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Original article

Hypothermia slows sequential and parallel steps initiated during caerulein pancreatitis

Vivek Mishra, Krutika Patel, Ram N. Trivedi, Pawan Noel, Chandra Durgampudi, Chathur Acharya, John A. Holmes, Sonya Narla, Vijay P. Singh*

Mayo Clinic, Scottsdale, AZ, USA

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ABSTRACT

Background and objectives: Multiple deleterious signaling cascades are simultaneously activated in acute pancreatitis (AP), which may limit the success of pharmacologic approaches targeting a single step. We explored whether cooling acinar cells slows distinct steps initiated from a stimulus causing pancreatitis simultaneously, and the temperature range over which inhibition of such deleterious signaling occurs.
Methods: Caerulein (100 nM) induced trypsinogen activation (TGA), CXCL1, CXCL2 mRNA levels, cell injury were studied at 37 °C, 34 °C, 31 °C, 29 °C and 25 °C in acinar cells. Trypsin, cathepsin B activities and cathepsin B mediated TGA were studied at 37 °C, 23 °C and 4 °C.
Results: There was >80% reduction in TGA, CXCL1 and CXCL2 mRNA levels at 29 °C, and in cell injury at 34 °C, compared to those at 37 °C. Trypsin activity, cathepsin B activity and cathepsin B mediated TGA at 23 °C were respectively, 53%, 64% and 26% of that at 37 °C. Acinar cooling to 31 °C reduced LDH leakage even when cooling was initiated an hour after caerulein stimulation at 37 °C.
Conclusions: Hypothermia synergistically and simultaneously slows parallel and distinct signaling steps initiated by caerulein, thereby reducing TGA, upregulation of inflammatory mediators and acinar injury.

1. Introduction

Acute pancreatitis (AP) is the most common gastrointestinal disorder leading to hospitalization in the United States [1], however, there is no specific therapy of AP apart from conservative management or management of its complications. Numerous signaling cascades are initiated in pancreatitis, including intra-acinar zymogen activation [2,3], cytokine generation [4–6] and acinar cell injury [7,8]. Despite several pharmacologic trials conducted to improve outcomes in AP patients using serine protease inhibitors [9–18], there is no conclusive data from such studies showing benefit. Similarly, while cytokines like TNF-α are increased in AP [19–23] there have been conflicting case reports of the effect of anti-TNF therapy on the course of AP [24–26].

Hypothermia has been shown to prevent inflammatory mediator generation and cell death in various systems. Cooling to 34 °C reduced lipopolysaccharide (LPS) induced IL-6 and IL-1β secretion and NF-κB activation in RAW263.7 cells [27]. Cooling to 30 °C prevented mitochondrial cytochrome C release, reduced active caspase-3, apoptosis, and necrosis due to ischemia in rat brain [28] and Fas ligand-induced apoptosis in hepatocytes [29]. Similarly, resting energy expenditure and oxygen consumption have been shown to decrease with hypothermia [30] and adenosine triphosphate (ATP) levels and glycogen stores have been noted to be conserved by hypothermia in ischemic myocardium [31] associated with a decrease in infarct size.

Previous studies in animal models of AP have noted reduced inflammatory markers, improved local injury and improved survival with hypothermia in caerulein, caerulein + LPS and taurocholate induced pancreatitis [32–34]. Noting the reduction in both inflammatory response and injury parameters reported in models of AP and also in other systems with moderate hypothermia, we decided to systematically explore if hypothermia can (i) affect different signaling pathways in acute pancreatitis and (ii) synergistically slow consecutive steps of a cascade. For this we used an *in vitro* acinar cell model, the temperature of which can be precisely controlled, to span the temperature range (37–25 °C) over which the improvement in parameters has been noted in the studies mentioned above, and went on to study the end points,

* Corresponding author. Division of Gastroenterology and Hepatology, Mayo Clinic, Scottsdale, AZ 85259, USA. Tel.: +1 480 301 4286; fax: +1 480 301 7017.
E-mail address: singh.vijay@mayo.edu (V.P. Singh).

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including trypsinogen activation, inflammatory mediator generation and cell death, which have been thought to be crucial to the initiation of AP.

2. Methods

2.1. Animals

4 to 6 week old CD-1/ICR mice (Charles River Laboratories, Wilmington, MA) were housed with a 12-h light/dark cycle at temperatures ranging from 21 to 25 °C, were fed standard laboratory chow and allowed to drink *ad libitum*. Caerulein was purchased from Bachem (King of Prussia, PA). All experimental protocols were approved by the Institutional Animal Use Committee of the University of Pittsburgh (Pittsburgh, PA).

2.2. Acinar cell preparation

This procedure was carried out as previously described [35,36]. Primary acinar cells were prepared freshly and used for all assays. Acinar viability was confirmed before use to be >95%, as indicated by trypan blue exclusion. Acini were incubated for 15 min in HEPES containing buffer prior to adding the stimulus (100 nM caerulein).

2.3. Use of acinar cells

2.3.1. Chemokine upregulation

Acini were cultured overnight in RPMI 1640 with 10% fetal bovine serum as described previously [4]. Next AM, these were transferred to CO_2 incubators with different temperatures. There was a control and caerulein (100 nM) group for each condition. These were then stimulated for 90 min as described previously [4], after which the acini were spun down at 300 g × 2 min, and the pellet was suspended and preserved in RNA later at −20C. These were the processed for real time PCR as described below.

2.3.2. Trypsin generation and cell death studies

For prophylactic studies (Figs. 2B and 3) Freshly harvested acinar cells were aliquoted and placed at the respective temperatures (i.e. 37, 34, 31, 28 and 25C) for 15 min prior to adding the stimulus. For studies on therapeutic hypothermia, acini were warmed to 37C for 15, minutes and further maintained at 37C for 30, 60, 90 min (either alone or after adding caerulein to a final concentration of 100 nM) prior to being transferred to 31C. The LDH leakage over these periods was measured as a percentage total. A >80% reduction in caerulein induced LDH leakage compared to basal conditions was, when statistically significant ($p < 0.05$, depicted as "*") regarded as relevant.

2.4. Trypsin activity measurement

This was done in the trypsin assay buffer [50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM $CaCl_2$, 0.01% BSA] using the fluorescent substrate Boc-Gln-Ala-Arg-MCA, trypsin 3135; Peptides International, Inc., Louisville, KY) as described previously [2]. Activity of bovine trypsin (T1426, Sigma–Aldrich) was first standardized from 0.01 to 1 unit/assay at 23 °C to confirm linearity over this range. To study the effect at different temperatures (37 °C, 23 °C, 4 °C), 0.1 unit trypsin was used per assay, and the assay was carried out at the respective temperatures. For cathepsin B mediated trypsinogen activation, 20 μg of bovine trypsinogen (T1143, Sigma–Aldrich) was added to 0.2 units of cathepsin B in 0.1 M potassium acetate buffer, containing 1 mM EDTA, 5 mM DTT, and 1 mg/ml BSA as described previously [37] and incubated at the respective temperatures for 30 min. This was then diluted and assayed in trypsin assay buffer with 25 mM calcium chloride at the same temperature. To determine cathepsin B mediated TGA, activity generated in the absence of cathepsin B at the same temperature was subtracted from the activity generated in the presence of cathepsin B. For trypsin measurements in acinar homogenates, these were prepared as described previously [2,3,35] using the buffer and substrate described above and depicted as arbitrary units per microgram of DNA in homogenate. The activities were measured spectrophotometrically (Bio-Versa, Bio-Rad).

2.5. Cathepsin B activity assay

Each assay contained 0.2 unit of bovine cathepsin B (C6286, Sigma Aldrich). Activity was determined flourometrically as described by McDonald and Ellis [38] using CBZ-arginyl-arginine-β-napthalamide as substrate. The enzyme was activated by incubating for 5 min in a 100 mM phosphate buffer (pH 6.0) containing 5 mM DTT and 5 mM EDTA. Activity was measured in arbitrary units (AU) as a change in fluorescence value and expressed as a percentage of maximal activity (2500AU/unit of enzyme/ml/30 s) obtained at 37 °C.

2.6. Lactate dehydrogenase assay

This assay to determine the extent of cell injury was carried out as previously described by us [39].

2.7. Real time quantitative PCR

These were done as previously described by us [4]. In short, total RNA was extracted using Trizol (Invitrogen) and quality was checked on 1% agarose gel stained with ethidium bromide. RNA quantification was carried out spectrophotometrically at 260 nm. A Superscript II reverse transcriptase kit (Invitrogen) using random primers (New England Biolabs) and 5 μg of non-degraded RNA was used for cDNA synthesis. Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using proprietary Taqman Gene Expression assays for KC (CXC11or Mm00433859_m1) and MIP-2 (CXC12, Mm00436450_m1). The relative expression levels were calculated after normalization to 18S (AM1718, Life Technologies) using the ΔΔCt method recommended by the manufacturer.

2.8. Statistical analysis

All data are representative of at least three independent experiments done in duplicate. Bar graphs depict mean and error bars depict standard error of mean (SEM). Pairwise comparisons between changes noted at 37 °C and other temperatures was done using the Mann Whitney-U test, and a $p < 0.05$ considered to indicate statistical significance. In experiments involving acini, the net difference of a parameter between basal and 100 nM caerulein at 37 °C was considered as 100%.

3. Results

3.1. Hypothermia synergistically slows consecutive steps in protease activation

Since cathepsin B mediated intra-acinar trypsin generation is thought to induce injury, we studied the effect of hypothermia on trypsin and cathepsin B activities in a cell free system. We also studied the effect of hypothermia on trypsin generation as a result of incubating trypsinogen with cathepsin B at different temperatures (37 °C, 23 °C and 4 °C, Fig. 1). Trypsin activity was 54% at 23 °C