**Title**

High-fat-diet induced inflammation and apoptosis *via* activation of Ire1α in liver and hepatocytes of black seabream (*Acanthopagrus schlegelii*)

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**Supplementary Material**

**Supplemental Methods**

**Diet preparation, fish feeding and sampling**

Fishmeal, soybean protein concentrate and soybean meal were used as protein sources, with fish oil, soybean oil and soybean lecithin used as the main lipid sources. All ingredients were purchased from Ningbo Tech-Bank Feed Co. Ltd., Ningbo, China. The experimental diets were produced as follows: ground ingredients were mixed in a Hobart type mixer and cold-extruded pellets produced with pellet strands cut into uniform sizes (2 mm and 3 mm diameter pellets). Pellets were heated for 30 min at 90 °C, and then air-dried to approximately 10 % moisture, sealed in vacuum-packed bags and stored at -20 ℃ until used in the feeding trial.

An 8-week feeding trial was conducted on juvenile black seabream (initial weight 2.08 ± 0.01 g) obtained from a local commercial hatchery at Xiangshan Bay, Ningbo, China. Prior to the start of the experiment, black seabream juveniles were acclimated to the experimental facilities and fed a commercial diet (40 % protein, 12 % crude lipid, Ningbo Tech-Bank Corp.) for two weeks. The feeding trial was carried out with a completely randomized design. A total of 180 black seabream juveniles were randomly allocated to 6 floating net cages (1.5 m × 1.5 m × 2.0 m) corresponding to triplicate cages for each of the two dietary treatments. Fish were hand-fed to apparent satiation twice daily at 05:00 and 17:00 for eight weeks. During the experimental period, seawater conditions including temperature (29.2-31.5℃), salinity (22.4-25.1 g/L), dissolved oxygen (6.28-7.13 mg/L) and pH (7.8 - 8.0) were measured with YSI Proplus (YSI, Yellow Springs, Ohio, USA).

At the end of feeding trial, fish were sampled 12 h after the last feed. All fish were anesthetized with Eugenol (Aladdin, China) and all fish in each tank were individually weighed and counted to determine specific growth rate (SGR) and calculate feed efficiency (FE). The experimental replicates (units) were tanks and so all analyses were performed on a per tank basis (n = 3) with samples from each tank being derived from pooled fish. Liver samples (pools of 3 fish per tank) were collected and stored at -80˚C for analyzing proximate composition. Liver, and intestine samples were also collected and stored at -80 ℃ until further analysis of biochemical indices (pools of 3 fish per tank), gene expression (pools of 3 fish per tank) and protein determination (pools of 2 fish per tank). Fresh liver tissues were collected into 4 % formaldehyde from one fish per tank for histological analyses. Blood samples were taken from the caudal vasculature of 8 fish per tank using 1 ml syringes, pooled, and stored at 4 ℃ for 24 h for analyzing serum biochemical indices.

**Supplemental Table 1**

Formulation and composition of the experimental diets (% dry matter).

|  |  |  |
| --- | --- | --- |
| Ingredients (%) | Diets | |
| Control | HFD |
| Fish meal | 26.00 | 26.00 |
| Soy protein concentrate | 10.00 | 10.00 |
| Soybean meal | 20.00 | 20.00 |
| Wheat flour | 24.50 | 24.50 |
| Fish oil | 4.00 | 4.00 |
| Soybean oil | 4.00 | 11.00 |
| Soybean lecithin | 1.00 | 1.00 |
| a Vitamin premix | 0.30 | 0.30 |
| b Mineral premix | 2.00 | 2.00 |
| Ca(H2PO4) | 1.00 | 1.00 |
| Choline chloride | 0.20 | 0.20 |
| Cellulose | 7.00 | 0.00 |
| Total | 100.00 | 100.00 |
| *Analyzed proximate composition (% dry weight)* |  |  |  |  |
| Dry matter | 89.80 | 90.08 |
| Crude protein | 40.50 | 40.97 |
| Crude lipid | 12.08 | 19.04 |
| Ash | 9.84 | 9.76 |

a Vitamin premix (IU or g kg-1 diet): retinol acetate, 400 IU; cholecalciferol, 300 IU; thiamin mononitrate, 8; riboflavin, 8; pyridoxine, 20; cobalamine, 0.1; α-tocopherol, 6; menadione, 10; d- calcium pantothenate, 35, niacin, 20; folic acid, 2.5; biotin, 0.04; inositol, 110; ascorbic acid, 300.

b Mineral mixture (g kg−1 premix): FeC6H5O7, 11.43; ZnSO4·7H2O, 11.79; MnSO~~4~~·H2O (99%), 2.49; CuSO4·5H2O (99%), 1.06; MgSO4·7H2O (99%), 27.31; KH2PO4, 233.2; NaH2PO4, 228.39; C6H10CaO6·5H2O (98%), 34.09; CoCl2·6H2O (99%), 0.54. KIO3 (99%), 0.06; zeolite, 449.66.

**Supplemental Table 2**

siRNA sequences for RNA interference.

|  |  |
| --- | --- |
| Primer | Sequence (5’ - 3’) |
| a Si*ire1α* | F: GCUCUGAUGGAGUCCUUUATT  R: UAAAGGACUCCAUCAGAGCTT |

a Si*ire1α*, small interfering RNA of inositol requiring enzyme-1 ɑ.

**Supplemental Table 3**

Primers for real-time quantitative PCR of black seabream (*Acanthopagrus schlegelii*)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gene | Nucleotide sequence (5’ - 3’) | Size (bp) | GenBank referenceor Publication | Functions |
| *sirt1 1* | F: TGGATGAAACTGTAGGAACC | 238 | MN871952 | Metabolic sensor |
| R: ACAACAATGGACTGGGAA |
| *pparα 2* | F: ACGACGCTTTCCTCTTCCC | 183 | KX066234 | Lipolysis pathway |
| R: GCCTCCCCCTGGTTTATTC |
| *srebp-1 3* | F: TGGGGGTAGGAGTGAGTAG | 247 | KX066235 | Lipogenesis pathway |
| R: GTGAAGGGTCAGTGTTGGA |
| *scd1 4* | F: CCACGGGAGAACAAGTTTG | 198 | MZ004439 | Lipogenesis |
| R: CCAGGTAGCACATCAGGTC |
| *fas 5* | F: AAGAGCAGGGAGTGTTCGC | 213 | KX066240 | Lipogenesis |
| R: TGACGTGGTATTCAGCCGA |
| *cpt1 6* | F: TGCTCCTACACACTATTCCCA | 203 | KX078572 | Lipolysis |
| R: CATCTGCTGCTCTATCTCCCG |
| *lpl 7* | F: CTGCTACTCCTCTGCCCA | 204 | KX078571 | Lipolysis |
| R: ACATCCCTGTTACCGTCC |
| *crt 8* | F: CATCCTGGCTTGTTTGC | 203 | OQ032506 | ERS pathway |
| R: TTTGGGTGTAGTTGCGG |
| *grp78 9* | F: AACCAGCTGACCTCTAACCC | 164 | MT451934 | ERS pathway |
| R: ATGTCTTCATCTGGCCACCA |
| *atf6 10* | F: CCTGTTGGGTTTCTCCTCAG | 222 | MT512507 | ERS pathway |
| R: CCGTTACTTCACAGTCAATCTGC |
| *xbp1 11* | F: TGATATCGGGGAAGCAGACC | 235 | MW589390 | ERS pathway |
| R: TTCCTGTCTCTGGCTGTCTG |
| *ire1α 12* | F: AGAGGTCTTGGGTCATGGTG | 181 | OL361769 | ERS pathway |
| R: GTCCCTCTCAGTGCAGAAGT |
| *perk 13* | F: ACGGTGACGGAGAAGAACAG | 108 | ON631219 | ERS pathway |
| R: GAGAGCCAGAGTAAGTGAGGTT |
| *nf-κb 14* | F: AGCCCAAGGCACTCTAGACA | 154 | MK922543 | Nuclear transcription factor |
| R: GTTCTGGGCAGCTGTAGAGG |
| *tnfα 15* | F: GGAGACAGACGAGGGCAAGA | 196 | AY335443 | Pro-inflammatory cytokine |
| R: TCAGCCGCAAGCGTTATCTC |
| *il-1β 16* | F: CATCTGGAGGCGGTGAA | 231 | JQ973887 | Pro-inflammatory cytokine |
| R: CGGTTTTGGTGGGAGGA |
| *tgfβ 17* | F: GGGTTTCCAACTTCGGC | 209 | OQ248005 | Anti-inflammatory cytokine |
| R: TTGTGTCCGTGGAGCGT |
| *il-10 18* | F: TGTCAAACGGTTCCTTGCAG | 172 | MK922542 | Anti-inflammatiory cytokine |
| R: GGCATCCTGGGCTTCTATCT |
| *caspase7 19* | F: GTTTGCCTACTCCACTGTGC | 152 | OL321593 | Pro-apoptotic pathway |
| R: TGGCCACCATGTAGTTGACT |
| *caspase9 20* | F: CCATTGTTTCTGCAGTGCCT | 214 | OL321594 | Pro-apoptotic pathway |
| R: GAGTAGTACTGGGTCTGGGC |
| *bax 21* | F: AAGTGGATGGACAGAGTGGG | 232 | OL321596 | Pro-apoptotic pathway |
| R: ATGCAATCTGGTGGTGGAGA |
| *jnk 22* | F: ATAGCGTGTGGTCTGGGAAA | 171 | OK315340 | Pro-apoptotic pathway |
| R: CGCAGACATGTAAACAGCCA |
| *bcl-2* 23 | F: GCTCCAACGACTGATCAACC | 203 | OL420679 | Anti-apoptotic pathway |
| R: TGACCTGAAGAACCCAGCTT |
| *gpx 24* | F: TCTGAAGTACGTCCGTCCTG | 247 | OL321587 | Antioxidation |
| R: TCTCAAAGTTCCAGGCCACA |
| *Cu / Zn sod 25* | F: CACGGTAAGAATCATGGCGG | 202 | OL321588 | Antioxidation |
| R: TCTCCTCGTTGCCTCCTTTT |
| *Mn sod 26* | F: TCTCTTTCTCGTAGCCCAGC | 247 | OL321589 | Antioxidation |
| R: GCAAAGGGAGATGTGACAGC |
| *β-actin* | F: ACCCAGATCATGTTCGAGACC | 212 | AY491380 | Housekeeping gene |
| R: ATGAGGTAGTCTGTGAGGTCG |

1 *sirt1*, silent regulator 1; 2 *pparα*, peroxisome proliferators-activated receptor alpha; 3 *srebp-1*, sterol regulatory element-binding protein-1; 4 *scd1*, stearoyl-CoA desaturase 1; 5 *fas*, fatty acid synthase; 6 *cpt1*, carnitine palmitoyltransferase 1; 7 *lpl*, lipoprotein lipase; 8 *crt* Calreticulin; 9 *grp78*, glucose regulated protein 78; 10 *atf6*, activating transcription factor 6;11 *xbp1*, X-box binding protein 1; 12 *ire1α*, inositol requiring enzyme-1; 13 *perk* protein kinase R-like ER kinase; 14 *nf-κb*, nuclear factor kappa B; 15 *tnfα*, tumor necrosis factor α; 16 *il-1β*, interleukin-1 β;17 *tgfβ*, transforming growth factor β; 18 *il-10*, interleukin-10; 19 *caspase7*, cysteinyl aspartate specific proteinase 7; 20 *caspas9*, cysteinyl aspartate specific proteinase 9; 21 *bax*, bcl2-associated X; 22 *jnk*, c-Jun N-terminal kinase; 23 *bcl-2*, B cell leukemia-2; 24 *gpx*, Glutathione peroxidase; 25 *Cu / Zn sod*, Cu, Zn-Superoxide dismutase; 26 *Mn sod*, Mn-Superoxide dismutase.



**Supplemental Fig.1.** Effects of different concentrations of oleic acid (OA) on viability (A) and triglyceride (TG) contents (B) of primary hepatocytes of black seabream (*Acanthopagrus schlegelii*). Values are means (n=3) with standard errors represented by vertical bars. Different letter on columns indicate mean values were significantly different (*P* < 0.05) by one-way ANOVA.



**Supplemental Fig. 2.** Weight gain (A), feed efficiency (B) and survival rate (C) of juvenile black seabream (*Acanthopagrus schlegelii*) fed Control and HFD diets for 8 weeks (Experiment 1). Values are means (n=3), with standard errors represented by vertical bars. Control, normal diet (12 % dietary lipid); HFD, high fat diet (19 % dietary lipid).



**Supplemental Fig. 3.** Contents of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HLD-C), triglyceride (TG) and total cholesterol (TC) in the serum of juvenile black seabream (*Acanthopagrus schlegelii*) fed Control and HFD diets for 8 weeks (Experiment 1). Values are means (n=3), with standard errors represented by vertical bars. “\*” represents significant difference (*P* < 0.05) between Control and HFD treatments by *t*-test. Control, normal diet (12 % dietary lipid); HFD, high fat diet (19 % dietary lipid).



**Supplemental Fig. 4.** RelativemRNA expression level of calreticulin (*crt*) related to calcium homeostasis in liver of juvenile black seabream (*Acanthopagrus schlegelii*) fed Control and HFD diets for 8 weeks (Experiment 1). Values are means (n=3), with standard errors represented by vertical bars. “\*” significant difference (*P* < 0.05) difference between Control and HFD treatments by *t*-test. Control, normal diet (12 % dietary lipid); HFD, high fat diet (19 % dietary lipid).



**Supplemental Fig. 5.** Oil red O staining of primary hepatocytes of *Acanthopagrus schlegelii* (A), relative mRNA expression levels of regulatory and transcriptional factor (B), relative mRNA expression levels of genes related to lipid metabolism (C), and relative protein expression levels of sterol regulatory element-binding protein-1c (Srebp-1c) and peroxisome proliferators-activated receptor alpha (Pparα) (D, E) in primary hepatocytes of *Acanthopagrus schlegelii* treated with OA, OA+NC, OA+ si*ire1α* and untreated (Control) (B). Values are means (n=3) with standard errors represented by vertical bars. Mean values for the same column with different letters were significantly different (*P* < 0.05) by one-way ANOVA. OA, incubation with oleic acid; OA+NC, incubation with oleic acid and transfection with non-silencing control siRNA (negative control); OA+ si*ire1α*, incubation with oleic acid and transfection with *ire1α*-small interfering RNA.