Prolonged High Fat / Alcohol Exposure Increases TRPV4 and Its Functional Responses in Pancreatic Stellate Cells

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Abstract (Word Count 250)

The present study investigated transient receptor potential vanilloid type 4 (TRPV4) ion channels in pancreatic stellate cells (PSCs) isolated from rats with high fat and alcohol diet (HFA)-induced chronic pancreatitis. TRPV4 is a calcium permeable non-selective ion channel responsive to osmotic changes, alcohol metabolites arachidonic acid, anandamide, their derivatives, and injury-related lipid mediators. Male Lewis rats were fed HFA for 6-8 weeks prior to isolation and primary culture of PSCs. Control pancreatic stellate cells were harvested from rats fed standard chow. Immunoreactivity for cytoskeletal protein activation product alpha-smooth muscle actin (α-SMA) and platelet-derived growth factor receptor β subunit (PDGFR-β) characterized the cells as PSCs. TRPV4 expression increased in pancreatic stellate cells of HFA fed rats and control cultures after alcohol treatment (50mM). Cell responses to activation of inducible TRPV4 were assessed with live cell calcium imagining. Three-fold increased and sustained intracellular calcium mobilization responses occurred in 70% of pancreatic stellate cells from HFA fed rats in response to TRPV4 activators arachidonic acid, lipid second messenger, phorbol ester 4 alpha-phorbol 12,13-didecanoate (4αPDD), and 50% hypoosmotic media compared to relatively unresponsive PSCs from control rats. Activation responses were attenuated by non-selective TRPV channel blocker, ruthenium red. Tumor necrosis factor alpha (TNFα, 1ng/ml, 16 hr) increased responses to 4αPDD in control PSCs. These findings implicate TRPV4 mediated calcium responses inducible after high fat/alcohol exposure and inflammation in reactive responses of activated PSCs that impair pancreatic function, such as responsiveness to cytokines and the deposition of collagen fibrosis that precipitates ductal blockage and pain.
Introduction

Pancreatic disease is precipitated by genetic and environmental factors such as alcohol, smoking and high fat diet (40-42). The pancreatic inflammation and fibrosis are promoted by activated pancreatic stellate cells (PSCs) in response to alcohol, alcohol oxidative metabolites such as acetaldehyde, and other injury related lipid mediators in the local microenvironment. Activated PSCs are innate immunity cells which enlarge, change shape, and initiate inflammatory processes. One of the key features of chronic pancreatitis is stellate cell deposition of collagen fibrosis in the gland that eventually leads to constrictive interference of secretory function (3). These spindle-shaped, α-smooth muscle actin (α-SMA)-positive myofibroblast-like cells were identified around the acini, pancreatic ducts and vessels of the human pancreas by Satome and colleagues (28). A method of isolating and culturing these cells from rat pancreas was established by Apte et al (4) and Bachem et al (10) who gave them the name pancreatic stellate cells. Activated PSCs are similar in function and morphology to hepatic stellate cells, and are principal effectors of progressive inflammation and fibrosis (6, 20, 32). These dendritic cells residing in both organs are derived from myeloid precursor cells in parallel with macrophages.

Evidence from both clinical and experimental studies indicate a role for PSCs in ethanol and high fat-induced pancreatic inflammation (3, 7-9, 19, 44). Activated PSCs have been identified in vivo in tissue from patients with chronic alcoholic pancreatitis and from animals with experimental chronic pancreatitis (21, 36). They are located within tissue areas with the collagen fibrosis they produce (2). Comparisons indicate that injury related microenvironment conditions can be mimicked in vitro by exposure of PSCs to ethanol, pro-inflammatory cytokines, fatty acids and acetaldehyde. In vitro studies have established that PSCs are activated directly by ethanol and its oxidative metabolites,
acetaldehyde, epoxyalcohols (HEETs) and epoxides (EETs). Also of particular interest is the observation that rat PSCs show alcohol dehydrogenase activity, indicating that, apart from hepatic and pancreatic acinar cells, ethanol can also be metabolized by the periacinar stellate cells in the pancreas. Persistently activated PSCs migrate to sites of tissue damage, undergo regulated contraction, proliferate, phagocytosize, and generate products that modulate the extracellular matrix either by facilitating repair or promoting fibrosis (25, 26, 30). For example, collagen is co-localized in human pancreatic stellate cells with lipid peroxidation-derived aldehydes in chronic pancreatitis (15). PSC activation is characterized by increased α-SMA and/or desmin expression and increased production of extracellular matrix proteins such as collagens I and III, fibronectin, and laminin which contribute to the pancreatic fibrosis that precipitates ductal blockage, pain, and impaired function (5, 37, 44).

Lipid peroxidation-derived aldehydes are activators of ion channel transient receptor potential (TRP) family member, TRPV4 (33). TRPV4 is the mammalian homologue of the Osm-9 C. elegans gene critical in osmotic and mechanical avoidance. The mammalian TRPV4 channel along with other TRP channels is emerging as a front line candidate for molecular detection and integration of chemical, thermal, osmotic, and mechanical stimuli particularly in sensory neurons (1, 12, 16, 18, 27). In the present study, the role of TRPV4 channels in the development of chronic pancreatitis was investigated in PSCs isolated from rats fed a liquid high fat and alcohol diet for 6-8 weeks. Activation of TRPV4 in response to specific agonists (arachidonic acid, 4αPDD) and 50% hypoosmotic media was assessed by live cell calcium microfluorimetry, and reduction by
a non-selective TRPV channel blocker tested. TRPV4 protein expression increase was
determined with Western blot and immunochemical analyses.

Methods

Animals and Diet

All procedures were consistent with the guidelines of the policies for Ethical Treatment of Research Animals published by the International Association for the Study of Pain and approved by the Animal Care and Use Committee at our institution. Male Lewis rats weighing between 200 -250 g (Harlan Sprague-Dawley, Indiana) were used for this study. Animals were kept in a temperature constant (23°± 2°C) room on a 12/12 hour dark-light reversed cycle. The liquid HFA diet (LD 101A Micro-stabilized alcohol rodent liquid diet mix, with LD 104 maltose, Test-Diet, Richmond, IN) was prepared fresh each day and consisted of 20% fat from corn oil, safflower oil and lard (39). The 30.3% protein, 5% fiber, vitamins and minerals were added as a dry powder to water, apple juice (10%), and alcohol (w/v, 95% ethyl alcohol). The dose of alcohol was progressively increased from 4% to 6% as follows: 4% alcohol for the first week, 5% for second week, and 6% for the third through the eighth week. Each rat consumed between 50 - 70g of liquid diet with alcohol per day. Body weight was monitored weekly. Animals were observed closely daily, and no evidence of alcohol intoxication (no ataxia or lethargy) was noted.

Pancreatic Stellate Cell Isolation and Culture

PSCs were isolated with a density gradient centrifugation (Nycodenz gradient) method adopted from Apte et al. (4). For each experiment, two 200 – 300 g rats were
sacrificed and pancreatic tissue was taken, minced with surgical scissors, and digested with Gey’s balanced salt solution (GBSS) containing 0.02% pronase, 0.05% collagenase P, and 0.1% DNAse at 37°C for 40 minutes. Digested tissue was pipetted vigorously and then filtered through a nylon mesh with 150 μm openings. After centrifugation, the supernatant was discarded and the cells resuspended in 9.5 ml GBSS containing 0.3% BSA. The cell suspension was mixed with 8 ml of 28.7% (wt/vol) of Nycodenz in GBSS without salt (NaCl). The gradient was prepared by layering the Nycodenz cell suspension beneath 6 ml GBSS with BSA in a 50 ml centrifuge tube. The gradient mixture was centrifuged for 20 minutes at 1400 g. The PSCs separated into a band at the interface of the Nycodenz cushion and the GBSS with BSA. The band was harvested, and the cells were washed and resuspended in Iscove’s modified Dulbecco’s medium containing 10% fetal calf serum, 4mM glutamine, and antibiotics (penicillin 100 units/ml; streptomycin 100 μg/ml). Cells were seeded in a density of 50,000 cells per well in plastic six well culture plates in Dulbecco’s medium with fetal calf serum, glutamine, and antibiotics as detailed above. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ air.

RT-PCR

RT-PCR was performed in primary cultured PSCs to confirm TRPV4 gene expression. PSCs were grown to confluence in 6-well cluster with culture medium. Total RNA was extracted from PSCs through TRIzol (Invitrogen, Grand Island, NY) suspension, chloroform separation and 2-propanol precipitation. Total RNA was treated with TURBO DNA-free (Invitrogen, Grand Island, NY) to eliminate DNA contamination and RNA concentration was measured. Reverse transcription was performed as following: 5x first-strand buffer (2 μl), 10mM dNTP (0.5 μl), 50μM random hexamer (1 μl), diethyl
pyrocarbonate H2O, 0.mM DTT (1 µl), 10 U/µl RNase inhibitor (1 µl), and 1µl SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY). Samples were incubated at 42°C for 50 min and inactivated at 70°C for 15 min. PCR was performed for 35 cycles using the following temperature protocol: 94°C (30 s), 56°C (60 s), and 72°C (30 sec), with an initial step at 94°C (2 min) to activate the platinum Taq polymerase (Invitrogen, Grand Island, NY) followed by 5 min extension at 72°C by PTC-100 programmable thermal controller (MJ Research Inc., Waltham, MA). TRPV4 Gene-specific primer is as follows: ATCAACTCGCCCTTCAGAGA (forward) and GGTGTTCTCTCGGGTGTTGT (reverse) (23). The predicted size of amplicon is 339bp for TRPV4. Reaction products were separated on 1% agarose gel in Tris-acetate EDTA buffer, stained with 0.5% ethidium bromide, and analyzed with Fotodyne software (Fotodyne, Hartland, WI).

Ratiometric Live Cell Imaging of Calcium Mobilization in Pancreatic Stellate Cells

Pancreatic stellate cells were plated at the density of 4x10⁴/cm² on a microscope coverslip (∅ 1.2 cm) and kept in normal cell culture media for 48 hours to form a single layer. Cells were loaded with a dual excitation Ca²⁺ indicator dye, acetoxy-methyl-ester Fura-2 (Fura-2AM, 5 µM, Molecular Probes, Eugene OR), which diffuses across the cell membrane in KREB’s solution for 1 hour at room temperature in the dark, prior to imaging. Cells were then washed three times with KREB’s solution. After a wash to remove extracellular fura-2, the indicator was allowed to de-esterify for 30 minutes. During this process, the charged Ca²⁺ indicator is trapped inside the cell body and any changes in the 340/380 emission ratio is an indication of intracellular Ca²⁺ mobilization. The coverslip with PSCs was mounted in a recording chamber containing 1 ml KREB’s solution attached to the stage of an inverted microscope at room temperature.
Fluorescence studies were performed on populations of cells with 10 - 20 cells in each microscope field of each plate using a Nikon TE-2000 inverted epi-fluorescence microscope with a 20X 0.9 quartz objective, a cool snap HQ digital camera connected to a computer with Nikon Element program software (Nikon). Fura-2 AM loaded PSCs were excited alternately at wavelengths of 340 nm and 380 nm with a Sutter Lambda LS high speed shutter system. The ratio of emitted light (340/380) detected at 510 nm was used as an indirect ratiometric measurement of intracellular Ca\(^{2+}\) activity. The composition of KREB’s solution was as follows (in mM): NaCl 130, KCl 5.5, CaCl\(_2\) 2.5, MgCl\(_2\) 1, HEPES 20, glucose 10, pH 7.2 -7.4.

All experiments were done at room temperature (22 - 23°C). Microscope fields with 10 - 20 cells each were globally selected to measure the changes of intracellular calcium activity. Cells were stimulated with arachidonic acid (AA), a lipid messenger and 4\(α\)PDD, a potent TRPV4 agonist, or hypotonic bath solution. AA and 4\(α\)PDD were diluted with KREB’s solution and applied by bath perfusion immediately after baseline recording. Ruthenium red (RR), a TRPV channel blocker, was applied in the bath 10 minutes before the agonist addition. Each experimental condition was repeated three times in different cell preparations.

**Immunofluorescent Study in Primary Cultured PSCs**

The cells, attached to the cover slip, were fixed with 4% paraformaldehyde for 20 minutes, washed in 0.1M PBS (pH7.3), permeabilized with 0.1% Triton X100, and incubated with primary antibody overnight at room temperature. Primary antibodies utilized included rabbit anti-PDGFR-β (1:500, Santa Cruz, CA); rabbit anti-TRPV4 ( 1:500, Abcam, Cambridge, MA); and mouse anti-\(α\) smooth muscle actin (1:200, Sigma, St. Louis,
MO). After washing, cells were incubated with a FITC- or Texas Red-labeled secondary antibody (1:1000 dilutions of goat anti-mouse IgG or goat anti-rabbit IgG, Invitrogen). Fluorescent signals were detected with a Nikon Eclipse E2000 microscope. Samples were excited at 488 nm or 594 nm and emitted fluorescence was recorded at 525 nm. The excitation levels were held constant between all samples. Quantification of PDGFR-β or TRPV4 expression was performed by Metamorph Imaging system offline on digital images collected under constant conditions between samples. The mean intensity of fluorescence was measured after subtraction of background fluorescence. Background level was set in regions from control cell samples and utilized throughout the session in which all fields were photographed during the same session using the same microscope settings. Cells were counted from four randomly selected fields on each slide from each treatment in 3 different preparations. A minimum of 20 cells appeared in the fields under study using the 20X objective lens. Data are expressed as relative fluorescence intensity values.

**In situ TRPV4 Immunohistochemical Study in Pancreatic Tissue**

Paraffin-embedded pancreas tissue sections (5μm) from control and HFA fed rats were batch processed immunostaining for TRPV4 and co-labeling with α-SMA. The sections were deparaffinized and rehydrated in citrosolv and concentration series of EtOH. The sections were washed with 0.1 M phosphate buffered saline (PBS, pH 7.3) and blocked with 3% normal goat serum (30 min, RT). Sections were incubated overnight at room temperature with rabbit anti-TRPV4 antibody (1:1000, Abcam, Cambridge, MA); mouse anti-α-SMA (1:1000, SIGMA, Saint Louis, MO) or mouse anti-desmin antibody
(1:500, SIGMA, Saint Louis, MO). Subsequently, sections were incubated with secondary antibodies, goat anti-rabbit IgG Alexa Fluor 594 and goat anti-mouse IgG Alexa Fluor 488 (1:1000, 1 h, Invitrogen). Sections were coverslipped with glycerol based mounting media (Vector Laboratories, Burlingame, CA). Sections incubated without primary antibody were included in each staining experiment as negative controls (data not shown). Histological stain of fibrotic collagen was achieved in defatted paraffin sections (5μm) with Sirius red (0.1%, 60 min, EMS Hatfield, PA) and H & E (21).

Staining was visualized and intensity analyzed using a Nikon E1000 microscope (Nikon Instruments, Inc., Melville, NY) equipped with the MetaVue and ACT-1 programs. Five randomly selected sections from each animal were photographed for study using the 20X objective lens. Background level was set in a region without staining in a pancreas section from the control group, the excitation levels were held constant between all samples, and the same microscope settings were maintained throughout the data collection session in which all fields and all groups were photographed. Data are expressed as relative fluorescence intensity values.

**Western Blot Analysis of TRPV4 Expression in Pancreatic Stellate Cells**

Pancreatic stellate cells isolated from HFA fed and control Lewis rats were cultured for 48 hours, washed with PBS (4°C) and lysed into RIPA buffer containing protease inhibitor and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein (15 μg) were size fractionated by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). After blocking with 5% (w/v) non-fat milk in washing buffer (0.1%Tween 20 in 0.1M PBS, pH 7.4) at 4°C for overnight, the membrane was incubated
with rabbit-anti-TRPV4 primary antibody (1:500 Abcam, Cambridge, MA) and mouse-anti-β-actin antibody from Santa Cruz (1:10,000, Santa Cruz, CA), diluted in 1% (w/v) non-fat milk in washing buffer at room temperature for 2 hours. The blots were then washed three times for 10 minutes each in washing buffer and incubated with horseradish peroxidase conjugated IgG, (1:10,000, GE Healthcare, Piscataway, NJ) diluted in 1% (w/v) non-fat milk in washing buffer at room temperature for 1 hour. The membrane was washed with washing buffer three times for 10 minutes each and incubated with Chemiluminescence reagents (ECL plus kit, GE Healthcare, Piscataway, NJ) for 5 minutes. The blots were exposed on CL XPosure film (Thermo Scientific, Rockford, IL), and the intensity of specific immunoreactive bands was quantified using densitometric scanning analysis and density detection software (Image J, NIH). The expression of β-actin was the internal control. The relative density of the immunoblot bands from the HFA fed and normal control animals were calculated by normalizing the density of TRPV4 blots over the density of β-actin blots for the same conditions, respectively.

**Statistical Analysis**

Paired treatment comparisons were analyzed with Student’s t-tests. Group data with different test reagent concentrations were analyzed with one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Multiple comparisons were analyzed with two-way ANOVA, followed by Bonferroni Post hoc test. P values ≤ 0.05 were considered statistically significant.
Results

1. Characterization of PSCs and Their Activation by Alcohol

Pancreatic stellate cell activation is characterized by the loss of autofluorescent vitamin A droplets and expression of α-SMA and PDGFR-β (Fig. 1) (24, 28).

1.1. Vitamin A in Quiescent PSCs in the Culture Model

Examination of the PSCs with a UV microscope filter revealed the presence of punctuate blue autofluorescent vitamin A droplets after 24 hours in culture indicating a quiescent state prior to the in vitro activation studies using alcohol and TNFα (Fig.1A). Vitamin A autofluorescence droplets disappeared after PSC activation with ethanol and TNFα treatment (data not shown).

1.2. PDGF-β Receptor Expressed in PSCs

The cytoplasm of the PSCs in culture stained positively for the β subunit of platelet-derived growth factor receptor (PDGFβ), a specific marker and transforming factor in pancreatic periacinar cells (Fig.1B). After PSCs were treated with 150mM (0.87%) EtOH for 24 hours, the cell bodies of the PSCs were enlarged and the cell body areas of PDGF-β receptor expression expanded (Fig.1C). After PSCs were treated with the cytokine TNFα (1ng/ml, 16 hours), the cell bodies of PSCs were also enlarged (Fig.1D). Histograms show the cell body area measurements (Fig. 1E) and relative staining density of PDGF-β receptor (Fig. 1F) for control PSCs (n=70) and PSCs treated 24 hours with 50mM EtOH (0.29%; n=60), 150mM EtOH (0.87%; n=50), or 1ng/ml TNFα (n=60). The cell body area of the PSC was significantly enlarged in a dose dependent manner suggesting cellular stress with the higher dose. The relative intensity
of PDGF-β receptor-like immunoreactivity remained constant as the cell body area expanded suggesting that overall the PDGFR-β content was significantly increased within the PSCs.

1.3. **TRPV4 Expression in Primary Cultured PSCs**

Immunohistochemical study data revealed that PSCs from control rats had a minimum level of TRPV4-like immunoreactivity. In the PSCs treated with 50mM (0.29%, v/v) EtOH for 24 hours, the average fluorescent intensity for TRPV4 expression was significantly increased in the cell cytoplasm (n=108) compared to TRPV4 expression in control PSCs (n=145) (Fig. 2A-C, p<0.001).

Western blot analysis was also utilized to compare TRPV4 in PSCs taken from HFA pancreatitis rats with PSCs from control rats fed normal chow (Fig. 2D). The bar graph summarizes the relative density of the immunoblot bands for TRPV4 expression normalized against β-actin in both control and HFA fed groups. The TRPV4 expression in PSCs from HFA fed rats was increased 20% over levels in PSCs from control rats (0.55±0.03 v.s. 0.34±0.05; n= 4 each group; Fig. 2E, p<0.05).

We evaluated TRPV4 channel gene expression by RT-PCR in primary cultured PSCs isolated from normal control and HFA fed rats. PSCs from both groups demonstrated a clear PCR product of TRPV4 (339 bp) (n= 4 each group; Fig 2. F).

1.4. **TRPV4 and α-Smooth Muscle Actin Protein Expression in Pancreas**

We verified activation of PSCs in animals fed HFA by examining immunohistochemical staining of α-smooth muscle actin (αSMA)(Fig. 3A). αSMA is
normally present in vessel and duct walls, as evidenced by immunostaining in normal pancreatic tissue. Despite variations from sample to sample, there was a marked increase in pancreatic α-SMA in the HFA fed group with chronic pancreatitis compared to groups fed normal chow. A low level of TRPV4 immunoreactivity appears on the vessel and duct walls in normal rat pancreas. TRPV4 immunoreactivity was increased on the vessel and duct walls, in some unidentified fibers of the interlobular area of the pancreas, as well as overall in pancreas from rats in the HFA fed group (Fig.3A).

1.5. HFA Induced Fibrosis in Rat Pancreas

Pancreatic sections from rats fed HFA diet for 6 and 8 weeks revealed the presence of glandular atrophy, intralobular, interlobular and periductal fibrosis, and inflammatory cells infiltrations evidenced by staining with Sirius red and H & E (Fig.3B). The presence of pancreatic fibrosis is provided as another indication of the chronic nature of the HFA pancreatitis model and the induced activation of PSCs.

2. Effects of Alcohol on Functional TRPV4 Ion Channels in PSCs

2.1. Alcohol Increases PSC Cytosolic Calcium Mobilization Responses to Arachidonic Acid

For in vitro calcium mobilization studies, pancreatic stellate cells were harvested from Lewis rats fed high fat/ethanol (6%) for 5 - 6 weeks, cultured for 2 days and pre-loaded with fura-2 (5μM, 1hour). Intracellular calcium mobilization responses evoked by bath application of arachidonic acid were classified as two types in distinct populations of pancreatic stellate cells: (1) irregular but sustained calcium responses which lasted for
hours (Fig. 4A) or (2) low level oscillatory calcium mobilization responses comprised of brief spike-like activity (data not show). The PSCs from rats with HFA pancreatitis were highly activated. There was a statistically significant increase in evoked calcium mobilization responses to arachidonic acid (AA, 10-100μM) for PSCs isolated from HFA fed rats compared to control PSCs which were relatively quiescent (Fig. 4A and C). In these experiments, 48 of 70 randomly selected cells were activated (68.57%, see table). The majority of the activated cells showed irregular, sustained calcium responses persisting over 1 hour (Fig. 4A). The F340/F380 ratio increased from a baseline of 0.2 - 0.3 to 1-1.5. Some of the activated cells showed multiple oscillatory spike calcium responses.

In contrast, pancreatic stellate cells taken from rats fed normal chow had minimal or no calcium activity in response to bath application of arachidonic acid (AA, from 10 - 100μM; Fig. 4B and C). In these experiments, 72 cells from control rats were randomly selected and only 16 cells appeared to be slightly activated (22.2%, Fig. 4C, see table).

2.2 Calcium Mobilization in PSCs Induced by TRPV4 Channel Activation

To determine if intracellular calcium mobilization responses were TRPV4 mediated, 4αPDD was bath applied to PSCs isolated from rats fed HFA. Bath application of 4αPDD (30μM) triggered an increase in intracellular calcium mobilization in stellate cells from HFA fed rats (Fig. 5A). In these experiments, 99 cells were tested and 60 of the cells were activated (60.6%). A sustained increase in calcium mobilization is seen in most PSCs in response to 4αPDD in a dose dependent manner (Fig. 5C). The F340/F380 ratio increased from a baseline average of 0.34± 0.006 to 0.74± 0.05. The activity was
effectively blocked at baseline level by bath application of ruthenium red (RR, 3µM 10 minutes), (Fig. 5B, D, p<0.001, one-way analysis of variance (ANOVA) followed by Tukey post hoc test).

2.3 TRPV4 is a Functional Osmosensor in PSCs

In light of the crucial role of TRPV4 in cell volume regulation in peripheral tissue osmosensation, we investigated the possibility that TRPV4 in PSCs are responsive to cell volume regulation. In primary cultured PSCs taken from HFA fed rats, responses to hypotonic saline challenge (≈50% osmolarity or 150mOsm) observed via live cell calcium imaging produced sustained calcium mobilization responses that were abrogated by ruthenium red (RR, 3 µM, 10 minutes) (Fig. 6 A, C. right, P<0.001, one-way analysis of variance (ANOVA) followed by Tukey post hoc test). In these experiments, 71 (53%) of the 134 cells tested were activated. The F340/F380 ratio increased from an average baseline of 0.33±0.01 to 0.73±0.02 (Fig. 6C. left, p<0.001, paired t-test).

2. TNFα Enhances TRPV4 Mediated Calcium Mobilization

PSCs isolated from naïve Lewis rat were pretreated with TNFα (1ng/ml) for 16 hours before calcium imaging in order to determine the impact of inflammatory factors on TRPV4 activation. Bath application of 4αPDD (30µM) significantly increased intracellular calcium mobilization (61.9%) compared to the baseline in 13 of 21 randomly selected PSCs (Fig.7A). The intracellular calcium activity was irregular with sustained repetitive increases persisting over 1 hour. The F340/F380 ratio increased from baseline (0.33±0.02 to 0.99±0.09, p<0.001, paired t-test, Fig. 7B). Repeated oscillatory calcium mobilization
responses were seen in some cells (data not shown). In untreated PSC cultures few cells responded to $4\alpha$PDD stimulation ($n=19$ tested, Fig 7A &B). These data suggest that TNF$\alpha$ potentiates downstream events resulting in increased TRPV4 mediated calcium mobilization in PSCs.

Discussion

In this study live cell calcium imaging, immunostaining, western blot and RT-PCR techniques were combined to investigate effects of a chronic HFA pancreatitis on isolated PSCs and pancreatic tissue. Levels of PDGFR-\(\beta\), a stellate cell transforming factor specific activator marker, and extracellular matrix protein marker $\alpha$-SMA expression levels increased positively after long term exposure to high fat/alcohol documenting that the cells under study were PSCs. This study demonstrated up-regulated expression of TRPV4 protein in pancreatic tissues and isolated PSCs. The inducible increase in TRPV4 mediated calcium mobilization in HFA activated PSCs compared to PSCs from control rats fed normal chow. Similar results were obtained in PSCs from control rats after in vitro treatments with alcohol or TNF$\alpha$. These results demonstrate that TRPV4 is a functional ion channel and a visceral cellular sensor in rat pancreatic stellate cells responsive to cellular level of alcohol/fatty acid metabolite accumulation, edema and inflammation typical in pancreatitis. The present experiments have determined that TRPV4 channel activation is an inducible, underlying cellular mechanism promoting prolonged pancreatic stellate cell activation.
There is an ever increasing body of evidence that TRPV4 is involved in visceral hypersensitivity and sensory integration. TRPV4 is found in many tissues, including visceral organs: kidneys, urinary bladder, colon and pancreas in rodent and humans (11, 13, 14, 16, 17). TRPV4 is expressed in sensory neurons and functional evidence has been correlated with intense enrichment of TRPV4 expression in splanchnic, pancreatic and pelvic colonic afferents traced to their cell bodies in the thoraco-lumbar and lumbo-sacral DRG (13, 17). The selective role of TRPV4 in high threshold afferents would be expected to translate into a role in visceral pain perception in vivo (12). Decreased abdominal electromyographic (EMG) responses to noxious colorectal distension have been shown in TRPV4 knockouts or in mice with down regulated TRPV4 expression (via intervertebral small interfering RNA (siRNA) delivery) (13, 29). Intracolonic administration of 4α-PDD in wild-type mice caused neuronal activation in the lumbar-sacral spinal cord and caused dose-dependent visceral hypersensitivity to colorectal distension (13). TRP channels are present in subsets of vagal afferent neurons that project to the stomach may confer temperature and mechanosensitivity on these cells (43) and contribute to acute pancreatitis pain as well (17). Interactive stress response events in conditions where alcohol metabolites and high fat up-regulate TRPV4 channels in gastrointestinal structures and innervating sensory nerves may confer and promote hypersensitivity and overactivation.

Arachidonic acid is a polyunsaturated fatty acid, a cellular second messenger, and an alcohol metabolite metabolized further to both pro-inflammatory and anti-inflammatory molecules, such as prostaglandin E2 (PGE2). Metabolized arachidonic acid is an endogenous chemical activator of TRPV4 via cytochrome P450 (CYP)-derived
epoxyeicosatrienoic acids (EETs) (38). TRPV4 is also activated by hypotonic solution induced cell swelling which shares the same cytochrome P450 pathway with AA (22). The PSC activators described which trigger calcium mobilization in the present study could be effectively inhibited by TRPV channel blocker, ruthenium red.

Chronic exposure to high fat (fatty acid) and alcohol causes PSCs to undergo molecular plasticity changes which maintain a hypersensitized state. Any additional stimuli including inflammatory insults such as an increase in blood level cytokines leads to tissue damage, deposition of collagen that can restrict ductal flow, and painful acute pancreatitis attacks. The present data suggest that both alcohol and TNFα potentiate events resulting in increased TRPV4 mediated calcium mobilization in PSCs. The studies thus provide additional evidence that cytokines can directly modulate calcium activated events in PSCs mediated through TRPV4 channel activation. Recent in vivo and in vitro studies from Apte and colleagues show that both LPS and alcohol exert anti-apoptotic effects on PSCs and promote their activation, thereby increasing the process of pancreatic fibrogenesis (34, 35). Importantly, PSCs are themselves capable of producing cytokines which might perpetuate their activation via an autocrine loop that includes up-regulation of TRPV4 channel expression and pathological interactions with sensory nerves. Thus, alcohol, high fat and the cytokines produced by the PSCs themselves play a critical role in activation of TRPV4 channels and calcium mediated downstream cellular activation events during pancreatic inflammation. It is now acknowledged that repeated episodes of acute pancreatitis can cause progressive damage to the pancreas: the necrosis - fibrosis sequence, a constant feature of chronic pancreatitis (31).
**Perspectives and Significance:** The present study more fully characterizes activation of TRPV4 on isolated PSCs taken from rats with HFA induced pancreatitis. Conditions similar to those found in the inflamed pancreatitis, including cell swelling and increases in alcohol metabolites and TNFα, promote increased TRPV4 expression and enhance TRPV4-mediated activation of PSCs as indicated by increases in intracellular calcium mobilization responses. The expression and responsively increases imply that TRPV4 plays a role in pancreatic stellate cell activation during pancreatic inflammation.

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**References:**


Figure Legends

Fig. 1. Pancreatic stellate cell activation is characterized by the loss of autofluorescent vitamin A droplets and expression of α-SMA and PDGFR-β.

A. Vitamin A fat droplets were evident as blue autofluorescence under UV light in the cytoplasm of pancreatic stellate cells (PSCs) after 24 hours in culture. This is a characteristic of quiescent stellate cells.

B. The cytoplasm of PSCs in culture stained positively for the β subunit of the PDGF receptor (PDGF-β).

C. After EtOH (50mM) stimulation for 24 hours, PSCs underwent activation with morphological and functional changes typical of myofibroblast-like cells. The cell body of PSCs expanded 2.5-fold, and PDGF-β receptor positively stained.

D. After TNFα (1ng/ml) exposure for 24 hours, the cell bodies of PSCs were enlarged.

E-F. Histograms show measurements of the soma area of PSCs (E) and the relative staining density (F) of PDGF-β receptor when treated with 50mM (n=60), 150mM EtOH (n=50) or 1ng/ml TNFα (n=60) for 24 hours. The cell body size of PSCs significantly increased with exposure to the high dose of EtOH and to TNFα. The intensity of the PDGF-β receptor-like immunoreactivity remained constant as the cell body area expanded, suggesting an overall increase in PDGF-β receptor in the PSCs soma as reported by others previously (EtOH vs. control (n=70); TNFα vs. control, *** p<0.001, Student’s t-test).

Fig. 2. A. Pancreatic stellate cells in the control group had minimal TRPV4 immunoreactivity (green) (n=145). DAPI (blue) nuclear stain.

B. TRPV4 expression increased in the cytoplasm (n=108) of PSCs after exposure to EtOH (50mM) for 24 hours.

C. The histogram shows the average fluorescent intensity of TRPV4 in alcohol treated PSCs was significantly increased (*** p<0.001, Student’s t-test).

D. Western immunoblot analysis was used to compare TRPV4 protein in PSCs pooled from rats chronically fed an alcohol and high fat diet (6%, 5-6 weeks; n=4) with TRPV4 in PSC from control rats fed regular rodent chow (n=4). TRPV4 expression was increased in the HFA fed group.

E. The bar graph summarizes the relative density of the TRPV4 expression in immunoblot bands for both HFA fed and control groups fed normal chow normalized with β-actin. There was approximately a 20% increase in expression for the PSCs isolated from HFA fed rats over cells from control rats fed a normal diet. (* p<0.05)

F. RT-PCR was performed with primary cultured PSCs from control and HFA fed rats. The predicted PCR amplicon sizes for TRPV4 is 339 bp. The PCR marker contains a 50-2000 bp ladder (Invitrogen, Grand Island, NY).

Fig. 3. A. Expression of α-smooth muscle actin protein (α-SMA), another indication of PSC activation and fibrinogenesis, was readily evident in pancreas of rats fed with HFA. A low level of TRPV4 immunoreactivity can be seen in blood vessels and ducts in normal pancreatic tissue section. TRPV4 immunoreactivity was increased
on the vessel and duct walls as well as in some unidentified fibers of interlobular area of the pancreas of HFA fed group.

B. H & E and Sirius Red staining of pancreatic tissue sections. The normal control pancreas sections showed clusters of healthy acinar and islet of Langerhans. In pancreatic tissue taken from rats fed HFA, the presence of glandular atrophy, intralobular, interlobular and periductal fibrosis (in red, white arrow) and inflammatory cell infiltration (black arrow) can be seen after 6 weeks (upper panel) and is greatly increased by 8 weeks (lower panel) on the HFA diet.

Fig. 4. A. A representative trace demonstrates the effect of arachidonic acid (AA, 100μM) on the Ca^{2+} fluorescence ratio in PSCs. AA produced a sustained and robust increase in intracellular calcium mobilization responses (F340/F380 ratio) in PSCs harvested from HFA fed rats (upper trace). Cells were isolated from male Lewis rats fed HFA for 6 - 8 weeks and placed in culture for 3 days. Calcium mobilization was seen in 48 out of 70 PSCs in response to bath application of arachidonic acid. In contrast, bath application of AA evoked no response or minimal response in PSCs (16 of 72) prepared from naïve control Lewis rats (lower trace).

B. Change in peak fluorescence ratio after AA was summarized as a concentration-response relationship. The strong activation response of PSCs taken from pancreas of HFA fed rats increased in a dose dependent manner ( ■ upper line), while a minimal response was recorded from PSCs isolated from pancreas of control rats ( □ lower line). (***p< 0.001, two way ANOVA, Bonferroni Post hoc test).

C. The table shows the total number of PSCs tested and percent of total cells activated by AA in both HFA fed and control rats.

Fig. 5. The 4αPDD evoked intracellular calcium response was significantly elevated in PSCs isolated from rats with chronic exposure to a diet with HFA.

A. A sustained increase in calcium mobilization was observed in all pancreatic stellate cells in response to bath application of 4αPDD (30μM). Cells were isolated from male Lewis rats fed HFA for 6 - 8 weeks and cultured for 3 days.

B. Ruthenium red (RR, 3μM, 10 min), a TRPV channel blocker, eliminated the F340/380 ratio elevation in response to 4αPDD (30 μM, 5 min). The response to 4αPDD was restored after a washout period.

C. Change in peak fluorescence ratio in response to 4αPDD was evident as a concentration dependent relationship (n=60, ■ upper line), while PSCs isolated from control rats fed a normal diet had a minimum response (n=62, □ lower line). (** p< 0.01, two way ANOVA, Bonferroni Post hoc test).

D. The histogram summarizes the 4αPDD activation and RR blockade in PSCs. The significantly increased response to 4αPDD was absent in PSCs when ruthenium red was present in the bath (n =19, baseline, RR and RR+4αPDD vs. 4αPDD, *** P< 0.001, one way ANOVA, Tukey’s multiple comparison test).

Fig. 6. Hypotonic experimental solution challenge induced a sustained calcium mobilization (F340/F380 ratio) in primary cultures of PSCs taken from rats fed HFA diet. A. A representative trace is shown illustrating the effect of hypotonic
experimental solution (50% osmolarity, ≈150 mOsm) on the calcium response in PSCs.

**B.** Hypotonic experimental solution induced calcium mobilization was abrogated by non-specific TRPV channel blocker, ruthenium red (RR, 3μM, 10 min).

**C.** The histogram summarizes the statistically significant increase in F340/F380 ratio after hypotonic solution challenge (left; n=71, Hypotonic vs. Baseline *** p<0.001, paired t-test). Pretreatment with ruthenium red effectively blocked the increased F340/F380 ratio (right; n=25, Baseline, RR and RR+hypotonic vs. Hypotonic *** P< 0.001, one way ANOVA, Turkey’s multiple comparison test).

**Fig. 7.** TNFα promoted the TRPV4 activation response to 4αPDD in PSC cultures from control rats. **A.** Pretreatment of control PSC cultures with TNFα (1 ng/ml) for 16 hours produced a robust and sustained intracellular calcium mobilization in response to bath application of 4αPDD (30μM) (upper trace). The activity was sustained over 1 hour with a single application of 4αPDD. In this experiment, 13 cells were activated from a total of 21 cells selected. In contrast, responses to 4αPDD were minimal for untreated control PSCs (lower trace).

**B.** The histogram summarizes the averaged F340/F380 ratio to bath application of 4αPDD after TNFα pretreatment (n=21). Statistically significant increases in responses were observed for cells with TNFα pretreatment compared to the untreated control group (n=19), *** p<0.001, Student’s t-test).
PDGF-β + TNFα + EtOH 25 μM

E

F
A

B

C

<table>
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<tr>
<th>Group</th>
<th># Cells Tested</th>
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