The cumulative effect of nuclear factor-κB (NF-κB) inhibition and endothelins in early cerulein-induced acute pancreatitis in rats

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Abstract

Purpose: **To assess effects of NF-κB activation inhibitor (pyrrolidine dithiocarbamate – PDTC) alone or with endothelins (ET-1, ET-2, ET-3) in early course of cerulein -induced acute pancreatitis (AP) in rats.**

Material and methods: **After 4 h of AP in Wistar rats, treated with PDTC 10 or 40 mg/kg or with PDTC 10 mg/kg and ET-1, ET-2 or ET-3, 0.5 or 1.0 nmol/kg twice i.p. in 1 h interval, free active trypsin (FAT), total potential trypsin (TPT) and lipase in 12000 x g supernatants of pancreatic homogenates, plasma α-amylase and histological changes were assayed. %FAT/TPT was an index of trypsinogen activation.**

Results: **%FAT/TPT significantly increased to 12.42±2.14%, lipase to 5.51±0.84 U/mg protein and α-amylase to 28.5±5.61 U/mL in AP vs 1.96±0.31%, 1.29±0.11 U/mg and 5.80±1.38 U/ml in healthy control. Higher dose PDTC attenuated trypsinogen activation to 3.01±0.53% and α-amylase to 15.3±1.38. PDTC and ET-1 attenuated %FAT/TPT to 2.55±0.18% with lower and 2.34±0.44% with higher dose. ET-3 was less effective than ET-1: 6.76±0.46% with lower dose. Lower doses of ET-1 and ET-2 with PDTC, diminished lipase activity to 2.60±0.36 and 2.94±0.33.**

Conclusions: **Cumulative attenuation of trypsinogen activation after lower dose of PDTC and ET-1 approximated the effect of higher dose of PDTC. Additional effect of ET-3 was weaker than ET-1, and ET-2 was ineffective in this respect. The combination of this NF-κB activation inhibitor and ET-1 could be beneficial in early course of edematous AP by attenuating of trypsinogen activation. However, it should be treated with caution because of some unfavorable effects on histological scores of pancreatic injury.**

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Introduction

Both, premature activation of trypsinogen and an impairment of pancreatic microcirculation are thought to play an important role in the pathogenesis of acute pancreatitis (AP) [1,2]. In recent years a pivotal role of transcriptional nuclear factor-κB (NF-κB) activation in the onset of acute pancreatitis with consecutive expression of inflammatory mediators has been postulated [3,4]. Rapid activation of NF-κB in acute taurocholate pancreatitis in rats, accompanied by an increase of TNF-α gene expression and its inhibition by blocking free radicals formation has been observed [5]. A direct activation of NF-κB within the pancreas increased the infiltration of neutrophils to the pancreas and caused extensive damage to the acinar cells [6]. On the contrary, the inhibition of NF-κB activation with antioxidants: pyrrolidine dithiocarbamate or resveratrol improved the course of taurocholate pancreatitis in rats [7,8].

In mild, cerulein (cholecystokinin analog)-induced AP, the NF-κB activation was correlated with the degradation of its inhibitor IκBα and followed by a rapid appearance of NF-κ B/Rel binding activity [9]. In NF-κB deficient mice, the cerulein-induced pancreatitis was attenuated as evidenced by the reduction of oxidative stress and enzymatic markers [10]. Selective inhibition of NF-κB binding activity or its nuclear import and unspecific inhibition of NF-κB activation, mostly by the reduction of lipid peroxidation, also ameliorated the tissue injury in cerulein-induced pancreatitis [11-14]. There are essential controversies on the relationship between NF-κB

and trypsinogen activation in cerulein- or cholecystokinin (CCK)-induced AP. Some authors [15,16] are of opinion, that NF-κB and trypsinogen activation, although temporally closely related, are independent events, whereas others suggest, that intracellular activation of trypsinogen may contribute to NF-κB activation [17].

In severe, necrotizing AP, a pancreatic capillary flow decreased by half during 6h, whereas in mild, cerulein-induced AP it increased almost twice after 3h and remained elevated throughout 6h experiment [18]. The role of endothelins (ETs), an endothelium-derived potent vasoactive peptides, in AP remains not fully elucidated. ETs family consists of three isoforms: ET-1, ET-2 and ET-3. ET-1 is a ligand of two receptors: ET_A – responsible for vasoconstriction and ET_B – linked mostly to vasodilation [19,20]. All forms of endothelins reduced significantly pancreatic blood flow in healthy dogs and rats, however ET-1 was much more effective than ET-2 or ET-3 on a molecular basis [21,22]. Some authors reported a beneficial effect of ET_A receptor blockade and harmful effect of exogenous ET-1 [23,24]. On the contrary, Kogire et al. [25] found a protective effect of ET-1 in cerulein-induced AP on histological changes and a detrimental effect of selective ET_A receptor antagonist in this model of AP. In our own study, both selective ET_A receptor antagonist and nonselective $ET_{A/B}$ antagonist do not exert any positive effects on early course of cerulein-induced AP and even some undesired effects on pancreatic histological changes were noted [26]. On the other side, all forms of endothelins (ET-1, ET-2 and ET-3) decreased inflammatory cell infiltration and attenuated trypsinogen activation in the pancreas in this model of AP [27].

Above data could suggest some positive effects of combined NF-κB inhibition and endothelins in early course of cerulein-induced AP. Therefore, the purpose of present study was to assess the cumulative effect of NF-κB activation inhibitor – pyrrolidine dithiocarbamate (PDTC) and exogenous endothelins (ET-1, ET-2 and ET-3) on trypsinogen activation, enzymatic (pancreatic lipase and plasma α-amylase) and histological changes in early course (4h) of cerulein-induced acute pancreatitis in rats.

Material and methods

Animals

The experiments were carried out on 65 male Wistar rats, weighing 240-300 g, housed individually in wire bottomed cages in a room temperature of 21±1°C using a 12 hours light – dark cycle. The animals were given a standard rat chow diet and fasted overnight before the experiment with free access to water. The care was provided in accordance with the current procedures for the care and use of laboratory animals. The protocol has been approved by the local Bioethical Commission.

Induction of acute pancreatitis

Acute cerulein pancreatitis was induced according to the method of Yamaguchi et al. [28]. The rats were injected i.p. with cerulein (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dose of 40 μg/kg of body weight (b.w.) twice in 1 hour interval. In control rats, only solvent of cerulein (0.9% NaCl) was given i.p. In the treated rats, the solution of respective endothelins in 0.9% NaCl was given i.p. twice, simultaneously with cerulein. A solution of pyrrolidine dithiocarbamate (PDTC) in 0.9% NaCl was added to the first injection of cerulein.

Experimental design

Rats were subdivided into 10 groups as follows:

Group I. Control group (C), healthy rats, receiving only saline (0.9% NaCl) i.p. at time 0 and 1 hour later (n=7).

Group II. Rats with cerulein-induced untreated acute pancreatitis (AP). The solution of cerulein in equivalent volume of saline was given i.p. at 0 time and 1 hour later (n=9).

Group III. Rats with cerulein-induced AP treated with PDTC at a dose of 10 mg/kg b.w. i.p. once, simultaneously with the first dose of cerulein (n=7).

Group IV. Rats with cerulein-induced AP treated with PDTC at a dose of 40 mg/kg b.w. i.p. once, simultaneously with the first dose of cerulein (n=6).

Group V. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once, as in the group III and ET-1 at a dose of 0.5 nmol/kg b.w i.p. twice, in 1 h interval, simultaneously with cerulein (n=6).

Group VI. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once, as in the group V and ET-1 at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group VII. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-2 at a dose of 0.5 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group VIII. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-2 at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group IX. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-3 at a dose of 0.5 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group X. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-3 at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group V (n=6).

The volume of 0.9% NaCl as a solvent was equilibrated in all rats to 3 ml/kg and 2 ml/kg b.w. during the first and the second injection respectively.

Preparation of pancreatic homogenate and the plasma

Four hours after the first cerulein injection (or 0.9% NaCl in C group) a general anesthesia was induced with i.p. ketamine at a dose of 40 mg/kg b.w., supported by pentobarbital at a dose of 20 mg/kg b.w. The blood samples were taken to the heparinized syringe by the cardiac puncture and the rats were sacrificed by decapitation. The pancreases were quickly excised, freed from the peripancreatic tissues and weighed. For light microscopy, representative specimens of the pancreas were fixed and the sections were stained with hematoxylin and eosin.

The remaining portion of the pancreas was processed according to Yamaguchi et al. [28], meaning it was homogenized

in four volumes of ice-cold, 50 mmol/L Tris-HCl buffer (pH 8.0), containing non-organic detergent Triton X-100, 0.5% (v/v) during 1 min by 3 full up and down strokes using a motor driven glass-Teflon homogenizer (Thomas Scientific, New Jersey, U.S.A.) cooled with ice. The resulting homogenate was sonified for 20 seconds in an ice bath using Vibra cell, model VC 50, Sonics and Materials Inc., Danbury, CT, U.S.A. (frequency 20 kHz and amplitude 70). The volumes were then adjusted giving 10% homogenates, placed on ice for 20 min for further extraction of the enzymes, and then centrifuged at 12000 x g for 20 min at 4°C. The supernatants were used for the assays of trypsin activity performed within 6 hours. The remaining portions of the supernatant were frozen at -80°C, for the assay of lipase activity and protein concentration.

The samples of heparinized blood were centrifuged at 4000 rpm with cooling to 4°C, the resulting plasma was collected and frozen at -80°C for the assay of α-amylase activity.

Biochemical assays

1. Trypsin activity: free active trypsin (FAT) and total potential trypsin (TPT) in the supernatants of pancreatic homogenates were estimated according to Yamaguchi et al. [28] with this exception that N_{α}-p-tosyl-L-arginine methyl ester hydrochloride (TAME) 1 mmol/L was used as a substrate and the absorbance of released product was estimated at 247 nm wave length in an automatic spectrophotometer Pye Unicam SP 505 (Cambridge, U.K.) as in our previous study [29].

Total potential trypsin (TPT) in the supernatants was estimated after activation of trypsinogen with enterokinase in 1:1 dilution in 50 mmol/L Tris-HCl buffer (pH 8.0) for 30 min at 37°C. The freshly prepared working solution of enterokinase contained 2 mg of enzyme/mL of the same buffer [28]. The time of this activation proved to be sufficient for maximal activation.

The activity was expressed in μg of trypsin/mg of protein by comparison with the calibration curve of increasing concentrations of bovine trypsin, type I. The %FAT/TPT ratio served as an index of trypsinogen activation [28].

2. Lipase activity in the supernatants of pancreatic homogenates was assayed with tributyrin (1,2,3-tributylglycerol) as a substrate and with the pehametric method using autotitrator (Radiometer, Copenhagen, Denmark) and 0.2 mol/L NaOH, as in our previous study [29].

3. α-amylase activity in the plasma was assayed with colorimetric method with soluble starch as a substrate as in our previous study [29].

All reagents, with the exception of soluble starch were purchased from Sigma Chemicals Co., St Louis, MO, U.S.A.

Histological examination

Ten slides from 5 rats from each group (50 slides per group) stained with hematoxylin and eosin (H&E) were evaluated at a magnification of 200x in light microscopy by an expert pathologist (A.A.), who was not familiar with the experimental code at this time. The edema, inflammatory infiltrate, necrosis and vacuolization were scored from 0 to 3 degrees of severity according to Kyogoku et al. [30]. Generally, the interstitial edema was scored as follows: 0=absent; 1=expansion of interlobular septa; 2=expansion of intralobular septa; 3=separation of acini. The inflammatory infiltrate: 0=absent; 1=less than 20 neutrophils per field; 2=20-50 neutrophils; 3=more than 50 neutrophils per field. Parenchymal necrosis: 0=absent; 1=less than approximately 5%; 2=5-20% ; 3=more than 20% of the involved area. The vacuolization: 0=absent; 1=less than 20% of acinar cells with vacuoles per field; 2=20-50%; 3=more than 50%.

Table 1. **Free active trypsin (FAT), total potential trypsin (TPT) and the index of trypsinogen activation (%FAT/TPT) in the supernatants of pancreatic homogenates in early (4h) cerulein-induced acute pancreatitis (AP) untreated and treated with NF-κB activation inhibitor pyrrolidine dithiocarbamate (PDTC) and different endothelins (ET-1, ET-2. ET-3) vs control group (C) in rats. Means ±S.E.M. are reported**

No	Group	FAT μg/mg protein	TPT μg/mg protein	%FAT/ /TPT
I	Control (C) (n=7)	0.219 ±0.029	11.80 ±1.27	1.96 ±0.31
II	AP untreated (n=9)	1.265••• ±0.103	11.51 ±1.18	12.42••• ±2.14
III	AP+PDTC 10 mg/kg (n=7)	1.507 ±0.486	15.92* ±1.26	10.50 ±3.70
IV	AP+PDTC 40 mg/kg (n=6)	0.434*** ±0.065	15.33 ±1.38	3.01 ±0.53
V	AP+PDTC 10 mg/kg + ET-1 2 x 0.5 nmol/kg (n=6)	0.394*** ±0.106	15.19* ±0.83	2.55*** ±0.18
VI	AP+PDTC 10 mg/kg + ET-1 2 x 1.0 nmol/kg (n=6)	0.385*** ±0.064	16.73** ±0.98	2.34*** ±0.44
VII	AP+PDTC 10 mg/kg + ET-2 2 x 0.5 nmol/kg (n=6)	1.169 ±0.241	11.95 ±1.21	10.78 ±2.56
VIII	AP+PDTC 10 mg/kg + ET-2 2 x 1.0 nmol/kg (n=6)	1.116 ±0.200	11.86 ±0.65	9.24 ±1.22
IX	AP+PDTC 10 mg/kg + ET-3 2 x 0.5 nmol/kg (n=6)	0.918 ±0.144	13.24 ±1.56	6.76* ±0.46
X	AP+PDTC 10 mg/kg + ET-3 2 x 1.0 nmol/kg (n=6)	0.773 ±0.197	11.13 ±0.62	6.88 ±1.68

Statistical significance of differences
between untreated AP group and control group: P<0.001•••, P<0.01••, P<0.05•;
between treated AP groups and untreated AP group: P<0.001***, P<0.01**, P<0.05*.

Statistical analysis

The results of biochemical assays are reported as means ±S.E.M. and after performing an F test for the equality of variances, the means were compared using the *t* test for unpaired data. The differences with P<0.05 were considered statistically significant.

Results

Tab. 1 illustrates the activities of trypsin and the index of trypsinogen activation in the supernatants containing enzymes extracted using organic detergent Triton X-100. FAT in cerulein--induced, untreated AP group has been shown to be 5.8 times higher than in the control group (P<0.001), whereas TPT was

similar in both groups. In the groups with AP treated with lower dose of PDTC, a slight decrease of FAT was not significant, whereas TPT was elevated by 38% (P<0.05) in comparison to AP untreated. In the group with AP treated with higher dose of PDTC, FAT achieved only 34% of its value from untreated AP group (P<0.001). A slight elevation of TPT was not significant. The combined treatment with lower dose of PDTC and lower or higher dose of ET-1 attenuated FAT elevation to about 30% of its value from untreated AP group (P<0.001). Simultaneously, TPT was increased by 32% (P<0.01) and by 45% (P<0.001) respectively in comparison to untreated AP. The combined treatment with lower dose of PDTC and both, lower and higher doses of ET-2 or ET-3 affected significantly neither FAT nor TPT in comparison to the untreated AP.

The index of trypsinogen activation (%FAT/TPT) in the untreated AP group was markedly (6.3 times) higher than in control group (P<0.001). Interestingly enough, lower dose of PDTC did not affect significantly the trypsinogen activation, whereas higher dose of PDTC attenuated it to 24% of its value from untreated AP (P<0.01). It is noteworthy, that combined treatment with lower dose of PDTC and both doses of ET-1 resulted in strong attenuation of trypsinogen activation to the values close to that seen after higher dose of PDTC (P < 0.001). The combined treatment of AP with lower dose of PDTC and both doses of ET-2 did not affect significantly the trypsinogen activation in comparison to untreated AP group. The attenuating effect of combined PDTC and ET-3 treatment at both doses on the trypsinogen activation to about 55% of its values from untreated AP was significant (P<0.05) only for lower dose of ET-3 (*Tab. 1*).

The activity of lipase in the supernatant of pancreatic homogenate from untreated AP was about four times elevated in comparison to control group (P<0.001). The treatment with lower dose of PDTC did not affect this activity, whereas after higher dose of PDTC, it was even higher than in untreated AP (P<0.05). Only combined treatment with lower dose of PDTC and lower doses of ET-1 or ET-2 attenuated significantly the increase of pancreatic lipase in AP to 47% (P<0.01) and to 53% (P<0.05) respectively in comparison to untreated AP group. The plasma α-amylase in untreated AP was five times higher as compared to control group (P<0.001). In treated AP groups, only higher dose of PDTC decreased plasma α-amylase activity to 54% (P<0.05) of its value from untreated AP group (*Tab. 2*).

As can be seen in *Tab. 3*, the increase of edema, inflammatory cell infiltration, necrosis and vacuolization scores after supramaximal cerulein stimulation supports the development of AP. Some shift towards higher scores of edema, necrosis and vacuolization in AP treated with both doses of PDTC can be observed. This trend was even more evident for the necrosis and vacuolization scores after higher dose of PDTC. However, it does not concern the inflammatory infiltration scores, where slight reverse tendency can be seen. The addition of ET-1 at both doses seemed to ameliorate the edema and vacuolization scores in AP treated with lower dose of PDTC, but not below AP untreated, without such an effect on the necrosis scores.

More evident positive shift of inflammatory infiltration scoring after addition of higher dose of ET-1 to PDTC can be seen.

Table 2. **Lipase activity in the supernatants of pancreatic homogenates and plasma α-amylase in caerulein-induced acute pancreatitis (AP) untreated and treated with NF-κB activation inhibitor pyrrolidine dithiocarbamate (PDTC) and different endothelins (ET-1, ET-2, ET-3) vs control group (C) in rats. Means ±S.E.M. are reported**

No	Group	Lipase U/mg protein	α-amylase U/mL
I	Control (C) (n=7)	1.29 ±0.11	5.8 ±1.38
II	AP untreated (n=9)	5.51••• ±0.84	28.5••• ±5.61
III	AP+PDTC 10 mg/kg (n=7)	5.41 ±0.80	24.6 ±3.62
IV	AP+PDTC 40 mg/kg (n=6)	8.69* ±0.77	15.3* ±1.38
V	AP+PDTC 10 mg/kg + ET-1 2 x 0.5 nmol/kg (n=6)	2.60** ±0.36	25.8 ±1.84
VI	AP+PDTC 10 mg/kg + ET-1 2 x 1.0 nmol/kg (n=6)	4.56 ±1.54	27.7 ±6.40
VII	AP+PDTC 10 mg/kg + ET-2 2 x 0.5 nmol/kg (n=6)	2.94 ±0.33	19.7 ±3.04
VIII	AP+PDTC 10 mg/kg + ET-2 2 x 1.0 nmol/kg (n=6)	3.61 ±0.72	26.8 ±2.69
IX	AP+PDTC 10 mg/kg + ET-3 2 x 0.5 nmol/kg (n=6)	4.46 ±0.95	30.1 ±2.86
X	AP+PDTC 10 mg/kg + ET-3 2 x 1.0 nmol/kg (n=6)	4.41 ±1.07	31.9 ±5.79

Statistical significance of differences
between untreated AP group and control group: P<0.001•••, P<0.01••, P<0.05•;
between treated AP groups and untreated AP group: P<0.01**, P<0.05*

Slight decrease of edema scoring in AP after addition of both doses of ET-3, but not ET-2 to PDTC can be observed, however it remains worse than in untreated AP. The addition of ET-2 and ET-3 does not improve generally the unfavorable effects of lower PDTC dose on the acinar cells necrosis and vacuolization scores in untreated AP. On the contrary, some amelioration of inflammatory infiltration scoring can be seen after addition of ET-2 and ET-3 to lower dose of PDTC in AP, especially after lower dose of ET-3 (*Tab. 3*).

Discussion

Our study shows, that pyrrolidine dithiocarbamate (PDTC) at a dose of 10 mg/kg, known to inhibit almost completely the NF-κB activation, does not attenuate significantly the trypsinogen activation in cerulein-induced AP in rats, but does it markedly at a dose of 40 mg/kg. This higher dose increases the lipase activity in the pancreas but decreases plasma α-amylase activity in AP – the effects not observed after lower dose of PDTC. Beside of enzymatic changes, both doses of PDTC slightly and to a similar extent aggravated the edema, acinar necrosis and vacuolization scores in AP, whereas the inflammatory cell infiltration seemed to be alleviated. The addition of ET-1 at two

Table 3. **The incidence of histological changes of the pancreas, scored at 0-3 scale* in early (4h) cerulein-induced acute pancreatitis (AP) untreated and treated with NF-κB activation inhibitor pyrrolidine dithiocarbamate (PDTC) and different endothelins (ET-1, ET-2, ET-3) vs control group (C) in rats**

Group	No	I **Control** (C) (n=7)	II **AP un-treated** (n=9)	III **AP+PDTC** 10 mg/kg (n=7)	IV **AP+PDTC** 40 mg/kg (n=6)	V **AP+PDTC** 10 mg/kg + ET-1, 2x0.5 nmol/kg (n=6)	VI **AP+PDTC** 10 mg/kg + ET-1, 2x1.0 nmol/kg (n=6)	VII **AP+PDTC** 10 mg/kg + ET-2, 2x0.5 nmol/kg (n=6)	VIII **AP+PDTC** 10 mg/kg + ET-2, 2x1.0 nmol/kg (n=6)	IX **AP+PDTC** 10 mg/kg + ET-3, 2x0.5 nmol/kg (n=6)	X **AP+PDTC** 10 mg/kg + ET-3, 2x1.0 nmol/kg (n=6)
Edema score	0	44	0	0	0	0	1	0	0	0	0
	1	6	12	7	5	5	4	6	0	7	7
	2	0	25	10	20	30	29	15	20	21	20
	3	0	13	33	25	15	16	29	30	22	23
Inflamma-tory infil-tration score	0	47	3	6	10	4	13	11	0	10	6
	1	3	22	27	25	27	24	25	41	33	32
	2	0	19	15	12	15	13	10	6	7	9
	3	0	6	2	3	4	0	4	3	0	3
Necrosis score	0	50	25	11	15	15	15	20	16	18	14
	1	0	21	37	24	32	31	29	31	29	31
	2	0	4	2	11	3	4	1	3	3	5
	3	0	0	0	0	0	0	0	0	0	0
Vacuoli-zation score	0	48	0	0	0	0	2	2	0	0	0
	1	2	21	0	0	9	23	8	12	4	3
	2	0	14	18	5	16	9	16	2	9	11
	3	0	15	32	45	25	16	24	36	37	36

*according to Kyogoku et al. [30] from 50 fields per group are reported

doses, 0.5 or 1.0 nmol/kg b.w. i.p. at 1 h interval to the treatment with lower dose of PDTC attenuated trypsinogen activation to that seen after higher dose of PDTC. Lower dose of ET-1 added to PDTC attenuated significantly the lipase activity in the pancreas without any evident effect on plasma α-amylase activity in AP. Lower dose of ET-1 added to lower dose of PDTC does not affect appreciably histological changes seen after PDTC alone. Higher dose of ET-1 added to lower dose of PDTC does not affect appreciably the necrosis scoring in comparison to PDTC alone, but some amelioration of edema, inflammatory infiltration and vacuolization can be seen.

The addition of ET-2 to PDTC treatment in AP does not exert evident favorable effects on the changes seen after PDTC alone, with the exception of decreasing influence on pancreatic lipase activity and a positive shift of necrosis score after lower dose of ET-2. The addition of ET-3 to PDTC treatment in AP resulted in the attenuation of trypsinogen activation, but to lesser degree than after ET-1 and in slight amelioration of necrosis and inflammatory scoring after lower dose of ET-3. Generally, the inhibition of NF-κB activation by PDTC in rats, despite the extent of trypsinogen activation, leads to slight aggravation of edema, pancreatic acinar cells necrosis and vacuolization but not the inflammatory infiltration in the early (4h) course of cerulein-induced AP in our present study.

Early changes occurring within the first 30 min after the supramaximal cerulein stimulation, leading to AP, include the colocalization of lysosomal hydrolases with digestive zymogens in cytoplasmic vacuoles, intraacinar cell activation of trypsinogen and activation of NF-κB [31,32]. In next hours of cerulein-induced AP, the accumulation of neutrophils [33] and oxidative stress [34] gain an increasing role in acinar cells injury. The activation of NF-κB depends on the phosphorylation and proteolytic degradation of its inhibitors IκBα and IκBβ. Beside early direct effect of cerulein hyperstimulation, another factor contributing to NF-κB activation could be the inflammatory cell infiltration, which becomes pronounced after 2-3 hours of cerulein AP. It is of interest, that known antioxidant, N-acetylcysteine inhibits both NF-κB and trypsinogen activation [3], suggesting an important role of the oxidative stress for both these mechanisms of the pancreatitis progression.

More extensive studies on isolated pancreatic acini have shown that either stimulation or inhibition of acinar cell NF-κB activation did not affect trypsinogen activation. On the other hand, the trypsin activity is not necessary for the NF-κB activation by supramaximal stimulation with cholecystokinin. However, the inhibition of NF-κ-B activation by preincubation with inhibitors of the trypsin activity suggests indirect role of this transcriptional factor in the development of cerulein-induced AP. Known antioxidant, pyrrolidine dithiocarbamate (PDTC) at the concentration of 10 mmol/L strongly inhibits both NF-κB and trypsinogen activation in isolated pancreatic acini [15,17]

Taking into account the key role of NF-κB in the expression of proinflammatory cytokines and their reciprocal effect on the NF-κB activation [6,11,17], it is obvious, that selective inhibition of NF-κB DNA-binding activity [11] or specific inhibition of its translocation into the nucleus by a nuclear import inhibitory peptide [12] could ameliorate the severity of cholecystokinin-induced AP. A controversy exists on the effect of less specific inhibitors of NF-κB activation, like PDTC. We have chosen this agent basing on its positive effects in taurocholate acute

pancreatitis (TCA) in rats at a dose of 10 and 100 mg/kg, abolishing the NF-κB activation in the peritoneal and alveolar macrophages. Lower dose of PDTC injected i.p. 1 hour or just before induction of TCA pancreatitis significantly improved the survival rate. Nevertheless, no significant differences were found in the increase of serum α-amylase or in the histological changes of the pancreas [7].

In cerulein-induced AP in rats, PDTC administered i.p. at a dose of 10 mg/kg, one hour before cerulein injection inhibited NF-κB activation in acinar cells. Unexpectedly, the serum α-amylase and LDH (lactic dehydrogenase) activities were significantly higher at 4h of AP, than after cerulein alone. Moreover, histological changes were aggravated after 24 hours. The authors suggest, that despite the proinflammatory effect of NF-κB/Rel activation, this factor can regulate a self-defending genetic program to prevent a higher degree of the damage to the pancreatic acinar cells. Therefore the final results of the PDTC dependent inhibition of NF-κB activation could be detrimental in rats [9]. On the contrary, in cerulein-induced AP in mice, pretreatment with PDTC at a dose of 30 mg/kg i.p., significantly ameliorated histological changes in the pancreas 6h after the induction of AP [14]. It means, that the effect of PDTC in cerulein-induced AP could be a species dependent.

Our present study have shown that PDTC slightly aggravated the scores of acinar cell injury already in early (4h) cerulein-induced AP in rats both at a dose of 10 and 40 mg/kg b.w., despite the strong attenuation of trypsinogen activation by higher dose of PDTC. Therefore, despite the beneficial effect of PDTC in taurocholate pancreatitis in rats [7] and it doubtless positive effect against NF-κB activation and mob-1 chemokine expression in isolated pancreatic acini [35], the application of this compound in the mild cerulein-induced AP to depress the NF-κB activation requires further evaluation.

In our previous study, we have found an attenuating effect of endothelins on trypsinogen activation in cerulein-induced AP in rats with concomitant improvement of inflammatory infiltration score after higher dose of ET-1 and to lesser extent after higher dose of ET-2 and ET-3. However, it did not prevent other morphological aspects of pancreatic acinar cell injury as slight elevation of necrosis and vacuolization scores after lower dose of ET-2 and ET-3 [27]. In this study, we have used the same doses of endothelins and additionally PDTC at a dose of 10 mg/kg. It appears that only cumulative effect of PDTC and lower dose of ET-1 towards attenuation of increased trypsinogen activation was higher than the effect of ET-1 alone ($P<0.001$). PDTC even depreciated the effect of both doses of ET-2 and higher dose of ET-3 against the trypsinogen activation. The accentuated cumulative effect of PDTC and higher dose of ET-1 to alleviate the inflammatory infiltration and pancreatic acinar cell vacuolization deserves further attention.

There are still controversies on the role of endothelins in AP. Liu et al. [24] observed, that ET-1 administered as a bolus after induction of cerulein AP caused a dose-dependent increase of pancreatic acinar cell damage. On the other hand, Kogire et al. [25] found, that the application of ET-1, infused simultaneously with supramaximal dose of cerulein, resulted in less advanced inflammatory cell infiltration and a decrease in the pancreatic edema index. A secondary stimulation of prostacyclin production by ET-1 has been suggested for this positive effect. In fact, in our previous study, we have found a protective effect of the prostacyclin analog in taurocholate AP in rats [36]. Our present study supports some protective effects of endothelins, which can be not sufficient to abrogate some unfavorable effects of PDTC administration, excluding some positive effects of higher ET-1 dose.

Lipase is known factor in the damage to isolated pancreatic acinar cells to a degree similar to the action of chymotrypsin [37]. The increase of its activity in pancreatic tissue of the rats with cerulein-induced AP treated with higher dose of PDTC over its value in untreated AP, in our study, could participate in the exacerbation of histological changes. On the contrary, its decrease after combined treatment with PDTC and lower dose of ET-1 could be observed, together with marked attenuation of trypsinogen activation. However, these positive trends had no appreciable beneficial influence on histological scores of the pancreatic injury.

In conclusion, the combined treatment of cerulein-induced acute pancreatitis in rats with PDTC and ETs resulted in the attenuation of trypsinogen activation, especially evident in the case of ET-1. The index of trypsinogen activation after ET-1 and lower dose of PDTC was especially diminished and close to the effect of higher dose of PDTC alone. A slight alleviation of the inflammatory cell infiltration in the pancreas was also noted in groups treated with PDTC alone and in combination with ETs. However, some unfavorable shift of the histological scores of edema and pancreatic acinar cell injury could be observed. Therefore, the application of PDTC to inhibit the NF-κB activation in cerulein-induced acute pancreatitis just in rats appears to be questionable, because of other unfavorable effects. Nevertheless, concomitant application of ET-1 could partly counteract these effects.

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