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Original Research

Ethacrynic acid inhibits pancreatic exocrine secretion[1]

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KEY WORDS ethacrynic acid; pancreas; exocrine glands; glutathione

ABSTRACT

AIM: The effect of ethacrynic acid on pancreatic exocrine secretion function and potential mechanisms of interference with the secretory process in pancreatic acinar cells were investigated. **METHODS**: After incubation with ethacrynic acid for 30 min, caerulein-stimulated amylase release and cholecystokinin (CCK) receptor binding characteristics were assessed in isolated rat pancreatic acini. The level of thiol groups (glutathione and protein thiols) and cytosolic free calcium were measured in pancreatic acinar cells. **RESULTS**: Ethacrynic acid decreased caerulein (0.1 nmol/L)-stimulated amylase release and the level of pancreatic acinar glutathione in a concentration-dependent fashion without a marked increase in cell damage. Ethacrynic acid also inhibited the caerulein (1 nmol/L)-induced Ca^{2+} mobilization in pancreatic acinar cells. But neither protein thiol nor CCK-receptor binding characteristics was altered by ethacrynic acid. **CONCLUSION**: Ethacrynic acid inhibit pancreatic exocrine secretion by depletion of glutathione and down-regulation of caerulein-induced Ca^{2+} mobilization. Glutathione might play a potential role in the secretory process in pancreatic acinar cells and in the secretory blockade observed in acute pancreatitis.

INTRODUCTION

Ethacrynic acid (EA) has been used for several decades as a highceiling (loop) diuretic, it is conjugated to glutathione through a nucleophilic attack at the α,β-unsaturated ketone, this reaction may occur both spontaneously or via the catalytic action of the major classes of glutathione *S*-transferase (α, μ, and π), with the π isozyme being the most active[1]. This allows its use in both experimental and clinical studies as an inhibitor of glutathione.

Glutathione (*L*-γ-glutamyl-*L*-cysteinyl-glycine, GSH) is the most abundant intracellular sulfhydryl and represents one of the major intracellular defense systems against mediators of oxidative stress[2]. Maintenance of the intracellular redox state by GSH contributes to proper protein folding in the acinar endoplasmic reticulum[3], the integrity of the cytoskeleton[4], and acinar stimulus-secretion coupling[5]. Disturbances of the GSH metabolism could result in impaired zymogen granule transport and reduce organelle integrity as well as thiol disulfid-mediated interactions with digestive enzyme activation[6]. However, the existence and significance of all of these potential mechanisms remains to be established.

Several previous clinical observations suggest that EA might lead to drug-induced pancreatitis[6,7]. In the state of pancreatitis in which the "secretion blockade" is typically observed, glutathione is depleted in pancreatic acinar cells[8,9]. Depletion of glutathione was suggested to inhibit pancreatic exocrine secretion[5], therefore it is hypothesized that EA induced pancreatitis at least partly caused by blocking pancreatic exocrine secretion and depleting glutathione. To prove this hypothesis, effect of EA on pancreatic exocrine secretion function and potential mechanism of interference with the secretory process were first studied *in vitro*. In this study, the effect of EA on pancreatic acinar amylase release was analyzed. Furthermore, the role of cholecystokinin (CCK) receptor affinity, pancreatic acinar thiols, and intracellular calcium following EA treatment were also investigated.

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MATERIALS AND METHODS

Isolation of pancreatic acinar cells After a 16 h fasting period, isolated pancreatic acinar cells were prepared by enzymatic digestion (collagenase, hyaluronidase, and chymotrypsin) from male Wistar rats (180 – 200 g), as previously described[10]. The viability of acinar cells and acini was assessed by Trypan blue exclusion test[11] and release of lactate dehydrogenase (LDH) by a commercially available test kit[12].

Preparation of isolated rat pancreatic acini and measurement of amylase release Experiments were performed on pancreas from overnight-fasted male Wistar rats (180 – 200 g). The rats were killed by decapitation, and the pancreas were immediately removed and trimmed of fat and mesentery. A suspension of acini were prepared as described by Niederau *et al*[12]. Amylase release of pancreatic acini was measured using Phadebas amylase test (Kabi Pharmacia, Uppsala, Sweden)[12].

Measurement of reduced GSH and protein thiols Pancreatic acinar cells were prepared and diluted to a cell density of 1×10^6/L. Aliquots of acinar cells were distributed into polycarbonate Erlenmeyer flasks, then incubated with various concentrations of EA at 37 ℃ for 30 min. After preincubation, all suspensions were transferred into tubes followed by centrifugation (10 000 × *g*, 4 ℃) for 5 min, then supernatants were discarded and cold extraction solution ($HClO_4$ 1 mol/L, edetic acid 2 mmol/L) were pipetted to each tube's pellet followed by 15 s vortexing. After centrifugation for 1 min, the supernatant of each tube was transferred into Eppendorf tube and kept on ice for the measurement of GSH. The pellets were also kept on ice and used for protein thiols measurement. For GSH measurement, acid extracts were neutralized. GSH content was then measured using a kinetic photometric assay as described previously[13]. Protein thiols were assayed according to Di Monte *et al*[14].

Measurements of intracellular calcium concentration Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with Fura 2 in pancreatic acinar cell suspension, as described by Klonowski-Stumpe *et al*[15]. Calibration of $[Ca^{2+}]_i$ signals was determined by applying the equation of Grynkiewicz *et al*[16].

Determination of CCK binding to its receptor To measure CCK binding to its receptors, ^{125}I-BH-CCK-8 (2.5 pmol/L) and increasing concentrations of unlabeled caerulein (10 pmol/L to 0.1 μmol/L) were added into acini suspension and incubated in a shaking water bath (60 cycles/min) at 37 ℃ for 30 min. After centrifugation at 10 000 × *g* for 5 min, the pellet was washed twice with 0.9 % saline at 4 ℃. Radioactivity associated with the pellet was measured in a gamma-scintillation counter. After the subtraction of unspecific binding, the specific binding to its receptors exposed to different EA was expressed as percent of control. Non-specific binding was determined by incubating acini with labeled CCK in the presence of an excess of unlabeled caerulein (125 nmol/L).

Statistical analysis Data were expressed as $x \pm s_x$. Statistical significance was determined by Student's *t*-test. A P value less than 0.05 was considered as significant.

RESULTS

Effect of EA on caerulein-stimulated amylase release The amylase release induced by caerulein 0.1 nmol/L was 22.4 % ± 0.6 % of total content of amylase. After incubation with increasing concentrations of EA, caerulein (0.1 nmol/L)-stimulated amylase release was inhibited in a concentration-dependent manner. The maximal inhibition was reached at 500 μmol/L of EA, stimulated amylase release was inhibited to 6.7 % ± 1.2 % (Fig 1).

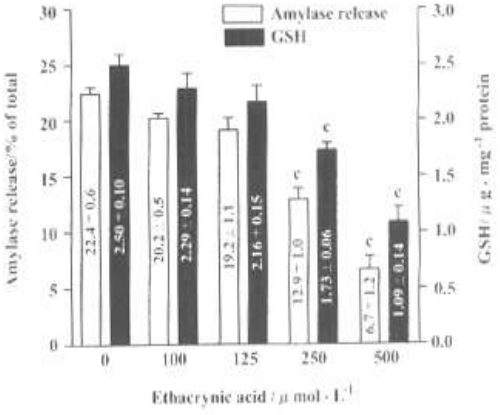

Fig 1. Effect of ethacrynic acid (EA) on caerulein (0.1 nmol/L)-stimulated amylase release from acini and pancreatic acinar GSH. $n = 4$ experiments. $x \pm s_x$. $^cP < 0.01$ *vs* EA 0 μmol/L group.

The effect of EA on concentration-response curve of caerulein-stimulated amylase release was also investigated. In normal acini, caerulein-stimulated

amylase release in a biphasic manner, with maximal release induced by caerulein 0.1 nmol/L and less secretion in response to both lower and higher concentrations. Pretreatment with EA 500 μmol/L decreased caerulein-induced amylase release over the whole range of caerulein concentrations (1 pmol/L to 1 nmol/L) without major alterations of the shape of the concentration-response curve (Fig 2).

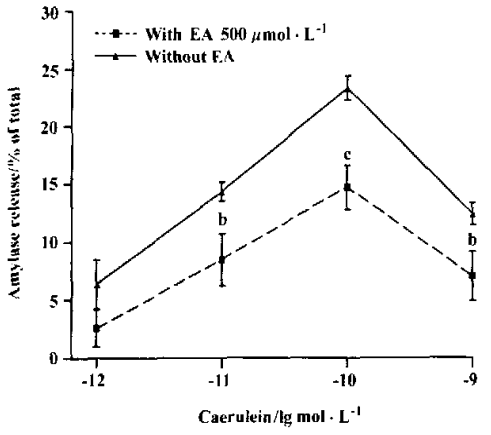

Fig 2. Effect of ethacrynic acid (EA) on amylase release stimulated by increasing concentrations (1 pmol/L to 1 nmol/L) of caerulein. $n = 4$ independent experiments. $\bar{x} \pm s_{\bar{x}}$. $^bP < 0.05$, $^cP < 0.01$ *vs* without EA treatment group.

Effect of EA on the CCK binding to its receptor ^{125}I-BH-CCK binding to isolated pancreatic acini was competitively inhibited by increasing concentrations unlabeled CCK (0 to 0.1 μmol/L). After incubation with EA, no changed characteristics of CCK binding to its receptor were found over the whole range of concentrations (data not shown).

Effect of EA on acinar GSH and protein thiols The amount of GSH in normal pancreatic acinar cell was (2.50 ± 0.10) μg/mg protein ($n = 4$). Pancreatic acinar cells incubated with various concentrations of EA for 30 min showed a dose-dependent decrease in GSH content, and incubation with EA 500 μmol/L for 30 min decreased GSH to (1.09 ± 0.14) μg/mg protein ($n = 4$), which was 43 % of control values (Fig 1). No obvious effects on protein thiol groups were found after treating pancreatic acinar cells with various concentrations of EA.

Effect of EA on intracellular calcium concentration In this study, the basal level, maximal increase of the curve, and the area under the curve were used to show the changes of resting level, initial transient phase, and sustained plateau phase, respectively. It has been suggested that the initial transient phase is probably related to Ca^{2+} release from intracellular stores (endoplasmic reticulum) and the followed sustained plateau phase is dependent on the presence of exocellular Ca^{2+} [17].

The basal Ca^{2+} value in pancreatic acinar cell suspensions containing cells about 2×10^9/L was (90.9 ± 2.2) nmol/L ($n = 6$). Addition of caerulein to the final concentration of 1 nmol/L immediately increased $[Ca^{2+}]_i$ to (408 ± 12) nmol/L ($n = 6$). The $[Ca^{2+}]_i$ decreased slowly in approximately 5 min to a new steady value above basal value (Fig 3). As Fig 3 shows, EA had no effect on the basal $[Ca^{2+}]_i$ level, but decreased maximal value induced by caerulein 1 nmol/L. Compared with control cell suspensions, the values of maximal increase of $[Ca^{2+}]_i$ in cell suspensions treated by EA 500 μmol/L decreased significantly ($P < 0.05$). The value of the area under the curve in cell suspensions treated by EA was obviously decreased, but no statistical difference was found.

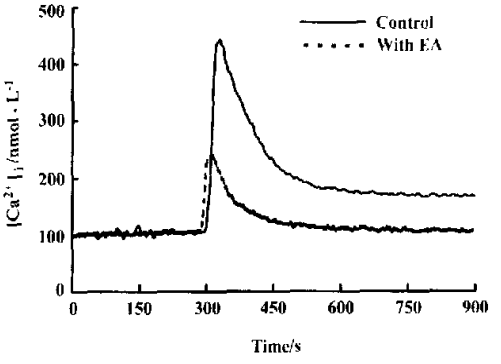

Fig 3. Effect of ethacrynic acid (EA) on caerulein-stimulated changes of intracellular calcium concentration ($[Ca^{2+}]_i$). Isolated pancreatic acinar cell suspension (2×10^9/L) was perfused with or without (control) EA 500 μmol/L, respectively, for 5 min, followed by addition of caerulein to a final concentration of 1 nmol/L, changes of $[Ca^{2+}]_i$ in cell suspension were recorded by fluorometry.

Effect of EA on viability of pancreatic acinar cell To exclude the possibility that the effect of inhibition on caerulein-stimulated amylase release was caused by gross cell damages induced by EA, the Trypan blue exclusion was assessed in pancreatic acinar cells,

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whereas LDH leakage was measured in acini. In acinar cells without incubation of EA, the Trypan blue uptake rate was below 2 %. After treatment with EA, the Trypan blue uptake cells minimally increased but remained below 5 %. The LDH leakage in normal pancreatic acini were 4 %. After incubation with EA (500 μmol/L), the value of LDH leakage in acini was increased only slightly and the increase was statistically not significant.

DISCUSSION

Because it is secreted in a form not requiring activation and a sensitive and colorimetric assay exists, amylase is the most commonly measured export protein to quantitate enzyme release[18].

As a cholecystokinin analogue, caerulein is widely employed to induce experimental acute pancreatitis in rats and be used as an agonist of CCK receptor[19].

The present results first demonstrate that EA significantly inhibits caerulein-induced amylase release in isolated rat pancreatic acini. However, it is difficult to determine the exact mechanism by which EA exerts its inhibitiory effect on pancreatic exocrine secretion. EA does not alter the characteristics of CCK-receptor affinity. This suggests that its effect on caerulein-induced amylase release must be caused by processes after caerulein binding to the CCK receptor.

It has been suggested that glutathione depletion inhibits pancreatic exocrine secretion in a previous publication[5]. This study was hampered by the effect that a very crude mode of oxidation was employed. Here we investigated the effect of EA on the thiol levels in pancreatic acinar cell. The present results show that EA can induce concentration-dependent glutathione depletion in pancreatic acinar cells similar to other types of cells[20,21]. Correlation analysis between the changes of pancreatic amylase release and glutatione strongly suggests that EA inhibits pancreatic exocrine secretion by depleting glutathione.

There is a close relation between glutathione status and the integrity of cytoskeleton[4]. In pancreatic acinar cells, microfilaments have been shown to be involved in the function of intercellular junction, the tensile strength of microvilli, and the final fusion-fission events that precede the extrusion of zymogen granule content during exocytosis[22]. Microtubules are suggested to transport secretory vesicles containing new synthesized protein from Golgi region to the cell surface[23]. Depletion of glutathione might be expected to induce damage or disruption of microfilament or microtubules, subsequently resulting in a blocked amylase secretion. Our next phase of research will focus on the effect of EA on pancreatic acinar cytoskeleton.

Intracellular calcium plays a predominant role in mediating caerulein-induced amylase release. In the present study, EA inhibited the caerulein-induced increase of intracellular Ca^{2+}, suggesting that inhibition of intracellular Ca^{2+} might be the mechanism for inhibition of caerulein- stimulated amylase release by EA, because an elevation of intracellular Ca^{2+} is considered to play a role in triggering the initial phase of secretion[24], and the failure to increase $[Ca^{2+}]_i$ to a certain level may impair enzyme secretion.

After treatment of EA 500 μmol/L, the initial transient phase and sustained plateau phase of caerulein-induced intracellular Ca^{2+} mobilization were inhibited, but no effect on resting levels was found. These observations imply that EA inhibits the release of Ca^{2+} from internal stores and the influx of extracellular Ca^{2+}. A potential candidate response for these decreases of Ca^{2+} may be the IP_3 receptor and its channels. It has been proposed that critical thiols play an important role in the mechanism of inhibition of IP_3-dependent release of calcium. IP_3-dependent calcium release is initiated by binding of IP_3 to a specific receptor, which has at least one essential free thiol group[25]. In the cell, glutathione acts as both a reducing agent and an antioxidant. Among its many physiological roles, glutathione plays an important role in the protein folding process and serves to protect intracellular constituents from oxidation by scavenging reactive oxygen species produced during normal cell metabolism[2]. So it might be speculated to conclude that treatment of EA may result in a decrease of IP_3 receptor or a decreased ability of IP_3 to release calcium from internal store by oxidizing the key thiol group after glutathione depletion.

Inhibition of Ca^{2+} uptake by a decrease of the activity of Ca^{2+}-ATPase is one other possible mechanism. EA was reported to inhibit activity of Ca^{2+}-ATPase involved in the intracellular sequestration of calcium[26]. Therefore, treatment of EA may disturb refilling of intracellular calcium store, resulting in a decrease of Ca^{2+} mobilization.

The present findings prove that EA inhibits pancreatic exocrine secretion. Depletion of glutathione

and down-regulation of caerulein-induced Ca^{2+} mobilization is involved in the effect of EA on secretory process in pancreatic acinar cells. Based on these findings, we can conclude that 1) EA is an effective thiol modulator and can be used to explore the pancreatic acinar thiols and 2) glutathione might play a potential role in the secretory process in pancreatic acinar cells and in the secretion blockade observed in acute pancreatitis. Together with previous reports which showed that GSH decreased the severity of experimental acute pancreatitis[9,27], our results suggest that replenishment of GSH might exert therapeutic effect on acute pancreatitis by amelioration of the pancreatic exocrine secretory blockade.

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依他尼酸抑制胰腺外分泌[1]

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