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Activation of AMP-activated protein kinase attenuates ethanol-induced ER/oxidative stress and lipid phenotype in human pancreatic acinar cells

Mukund P Srinivasan^a, Kamlesh K Bhopale^a, Anna A Caracheo^a, Samir M Amer^{a,e}, Shamis Khan^a, Lata Kaphalia^b, Gopalakrishnan Loganathan^c, Appakalai N Balamurugan^{c,d}, Bhupendra S Kaphalia^{a,*}

^aDepartment of Pathology, The University of Texas Medical Branch, Galveston, TX, 77550, USA

^bDepartment of Internal Medicine, The University of Texas Medical Branch, Galveston, TX, 77550, USA

^cDepartment of Surgery, University of Louisville, Louisville, KY, 40202, USA

^dIslet Biology Laboratory, Division of Pediatric General and Thoracic Surgery, Cincinnati Children's Hospital Medical Center, Department of Surgery, University of Cincinnati, Cincinnati, OH 45229, USA

^eDepartment of Forensic Medicine and Clinical Toxicology, Tanta University, Tanta, Egypt

Abstract

Primary toxicity targets of alcohol and its metabolites in the pancreas are cellular energetics and endoplasmic reticulum (ER). Therefore, the role of AMP-Activated Protein Kinase (AMPK α) in amelioration of ethanol (EtOH)-induced pancreatic acinar cell injury including ER/oxidative stress, inflammatory responses, the formation of fatty acid ethyl esters (FAEEs) and mitochondrial bioenergetics were determined in human pancreatic acinar cells (hPACs) and AR42J cells incubated with/without AMPK α activator [5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)]. EtOH treated hPACs showed concentration and time-dependent increases for FAEEs and inactivation of AMPK α , along with the upregulation of ACC1 and FAS (key lipogenic proteins) and downregulation of CPT1A (involved β -oxidation of fatty acids). These cells also showed significant ER stress as evidenced by the increased expression for GRP78, IRE1 α , and PERK/CHOP arm of unfolded protein response promoting apoptosis and activating p-JNK1/2 and p-ERK1/2 with increased secretion of cytokines. AR42J cells treated with EtOH showed increased oxidative stress, impaired mitochondrial biogenesis, and decreased ATP production rate. However, AMPK α activation by AICAR attenuated EtOH-induced ER/oxidative stress, lipogenesis, and inflammatory responses as well as the formation of FAEEs and restored mitochondrial function in

***Corresponding Author:** Bhupendra S Kaphalia, Ph.D., Professor, Department of Pathology, The University of Texas Medical Branch, 301 University Blvd, Galveston, Texas, USA 77550, Tel.: +1 409 772 4995; bkaphali@utmb.edu.
Author Contributions

Conception and design: MPS and BSK; Data curation and elaboration, analysis and/or interpretation: MPS, KKB, AAC, SA, and SK; Formal analysis and software: MPS; Data validation and discussion: MPS and BSK; Manuscript writing: MPS and BSK; Funding acquisition: BSK; Resources: GL, ANB, and LK. All authors contributed for manuscript editing and final approval

Conflict of Interest

The authors declare no conflicts of interest.

hPACs as well as AR42J cells. Therefore, it is likely that EtOH-induced inactivation of AMPK α plays a crucial role in acinar cell injury leading to pancreatitis. Findings from this study also suggest that EtOH-induced inactivation of AMPK α is closely related to ER/oxidative stress and synthesis of FAEEs, as activation of AMPK α by AICAR attenuates formation of FAEEs, ER/oxidative stress and lipogenesis, and improves inflammatory responses and mitochondrial bioenergetics.

Graphical Abstract

A link between AMPK α inactivation and ER/oxidative stress is established in understanding metabolic basis of alcoholic chronic pancreatitis.

Further, activation of AMPK α by AICAR attenuated EtOH-induced pancreatic acinar cell injury.

Keywords

Alcoholic pancreatitis; Human pancreatic acinar cells; Fatty acid ethyl esters; ER stress; AMPK α ; Mitochondrial stress

1. Introduction

Chronic alcohol abuse costing ~\$250 billion to the U.S. economy as well as ~100,000 deaths each year [1], is the primary cause of chronic pancreatitis (CP) progressing to fibrosis with/without overt co-morbidities such as diabetes and pancreatic cancer. It is well known that, many individuals with a history of chronic alcoholic intake die even before the disease becomes clinically manifested. The exocrine pancreas is one of the target tissues commonly damaged during chronic alcohol abuse. Although activation of trypsinogen (one of the key zymogens synthesized and stored in the pancreatic acinar cells) in the pancreatic gland itself is central to the initiation and propagation of inflammatory processes and necrotic cell death [2–4], the mechanism and metabolic basis of alcoholic chronic pancreatitis (ACP) are not clearly understood. Both oxidative and non-oxidative pathways of ethanol (EtOH) metabolism have been described in pancreatic acinar cells [5, 6]. However, non-oxidative metabolism of EtOH to fatty acid ethyl esters (FAEEs) catalyzed by FAEE synthase is a predominant pathway for EtOH disposition in the pancreas during chronic alcohol abuse [7]. The expression of FAEE synthase is reported much higher in the pancreas than several other organs and significantly induced upon EtOH exposure [5–8]. FAEEs can be detected in plasma and other tissues after alcohol consumption [7, 9]. On the other hand, pancreatic alcohol dehydrogenase (ADH) and cytochrome P450E1 (CYP2E1) activities involved in the canonical oxidative pathway of EtOH metabolism are relatively low or negligible [5]. Inhibition of hepatic ADH1 (a major enzyme involved in EtOH oxidation) leads to increased biosynthesis of FAEEs in the pancreas and toxicity to the pancreatic acinar cells [2, 5, 6, 8, 10–15]. Therefore, cytotoxicity of FAEEs, especially to the pancreatic acinar cells, is of interest to investigate the metabolic basis of alcoholic pancreatitis.

In comparison to other cell types, the pancreatic acinar cells have a vast network of the endoplasmic reticulum (ER) to enable their highest rate of protein and lipid synthesis required for various metabolic and digestive activities [16]. This unique feature, however, also makes acinar cells particularly susceptible to EtOH-induced metabolic perturbations, resulting in unfolding/misfolding of de novo synthesized proteins. Such unfolded/misfolded proteins accumulate in the ER lumen and cause ER stress. A sustained ER stress subsequently activates the unfolded protein response (UPR) required for ER homeostasis mediated by three transmembrane proteins; protein kinase RNA-like ER kinase (PERK), inositol requiring enzyme 1 α (IRE1 α) and activating transcription factor 6 (ATF6) [16–19]. Each of them activates a unique UPR signaling pathway to mitigate ER stress and promotes cellular homeostasis. However, unresolved/prolonged ER stress underlies the pathology of many chronic diseases, including EtOH-related disorders and might activate mitogen-activated protein kinases (MAPKs) leading to inflammation and cell death [17, 20, 21].

Various such factors as EtOH metabolism and its metabolites (acetaldehyde and FAEEs) and increased lipid synthesis could induce ER stress and oxidative stress in acinar cells [19, 22].

AMPK α is a crucial regulator of energy metabolic homeostasis, and its inactivation plays a significant role in key cellular events that are involved in the pathogenesis of various diseases [23]. More recently, EtOH-induced AMPK α dysregulation has been linked to ER stress in alveolar macrophages and the liver [24, 25]. However, the role of AMPK α in ER stress and its link towards the initiation and progression of ACP is not well understood. Therefore, we performed an in vitro study using freshly isolated human pancreatic acini from brain dead pancreas donors, to link EtOH-induced dysregulated AMPK α signaling with ER/ Oxidative stress and understand the metabolic basis of EtOH-induced pancreatic acinar cell injury.

2. Materials and Methods

2.1 Antibodies and reagents

The primary antibodies for AMPK α (62 kDa; Cat # 5831), phospho (p)-AMPK α (Thr 172) (62 kDa; Cat # 2535), acetyl CoA carboxylase 1 (ACC1; 265 kDa; Cat # 4190), p-ACC1 (Ser 79; 280 kDa; Cat #3661), fatty acid synthase (FAS; 273 kDa; Cat # 3189), liver kinase B1 (LKB1; 54 kDa; Cat #3050), p-LKB1 (Ser 428; 54 kDa; Cat #53482), glucose regulated protein 78 (GRP78; 78 kDa; Cat # 3177), eukaryotic translation initiation factor 2A (eIF2 α ; 38 kDa; Cat # 5324), p-eIF2 α (Ser 51; 38 kDa; Cat # 3398), p38 mitogen-activated protein kinases (p38MAPK; 40 kDa; Cat # 8690), p-p38MAPK (40 kDa; Cat # 4511), p44/42 MAP Kinase (ERK 1/2, 42, 44 kDa; Cat # 4695), p-p44/42 MAP Kinase (p-ERK 1/2, 42, 44 kDa; Cat # 4370), c-Jun N-terminal kinase 1/ 2 (JNK; 46, 54 kDa; Cat # 4970), p-JNK 1/2 (46, 54 kDa; Cat # 4668), alcohol dehydrogenase 1 (ADH1; 40 kDa; Cat # 5295) and β -actin (45 kDa; Cat # 4970) were purchased from Cell Signaling Technology (Danvers, MA).

Antibodies for spliced X-box binding protein1 (sXBP1; 40 kDa; Cat # 37152), unspliced XBP1 (uXBP1; 29 kDa; Cat # 37152), carnitine palmitoyltransferase 1A (CPT1A; 88 kDa; Cat # 128568), 4 hydroxynonenal (4HNE; Cat# ab46545) and cytochrome P450 2E1 (CYP2E1; 50 kDa; Cat# ab28146) were purchased from Abcam Inc (Cambridge, MA, USA). Antibodies to inositol-requiring transmembrane kinase/endoribonuclease 1 α (IRE1 α ; 110 kDa; Cat # NB100–2324), p-IRE1 α (Ser 724) (110 kDa; Cat # NB100–232), activating transcription factor 6 (ATF6; 88 kDa; Cat #IMG273), sterol regulatory element-binding protein 1c (SREBP1c; 120 kDa; Cat # NB600–582) were from Novus Biologicals (Littleton, CO, USA). Antibodies to protein kinase RNA-like endoplasmic reticulum kinase (PERK; 150 kDa; Cat # 100-401-962), CHOP (31 kDa; Cat #MA1–250) and carboxyl ester lipase (CEL; 74 kDa; Cat# sc-377130) were from Rockland (Limerick, PA), Thermo Fisher Scientific (Houston, TX), and Santa Cruz Biotechnology, Inc (Dallas, TX), respectively.

The chemicals, reagents, and HPLC and GC grade solvents used in the present study were obtained from Sigma Aldrich (St Louis, MO) and/or Thermo Fisher Scientific (Houston, TX).

Human pancreatic acinar cells (hPACs) were obtained from Prodo Laboratories (Cat # HAR-001, Irvine, CA) and Department of Surgery, University of Louisville, Kentucky. Pancreatic acinar cells were the byproduct obtained during islet isolation procedure from pancreata of brain-dead donors [26, 27]. The isolated acinar cells were washed and transported in cold CMRL 1066 media containing human serum (Cat# 99-785-CV, Corning, NY) to our laboratory [28]. An experimental approach for the separation of acinar cells was similar at both the centers. The human pancreatic acini are known to maintain most of the physiological functions suitable to investigate the mechanism of ACP [28, 29].

The pancreatic acinar cells were obtained from the male (n = 6) and female (n = 5) Caucasians who were non-diabetic, average age 42 years, with no history of chronic alcohol use, smoking, drug abuse, and gastrointestinal disorders (Table 1).

Rat pancreatic tumor (AR42J) cell line obtained from ATCC® (CRL- 14092™, Rockville, MD) were cultured to confluence and used thereafter, as described previously [30].

2.2 Cell culture studies with hPACs

Upon receipt, the cells were washed twice with pancreatic cell media (PIM®) and centrifuged at 180g. The sedimented cell pellet was re-suspended in PIM® containing PIM (ABS)®, PIM (G)®, and mixed with ciprofloxacin antibiotic as per manufacturer's instructions (Cat # PIM-ABS001GMP, Prodo Laboratories INC, Aliso Viejo, CA). The cells were distributed into cell culture flasks (0.5×10^6 cells/flask) and treated with 1, 3, or 6 mg/ml isotopic 1, 2-¹³C-EtOH (Cat # 427039, Sigma-Aldrich, St. Louis, MO) or non-isotopic EtOH for 6 hr. The range of EtOH concentrations used for this study is relevant to those reported in acute and chronic alcoholic subjects [9, 31, 32]. The isotopic EtOH was used to assess the formation of FAEEs and non-isotopic EtOH for the biochemical/molecular studies. All the in vitro studies conducted in hPACs were completed within 30 hr of their isolation, beyond which they may lose their characteristic phenotype and viability [29, 33, 34]. The cells were incubated at 37 °C supplied with purified air containing 5% CO₂ as described earlier [30].

For the time-dependent studies, hPACs were incubated with 3 mg/ml EtOH for 1 hr, 3 hr, and 6 hr. For AMPK α activation and inactivation studies, hPACs were pre-incubated with/without 5-aminoimidazole-4- carboxamide-1- β -D-ribonucleotide, (AICAR, AMPK α agonist, 1 mM, Cat # 123040, Cal Biochem, San Diego, CA) or dorsomorphin 2HCl (Compound C, AMPK α antagonist, 50 μ M, Cat # S7306, Selleckchem, Houston, TX) for an hour followed by incubation with EtOH (3 mg/ml) for 6 hr. EtOH concentration of 3 mg/ml for the studies mentioned above for hPACs was preferred because it is comparable to the median blood alcohol concentration (BAC) reported in the chronic alcoholic subjects [9].

2.3 Viability Assessment

The purity of acinar cell preparations was assessed indirectly by measuring the purity of isolated islets by dithizone staining [35]. Therefore, upon receipt, the viability of the acinar cells was measured by fluorescein diacetate (FDA) and ethidium bromide (EB) staining, a simple and sensitive assay to differentiate living and dead cells, respectively [36]. The live cells fluoresce green with FDA, and the dead cells fluoresce red with EB under a

fluorescence microscope. The intensity of observed fluorescence was calculated using Image J (version 1.50i; Bethesda, NIH) and viability is expressed as percent of total number of cells.

2.4 Amylase and Lipase assays

Amylase and lipase, synthesized and stored in pancreatic acinar cells, are the most widely used clinical markers of pancreatic injury. The secretion of these digestive enzymes was assayed in the culture medium (10 μ l) of hPACs treated with EtOH using respective assay kits from BioVision (Cat# K711, Milpitas, CA) and Cayman Chemical Company (Cat# 700640, Ann Arbor, MI), respectively.

The α -amylase assay uses ethylidene-pNP-G7 as the substrate, which is specifically cleaved by α -amylase. The smaller fragments produced can be acted upon by α -glucosidase ultimately to release the chromophore which was measured at 405 nm using Bio-Tek Epoch 2 microplate reader (Winooski, VT). The amylase activity is expressed as nmol/min/mg protein.

A fluorescence-based method was used for detection of lipase activity. In the assay, lipase hydrolyzes arachidonoyl-1-thioglycerol to thioglycerol and arachidonic acid. Thioglycerol reacts with thiol fluorometric detector to yield a highly fluorescent product which was measured at excitation and emission wavelengths of 380–390 nm and 510–520 nm, respectively, using Bio-Tek synergy/HTX multi-mode reader, respectively. The lipase activity is expressed as nmol/min/mg protein.

2.5 Cytotoxicity and cell death assays

Lactate dehydrogenase (LDH, a cytosolic enzyme) is released into the culture medium during cell injury/death. Thus, LDH release in the culture medium of EtOH treated hPACs as an index of acinar cell injury was measured using the LDH cytotoxicity assay kit (Cat# 601170, Cayman Chemical Company), as per the manufacturer's instructions. The kit uses a coupled two-step reaction for measuring LDH cytotoxicity. In the first step, LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses de novo formed NADH and H^+ to catalyze the reduction of tetrazolium salt to highly-colored formazan. The amount of formazan produced is proportional to the amount of LDH released into the culture medium. The absorbance was measured at 490–520 nm using Bio-Tek Epoch 2 microplate reader and presented as a percent of the total cellular LDH activity.

For measuring apoptosis, CellEvent® Caspase-3/7 Green Ready Probes® reagent (Cat# R37111, Thermo fisher scientific, Houston, TX) was added to the EtOH-treated acinar cell suspension at 2 drops/ml. The reagent is a four amino acid peptide (DEVD, Asp-Glu-Val-Asp) conjugated to a non-fluorescent nucleic acid-binding dye. DEVD peptide inhibits binding of the dye to DNA. Upon activation of caspase-3/7 in apoptotic cells, the DEVD peptide is cleaved and the free dye binds to DNA generating a bright green fluorescence monitored using an Olympus IX71 microscope with FITC filter. The fluorescence intensity was quantified by ImageJ and represented as an arbitrary unit (a.u.).

2.6 Cytokine Secretion

Cytokines secreted into the culture medium from hPACs incubated with EtOH for 6 hr in the presence/absence of AMPK α activator was measured using Human Cytokine Antibody Array kit (Cat # ab133997, Abcam, Cambridge MA) following manufacturer's instructions. Briefly, an aliquot of media was incubated on the array membrane, and the target cytokines were visualized using the Chemiluminescence detection system. The intensity of cytokine spot was measured using ImageJ. Data is normalized to the control and presented as a.u.

2.7 Western blot analysis

The hPACs and AR42J cells treated with non-isotopic EtOH with/ without AICAR or Compound C, as described earlier (Section 2.2); were harvested then lysed by mammalian protein extraction reagent (Cat # 78501, Thermo Fisher Scientific, Houston, TX) containing phosphatase and protease inhibitors. AMPK α and ER stress signaling molecules were analyzed by Western blot analysis using their respective antibodies and standard immunoblotting protocols. Briefly, cell lysate was prepared, and protein concentration was measured by Bio-Rad protein assay (Cat # 5000006, Bio-Rad Laboratories, Hercules, CA). An equal amount of lysate was mixed with sample buffer and boiled for 10 min, and ~40 μ g of protein was subjected to SDS PAGE [2]. The membrane was incubated with antibodies (rabbit; and isotype controls, 1:1000 dilution) followed by incubation with anti-rabbit/mouse IgG conjugated with horseradish peroxidase (secondary antibodies, 1:2000 dilution). Protein bands were developed with an ECL detection reagent (Amersham, Buckinghamshire, England), and their intensities determined and compared using ImageJ.

2.8 FFAE and Triglyceride analyses

Extraction and analysis of FFAEs formed by hPACs treated with 1,2-¹³C-EtOH in the presence/absence of AMPK α activator (AICAR) were performed as described previously [24]. Triglyceride content in hPACs treated with EtOH with/ without AICAR was measured using a triglyceride colorimetric assay kit as per manufacturer's instructions (Cat # 10010303, Cayman Chemical, Ann Arbor, MI). This assay uses enzymatic hydrolysis of triglycerides by lipase to produce glycerol and free fatty acids. The glycerol is phosphorylated to glycerol-3-phosphate, which is further oxidized by glycerol phosphate oxidases producing dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalyzes the redox-coupled reaction of hydrogen peroxide with 4-aminoantipyrine and N-Ethyl-N-(3-sulfoethyl)-m-anisidine producing a brilliant purple color which absorbs at 530–550 nm. The absorbance was measured using Bio-Tek Epoch 2 microplate reader and the concentration of triglycerides expressed as μ g/mg protein.

2.9 Cell culture studies with AR42J cells

For cellular bioenergetics and long-term EtOH exposure studies (up to 72 hours), rat pancreatic tumor cells (AR42J, ATCC CRL1492; ATCC, Rockville, MD) cultured to confluence were used (28). For long-term EtOH exposure studies, AR42J cells were treated with 3 mg/ml of EtOH for 6, 24, and 72 hr, respectively. For the studies with AR42J cells, an EtOH concentration of 3 mg/ml and AICAR (1mM) were used as described for the hPACs studies.

2.10 Mitochondrial function and ATP Production Rate

All the experiments to determine the mitochondrial function and ATP production rate were conducted in AR42J cells treated with EtOH. Mitochondrial function and Real-time ATP production rate were determined using Seahorse XFp extracellular flux analyzer (Agilent, Santa Clara, CA) as per the manufacturer's instructions.

Prior to bioenergetics assay, AR42J cells were seeded into Seahorse XFp cell culture mini plates at an initial density of ~30,000 cells/well. After overnight culture, the cells were treated with EtOH (3 mg/ml). Untreated cells were used as controls. In brief, the cell culture medium was changed to Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 10 mM glucose, 2 mM L-glutamine and 2 mM pyruvate, followed by incubation in a non-CO₂ incubator at 37 °C for 60 min which is necessary for de-gassing the plate, allowing for CO₂ diffusion from the cells, medium, and the plate which could interfere with the assay.

A mitochondrial respiratory function "stress" test protocol (Cat # 103010–100, Agilent, Santa Clara, CA) was implemented to measure indices of mitochondrial function with/without EtOH. Oligomycin, trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), antimycin A and rotenone mix were injected sequentially through ports of the Seahorse Flux cartridges to achieve final concentrations of 2 µM, 1 µM and 0.5 µM, respectively, to determine the basal oxygen consumption rate (OCR), oxygen consumption linked to ATP production, level of non-ATP-linked oxygen consumption (proton leak), maximal and spare respiration capacity and non-mitochondrial oxygen consumption.

Similarly, the cellular ATP production rate was determined using real-time ATP rate assay (Cat # 103591–100, Agilent, Santa Clara, CA), which measures total cellular ATP production rate from two main metabolic pathways; oxidative phosphorylation (OXPHOS) and glycolysis. This assay uses metabolic modulator (oligomycin and a mix of rotenone and antimycin A) that when serially injected, allows the calculation of the mitochondrial and glycolytic ATP production rates in situ in live cell. Injection of oligomycin results in an inhibition of mitochondrial ATP synthesis that results in a decreased oxygen consumption rate (OCR), allowing the mitochondrial ATP Production Rate to be quantified. Extracellular acidification rate (ECAR) data combined with the buffer factor of the assay medium (2.5 mmol/L/unit of pH) allows calculation of total Proton Efflux Rate (PER). Complete inhibition of mitochondrial respiration with rotenone plus antimycin A allows accounting for mitochondrial-associated acidification, and allows calculation of the glycolytic ATP Production Rate when combined with PER data. The test was performed by injecting oligomycin and antimycin A/rotenone mix to achieve final concentrations of 1.5 µM and 0.5 µM, respectively, through ports of the Seahorse Flux cartridges.

2.11 EtOH-induced oxidative stress

To determine EtOH-induced oxidative stress, AR42J cells were grown in chamber slides (Cat # 177399, Thermo Fisher Scientific, Houston, TX) and incubated with EtOH (3 mg/ml) for 6 hr, 24 hr, and 72hr. After the treatment, cells were fixed in freshly prepared 4% paraformaldehyde for 10 min at room temperature followed by permeabilization with 0.1%

Triton-X 100 prepared in 0.01M phosphate-buffered saline (1X PBS, pH 7.4) for 10 min. Non-specific binding was blocked with 1% bovine serum albumin prepared in PBS and Tween-20 for 30 min at room temperature. After a series of washes, the cells were incubated overnight at 4°C with a primary antibody for 4-hydroxynonenal (4HNE, 1:500 dilution, Cat# ab46545, Abcam Inc, Cambridge, MA). The cells were then incubated with Goat Anti-Rabbit IgG (H+L) - FITC conjugated secondary antibody (2 µg/ml, Cat# 4050-02, Southern Biotech, Birmingham, AL) for 1hr at room temperature in the dark followed by counterstain with 4', 6-diamidino-2-phenylindole (DAPI). The fluorescent images were acquired using an Olympus IX71 microscope with FITC filter, and relative intensities were analyzed using ImageJ.

2.12 Statistical analysis

The data sets were analyzed for the statistical significance using One Way ANOVA followed by the Tukey Multiple Comparison test. The results are expressed as Mean $\pm$ SEM (standard error of mean). A p-value $\leq$ 0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism (v 7.0, San Diego, CA).

3. Results

Cell culture studies with hPACs

3.1 Viability and markers of acinar cell injury—Overall cell viability as a percentage of the total number of cells used in all the experiments was found to be ~95% (Fig. 1 A–D).

No significant changes in the activities of amylase (Fig. 2A) and lipase (Fig. 2B) [key enzymatic markers of pancreatic injury] were observed in the culture medium of EtOH treated hPACs as compared to their respective controls. Surprisingly, a significant concentration-dependent increase in LDH leakage was found from the cells treated with 3 and 6 mg/ml EtOH (Fig. 3A). Similarly, a significantly increased number of apoptotic cells as measured by an increase in fluorescence was also noted at 3 and 6 mg/ml of EtOH as compared to the respective controls (Fig. 3 B, C).

3.2 EtOH-induced AMPK α dysregulation and lipogenesis—The p-AMPK α levels were significantly decreased in hPACs treated with 3 and 6 mg/ml of EtOH as compared to the controls (Fig. 4A). As expected increased expression of key proteins [ACC1 (Fig. 4B), FAS (Fig. 4C)] and transcription factor [SREBP1c (Fig. 4D)] involved in lipogenesis was noted at 3 and 6 mg/ml of EtOH along with a significant concentration-dependent decrease for p-ACC1 (Fig. 4B), a substrate for AMPK α and CPT1A, a key protein involved in β -oxidation of fatty acids (Fig. 4E). Of importance, the levels of p-LKB1 (Fig. 4F) (upstream kinase regulating AMPK α), were significantly decreased in a concentration-dependent manner in hPACs treated with EtOH. Overall, an increased lipogenesis found in the hPACs treated with EtOH could also be associated with the inactivation of AMPK α and decreased oxidation of fatty acids.

3.3 EtOH-induced ER stress signaling—ER stress as evaluated by increased expression of GRP78 and the unfolded protein responses, p-IRE 1 α , uXBP1, pEIF2 α , and CHOP was found to be concentration-dependent in hPACs incubated with EtOH (Fig. 5 A–F). Of importance, EtOH treatment resulted in a significantly decreased expression of total PERK (Fig. 5D). However, significant changes for ATF6 were not found in EtOH treated cells (data not shown).

3.4 EtOH-induced activation of inflammatory pathways, oxidative stress, and cytokine secretion—Mitogen-activated protein kinases (MAPKs), a key pathway activated during oxidative stress/inflammation, are significantly upregulated in hPACs treated with 3 and 6 mg/ml EtOH as evidenced by a concentration-dependent increased expression of JNK1/2, ERK1/2, and P38MAPK in EtOH treated cells (Fig. 6A–C)

Out of 42 human cytokines and chemokines assayed in the culture medium of acinar cells treated with EtOH, only 14 (5 inflammatory cytokines and 9 chemokines) were secreted and detected (Fig. 6D). Levels of secreted cytokines were estimated to be 2 to 3-fold higher in the cells treated with 3 and 6 mg/ml EtOH as compared to the respective controls.

3.5 Time-dependent effect of EtOH on AMPK α /ER stress signaling, and inflammatory pathway(s)—Although levels of p-AMPK α were increased at 1 and 3 hr as compared to the respective controls, inactivation of AMPK α followed by increased expression of GRP78, JNK1/2, and ERK1/2 markers for ER/oxidative stress were observed only at 6 hr as compared to their respective controls (Fig. 7A–D). Thus, the optimum time to assess acinar cell injury, due to EtOH exposure/metabolism, appears to be at 6 hr, under the experimental conditions used in this study.

3.6 AICAR attenuates EtOH-induced AMPK α inactivation, ER stress, inflammatory signaling, and cytokine/chemokine secretion—AICAR pretreatment in hPACs attenuated EtOH-induced AMPK α inactivation (Fig. 8A), decreased lipogenic protein expressions (ACC1, FAS) (Fig. 8B, C), and increased p-ACC1 and CPT1A expressions (Fig. 8B, D).

Subsequently, activation of AMPK α by AICAR ameliorated EtOH mediated ER/oxidative stress and inflammatory signaling in hPACs, as indicated by decreased expression of GRP78, CHOP, and JNK (Fig. 8E–G).

Of importance, the treatment of hPACs with compound C (AMPK α inhibitor) alone, resulted in dysregulation of AMPK α and ER stress signaling (Fig. 8A–G). However, no significant changes were observed in the signaling of AMPK α and ER stress in hPACs incubated with combination of AICAR and compound C (Fig. 8A–G).

As summarized in Fig. 8H, activation of AMPK α by AICAR decreased EtOH-induced cytokine/chemokine secretion from hPACs. A significant decrease in EtOH-induced secretion of 8 cytokines (5 inflammatory cytokines and 3 chemokines) out of 14 detected, was observed in the culture medium of hPACs pretreated with AICAR. Overall, activation of AMPK α attenuated EtOH-induced ER stress and inflammatory responses in hPACs.

3.7 AICAR treatment attenuates EtOH-induced formation of FAEEs and Triglycerides

—As shown in Fig. 9A, a concentration-dependent increase in the expression of carboxyl ester lipase (CEL, a key enzyme involved FAEE synthesis) was observed in EtOH treated hPACs. About ~1.5 and 3 fold increases for CEL expression were noted in cells treated with 3 and 6 mg/ml of EtOH compared to the respective controls. Of importance, the expression/induction of either alcohol dehydrogenase (ADH1) or cytochrome P450 2E1 (CYP2E1), proteins involved in oxidative EtOH metabolism were not observed in EtOH treated hPACs (data not shown).

As shown in Fig. 9B, the formation of FAEEs increased exponentially with increasing concentration of EtOH, average levels being 22, 48, and 160 ng/ 0.5×10^6 cells incubated with 1, 3, and 6 mg/ml 1, 2-¹³C EtOH, respectively. The total FAEE levels were ~2 and ~7 fold higher in acinar cells incubated with 3 and 6 mg/ml EtOH, respectively than those formed with 1 mg/ml EtOH. Among all the FAEEs, ethyl palmitate and ethyl oleate were the most abundant esters formed in hPACs treated with EtOH. The hPACs treated with 6 mg/ml EtOH for 6 hr showed the highest level of FAEEs, which was attenuated by ~1.3-fold with AICAR pretreatment (Fig. 9B). Besides, AICAR pretreatment also decreased EtOH-induced formation of ethyl palmitate and ethyl oleate by ~1.3 and 1.4 folds, respectively (Fig. 9B).

As summarized in Fig. 9C, the triglyceride levels were significantly elevated with increasing concentrations of EtOH; ~2 and ~4 folds greater in acinar cells incubated with 3 and 6 mg/ml EtOH as compared to the respective controls. Activation of AMPK α by AICAR decreased EtOH-induced triglyceride biosynthesis/accumulation in hPACs by ~1.3-fold (Fig. 9C).

Overall, AICAR treatment decreased the formation of FAEEs and biosynthesis/accumulation of triglycerides in hPACs; key putative lipid mediators in EtOH-induced acinar cell injury.

Cell culture studies with AR42J cells

3.8 Long term effect of EtOH on AMPK α /ER Stress signaling—EtOH exposure increases AMPK α inactivation and ER/Oxidative stress in a time-dependent manner in AR42J cells. Inactivation of AMPK α (Fig. 10A) and p-ACC1 (Fig. 10B) was parallel to the increased expression of ACC1 (Fig. 10B) and FAS (Fig. 10C), proteins involved in lipogenesis, all peaked at 72 hr. Furthermore, elevated expression of GRP78 and CHOP (markers for ER stress) was observed at 72 hr as compared to that at 6 and 24 hr, respectively (Fig. 10D, E). Immunofluorescence study using antibodies against 4HNE showed significant oxidative stress in AR42J cells treated with EtOH. The fluorescence intensity was maximum at 72 hr of EtOH treatment (Fig. 10F). Thus, long term EtOH exposure resulted in sustained inactivation of AMPK α with increased ER/oxidative stress in AR42J cells.

3.9 Effect of EtOH on cellular bioenergetics—As shown in Fig. 11A, the mitochondrial-stress test using Seahorse flux analysis in AR42J cells showed a basal oxygen consumption rate (OCR) of ~79 pmol/min, with a spare respiratory capacity of ~72 pmol/min indicating the presence of a significant reserve available in AR42J cells against increased bioenergetics demand. The maximal respiration and non- mitochondrial oxygen

consumption were found to be ~150 pmol/min and ~55 pmol/min, respectively. AR42J cells exhibited a basal extracellular acidification rate (ECAR) of ~9 mpH/min. Upon EtOH exposure (3 mg/ml), a decreased basal OCR of ~66 pmol/min, with a subsequent reduction in spare respiratory capacity/mitochondrial reserve of ~59 pmol/min was observed. In addition, a reduction in maximal respiration (~125 pmol/min) and non-mitochondrial oxygen consumption (~38 pmol/min) was observed in EtOH treated AR42J cells. Furthermore, a ~1.7-fold increase in basal ECAR (~16 mpH/min) was observed in AR42J cells treated with EtOH. Of note, a significant reduction in mitochondrial ATP production was observed in EtOH treated cells compared to the controls (~58 pmol/min vs. ~71 pmol/min).

As shown in Fig. 11B, the real-time total ATP production rate assay using Seahorse flux analysis in control AR42J cells showed a total ATP production rate of ~472 pmol/min. The ATP production rate from mitochondria and glycolysis was found to be ~468 pmol/min and ~4 pmol/min, respectively. Upon EtOH exposure, the total ATP production rate was decreased by ~1.8 fold (~250 pmol/min), and the mitochondrial ATP production rate reduced by ~2.3 fold (~202 pmol/min), respectively. Of importance, the ATP production from glycolysis was increased by ~13 fold (~48 pmol/min) upon EtOH treatment. Thus, EtOH exposure increased the rate of glycolysis with subsequent reduction of oxidative phosphorylation in AR42J cells. Overall, EtOH resulted in impaired mitochondrial function and decreased total ATP production rate.

3.10 AICAR improves EtOH-induced mitochondrial bioenergetics and oxidative stress in AR42J cells—Seahorse flux analysis showed that AICAR pretreatment in AR42J cells exposed to EtOH improves the basal oxygen consumption rate from ~84 to 104 pmol/min (Fig. 12A). A significant increase in mitochondrial reserve / spare respiratory capacity was observed from ~7.0 pmol/min to 33 pmol/min. Furthermore, AICAR pretreatment improved maximal respiration (~91.0 pmol/min to 137 pmol/min) and non-mitochondrial oxygen consumption (~37.0 pmol/min to 47 pmol/min) in EtOH treated AR42J cells.

Of note, the mitochondrial ATP production rate was elevated by ~1.2-fold in EtOH treated AR42J cells by AICAR pretreatment. Furthermore, cells treated with EtOH (3 mg/ml) significantly elevated basal ECAR, an index of glycolytic rate/lactate production, was reduced by ~1.6 fold (~16 mpH/min to 10 mpH/min) with AICAR pretreatment.

As illustrated in Fig. 12B, EtOH mediated oxidative stress was attenuated in AR42J cells pre-incubated with AICAR.

4. Discussion

The exocrine pancreas is the primary target organ of injury during chronic alcohol abuse, which is a predisposing risk factor for the initiation and progression of acute and chronic pancreatitis [2, 19]. The pancreas appears to be the most favored organ for EtOH disposition via non-oxidative metabolism to FAEEs [7]. Deficiency/Inhibition of the key alcohol oxidizing enzyme (alcohol dehydrogenase) as commonly seen in chronic alcoholic subjects,

further enhances the formation of FAEEs in the pancreas [2,10,15]. To our knowledge, this is the only study showing a significant concentration-dependent formation of FAEEs in hPACs incubated with EtOH. In the present study, ethyl palmitate and ethyl oleate were the most abundant FAEEs in hPACs as also reported in the pancreas of humans and rodents exposed to EtOH [7, 37].

Of note, the FAEE synthase (a group of enzymes involved in FAEE synthesis) was reported to be higher in patients with alcohol-related pancreatitis [38]. Formation of FAEEs in EtOH treated hPACs was observed with a concomitant increased expression of carboxyl ester lipase (CEL, one of the significant FAEE synthases involved in FAEE synthesis in the pancreas). Irrespective of the overexpression of CEL, AICAR showed the potential to attenuate the formation of FAEEs in hPACs treated with EtOH. Thus, the formation of FAEEs is likely to be dependent upon the endogenous concentration of fatty acids, key substrates for FAEE synthesis. However, a lack of expression/induction of ADH1 (major isozyme responsible for EtOH oxidation) and CYP2E1 observed in these cells further supports the prevalence of non-oxidative metabolism of EtOH in hPACs. Therefore, FAEEs are the major EtOH metabolites formed in the pancreas and likely to contribute significantly towards pancreatic acinar cell injury and subsequently to the process of pathogenesis of ACP.

AMPK α is a crucial regulator of cellular homeostasis against metabolic stress, and its activation could be a key therapeutic strategy to attenuate cellular events that are involved in the pathogenesis of various diseases [23, 39]. Of note, the expression of p-AMPK α /AMPK α has been well characterized in the human pancreas [40] and rat pancreatic acini [41]. Decreased levels of p-AMPK α are evident in pancreatic adenocarcinoma [40] and cerulein-induced acute pancreatitis [41] suggesting, that AMPK α activity is essential for normal cellular functioning. The inactivation of AMPK α with increasing concentration of EtOH (> 3 mg/ml) as found in this study is relevant to individuals with a history of chronic alcohol abuse [9]. Increased expression of ACC1, FAS, and SREBP1c with decreased expression of CPT1A further supports our finding of accumulation of triglycerides in hPACs treated with EtOH. Furthermore, the activity of AMPK α is also regulated by an upstream kinase LKB1, related to oxidative/cellular stress. Therefore, an inhibition of LKB1 as observed in EtOH treated hPACs could also deactivate AMPK α [42, 43]. Therefore, dysregulated AMPK α signaling followed by triglyceride accumulation and formation of FAEEs in acinar cells could be linked to pancreatic exocrine dysfunction and fatty pancreas (an early lipid phenotype of alcoholic pancreatic disease).

EtOH-induced ER stress as found in hPACs could be attributed to such various factors as EtOH oxidative and non-oxidative metabolites, and generation of reactive oxygen species. In pancreatitis, ER stress is manifested by increased phosphorylation of PERK, splicing of XBP1 (sXBP1), and CHOP expression [16, 18, 44]. An increase in sXBP1 may protect the pancreas against injury [18], whereas activation of CHOP is associated with acinar cell injury/death and inflammatory responses [44]. Increased expression of GRP78 (a marker for ER stress) in hPACs with an increasing concentration of EtOH followed by subsequent activation of UPRs (IRE1 α and PERK/CHOP pathways) indicates a systemic response to ER stress. However, reduced expression of sXBP1 as observed in hPACs treated with 3 and

6 mg/ml EtOH suggests a lack of adaptive UPR to maintain ER homeostasis in acinar cells. Besides, long term EtOH exposure in AR42J cells resulted in sustained activation of GRP78 and CHOP, indicating prolonged ER stress can activate cell death pathways and inflammatory processes. Overall, EtOH-induced upregulation of the PERK/CHOP pathway and decreased sXBP1 expression converge to ER dysfunction and acinar cell pathology, which mimics several features of human alcoholic chronic pancreatitis.

A concentration and time-dependent activation of three major classes of MAPKs; the extracellular signal-regulated kinases (ERKs) and the two- stress activated protein kinase (SAPKs) families, c-jun N-terminal kinase (JNK1/2) and p38MAPK as found in hPACs treated with EtOH could be linked to oxidative stress related to EtOH metabolism and/or ER stress [45,46]. These activated MAPKs mediate an inflammatory response involved in the development of pancreatitis [47,48], as indicated by an increased secretion of inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF α) and chemokines (MCP-1, RANTES) in the culture medium of hPACs incubated with 3 mg/ml and 6 mg/ml of EtOH. Of note, these secreted inflammatory cytokines and chemokines have been reported in patients with severe acute pancreatitis and animal models of acute pancreatitis [49–54]. Besides, increased secretion of growth mediated chemokines like growth-related oncogene alpha (GRO α), angiogenin, epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) observed in hPACs treated with 3 and 6 mg/ml EtOH can be associated with various pancreatic pathologies including pancreatic cancer [55]. These seminal findings in hPACs, further strengthen a case for the metabolic basis of EtOH-mediated acinar cell injury at concentrations of EtOH like those reported in alcoholic subjects.

EtOH and FAEs primarily impair mitochondrial function in pancreatic acinar cells [11, 12] and decrease ATP production [3]. Spare respiratory capacity (SRC, an index of mitochondrial function) is considered a vital reserve of the mitochondria, a key bioenergetic parameter utilized in response to stress but is reduced in several pathological conditions [56]. A diminished SRC observed in AR42J cells could be attributed to EtOH-induced oxidative stress, suggesting incompetency of the cells to meet its metabolic challenges under conditions of elevated stress. Besides, a decreased mitochondrial ATP production rate as found in this study could contribute to the loss of secretory capacity of acinar cells and shift pancreatic cell death from apoptosis to necrosis [57].

A robust increase for extracellular acidification rate (ECAR) in response to EtOH exposure with an increase in ATP turnover from glycolysis, suggest a compensatory metabolic shift towards glycolysis under impaired oxidative phosphorylation. Such findings from AR42J cells could be extrapolated to hPACs in terms of cytotoxicity as determined by increased LDH leakage from the cells treated with EtOH. However, impaired secretory capacity for amylase and lipase as observed in EtOH treated hPACs could be linked to reduced mitochondrial ATP production. A decreased level of sXBP1, as found in hPACs treated with EtOH, could also decrease the secretory capacity of acinar cells [29]. Of note, an impaired endolysosomal pathways, apical and basolateral exocytosis process due to dysregulated exocytotic complex proteins by EtOH and its metabolites can reduce the secretory capacity of acinar cells [58, 59].

AMPK α has emerged as a key regulator of ER stress, and its activation is known to attenuate ER stress [24, 60, 61]. We expect an underlying link between AMPK α inactivation and ER/oxidative stress in hPACs from the data presented herein with AMPK α agonist (AICAR) and antagonist (compound C). AMPK α inactivation by compound C alone resulted in an increased expression of GRP78, CHOP, and JNK1/2 markers for ER/oxidative stress, suggesting a strong link between AMPK α inactivation and ER/oxidative stress. Time-dependent dysregulated AMPK α signaling with a concomitant increase in ER/oxidative stress again supports an interrelationship between AMPK α inactivation and ER/oxidative stress. Our findings collectively suggest that AMPK α activation protects acinar cells from EtOH-induced injury and associated oxidative/ER stress, as an optimal basal AMPK α is necessary for maintaining normal acinar cell functions and modulation of ER stress [41].

To our knowledge, this is the first study, where AMPK α activation by AICAR attenuated FAEEs formation and related toxicity in the acinar cells treated with EtOH. Further restoration of mitochondrial function and ATP production rate in AR42J cells by AICAR supports the therapeutic role of AICAR on mitochondrial biogenesis [61], and could be attributed to direct activation of AMPK α by AICAR.

AMPK α inhibition, along with decreased ATP and impaired mitochondrial/ER function, as observed in EtOH treated hPACs, is noteworthy. Of importance, a novel mechanistic study has shown that AMPK α activity is inhibited not only under the condition of oxidative stress but also during conditions of decreased ATP [62]. AMPK α is known to be covalently modified by reactive aldehydes reducing its activity following chronic ethanol consumption [62]. Thus, increased oxidative stress caused by EtOH appears to be one of the critical factors that could explain the mechanism of EtOH-induced AMPK α inhibition in these hPACs. Since AICAR treatment mitigated the effects of EtOH in hPACs, AMPK α activation could serve as a potential therapeutic target for alcoholic pancreatitis using AMPK α agonists including, metformin.

It is important to acknowledge the limitation of the present study. The use of AICAR for regulating AMPK α activity, as this agent, could have non-specific effects. However, AICAR does not interfere with mitochondrial bioenergetics like other AMPK α activators and can directly activate AMPK α in most of the cells. AICAR monophosphate can readily accumulate within the cells in millimolar concentrations. Thus, AICAR seems to be a more suitable therapeutic agent/model for the studies in the hPACs. Further, an in vivo experiment showing protection against EtOH-induced pancreatic injury by AICAR would significantly strengthen the current in vitro findings.

In conclusion, EtOH-induced inactivation of AMPK α and upregulation ER stress, most likely via non-oxidative metabolism of EtOH, may play a key role in acinar cell injury leading to pancreatitis. Impaired secretion of amylase and lipase together with a reduction in sXBP1 levels highlights the role of sXBP1 required for exocrine pancreatic functions and exocytosis of digestive zymogens. The actions of EtOH on cellular bioenergetics demonstrated a compensatory metabolic shift from oxidative phosphorylation to glycolysis to meet cellular ATP levels under elevated stress conditions. However, impaired mitochondrial function and decreased cellular ATP production rate observed in this study

might impair several cellular functions and decide the cell death fate from apoptosis to necrosis. In view of attenuation of ER stress, lipogenesis, and associated formation of FAEEs with AICAR in hPACs and AR42J cells treated with EtOH, further studies are warranted to explore the role of individual oxidative and non-oxidative metabolites of EtOH in pancreatic acinar cell injury, to identify the putative etiology of alcoholic pancreatitis.

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Abbreviations:

ACC1	Acetyl CoA carboxylase
ACP	Alcoholic chronic pancreatitis
ADH1	Alcohol dehydrogenase 1
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPKα	AMP activated protein kinase α
AP	Acute pancreatitis
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BAC	Blood alcohol concentration
CEL	Carboxyl ester lipase
CP	Chronic pancreatitis
CPT1A	Carnitine palmitoyltransferase 1A
CYP2E1	Cytochrome P450 2E1
ECAR	Extracellular acidification rate
EIF2α	Eukaryotic translation initiation factor 2 α
EtOH	Ethanol
ER	Endoplasmic reticulum
FAEE	Fatty acid ethyl ester(s)

GRP78	Glucose regulated protein 78
hPACs	Human pancreatic acinar cells
IRE1α	Inositol-requiring enzyme 1 α
LDH	Lactate dehydrogenase
LKB1	Liver kinase B1
MAPK	Mitogen activated protein kinase
OCR	Oxygen consumption rate
PERK	Protein kinase RNA-like ER kinase
SREBP1c	Sterol regulatory element-binding protein 1c
TG	Triglycerides
XBP1	X-Box binding protein 1
UPR	Unfolded protein response

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Fig. 1.

Morphology of isolated human pancreatic acinar cells (hPACs) as observed under a bright-field microscope (A). Viability of the cells, determined by dual staining using fluorescein diacetate (FDA, green fluorescence for live cells) (B) and ethidium bromide (EB, red fluorescence for dead cells) (C). Quantification of fluorescence intensity was performed by using Image J (D). Overall, cell viability is shown as a percentage of the preparation (original magnification $\times 20$). Values are expressed as Mean $\pm$ SEM (n =6 replicates).

Fig. 2. Amylase (**A**) and Lipase (**B**) activities, in the culture medium of hPACs incubated at 37°C for 6 h with different concentrations of EtOH. Values are expressed as Mean $\pm$ SEM (n =16 replicates).

Fig. 3.

EtOH-induced acinar cell injury as measured by the percentage of LDH release (**A**).

Representative light microscopic images of hPACs (**B**, Upper Panel) and Caspase-3/7 green stained fluorescence images of apoptotic hPACs incubated with different concentrations of EtOH (**B**, Lower Panel) and their relative intensities (**C**), as quantified by Image J and are normalized to increase in fluorescence from baseline and expressed as Mean $\pm$ SEM (n =6 replicates). * p < 0.05 compared to the control.

Fig. 4.

EtOH-induced AMPKα signaling in hPACs. Western blots, along with respective bar diagrams, show relative intensities of p-AMPKα/AMPKα (A), p-AMPKα (A1), AMPKα (A2), p-ACC1/ACC1 (B), p-ACC1 (B1), ACC1 (B2), FAS (C), SREBP1c (D), CPT1A (E) and p-LKB1/LKB1 (F). Intensities were normalized to β-actin (loading control). Values are expressed as Mean ± SEM (n = 16 replicates). *, #, † p-value < 0.05.

Fig. 5. EtOH-induced ER stress and UPR signaling in hPACs. Immunoblots along with respective bar diagram show expression levels of GRP78 (**A**), p-IRE1α/ IRE1α (**B**), sXBP1/uXBP1 (**C**), PERK (**D**), p-EIF2α/EIF2α (**E**) and CHOP (**F**). Intensities were normalized to β-actin (loading control). Values are expressed as Mean ± SEM (n =16 replicates). *, #, † p-value 0.05.

Fig. 6.

Immunoblots, along with respective bar diagrams, show levels of p-JNK1/2/JNK1/2 (**A**), p-ERK1/2/ ERK1/2(**B**), and p-P38MAPK/P38MAPK (**C**), key proteins upregulated by EtOH-induced oxidative stress and involved in activation of inflammatory pathway in hPACs. Intensities were normalized to β -actin (loading control). Values are expressed as Mean $\pm$ SEM (n =16 replicates). *, #, $\dagger$ p-value $\leq$ 0.05. Cytokines/chemokines secreted into the culture medium from hPACs incubated with different concentrations of EtOH. The optical density of 14 detected cytokines (out of 42) (**E**). Values are expressed as Mean $\pm$ SEM (n =4). * p-value $\leq$ 0.05 compared to the control.

MCP, Monocyte Chemoattractant Protein; TNF, tumor necrosis factor; EGF, Epidermal growth factor; IGF-1, Insulin-like growth factor-1; IL-, Interleukin. Following cytokines/chemokines were tested but not detected in the culture medium. IL-2; IL-3, IL-4; IL-5; IL-7; IL-10; IL-12 p40/p70; IL-13; IL-15; IFN- γ ; ENA-78; GCSF; MCSF; MDC; MIG;

Oncostatin M; I-309; IL-1 α ; MIP-1 δ ; SCF; SDF-1; TARC; VEGF; PDGF BB; TGF- β 1; Thrombopoietin and Leptin.

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Fig. 7. Time-dependent EtOH- induced AMPKα inactivation and ER stress and induction of inflammatory pathway in hPACs incubated at 37°C with 3 mg/ml EtOH for 1 hr, 3 hr, and 6 hr respectively. Immunoblots are shown along with respective bar diagrams for p-AMPKα/AMPKα (A), GRP 78 (B), p-JNK1/2/JNK1/2 (C) and p-ERK1/2/ERK1/2 (D). Intensities were normalized to β-actin (loading control). Values are expressed as Mean ± SEM (n =6 replicates). * p-value < 0.05 compared to the respective controls.

Fig. 8.

Amelioration of EtOH-induced lipogenesis, ER/oxidative stress, and inflammation by activation of AMPK α . Immunoblots are shown along with respective bar diagrams for p-AMPK α /AMPK α (A), p-AMPK α (A1), AMPK α (A2), p-ACC1/ACC1 (B), p-ACC1 (B1), ACC1 (B2), FAS (C), CPT1A (D), GRP 78 (E), CHOP (F) and p-JNK1/2/JNK1/2 (G) in hPACs incubated with EtOH (3 mg/ml) with or without AICAR (1 mM) and compound C (AMPK α inhibitor, 50 μ M) for 6 h at 37°C. Intensities were normalized to β -actin (loading control). Values are expressed as Mean $\pm$ SEM (n = 6 replicates). * p-value $\leq$ 0.05 compared to the control. # p-value $\leq$ 0.05 compared to EtOH.

An optical density of 14 detected inflammatory cytokines/chemokines secreted into the culture medium from hPACs incubated with EtOH (3 mg/ml) with or without AICAR (1 mM) for 6 h at 37°C (H). A significant decrease in the secretion of 8 cytokines/chemokines (out of 14 detected) was observed in culture medium upon AICAR pretreatment. Values are

expressed as Mean $\pm$ SEM (n = 4 replicates). * p-value 0.05 compared to the control. # p-value 0.05 compared to EtOH.

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Fig. 9.

Immunoblot, along with the respective bar diagram, shows the expression of carboxyl ester lipase (CEL) in EtOH treated hPACs (A). Intensities were normalized to β -actin (loading control). Values are expressed as Mean $\pm$ SEM ($n = 3$ replicates). *, #, p -value < 0.05 . Levels of FFAEs (B) and triglycerides (C) in hPACs incubated with 1, 2- ^{13}C -ethanol/ non-isotope EtOH with/without AICAR (1mM) at 37°C for 6 h. Values are expressed as Mean $\pm$ SEM ($n = 4$ replicates). * p -value < 0.05 compared to the respective EtOH concentration.

Fig. 10.

Long term EtOH exposure-induced AMPK α inactivation and ER stress in AR42J cells incubated at 37 °C with 3 mg/ml EtOH for 6, 24, and 72 hrs, respectively. Immunoblots are shown along with respective bar diagrams for p-AMPK α /AMPK α (A), p-AMPK α (A1), AMPK α (A2), p-ACC1/ ACC1 (B), p-ACC1 (B1), ACC1 (B2), FAS (C), GRP 78 (D), and CHOP (E). Intensities were normalized to β -actin (loading control). Oxidative stress was measured by immunofluorescence microscopy using antibodies against 4-HNE in AR42J cells. Upper panel -DAPI, Lower panel - immunofluorescence (F), relative intensities of immunofluorescence are shown by bar diagram using Image J (G). Values are expressed as Mean $\pm$ SEM (n = 3 replicates). * p-value = 0.05 compared to the respective controls.

Fig. 11.

Acute effects of EtOH on mitochondrial bioenergetics and ATP production rate in AR42J cells incubated with 3 mg/ml EtOH using Seahorse extracellular flux analyzer. Mito stress test showing basal oxygen consumption rate (OCR), spare respiratory capacity, Maximal respiration, and Non-mitochondrial oxygen consumption in AR42J cells (A). Data are shown as the Mean $\pm$ SEM ($n = 3$ independent experiments). * p -value < 0.05 compared to the respective controls.

Real-time ATP rate assay showing total ATP production rate, mitochondrial ATP rate, and Glycolytic ATP rate in AR42J cells (**B**) Data are shown as the Mean $\pm$ SEM (n = 3 independent experiments). * p-value = 0.05 compared to respective controls.

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Fig.12.

Mitochondrial bioenergetics in AR42J cells incubated with 3 mg/ml EtOH in the presence of AMPK α activator (AICAR, 1mM). Mito stress test showing baseline changes of mitochondrial respiration including basal oxygen consumption rate (OCR), spare respiratory capacity, Maximal respiration, and Non-mitochondrial oxygen consumption in AR42J cells (A). Data are shown as the Mean $\pm$ SEM (n = 3). *p-value $\leq$ 0.05 compared to the control, #p-value $\leq$ 0.05 compared to EtOH.

Immunofluorescence microscopy of oxidative stress using antibodies against 4-HNE in AR42J cells incubated with 3 mg/ml EtOH at 37 °C for 24 hours in the presence of AMPK α activator (AICAR, 1 mM) (B). Upper panel - DAPI; Lower panel- immunofluorescence relative intensities of immunofluorescence are shown by bar diagram using Image J (C).

Values are expressed as Mean $\pm$ SEM (n = 3 independent experiments). * p-value 0.05 compared to EtOH.

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Table 1:

Donor Characteristics

Characteristics	Description
Gender	Male (n = 6) Female (n = 5)
Race	Caucasians (n = 11)
Average age [years $\pm$ standard error of mean (SE)]	Male donors: 41.2 ± 7.2 Female donors 43.25 ± 6.4
Body mass index (BMI $\pm$ SE BMI), kg/m ²	Male donors 25.2 ± 3.4 Female donors 28.4 ± 4.4
Diabetic status	None- with HbA1C < 6.0
Alcohol intake history	3 Donors with social drinking status
Smoking Status	None
Recreational drug use	None
Cause of Death	Stroke (n = 3) Head trauma (n = 6) Anoxia (n = 2)