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Lasting inhibition of receptor-mediated calcium oscillations in pancreatic acini by neutrophil respiratory burst – A novel mechanism for secretory blockade in acute pancreatitis?

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ABSTRACT

Although overwhelming evidence indicates that neutrophil infiltration is an early event in acute pancreatitis, the effect of neutrophil respiratory burst on pancreatic acini has not been investigated. In the present work, effect of fMLP-induced neutrophil respiratory burst on pancreatic acini was examined. It was found that neutrophil respiratory burst blocked calcium oscillations induced by cholecystokinin or by acetylcholine. Such lasting inhibition was dependent on the density of bursting neutrophils and could be overcome by increased agonist concentration. Inhibition of cholecystokinin stimulation was also observed in AR4-2J cells. In sharp contrast, neutrophil respiratory burst had no effect on calcium oscillations induced by phenylephrine (PE), vasopressin, or by ATP in rat hepatocytes. These data together suggest that inhibition of receptor-mediated calcium oscillations in pancreatic acini by neutrophil respiratory burst would lead to secretory blockade, which is a hallmark of acute pancreatitis. The present work has important implications for clinical treatment and management of acute pancreatitis.

1. Introduction

Neutrophil is an essential component of innate immune system [1]. It has been reported that neutrophil content in blood increased in patients with inflammation, with concurrent neutrophil infiltration of inflamed tissues [2–4]. Respiratory burst of neutrophils frequently leads to damage to surrounding tissues. Neutrophil contents both in the whole blood and in inflamed tissues could be used for diagnosis of inflammatory diseases [5–8]. Reports have suggested that neutrophil infiltration is a key event in the initiation and development of acute pancreatitis [3,9,10].

Reports both on pancreatitic patients [5–7] and on animal models have suggested that neutrophils play a pivotal role in the pathogenesis of acute pancreatitis [3,11,12]. The activity of neutrophil myeloperoxidase and elastase in the blood has been used as makers of acute pancreatitis [5,7]. Myeloperoxidase and neutrophil elastase contents were at peak values on day 1 of acute pancreatitis [5–7], but the blood contents of other markers such as C-active protein did not reach peak levels until on days 2–4 or even later [5–7]. It is possible to measure neutrophil infiltration in greater temporal detail in animal models of acute pancreatitis. Different methods to measure neutrophil content in inflamed pancreas all suggested that neutrophil infiltration start within 1 h of the onset of acute pancreatitis [3,12]. Chemiluminescent measurements of superoxide anion on the surface of pancreas further confirmed that neutrophil infiltration started early in acute pancreatitis [11,13]. Therefore abundant evidence points to early neutrophil infiltration in acute pancreatitis. These understandings notwithstanding, how neutrophils might regulate pancreatic acinar cell function is not known.

With the above in mind, we have investigated the effect of respiratory burst of neutrophils on pancreatic acinar cell function in a co-incubation system. Real time functional changes in pancreatic acini after neutrophil respiratory burst were examined by monitoring calcium concentration in perifused pancreatic acini. It was found that neutrophil respiratory burst blocked calcium oscillations induced by physiological concentrations of CCK and ACh. The inhibition of CCK1 receptor in pancreatic acini by neutrophil respiratory burst was reproduced in pancreatic tumor cell AR4-2J. Such inhibition of receptor-mediated cytosolic calcium oscillations by neutrophil respiratory burst was not seen in isolated rat hepatocytes. Calcium oscillations induced by activation of α1 adrenergic receptors, V1a vasopressin receptors, or P2Y purinergic receptors were not inhibited by neutrophil respiratory burst. To the best of our knowledge, this is the first report to show that neutrophil respiratory burst blocks cell surface receptors in pancreatic acini. This has important implications for the understanding of pathogenesis of acute pancreatitis and may provide guidelines for the clinical treatment of acute pancreatitis.

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2. Materials and methods

2.1. Reagents

Cholecystokinin octapeptide (CCK), bethanechol (Beth), acetylcholine chloride (ACh), soybean trypsin inhibitor, arginine vasopressin (VP), L-phenylephrine (PE), L-glutamine, N-formyl-Met-Leu-Phe (fMLP), ATP and heparin sodium were from Sigma–Aldrich (St. Louis, MO, USA). Collagenase P and collagenase H were from Roche (Mannheim, Germany). Bovine serum albumin (BSA), 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) were bought from Calbiochem (Darmstadt, Germany). Fura-2 AM was from AAT Bioquest Inc. (Sunnyvale, CA, USA). Dextran T-500 was from Fluka. Concentrated MEM amino acids mixture, DMEM/F12 (1:1) medium, 0.25% trypsin (with EDTA) were from Invitrogen. Cell-Tak was from BD Biosciences (Bedford, MA, USA).

2.2. Isolation of rat pancreatic acini

Male Sprague-Dawley rats (250 ± 50 g) were used for pancreatic acini isolation [14]. Excised pancreas was digested by collagenase P (2 g L^{-1}) at 37 °C in a shaking water bath for 30 min (120 cycles per min) then dispersed with a plastic pipette. Isolated acini were filtered and washed before use.

Buffer for acini isolation had the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.13 $MgCl_2$, 1.0 NaH_2PO_4, 5.5 D-glucose, 10 HEPES, 2.0 L-glutamine, and 2% BSA, 2% minimum essential medium amino acids mixture, 0.1 g L^{-1} soybean trypsin inhibitor (SBTI). Buffer pH was adjusted to 7.4 with 4 M NaOH, and buffer was oxygenated with O_2. Buffer for acini perfusion had the same composition, but SBTI, amino acids mixture, glutamine and BSA were omitted.

2.3. Isolation of rat neutrophils

Rat neutrophils were isolated from heparinized blood [15]. Blood was collected from Sprague-Dawley rats (250–400 g). Neutrophils were dextran sedimented (4.5% dextran in 0.9% NaCl solution in 40 min at 4 °C), centrifuged through a solution for lymphocyte separation (500 g, 15 min). Residual erythrocytes were broken by hypotonic lysis. Isolated neutrophils had a purity of >95% as determined by Wright's stain. Cell viability was >95% as checked by trypan blue exclusion.

2.4. AR4-2J cell culture

AR4-2J cells were cultured in a mixed medium (DMEM/F12 by 1:1) containing FBS (20% v/v) and antibiotics (100 μg/ml streptomycin, 100 unit/ml penicillin) as reported [16].

2.5. Measurement of cytosolic calcium concentration

Cells were loaded with Fura-2 AM (at 10 μM) for 30 min (60 min for AR4-2J) in a shaking water bath (37 °C, 50 cycles per min). Fura-2 AM-loaded cells were attached to Cell-Tak-coated cover-slips, which formed the bottom of Sykes-Moor perfusion chambers. Cells were perfused at a rate of 1 mL min^{-1}, and stimulants were introduced by changing buffers. Calcium concentration was measured on an inverted fluorescence microscope (Nikon TE 2000U), which was coupled to a calcium imaging system (EasyRatioPro, Photon Technology International, New Jersey, USA). Fura-2 was excited alternately at 340/380 nm (monochromator slit width 1 nm), fluorescence images were captured (after passing through a band pass filter 510 ± 40 nm) on a CCD camera (QuantEM 512SC, Roper Scientific) (Figs. 1–4, S1 and S2). Data in Figs. 3D, 4D, S3 were obtained in a PMT-based system [14]. Fluorescence ratios (F340/F380) were taken as indicative of calcium concentration [17,18]. Calcium oscillatory frequencies induced by the 1st and 2nd agonist stimulations in *N* experiments were analyzed and presented as mean ± SEM next to the calcium traces. Asterisk (*) indicates $P < 0.05$.

3. Results

3.1. Neutrophil respiratory burst blocked CCK-induced calcium oscillations in rat pancreatic acini

Bacterial peptide fMLP triggers respiratory burst [19]. fMLP ($10^{-8} - 10^{-4}$ M) induced respiratory burst dose-dependently in isolated rat neutrophils, with maximum effect at 10^{-5} M (data not shown). fMLP 10 μM was therefore used to trigger respiratory burst. Neutrophils (at a density of 5×10^5/ml) alone or after incubation with fMLP had no effect on basal calcium in isolated rat pancreatic acini (Fig. 1A). Two sequential doses of CCK (10 pM) induced reproducible calcium oscillations (Fig. 1B). fMLP (10 μM) alone had no effect on either basal calcium or on the second dose of CCK stimulation (Fig. 1C). After simultaneously additions (with a 10 s gap) of neutrophils (5×10^5/ml) and fMLP (10 μM), however, a second dose of CCK (10 pM) failed to induce any calcium oscillations (Fig. 1D). In this latter experiment (Fig. 1D), to ensure full contact between stimulated neutrophils and pancreatic acin, perfusion was stopped for 4 min immediately after the addition of neutrophils. Perfusion was re-started 4 min later. The multiple color-coded calcium traces in each panel (Fig. 1A–D) indicate calcium changes in individual pancreatic acinar cells in one typical experiment, the *N* numbers in each figure indicate number of rats used to perform identical experiments.

Time-matched parallel experiments were done as shown in Fig. 2A–C. Two sequential doses of CCK induced reproducible calcium oscillations (Fig. 2A). After respiratory burst (neutrophils, 5×10^5/ml plus fMLP 10 μM), a second CCK dose no longer induced any calcium oscillations (Fig. 2B). Pancreatic acini exposed to respiratory burst remained perfectly healthy, because a subsequent dose of bethanechol (Beth, 5 μM) induced a robust calcium increase (Fig. 2B). Respiratory burst from a reduced amount of neutrophils (1.67×10^5/ml, 1/3 of that in Fig. 1D and Fig. 2B) did not block calcium oscillations induced by a second CCK dose (Fig. 2C). Non-bursting neutrophils (10^6/ml, double density of Fig. 1D and Fig. 2B) alone, however, had no effect on calcium oscillations induced by a second dose of CCK 10 pM (Fig. 2D). Therefore inhibition exerted by respiratory burst was dependent on the density of bursting neutrophils.

3.2. Neutrophil respiratory burst blocked ACh-induced calcium oscillations in rat pancreatic acini

Similarly, ACh-induced calcium oscillations were also inhibited by neutrophil respiratory burst (Fig. 3). In these experiments, sequential doses of ACh (20 nM) induced reproducible calcium oscillations in isolated rat pancreatic acini (Fig. 3A). The pattern of calcium oscillations induced by ACh was slightly different from CCK (compare Fig. 3 with Figs. 1 and 2). A higher ACh dose (100 nM) induced plateau calcium increase (Fig. 3A). fMLP 10 μM alone had no effect on either basal calcium or calcium oscillations induced by a second dose of ACh 20 nM(Fig. 3B); ACh 100 nM still induced a long-lasting calcium plateau (Fig. 3B). After neutrophil respiratory burst, the second dose of ACh 20 nM failed to induce any calcium oscillations; even ACh 100 nM had no effect (Fig. 3C). An ACh dose of 200 nM was required to induce regular calcium oscillations (Fig. 3C). Therefore inhibition of calcium