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BASIC AND TRANSLATIONAL—PANCREAS

Inhibitors of ORAI1 Prevent Cytosolic Calcium-Associated Injury of Human Pancreatic Acinar Cells and Acute Pancreatitis in 3 Mouse Models

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BACKGROUND & AIMS: Sustained activation of the cytosolic calcium concentration induces injury to pancreatic acinar cells and necrosis. The calcium release–activated calcium modulator ORAI1 is the most abundant Ca^{2+} entry channel in pancreatic acinar cells; it sustains calcium overload in mice exposed to toxins that induce pancreatitis. We investigated the roles of ORAI1 in pancreatic acinar cell injury and the development of acute pancreatitis in mice. **METHODS:** Mouse and human acinar cells, as well as HEK 293 cells transfected to express human ORAI1 with human stromal interaction molecule 1, were hyperstimulated or incubated with human bile acid, thapsigargin, or cyclopiazonic acid to induce calcium entry. GSK-7975A or CM_128 were added to some cells, which were analyzed by confocal and video microscopy and patch clamp recordings. Acute pancreatitis was induced in C57BL/6J mice by ductal injection of taurolithocholic acid 3-sulfate or intravenous' administration of cerulein or ethanol and palmitoleic acid. Some mice then were given GSK-7975A or CM_128, which inhibit ORAI1, at different time points to assess local and systemic effects. **RESULTS:** GSK-7975A and CM_128 each separately inhibited toxin-induced activation of ORAI1 and/or activation of Ca^{2+} currents after Ca^{2+} release, in a concentration-dependent manner, in mouse and human pancreatic acinar cells (inhibition >90% of the levels observed in control cells). The ORAI1 inhibitors also prevented activation of the necrotic cell death pathway in mouse and human pancreatic acinar cells. GSK-7975A and CM_128 each inhibited all local and systemic features of acute pancreatitis in all 3 models, in dose- and time-dependent manners. The agents were significantly more effective, in a range of parameters, when given at 1 vs 6 hours after induction of pancreatitis. **CONCLUSIONS:** Cytosolic calcium overload, mediated via ORAI1, contributes to the pathogenesis of acute pancreatitis. ORAI1 inhibitors might be developed for the treatment of patients with pancreatitis.

Keywords: STIM1; SOCE; Calcium Entry Inhibition; Drug Development; Experimental Pancreatitis.

Sustained increase of the cytosolic calcium concentration ($[Ca^{2+}]_C$) is a critical trigger for pancreatic acinar cell injury and necrosis, which depends on store-operated calcium entry (SOCE).[1–4] ORAI1 is the principal SOCE channel in the pancreatic acinar cell,[5] the opening of which is coordinated by stromal interaction molecule (STIM)1 and STIM2, after decreases in endoplasmic reticulum calcium store concentrations.[3,5–7] GSK-7975A and CM_128 were developed independently by GlaxoSmithKline (Stevenage, United Kingdom)[3,7,8] and CalciMedica (La Jolla, CA), respectively, to block ORAI1 channels, although only CM_128 continues toward clinical development. GSK-7975A inhibits SOCE induced by thapsigargin in isolated murine pancreatic acinar cells over the range of 1–50 μmol/L (half-maximal inhibitory concentration [IC_{50}], ∼3.4 μmol/L),[3] inhibits endocytic vacuole formation[9] and reduces necrosis induced by toxins that cause acute pancreatitis.[3,9] CM_128 is a new molecular entity. ORAI1 inhibition could inhibit SOCE and necrosis in human pancreatic acinar cells and ameliorate acute pancreatitis.

Genetic knockout of the transient receptor potential canonical 3 channel,[10] a nonselective cation channel regulated in part by STIM1 via transient receptor potential canonical 1,[11] resulted in an approximately 50% reduction of in vivo serum amylase increase and edema formation induced by 4 injections of cerulein.[10] These experiments supported some role for SOCE in acute pancreatitis, but in a single mild model with few parameters of response.

Abbreviations used in this paper: AP, acute pancreatitis; $[Ca^{2+}]_C$, cytosolic calcium concentration; CCK, cholecystokinin; CER, cerulein; FAEE, fatty acid ethyl ester; IC_{50}, half-maximal inhibitory concentration; I_{CRAC}, Ca^{2+} release-activated Ca^{2+} currents; IL, interleukin; MPO, myeloperoxidase; PI, propidium iodide; SOCE, store-operated calcium entry; STIM, stromal interaction molecule; TLCS, taurolithocholate acid sulfate.

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Here, we defined the concentration-dependent inhibitory effects of GSK-7975A and CM_128 on SOCE and necrosis in murine and human pancreatic acinar cells induced by taurolithocholic acid 3-sulfate (TLCS)[2,12] or cholecystokinin (CCK) 8.[1,10] The effects of CM_128 on ORAI1 were substantiated by examination of its effect on Ca^{2+} release-activated Ca^{2+} currents (I_{CRAC})[3,6,7] in ORAI1/STIM1-transfected HEK 293 cells.[7] Our in vitro work informed in vivo pharmacokinetic analysis. GSK-7975A was given at selected doses after induction of acute pancreatitis (AP) with TLCS (TLCS-AP),[13] 7 injections of cerulein (CER-AP)[14] or ethanol and palmitoleic acid (FAEE-AP).[15] Because GSK-7975A markedly reduced all parameters of pathobiologic response in a dose-dependent manner, a high dose of GSK-7975A and separately CM_128 was begun at 2 different time points after disease induction to determine the effect of early vs late drug administration. Drug administration that was begun 1 hour after disease induction was highly effective in reducing parameters of pathobiologic response, significantly more so than when begun 6 hours after disease induction, in all models. These data provide thorough preclinical validation for ORAI channel inhibition as a potential early treatment for acute pancreatitis.

Materials and Methods

Human Specimen Sampling

Human pancreas was sampled and cells were isolated as described.[16] The time from sampling to the start of cell isolation was fewer than 10 minutes.

Cell Culture and Transfection

HEK 293 cells were cultured and transfected as described.[7] HEK 293 cells stably transfected with complementary DNAs encoding human ORAI1 and STIM1 were used in patch-clamp recording.

Animals

CD-1 and C57BL/6J mice were from Charles River UK, Ltd (Margate, Kent, UK). Pancreatic acinar cells were isolated from CD-1 mice as described.[1,3,12,15] For in vivo experiments, 10-week-old male C57BL/6J mice (25 g) were used.

Confocal Fluorescence Microscopy and Video Imaging

Isolated pancreatic acinar cells were imaged using a Till Photonics System (Munich, Germany) to assess $[Ca^{2+}]_C$ with Fura-2 (5 μmol/L; excitation, 340 and 380 nm; emission, $>$490 nm; ratio of fluorescence recorded from excitation, 340 and 380 nm) and using LSM710 systems (Carl Zeiss, Jena GmbH) to assess necrotic cell death pathway activation with propidium iodide (PI) (1 μmol/L; excitation, 488 nm; emission, 630–693 nm).

Necrotic Cell Death Pathway Activation Measurement

Cells were treated with GSK-7975A or CM_128 together with TLCS (500 μmol/L) for 30 minutes, gently shaking at 1000 rpm at room temperature. After washing, cells were stained with PI and Hoechst 33342, distributed into 96-well glass bottom plates (150 μL/well), and imaged using LSM710 systems. Hoechst 33342 (50 μg/mL; excitation, 364 nm; emission, 405–450 nm) was used to stain nuclei and count the total number of cells. PI was used to assess plasma membrane rupture: the total number of cells showing PI uptake was counted in 3 or more wells and in 12 or more random fields of each differently treated group of each isolate to provide a percentage, averaged across fields, as the mean $\pm$ SEM field percentage PI uptake with 3 or more isolates per group, except where stated.

Patch-Clamp Current Recording

The whole-cell configuration was used to record I_{CRAC} from hORAI1/hSTIM1 HEK 293 cells.[7] Patch pipettes were pulled from borosilicate glass capillaries (Sutter Instruments) with a resistance of 2–5 MΩ when filled with an extracellular solution of 120 mmol/L NaCl; 10 mmol/L TEA-Cl; 10 mmol/L HEPES; 10 or 0 mmol/L $CaCl_2$; 2 or 12 mmol/L $MgCl_2$, and 10 mmol/L glucose, pH 7.2. I_{CRAC} was activated by passive depletion of intracellular Ca^{2+} stores using the intracellular solution of 105 mmol/L Cs-glutamate; 10 mmol/L HEPES; 20 mmol/L 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; 8 mmol/L $MgCl_2$, pH 7.2. Patched cells were exposed to Ca^{2+}-free buffer to establish stable baseline (for 5 min), then 10 mmol/L $CaCl_2$ to develop I_{CRAC} (for 10 min), and then CM_128 (0.001, 0.01, 0.1, and 1 μmol/L for 10 min). External recording saline with no Ca^{2+} then was perfused for 2 minutes to determine the background current in the absence of I_{CRAC}. Whole-cell currents were sampled at 10 KHz and filtered at 2 KHz (Multiclamp 700B amplifier and PClamp software; Axon Instruments). The voltage clamp protocol included a cycle of steps to 0 mV (for 10 ms to evaluate zero current), then -100 mV (for 10 ms to measure I_{CRAC}), and a ramp from -100 mV to $+$100 mV over 50 ms for I-V relationship followed by step to $+$50 mV (for 10 ms to estimate leak current). The voltage between sweeps was $+$30 mV (for 12 s). Whole-cell capacitive compensation was used. Data analysis was performed using Clampfit software. I_{CRAC} was measured at -100 mV and current was measured at approximately 6 minutes and was used as the baseline control. The current measured after a 10-minute application of test compound was normalized to the baseline current (expressed as the percentage of control). The current measured in zero Ca^{2+} buffer was used to subtract the background leak current. Data points were fitted by nonlinear regression analysis with variable slope (SigmaPlot software) to determine the IC_{50} and Hill slope. The IC_{50} was taken as the point on the nonlinear regression halfway between the extrapolated baseline (control) and maximum inhibition produced by the compound.

Experimental Acute Pancreatitis

TLCS-AP was induced by retrograde pancreatic ductal injection with 3 mmol/L TLCS (5 μL/min over 10 minutes by infusion pump)[13]; humane killing was 6 or 24 hours later. CER-AP was induced by 7 hourly intraperitoneal cerulein injections (50 μg/kg)[14]; humane killing was 12 hours after the first. FAEE-AP was induced by 2 hourly intraperitoneal injections of 150 mg/kg palmitoleic acid and 1.35 g/kg ethanol[15]; humane killing was 6 or 24 hours later. GSK-6288B, the prodrug of

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