Alcohol predisposes obese mice to acute pancreatitis via adipose triglyceride lipase dependent visceral adipocyte lipolysis

Supplementary methods

Animals and ethics

C57BL/6J male mice were purchased from Beijing Huafukang Bioscience Corporation (Beijing, China). Mice were housed at 22°C with a 12-h light-dark cycle and had free access to food and water throughout experiments. All animal procedures were assessed and authorised by the Ethics Committee of West China Hospital of Sichuan University (20211086A).

Obesity and alcohol-induced acute pancreatitis

Mice of 4-5 weeks old mice were randomly divided into chow diet (CD) group and high-fat diet (HFD) group, and respectively fed a CD (10 kcal % fat, H10010) and HFD (60 kcal % fat, H10060 Huafukang Bioscience Corporation; Beijing, China) for 12 weeks. Then, alcohol stimulation was conducted by two hourly intraperitoneal injections of ethanol at a dose of 2 g/kg. Ethanol (459836, Sigma, Shanghai China) was dissolved in sterile normal saline at 37.5% concentration (v/v). Control mice were given equal-volume saline injections. Animals were sacrificed at 3, 6, 12, 24, and 48 h after the first injection.

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Adipocytes and alcohol-induced acute pancreatitis

Adipocytes were freshly isolated from HFD obese mouse as previously described with minor modifications.[1] Briefly, mouse white adipose tissue fat pads were dissected, weighted, and minced thoroughly until no obvious tissue pieces were visible. The minced tissue was incubated at 37°C with shaking at ~220 rpm for 1 h with collagenase solution (Krebs Ringer Bicarbonate HEPES [KRBH] buffer containing 2% bovine serum albumin [BSA], 500 nM adenosine, and 3 mg/ml Collagenase I; 3 ml per gram of tissue). After digestion, the mixture was filtered through a 300 mm nylon mesh strainer, where after adipocytes were washed 3 times by free floating using a total of 40 ml wash buffer (2% BSA, 500 nM adenosine in KRBH) at room temperature. For each round of washing cells were allowed to float for > 5 min and the infranatant removed with a long needle and syringe. After the final wash, about 1.5-2 x 10^7 in 0.3 ml volume was intraperitoneally injected into 8-10 weeks-old mice using an 18-gauge needle. After 1 h, alcohol stimulation was conducted as above described. Animals were sacrificed at 12 h after the first alcohol injection.

Caerulein-induced acute pancreatitis

CD or HFD mice were given seven intraperitoneal injections of caerulein (50 μg/kg, Tocris, Shanghai) at hourly intervals as described previously.[2, 3] Control mice were given equal-volume saline injections. Animals were sacrificed at 12 h after the first injection.

Atglistatin and orlistat treatment

Atglistatin (HY-15859, MedChemExpress, Shanghai) was dissolved in dimethyl sulfoxide at a stock (0.3 M), and then further dissolved in olive oil. Atglistatin treatment was administrated orally by gavage (200 μmol/kg) before 1 h of first ethanol or caerulein injection.[4] Orlistat (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol at a stock (20 mg/ml). Orlistat treatment (50 mg/kg) was administered intraperitoneally 2 h after the first ethanol or caerulein injection. Animals were sacrificed at 12 h after induction of acute pancreatitis.

Histology

The tissues were collected and fixed in 10% formalin for 24 hours, embedded with paraffin, and cut into slices before haematoxylin and eosin (H&E) staining. The severity of pancreatic injury was evaluated by two independent, blinded investigators grading oedema, inflammatory cell infiltration, and acinar necrosis (scale, 0-4) on × 10 high-power fields/slide/mouse.[5]

Serum biochemical marker measurement

Biochemical parameters (amylase, lipase, alanine aminotransferase, aspartate aminotransferase, urea, and creatinine) were measured by an automatic biochemical analyser (Roche Cobas 8000, Shanghai, China). Interleukin-6 (IL-6) was determined by enzyme-linked immunosorbent assay (R&D Systems, Shanghai, China), free fatty acids (FFA) were determined by a commercial kit

(Abcam, Shanghai, China).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured as described.[6] Pancreatic or lung tissue was homogenised, resuspended in 100 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethyl ammonium bromide, 10 mM ethylene diamine tetraacetic acid and protease inhibitors, freeze-thawed three times, sonicated for 30 sec and centrifuged for 15 min at 16,000 × g. MPO activity was measured in supernatants mixed with 3,3,5,5-tetramethylbenzidine as substrate (1.6 mM, final) with freshly added H_2O_2 (3 mM, final). Absorbance was measured at 655 nm and MPO was calculated as the difference between absorbance at 0 and 3 minutes. The activity was calculated by standard curve and expressed as units/mg protein or normalised to control.

Ex vivo lipolysis measurement

Lipolysis of adipose tissue was measured as the release of glycerol and FFA into the culture medium ex vivo.[7] Briefly, the fresh epididymal adipose tissue explants were isolated from mice. Approximately 30 mg of adipose tissue was transferred in 1 ml KRBH medium containing 2% FFA-free BSA, minced into small pieces, and then incubated at 37°C for 3 h. The contents of glycerol and FFA (Abcam) in incubation media were quantified according to the manufacturer's protocols.

Western blot analysis

After collection, the adipose tissues were centrifuged at 250 × g for 5 min, followed by removal of supernatant and addition of RIPA lysis buffer with protein extraction cocktail (100 μl). Protein was extracted by ultrasonication and centrifuged at 12000 × g for 15 min 4°C. The protein concentration was quantified by a bicinchoninic acid assay. Proteins (20 μg) were subjected to 8% or 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were blocked with 5% non-fat milk for 1 h at room temperature. Then the membranes were further incubated overnight at 4°C with respective primary antibodies: adipose triglyceride lipase (ATGL; Santa Cruz Biotechnology, Shanghai, China), and β-actin (Proteintech, Wuhan, China). After primary antibody incubation, membranes were washed with phosphate-buffered saline containing 0.1% Tween 20 and followed by the incubation of secondary goat anti-mouse IgG-HRP antibody (Proteintech) for 1 h at room temperature. Protein bands were visualised using a Chemiluminescent Detection System (Bio-Rad, Hercules, CA). β-actin was used as the internal reference control.

Statistical analysis

Data were presented as mean ± SEM and analysed using GraphPad Prism 8.4.1 (San Diego, CA). For two-group comparisons, mean differences were analyzed by 2-tailed Student's *t* test. For multi-group comparisons, mean differences were analysed by one-way ANOVA analysis of variance with the

Tukey's multiple comparison post-test. *P* value < 0.05 was considered significant.

References

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