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Pancreatic stellate cell activation is regulated by fatty acids and ER stress☆

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ABSTRACT

Introduction: Pancreatic pathologies are characterized by a progressive fibrosis process. Pancreatic stellate cells (PSC) play a crucial role in pancreatic fibrogenesis. Endoplasmic reticulum (ER) stress emerges as an important determinant of fibrotic remodeling. Overload of fatty acids (FA), typical to obesity, may lead to lipotoxic state and cellular stress.

Aim: To study the effect of different lipolytic challenges on pancreatic ER stress and PSC activation.

Methods: Primary PSCs were exposed to different FAs, palmitate (pal) and oleate (ole), at pathophysiological concentrations typical to obese state, and in acute caerulein-induced stress (cer). PSC activation and differentiation were analyzed by measuring fat accumulation (oil-red staining and quantitation), proliferation (cells count) and migration (wound- healing assay). PSC differentiation markers (α-sma, fibronectin, tgf-β and collagen secretion), ER stress unfolded protein response and immune indicators (Xbp1, CHOP, TNF-α, IL-6) were analyzed at the transcript and protein expression levels (quantitative RT-PCR and western blotting).

Results: PSC exposure to pal and ole FAs (500 μM) increased significantly fat accumulation. Proliferation and migration analysis demonstrated that ole FA retained PSC activation, while exposure to pal FA significantly halted proliferation rate and delayed migration. Cer significantly augmented PSC differentiation markers α-sma, fibronectin and collagen, and ER stress and inflammation markers including Xbp1, CHOP, TNF-α and IL-6. The ole FA treatment significantly elevated PSC differentiation markers α-sma, fibronectin and collagen secretion. PSC ER stress was demonstrated following pal treatment with significant elevation of Xbp1 splicing and CHOP levels.

Conclusion: Exposure to pal FA halted PSC activation and differentiation and elevated ER stress markers, while cer and ole exposure significantly induced activation, differentiation and fibrosis. Thus, dietary FA composition should be considered and optimized to regulate PSC activation and differentiation in pancreatic pathologies.

1. Introduction

Acute pancreatitis (AP) is the second most common indication for hospitalization due to a gastrointestinal disease in the U.S [1]. Transition from acute to chronic pancreatitis (CP) is characterized by a process of pancreatic fibrosis, which might lead to exocrine pancreatic insufficiency and pancreatic cancer [2,3]. CP is a fibro- inflammatory syndrome of the pancreas. It typically involves background risk factors, both genetic and environmental, such as alcohol abuse, drugs use and smoking, partly through generation of reactive oxygen species (ROS) and altered metabolism. Those, in turn, lead to the development of persistent pathologic responses to parenchymal injury or stress, promoting deposition of extracellular matrix (ECM) protein by activated pancreatic stellate cells (PSC) [4–7].

PSCs compromise 4–7% of the total pancreas cell mass and are located predominantly in the periacinar, periductal and perivascular spaces [8–10]. In normal physiological state, PSCs have a quiescent (non-activated) phenotype characterized by vitamin A-containing lipid droplets in the cytoplasm, and function in preservation of normal tissue architecture and regulation of ECM turnover [11,12]. Upon pancreatic injury, PSCs undergo activation, and trans-differentiate phenotypically to myofibroblast-like cells, with loss of cytoplasmic lipid droplets, enhanced proliferation and migration, as well as augmented synthesis of large amounts of ECM proteins and secretion of growth factors and inflammatory cytokines [8,13,14].

The endoplasmic reticulum (ER) is an important organelle required for cell survival and normal cellular function. Imbalance between ER capacity and protein folding load cause ER stress and increase secretory load with accumulation of misfolded proteins, causing cells to activate the unfolded protein response (UPR) [15–17]. The UPR is a

Abbreviations: PSC, Pancreatic stellate cells; ER, Endoplasmic reticulum; cer, caerulein; pal, palmitic acid; ole, oleic acid

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complex and coordinated adaptive signaling mechanism aimed at re-establishing homeostasis of ER function. UPR consists of 3 main signaling systems initiated by 3 ER transmembrane proteins (PERK, IRE1 and ATF6) that dissociate from Bip chaperone protein followed by activation of several transcription factors, including; CHOP and XBP1 (XBP1 is activated by mRNA splicing) [17,18]. ER stress and UPR pathways are emerging as important determinants of fibrotic remodeling in tissue fibrosis of internal organs, including lungs, liver, kidney, GI tract and heart [19,20], by enhancing the susceptibility of structural cells, in most cases the epithelium, to pro-fibrotic stimuli. Additionally, ER stress is involved in the progression of chronic disease and has been demonstrated to occur in pancreatitis; however, the mechanism through which the fibrogenic phenotype of activated PSCs induces ER stress and UPR is not fully known and might be associated with other environmental insults, such as overload of dietary fatty acids typical to obesity [21,22].

Obesity is a risk factor for many chronic diseases, including pancreatitis and pancreatic cancer. Chronic consumption of high fat diet (HFD) with augmented blood concentrations of FFAs is associated with the development of chronic diseases and pancreatic diseases [23–25]., One of the proposed mechanisms through which obesity enhances the risk of such diseases is that overload of dietary FAs causes accumulation of fat in non- adipose tissues, resulting in lipotoxicity state, characterized by cellular and tissue stress [25–27]. In recent years, obesity-related FFAs generated from the breakdown of excess intra-pancreatic fat were shown to be associated with inflammation and pancreatic organ failure. Beyond the lipotoxic effect of the FFAs, it was suggested that the accumulating FFAs also induce a low grade chronic inflammatory process, which leads to accumulation of misfolded proteins in the ER, causing ER stress and pancreatic damage [23,25].

The most abundant dietary FAs are palmitic acid (pal) - a saturated FA, and oleic acid (ole) - a mono unsaturated FA. Little is known regarding the metabolic effects of different FAs on PSC activation and differentiation, especially in relation to ER stress [28–30]. We now demonstrate differential effects of different FAs on cellular and molecular pathways regulating PSC activation and fibrosis.

2. Materials and methods

2.1. Isolation of primary pancreatic stellate cells

Male rats (250 gr, Sprague-Dawley, INVIVOGEN Laboratories) were housed and treated according to the institutional ethics approval and guidelines. Isolation of primary PSC was done by density gradient centrifugation according to the protocol of Minoty Apte et al. [31]. In brief, the pancreas was removed and digested by Hank's balanced salt solution (HBSS, Sigma-Aldrich), containing 0.55% Pronase, 0.13% collagenase P, 2.75% Dnase, 1.1% HEPES buffer with 18 ml of HBSS (Rocsh) at 37 °C for 12 min. The resultant suspension of pancreatic cells was separated to PSC at 1400 G for 12 min using Optiprep-density gradient. Cell suspensions containing primary PSC were re-suspended in 10 ml complete media (2% FCS, 1% glutamine, 1% penicillin and streptomycin; Biological industries, Israel). Isolated PSC were cultured at 37 °C (5% CO_2).

2.2. Cell culture

Medium was replaced every 2 days (complete medium: Dulbecco's Modified Eagle Medium, 1.5% HEPES 1 M buffer (Rhenium, Israel), 20% Fetal Bovine Serum (Biological industries, Israel) 1% (v/v) Penicillin-Streptomycin, 2% L-glutamine (Biological industries, Israel). Experiments were performed using culture activated cells (passages 2–4).

2.3. Cell proliferation assays

Cells were collected using Trypsin EDTA 0.05% (Biological Industries, Israel) into fresh medium and centrifuged at 300 G for 5 min. Cell pellets were re-suspended in 1 ml of medium. Cell samples (10 μl) were loaded on hemicytometer (Marienfeld, Germany) and counted using light microscopy.

2.4. Free fatty acid load and PSC treatments

Fatty acid supplemented medium was prepared with slight modification of the protocol of Spector [32]. Briefly, Palmitic acid and Oleic acid (Sigma Aldrich, Israel) were dissolved in ethanol and gently mixed until completely dissolved, after which the clear FAs solution was complexed with fatty acid free BSA (Roche) at a 1:10 FA to BSA ratio (7 μg/μl). The complexed FAs solution was added to the cell culture medium to obtain the indicated final FFA concentration (500 μM). The control untreated cells were treated with the same vehicle solution without the FAs. PSCs were treated with tm (an ER stress inducer) 5 μg/ml for 24 h and cer (an AP inducer) 10nmol/L for 4 h.

2.5. Fat accumulation – oil red O staining

Oil red O (ORO) staining was performed using the protocol of Koopman [33] with slight modifications. Briefly, cells were grown on 12 wells plates and subjected to different treatments. Cells were chemically fixed by 4% formaldehyde solution for 20 min and then rinsed with PBS solution. They were then rotated at room temperature in ORO solution (Sigma, Israel) for 30 min and washed (3× 30 s) in PBS. ORO staining was analyzed using light microscopy (Nikon ECLIPSE TE2000-U) and quantified by isopropyl alcohol extraction using ELISA Reader (TECAN Infinite F200, 495 nm O.D).

2.6. RNA Isolation of pancreatic stellate cells

PSC were cultured in six-well plates. Following treatments, total RNA was isolated using Trizol (Rhenium, Israel) in a ratio of 1 ml per 10 cm^2 of culture dish area, according to manufacturer's instructions. RNA integrity was analyzed by 1% agarose gel electrophoresis with ethidium bromide (Mercury, Israel) staining. RNA was quantified by UV absorption at 260 nm (NanoDrop ND-1000 UV–Vis; NanoDrop Technologies). Samples were stored at − 80 °C.

2.7. Reverse transcription (RT)

cDNA was synthesized using Tetro Reverse Transcriptase kit, according to the manufacturer's protocol (Origolab, Israel). Briefly, mRNA samples (1 μg) were subject to first strand cDNA synthesis by random hexamer using a cDNA mix. Samples were incubated at 70 °C for 5 min after which 1 μl dNTP, 4 μl Tetro RT buffer and 1 μl Tetro RT enzyme were added. The samples were then incubated at 42 °C for 60 min, followed by incubation at 70 °C for 10 min. Samples were stored at − 20 °C.

2.8. Quantitative real time PCR (qPCR)

Transcript levels were determined by qPCR using SYBR® Green PCR Master Mix (Rhenium, Israel). Reactions were carried out using the MxPro3000 apparatus (Stratagene, Santa Clara, CA) according to the instructions of the manufacturer. Gene-specific primers were designed using the primer 3 online software. The qPCR primer pairs were designed across exons to avoid false positive signals from potentially contaminating genomic DNA. Primer and cDNA concentrations were optimized following the guidelines of the supplier. Each 20 μl reaction contained 2 μl cDNA, 10 μl PCR Master Mix (Rhenium, Israel), primers at 100–500 nM concentration of each forward and