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ALCOHOL AND HIGH FAT INDUCED CHRONIC PANCREATITIS: TRPV4 ANTAGONIST REDUCES HYPERSENSITIVITY

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Abstract—The pathogenesis of pain in chronic pancreatitis is poorly understood, and its treatment can be a major clinical challenge. Surgical and other invasive methods have variable outcomes that can be unsatisfactory. Therefore, there is a great need for further discovery of the pathogenesis of pancreatitis pain and new therapeutic targets. Human and animal studies indicate a critical role for oxidative stress and activation of transient receptor potential (TRP) cation channel subfamily members TRPV1 and TRPA1 on pancreatic nociceptors in sensitization mechanisms that result in pain. However, the *in vivo* role of transient receptor potential cation channel subfamily V member 4 (TRPV4) in *chronic* pancreatitis needs further evaluation. The present study characterized a rat alcohol/high fat diet (AHF)-induced chronic pancreatitis model with hypersensitivity, fibrotic pathology, and fat vacuolization consistent with the clinical syndrome. The rats with AHF-induced pancreatitis develop referred visceral pain-like behaviors, i.e. decreased hindpaw mechanical thresholds and shortened abdominal and hindpaw withdrawal latency to heat. In this study, oxidative stress was characterized as well as the role of TRPV4 in chronic visceral hypersensitivity. Lipid peroxidase and oxidative stress were indicated by increased plasma thiobarbituric acid reactive substances (TBARS) and diminished pancreatic manganese superoxide dismutase (MnSOD). The secondary sensitization associated with AHF-induced pancreatitis was effectively alleviated by the TRPV4 antagonist, HC 067047. Similarity of the results to those with the peripherally restricted μ-opiate receptor agonist, loperamide, suggested TRPV4 channel activated peripheral sensitization. This study using a reliable model that provides pre-clinical correlates of human chronic pancreatitis provides further evidence that TRPV4 channel is a potential therapeutic target for treatment of pancreatitis pain.

Key words: TBARS, rat, HC 067047, visceral pain, behavior, loperamide.

INTRODUCTION

Chronic pancreatitis features failure of its exocrine gland function and in some cases even its endocrine function (diabetes type 3c). Pain present in up to 90% of patients is another major clinical challenge and a primary cause of hospitalization. The pathogenesis of pain in this disorder is poorly understood and effectiveness of treatments long-term largely unsatisfactory. Therefore, there is great need for discovery of the pathogenesis of chronic pancreatitis pain and new therapeutic targets. Chronic pancreatitis can be due to long-term excessive alcohol/fat intake (Ammann et al., 1984). Human and animal studies indicate a critical role for neurogenic mechanisms since pancreatic nociceptors reportedly are particularly prone to sensitization (Bhutani and Pasricha, 2003; Xu et al., 2006; Li et al., 2013).

Activation of somatic and visceral nociceptors through transient receptor potential (TRP) channel family members has drawn attention due to their unique physiological functions and distributions in a wide range of tissues. Approximately 20 of the 30 mammalian TRP channel subunits are expressed by specific neurons and non-neuronal cells within the digestive system (Holzer, 2011b). TRP channel activation plays an important role in mechanosensation and hyperalgesia, as well as chemesthesis, taste, regulation of gastrointestinal motility, absorptive and secretory processes, blood flow, and mucosal homeostasis. At the cellular level, TRP channels operate either as primary detectors of chemical and physical stimuli, as secondary transducers of ionotropic/metabotropic receptors, or as ion transport channels (Holzer, 2011a,b). Implication of some TRP channels in pathological processes has raised enormous interest in exploring them as therapeutic targets. This is particularly true for TRPV1, TRPA1, and TRPV4. While the roles of TRPV1 and TRPA1 as sensors in nerve terminals of C and Aδ fibers promoting somatic and/or visceral hypersensitivity have been extensively investigated (Bartho et al., 2004; Xu et al., 2007; Kondo et al., 2009; Blackshaw et al., 2010; Schwartz et al., 2013), the present study examines the role of TRPV4.

TRPV4 channels, as multimodal sensors, are reported to be involved in somatic and visceral nociception after activation by heat (27–34 °C threshold), chemicals, and mechanical insult and stretch, including hypotonicity

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Abbreviations: ABC, avidin–biotin complex; ABWL, abdominal withdrawal latency; AHF, alcohol/high fat diet; ANOVA, analysis of variance; CNS, central nervous system; ENK, Enkephalin; IBD, inflammatory bowel diseases; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; MOR, μ-opioid receptors; PWT, paw withdrawal threshold; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TRPV4, transient receptor potential cation channel subfamily V member 4.

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(Alessandri-Haber et al., 2004; Cenac et al., 2008; Blackshaw et al., 2010). Intraductal administration of a TRPV4 channel agonist to the murine pancreas induces c-Fos expression in the spinal cord (Ceppa et al., 2010). In the same study deletion of the *trpv4* gene inhibited transmission of input to the spinal cord and pain-related behaviors associated with acute experimental pancreatitis induced by subcutaneous injection of caerulein.

In our previous *in vitro* study, TRPV4 channels were overexpressed in pancreatic stellate cells isolated from rats with AHF induced chronic pancreatitis. Overresponsiveness was reported to hypotonic stimuli, mimicking stretch as in edema during the course of cellular injury, and to biologically active compounds such as the lipid messenger, arachidonic acid. Activation resulted in intracellular calcium overload, initiating signaling cascades leading to sensitization (Ceppa et al., 2010; Zhang et al., 2013). The role of TRPV4 channels in chronic pancreatitis *in vivo* was under further study in the current study.

In the present study, the alcohol/high fat diet (AHF)-induced chronic pancreatitis rat model was utilized to investigate oxidative stress and the ability of a TRPV4 antagonist to reduce behavioral hypersensitivity. We hypothesized that TRPV4 channels would be activated in the alcohol and fatty acid metabolite rich environment. In animals with AHF induced pancreatitis referred hypersensitivity was alleviated by both a TRPV4 antagonist and the peripherally restricted mu opioid receptor, loperamide.

EXPERIMENTAL PROCEDURES

This study was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Induction of chronic pancreatitis

A total 18 male Fischer 344 rats weighing between 240 and 250 g (from Harlan Sprague–Dawley Inc, Indianapolis, IN, USA) were used for this study. The number of animals required to achieve statistical significance is specified in each test description and represents reduction of animal use mandated by our IACUC. Animals were single caged and kept in a temperature constant (23° ± 2 °C) room on a 12/12-h reversed dark–light cycle. Rats were randomly divided into two groups and fed either an alcohol and high-fat liquid diet (AHF) (n = 12) or control rodent chow (Harlan Teklad 8626 with low soy content) (n = 6). Chronic pancreatitis was induced with a high fat liquid diet (AHF) made from micro-stabilized rodent diet mix (LD 101A; Test-Diet, Richmond, IN, USA). The commercial diet provides protein, fat, fiber, vitamins and minerals. Maltodextran (90 g), water (770 g), apple juice (100 g), and alcohol (w/v, 95% ethyl alcohol) are added to the mix. The dose of alcohol was progressively increased weekly from 4% alcohol for the first week, 5% for second week, and 6% for the third week, adjusting the mixture percentage with addition of less water. Corn oil (33 g) and 6% alcohol were added to the liquid diet in all subsequent weeks through the end of the experiment. A lard supplement (8 g/rat/day) was also given daily in a stainless steel condiment dish. The control group was fed standard rodent chow (Teklab 8626, Harlan, Indiana). All rats were given access to food and water *ad libitum*. Animals were observed closely, and no evidence of alcohol intoxication (no ataxia or lethargy) was noted. Food consumption was monitored daily and body weight monitored weekly. Rats fed with AHF diet consumed an average of ≈50-ml liquid diet per day and the lard supplement (⩽8 g/rat/per day). The body weight gain of rats fed AHF diet progressed more slowly than rats fed standard chow in the later experimental weeks but did not exceed 20% weight difference between groups. Rats fed standard rodent chow gained 10-g body weight each week. The rats fed the AHF diet gained 2–5-g body weight each week.

Detecting oxidative stress

Blood plasma lipid peroxide analysis. Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation (i.e. as degradation products of fat) which can be detected by the TBARS assay using TBA as a reagent. The TBARS assay measures malondialdehyde (MDA) present in blood plasma samples as an index of lipid peroxidation and systemic oxidative stress. A 90-μl rat blood plasma sample was added to 1 ml of 20% Trichloroacetic acid (TCA), mixed gently and incubated on ice for 15 min. The mixture was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was discarded and the sediment mixed with 1.25 ml of 0.05 M H_2SO_4 and 1.5 ml of 0.2% TBA. The glass test tubes were caped, vortexed and kept in a boiling water bath, 100 °C, for 30 min. After cooling down, 2 ml of butanol was added to each tube and the color extracted in the butanol phase was read at 530 nm with a Spectrophotometer (SmartSpec Plus, BIO-RAD, Hercules, CA, USA). The lipid peroxide content was calculated and expressed as nanomoles of TBA reactants/ml plasma (Buege and Aust, 1978; Deevska et al., 2012).

Immunohistochemical localization of MnSOD in pancreas tissue. Manganese superoxide dismutase (MnSOD, SOD2) protein utilized during oxidative stress was immunolocalized in defatted paraffin sections of paraformaldehyde immerse fixed pancreas with the avidin–biotin complex (ABC) method (VECTASTAIN, ABC kit, VECTOR Laboratories, Inc., Burlingame, CA, USA). Tissue sections from all groups were simultaneously processed allowing quantitative analysis. After deparaffinization, antigen retrieval (10 mM sodium citrate buffer, pH 6.0) and blocking, the sections were incubated with the primary rabbit polyclonal antibody against human MnSOD (1:1000; Enzo Life Sciences, Inc., Farmingdale, NY, USA) overnight at room temperature, followed by incubation with goat anti-rabbit biotinylated antibody for 1 h. The sections were then