuries, endotoxic and circulatory shock.

Melatonin exerts its antioxidant effects by directly detoxifying free radicals, influencing the activity of scavenger enzymes like glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) and by inhibiting the activity of NOS. Moreover, melatonin can inhibit the activation of NF- κ B, thereby it reduces the synthesis of inflammatory cytokines such as IL-6 and TNF- α .

In an earlier study, Qi and colleagues showed that in mild, cerulein-induced edematous pancreatitis pharmacological doses of melatonin protected against the injury caused by FRs. Melatonin decreased the edema in the pancreas and stomach, and also the extent of lipid peroxidation in the pancreas. Jaworek et al. demonstrated that even the circadian changes in the physiological levels of melatonin reduced the severity of experimental pancreatitis. The protective effect of melatonin in severe L-Arg-induced pancreatitis has not been investigated to date

Resveratrol

Resveratrol (trans-3,5,4'-trihydroxystilbene), a naturally occurring phytoalexin, is found in numerous plants such as grapes, peanuts and soya beans, and it can reach especially high concentrations in red wine. Plants produce resveratrol in response to fungal infections or UV irradiation. Resveratrol had long been used in the traditional Chinese medicine as a drug against inflammation, allergy and hyperlipidemia before its beneficial effect was proven in experimental settings. It exerts antioxidant, anti-inflammatory and also anti-cancer effects in various organs like heart, brain, lung and intestines. The protective properties of resveratrol observed *in vitro* and *in vivo* and the high amount of it present in red wine had led some scientists to believe that this is the substance responsible for the "French paradox": the

phenomenon that the frequent consumption of red wine in France is associated with a reduced mortality due to coronary heart disease and cancer as compared with other European countries.

The molecular background of the effects of resveratrol appears to be its ability to eliminate hydroxyl, superoxide and metal-induced radicals and thereby protect the lipid membranes of the cell against the lipid peroxidation otherwise caused by these radicals. The suppression of COX-2 and iNOS activities also contributes to its anti-inflammatory and antioxidant effects. Resveratrol additionally inhibits platelet aggregation and endothelial activation, and blocks all three phases of tumor development including initiation, promotion and progression. Resveratrol blocks the activity of I- κ B kinase, thereby inhibiting the activation of NF- κ B and the production of inflammatory cytokines. Our experiment investigated the protective effects of resveratrol pretreatment in acute CCK-8-induced experimental pancreatitis.

Zerumbone

Zerumbone (2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one) is a sesquiterpenoid found in large amounts in the rhizome of *Zingiber zerumbet*, a plant traditionally used as a condiment. In the South-east Asian countries *Zingiber zerumbet* is used for the treatment of swellings, sores, loss of appetite, worm infestation, toothache and indigestion. Sesquiterpenes such as zerumbone isolated from this plant also possess anti-allergenic and anti-spasmodic properties. Only few literary data exist about the molecular effects of zerumbone. However *in vitro* experiments with zerumbone have demonstrated a number of beneficial effects of the drug. Most of these studies were focused on the chemopreventive and chemotherapeutic effects of zerumbone in tumor cell lines. Further *in vitro* experiments have shown that zerumbone inhibits the activation of Nf- κ B and also the *de novo* synthesis

of iNOS and COX-2. Intrinsic antioxidant properties of zerumbone are believed to contribute to its anti-tumor action.

The *in vivo* effects of the compound on diseases associated with inflammation and oxidative stress are not well described. The *in vivo* study by Somchit et al showed that an extract of *Zingiber zerumbet* reduces the extent of experimentally induced hind paw edema in rats. Murakami et al. detected the beneficial effects of the substance *in vivo* in a model of dextran sulfate-induced colitis in mice. Prolonged oral administration of the drug reduced the levels of IL-1 β , TNF- α and prostaglandin E2 in the colonic mucosa of the animals and reduced the extent of histological damage to the tissue. To date, the influence of zerumbone on the severity of pancreatitis has not been investigated. As transcription factor Nf- κ B, mediators such as IL-6 and TNF- α and also enzymes such as iNOS and COX-2 play important roles in the development of acute pancreatitis, the drug is a promising candidate for the prevention or treatment of acute pancreatitis. Therefore we intended to detect the effects of zerumbone in CCK-8-induced experimental pancreatitis.

Aims

The aim of this study is to enlarge our knowledge about the pharmacological action of melatonin, resveratrol and zerumbone, three natural substances with antioxidant and anti-inflammatory features, and particularly to investigate the impact of these substances on the severity of acute experimental pancreatitis.

METHODS

Animals

Male Wistar rats weighing (200-300g) were used in our experiments. Tissue samples were collected from the animals after exsanguinations in pentobarbital anesthesia. The samples were quickly removed and frozen at -70°C until

use. These studies were approved by the Ethical Committee on Animal Experiments of the University of Szeged, in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983).

Protocol of the melatonin study

Rats were divided into five groups (n=5 per group). In group A, pancreatitis was induced with 3.2 g/kg body weight L-Arg i.p. twice at an interval of 1 h. Rats in group MA were treated with a single dose of 50 mg/kg body weight melatonin i.p. 30 min prior to L-Arg administration. Rats of group AM received the same dose of melatonin 1 h after the second injection of L-Arg. In group M, a single dose of melatonin was administered. The rats in group C served as control animals and received i.p. injections of physiological saline.

Protocol of the resveratrol study

Rats were divided into three groups (n=5 in each group). The rats in group RP were treated with a single dose of 10 mg/kg body weight resveratrol i.p. 30 min prior to the induction of pancreatitis by three s.c. injections of 75 μ g/kg CCK-8 at intervals of 1 h. In group P, the rats received an injection of physiological saline instead of resveratrol, followed by the three CCK-8 injections. The rats in group C served as control animals and received one i.p. and three s.c. injections of physiological saline.

Protocol of the zerumbone study

Rats were divided into four. The rats in group ZP were treated i.p. with a single dose of 20 mg/kg zerumbone 30 min prior to the induction of pancreatitis by three s.c. injections of 100 μ g/kg CCK-8 at intervals of 1 h (n=10). In group P, the rats received an injection of DMSO (vehicle) instead of zerumbone, followed by the three CCK-8 injections (n=10). The rats in group Z were treated i.p. with a single injection of

20 mg/kg zerumbone followed by three s.c. injections of physiological saline (n=5). The rats in group C served as control animals and received one i.p. and three s.c. injections of physiological saline (n=5).

Assays

The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema. The serum amylase, lipase and aspartate aminotransferase (ASAT) activities, and the triglyceride, creatinine, urea nitrogen, calcium and glucose concentrations were determined by colorimetric methods (Dialab, Vienna, Austria, and FaBio, Hungary). The concentration of the lipid peroxidation marker malonyl dialdehyde (MDA) was measured as described by Placer. Total SOD activity was determined on the basis of the inhibition of epinephrine-adenochrome autoxidation. Mn-SOD activity was measured via the extent of autoxidation in the presence of 5×10^{-3} M KCN. The Cu/Zn-SOD activity was obtained by deducting the Mn-SOD activity from the total SOD activity. CAT activity was determined spectrophotometrically at 240 nm and expressed in Bergmeyer units. GPx activity was determined using cumene hydroperoxide and reduced glutathione (GSH) as substrates of GPx. Total GSH was measured spectrophotometrically with Ellman's reagent. The degree of leukocyte infiltration was quantified by the measurement of pancreatic myeloperoxidase (MPO) activity. The activities of iNOS and cNOS, were determined by the method described by Knowles and Salter. For the evaluation of pancreatic TNF- α IL-6 concentrations, tissues were homogenized by the method of Dignam et al and measured with an Enzyme-Linked Immunosorbent Assay kit according to the manufacturer's instructions and corrected for the protein content of the tissue. The protein concentration of the tissues was determined by the method of Goa. A histological investigation was performed on hematoxylin and

eosin stained slices. The nuclear translocation of the P65 subunit of NF- κ B was investigated by immunohistochemistry, according to the manufacturer's instruction.

The I- κ B concentration of the cytosol fraction was measured in after homogenization by Western blot according to the method of Laemmli.

Statistical analysis

One-way analysis of variance (ANOVA) and a least significant difference (Fisher's LSD) *post hoc* analysis were performed to test for significant differences between the three experimental groups. $P < 0.05$ was accepted as indicating a statistically significant difference.

RESULTS

Melatonin

Histological investigation confirmed the development of severe necrotizing pancreatitis in all rats given L-Arg, with no discernible differences between the groups. Melatonin pre-treatment failed to influence the pancreas weight/body weight ratio, the serum amylase and the pancreatic SOD activity, but it significantly reduced the pancreatic CAT activity, the hepatic CAT and Cu/Zn-SOD activities as compared with group A and prevented the significant elevation of hepatic GPx activity and pancreatic MDA, MPO and IL-6 content and the reduction of hepatic Mn-SOD activity. Melatonin post-treatment significantly reduced the serum amylase activity versus group A and ameliorated the changes of hepatic MDA, Mn-SOD, Cu/Zn-SOD activities and the pancreatic MPO and IL-6 content.

Resveratrol

The development of edematous pancreatitis in the CCK-8-treated animals was confirmed by histology. Resveratrol significantly reduced the edema and acinar

necrosis in the pancreas, it reduced the elevation of pancreatic weight/body weight ratio and serum amylase and lipase activities and ameliorated the changes of serum glucose, calcium, ASAT and creatinine levels relative to group P. Resveratrol pretreatment could not influence the reduction of the Cu/Zn-SOD and GPx activities in the pancreas. Administration of resveratrol in group RP significantly increased the amount of GSH in the liver vs. group C and P, and prevented the significant reduction of CAT activity compared to group C. The immuno-histochemistry of the pancreas revealed increased numbers of P65 nuclear-positive cells in groups P and RP versus group C, but there was no significant difference in nuclear staining between groups RP and P.

Zerumbone

The development of acute pancreatitis was confirmed by the significant elevation of the pancreatic weight/body weight ratio, serum amylase and lipase activities in group P and RP vs group C, but the histology revealed only slight changes referring to the mildness of edematous pancreatitis. Zerumbone treatment significantly reduced the elevation of pancreatic weight/body weight ratio, serum amylase and lipase activities but it failed to influence the histology and increased the concentration of ASAT versus group C and P. Zerumbone pretreatment significantly reduced the changes of pancreatic iNOS, cNOS, Mn-SOD and Cu/Zn-SOD activities and reduced the elevation of pancreatic TNF- α and IL-6 concentrations. The amount of cytosolic I- κ B was significantly reduced in group P, but not in group ZP, as compared with group C. The pancreatic I- κ B content in group Z was significantly higher than in group C.

CONCLUSION

The aim of our work was to demonstrate the effects of three substances on the development of acute experimental pancreatitis. The common features of these drugs, namely melatonin, resveratrol and zerumbone are their antioxidant properties and their ability to exert anti-inflammatory effects. The source of the three natural substances is different. Melatonin is a pineal hormone present in the human organism, whereas resveratrol and zerumbone are found in plants that can be the constituents of our diet. Melatonin is available as a drug against jet-lag or depression, whereas resveratrol is used in the herbal medicine as a prophylactic drug against cardiovascular diseases. Zerumbone is a recently isolated substance and its *in vivo* effects are poorly described, therefore the medical use of the pure isolated drug requires further investigations. However *Zingiber zerumbet*, the main source of zerumbone and also resveratrol have long been used in the traditional Southeast Asian or Chinese medicine as anti-inflammatory drugs. Our experiments demonstrated the beneficial effects of these compounds in experimental pancreatitis. Because the protective abilities of melatonin were already examined in mild edematous pancreatitis, our aim was to investigate the effects of the drug in the L-arginine-induced necrotizing pancreatitis model. Our study showed that melatonin is potent enough to influence some of the parameters of L-arginine-induced pancreatitis; however the histological changes were not mitigated by the drug. The principle observation was the hepato-protective effect of melatonin pretreatment. The basal activities of the scavenger enzymes are 10-fold higher in the liver than in the pancreas, the influence of melatonin on the scavenger enzyme activities can therefore predominate in this tissue. The low pancreatic scavenger activities are probably one reason for the high mortality associated with acute pancreatitis.

The effects of resveratrol on acute pancreatitis were not described by others at the time our study was carried out.

Therefore we chose the CCK-8-induced pancreatitis model and examined the effects of resveratrol pretreatment in this model. Our findings demonstrated the antioxidant effects of resveratrol in the liver, the considerable beneficial effect of the drug on the serum parameters of pancreatitis and the histological protection due to resveratrol pretreatment. Meanwhile Meng et al. demonstrated the beneficial effect of resveratrol in taurocholate-induced necrotizing pancreatitis and Lawinski et al. reported the antioxidant effect of resveratrol in tert-butyl-hydroperoxide-induced pancreatitis. In contrast to the results of Meng et al inhibition of NF-kappa B activation could not be detected in our study.

We were the first to detect the positive effects of zerumbone in acute experimental pancreatitis. The substance has not been used earlier in the therapy of experimental pancreatitis and only few literary data exist about the in vivo effects of the drug. Therefore we decided to investigate the efficacy of zerumbone pretreatment in CCK-8-induced pancreatitis to decide if the compound is a promising candidate for further studies in the field of acute pancreatitis. Our results clearly demonstrated the antioxidant and anti-inflammatory effects of zerumbone in acute pancreatitis, however a beneficial effect of the drug on the histological scores was not detected. Inhibition of I-kappaB degradation by zerumbone refers to the NF-kappaB inhibitory effect of the drug, being probably the key mechanism of the action of zerumbone.

NEW FINDINGS

- I. Compared to previous publications where melatonin protected against mild edematous pancreatitis, our results showed that in severe acute necrotizing pancreatitis this substance can exert only moderate protective effects. The antioxidant effects of melatonin predominate in the liver tissue.

- II. The beneficial effect of resveratrol pretreatment on CCK-8-induced acute pancreatitis was clearly demonstrated in our study. The substance significantly attenuated the serum parameters of acute pancreatitis, reduced the histological damage of the pancreatic tissue and improved the antioxidant state of the liver, without influencing the activation of NF-kappaB.
- III. Zerumbone pretreatment was shown to express its anti-inflammatory and antioxidant effects in the pancreas of animals with CCK-8-induced acute pancreatitis. The background of the observed beneficial effects is probably the inhibition of NF-kappaB activation.

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Scientific publications in the topic of the present work

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Scientific publications related to the present work

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