



King's Research Portal

DOI:

[10.1007/s00018-019-03263-6](https://doi.org/10.1007/s00018-019-03263-6)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Zhao, T., Wu, K., Hogstrand, C., Xu, Y. H., Chen, G. H., Wei, C. C., & Luo, Z. (2020). Lipophagy mediated carbohydrate-induced changes of lipid metabolism via oxidative stress, endoplasmic reticulum (ER) stress and ChREBP/PPAR γ pathways. *Cellular and Molecular Life Sciences*, 77, 1987–2003.
<https://doi.org/10.1007/s00018-019-03263-6>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

[Click here to view linked References](#)

1 **Lipophagy mediated carbohydrate-induced changes of lipid metabolism via**
2 **oxidative stress, endoplasmic reticulum (ER) stress and ChREBP/PPAR γ**
3 **pathways**

4
5 Tao Zhao¹, Kun Wu¹, Christer Hogstrand³, Yi-Huan Xu¹, Guang-Hui Chen¹,
6 Chuan-Chuan Wei¹, Zhi Luo^{1,2,*}

7
8 ¹ Key Laboratory of Freshwater Animal Breeding, Ministry of Agriculture, Fishery
9 College, Huazhong Agricultural University, Wuhan 430070, China

10 ² Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao
11 National Laboratory for Marine Science and Technology, Qingdao 266237, China

12 ³ Diabetes and Nutritional Sciences Division, School of Medicine, King's College
13 London, United Kingdom

14 *Corresponding author. Prof. Zhi Luo, Tel.: +86-27-8728-2113; Fax:
15 +86-27-8728-2114; Email address: luozhi99@mail.hzau.edu.cn;
16 luozhi99@aliyun.com (Z. Luo).

17
18 **Abstract:**

19 High carbohydrate diets (HCD) can induce the occurrence of nonalcoholic fatty liver
20 disease (NAFLD), characterized by dramatic accumulation of hepatic lipid droplets
21 (LDs). However, the potential molecular mechanisms are still largely unknown. In
22 this study, we investigated the role of autophagy in the process of HCD-induced
23 changes of hepatic lipid metabolism, and to examine the process of underlying
24 mechanisms during these molecular contexts. We found that HCD significantly
25 increased hepatic lipid accumulation and activated autophagy. Using primary
26 hepatocytes, we found that HG increased lipid accumulation and stimulated the
27 release of NEFA by autophagy-mediated lipophagy, and that lipophagy significantly
28 alleviated high glucose (HG)-induced lipid accumulation. Oxidative and endoplasmic
29 reticulum (ER) stress pathways played crucial regulatory roles in HG-induced
30 lipophagy activation and HG-induced changes of lipid metabolism. Further
31 investigation found that HG-activated lipophagy and HG-induced changes of lipid
32 metabolism were via enhancing carbohydrate response element binding protein
33 (ChREBP) DNA binding capacity at PPAR γ promoter region, which in turn induced
34 transcriptional activation of the key genes related to lipogenesis and autophagy. The

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

present study, for the first time, revealed the novel mechanism for lipophagy mediating HCD-induced **changes of lipid metabolism** by oxidative stress and ER stress, and ChREBP/ PPAR γ pathways. Our study provided innovative evidence for the direct relationship between carbohydrate and lipid metabolism via ChREBP/ PPAR γ pathway.

Keywords: Dietary carbohydrate; Lipid deposition; Lipid metabolism; Regulatory pathways; Lipophagy

Abbreviations: 3-MA, 3-methyl adenine; 4-PBA, 4-Phenylbutyric acid; 6PGD, 6-phosphogluconate dehydrogenase; ACCa, acetyl-CoA carboxylase a; ACSL, acyl-CoA synthetase long-chain; AO, acridine orange; ATF, activating transcription factor; ATG, autophagy related gene; BSA, bovine serum albumin; CF, condition factor; ChREBP, carbohydrate response element-binding protein; CPT-1, carnitine palmitoyltransferase-1; CQ, chloroquine; DCFH2-DA, 2', 7'-dichlorodihydrofluorescein diacetate; eIF2 α , eukaryotic translation initiation factor 2 α ; ERS, endoplasmic reticulum stress; FABPL, fatty acid-binding protein liver; FAS, fatty acid synthase; FBW, final mean body weight; FCR, feed conversion rate; FFA, free fatty acids; FI, feed intake; G6PD, glucose 6-phosphate dehydrogenase; GLUT, glucose transporter; GRP78, Glucose-regulated protein 78; GSH, glutathione; GSSG, glutathione disulfide; HCD, high carbohydrate diet; H&E, hematoxylin and eosin; HG, high glucose; HSI, hepatosomatic index; HSL, hormone-sensitive lipase; ICD, intermediate carbohydrate diet; ICDH, isocitrate dehydrogenase; IRE1 α , inositol requiring 1 α ; LCD, low carbohydrate diet; LD, lipid droplet; LXR a, liver x receptor a; MAP1LC3B, microtubule-associated proteins 1A/1B light chain 3B; IBW, initial mean body weight; M199, medium-199; MDA, malondialdehyde; MDC, monodansylcadaverine; ME, malic enzyme; NAC, N-acetyl-L-cysteine; NAFLD, nonalcoholic fatty liver disease; NEFA, nonesterified fatty acid; OD, optical density; ORO, oil red O; PERK, protein kinase R (PKR)-like ER kinase; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; S.E.M, standard error of the mean; SGR, specific growth rate; SOD, superoxide dismutase; SREBP-1, sterol regulatory element binding proteins-1; TG, triglyceride; UPR, unfolded protein response; VAI, visceral adipose index; VSI, viscerosomatic index; WG, weight gain; XBP1, x-box binding protein 1.

69

70 **Introduction**

71 Non-alcoholic fatty liver disease (NAFLD), characterized by the excessive
72 triglyceride (TG) accumulation in the livers, has been increasing in recent years [1].
73 The spectrum of NAFLD ranges from simple fatty liver to nonalcoholic
74 steatohepatitis (NASH), which may result in liver fibrosis and eventually the
75 development of hepatocellular carcinoma [2]. At present, treatment approaches are
76 limited because of the unclear pathological mechanism of NAFLD. It is well known
77 that the diet rich in carbohydrate **along with other factors** can induce hepatic NAFLD
78 and NASH [3, 4]. However, the **additional** mechanisms of dietary carbohydrate
79 leading to the NAFLD are not fully elucidated.

80 Autophagy is a highly conserved self-renewal process in eukaryotic cells,
81 characterized by the engulfment of cytoplasmic materials into double-membrane
82 vesicles (autophagosomes) for subsequent degradation in lysosomes [5]. Studies
83 suggested that autophagy regulates lipid metabolism by eliminating TG and **its**
84 **activation** plays a key **inhibitory** role in the development of NAFLD [6]. **Moreover,**
85 **lipophagy, one kind of autophagy, regulates lipid metabolism by breaking lipid**
86 **droplets down and enhancing the rate of mitochondrial β -oxidation [6-8].** Impaired
87 lipophagy will increase cellular lipid storage and result in the occurrence of NAFLD
88 [9, 10].

89 Several signaling pathways were reported to regulate the autophagic process,
90 such as oxidative stress, endoplasmic reticulum (ER) stress and PPAR γ pathways.
91 Oxidative stress, which results from an imbalance between free radical production and
92 scavenging, **induces the formation of autophagy [11]. Oxidative stress also accelerates**
93 **lipid accumulation by disrupting mitochondrial functions and reducing oxidation of**
94 **fatty acids [12-14].** The ER is the intracellular important organelle for the synthesis
95 and folding of proteins. **ER stress will occur if ER homeostasis is disrupted and**
96 **unfolded /unprocessed proteins are accumulated** within the ER. Increasing evidences
97 demonstrated that ER **stress** might be associated with autophagy [15, 16], and
98 involved in the development of NAFLD [17, 18]. The PPAR γ is the important
99 transcriptional factor which regulates autophagic process and lipid metabolism [19,
100 20].

101 **Thus, in view of the important role of autophagy in regulating lipid metabolism,**
102 **and the relevant signaling pathways can activate autophagy, we hypothesis that these**

103 molecular events mediated high carbohydrate diets-induced changes of lipid
104 accumulation in the liver. If our hypothesis can be confirmed, **our results will provide**
105 **insight into key pathological mechanisms contributing towards fatty liver occurrence.**

106 Fish are the largest group of vertebrates in the world. **During the evolution, fish**
107 **were considered to experience the fish-specific genome duplication event (FSGD)**
108 **[21]. By analyzing whole-genome sequence information, Gong et al. [22] found the**
109 **FSGD in yellow catfish *Pelteobagrus fulvidraco*, an important freshwater omnivorous**
110 **fish in China and other countries [23]. Some duplicated genes evolve new functions**
111 **that in turn result in novel regulatory mechanism [21]. Therefore, using yellow catfish**
112 **as a model, we hope to find some novel regulatory mechanism of metabolism.**
113 **Moreover,** previous study in our laboratory pointed that high dietary carbohydrate
114 increased liver lipid deposition and developed fatty liver symptom in juvenile yellow
115 catfish [23]. **Accordingly, the present study investigated the mechanism of dietary**
116 **carbohydrate influencing lipid metabolism and dissected the roles of lipophagy**
117 **mediating carbohydrate-induced changes of lipid metabolism via oxidative stress, ER**
118 **stress and ChREBP/PPAR γ pathways.**

119 **Materials and Methods**

120 **Animals feeding and sampling**

121 The protocols of all animal and cells experiments were approved by the ethical
122 guidelines of Huazhong Agricultural University for the care and use of laboratory
123 animals. The experimental protocols were similar to those in our recent study [24], as
124 described in Yang et al. [25]. Briefly, 270 uniformly-sized yellow catfish (4.1 ± 0.01 g,
125 mean \pm SEM) were randomly stocked in 9 circular fiberglass tanks, 30 fish for each
126 tank. They were fed with three experimental diets with dietary carbohydrate levels at
127 17.2% (low carbohydrate diet, LCD), 22.8% (**intermediate** carbohydrate diet, ICD)
128 and 30.2% (high carbohydrate diet, HCD), respectively, and corn starch was used as
129 the carbohydrate source (**Supplementary Table 1**). Each diet was assigned to 3 tanks
130 in a completely randomized manner and fed to apparent satiation twice daily at 8:00
131 am and 4:00 pm for 10 weeks. During the experiment, water temperature ranged from
132 25.7°C to 28.6°C; dissolved oxygen and NH₄-N were 6.07 ± 0.01 mg/L and 0.11 ± 0.01
133 mg/L, respectively.

134 At the end of the 10-week feeding trial, all fish were fasted for 24h before
135 sampling. Fish were anesthetized with MS-222 (100 mg/L water), and then, counted
136 and weighed to determine survival, WG (**weight gain**) and SGR (**specific growth rate**).

137 After obtaining the final total weight of fish in each tank, ten fish per tank were
138 randomly collected, weighed and dissected on ice to obtain the liver and viscera
139 samples for the calculation of VSI (**viscerosomatic index**), VAI (**visceral adipose**
140 **index**), and HSI (**hepatosomatic index**). Another six fish were randomly selected from
141 per tank, and the liver and blood were immediately collected. The livers were quickly
142 frozen in liquid nitrogen and stored at -80°C for RNA and protein isolation. The blood
143 was centrifuged at 3500 g min⁻¹ for 10 mins, and then serum was separated to
144 determine glucose content. Another three fish per tank were randomly selected and
145 the liver were collected, and then fixed in 10% neutral buffered formalin and 2.5%
146 paraformaldehyde for histological, histochemical and ultrastructural observation,
147 respectively. For analyzing hepatic enzyme activity and TG content, another six fish
148 per tank were randomly selected and the liver were collected, and then were
149 immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

150 **Cell culture and treatments**

151 Primary hepatocytes were isolated from *P. fulvidraco* liver and cultured as
152 described previously [24]. In order to determine the mechanism of autophagy
153 influencing the glucose-induced lipid deposition, we used the pharmacological
154 autophagy–lysosomal pathway inhibitor 3-MA (S2767; Selleck Chemicals, Houston,
155 TX, USA) or CQ (C6628; Millipore Sigma, Burlington, MA, USA), respectively, to
156 incubate the hepatocytes. To illuminate the mechanism by which glucose induced
157 autophagy and lipid accumulation, the corresponding inhibitors of the signaling
158 pathways were used, including 4-PBA (ER stress inhibitor, SML0309;
159 MilliporeSigma), NAC (ROS scavenger, S1623; Selleck Chemicals) and T0070907
160 (PPAR γ inhibitor, S2871; Selleck Chemicals). The concentrations and incubation time
161 for all experiments are reported in the Figure legends.

162 **MTT assay for cell viability**

163 The protocols for MTT assay was based on these described in our recent
164 publication [26].

165 **Histological, histochemical analysis, transmission electron microscopy (TEM)** 166 **observation and Bodipy 493/503 staining**

167 **Hematoxylin-eosin** (H&E staining), histochemical (Oil Red O staining) and
168 Bodipy 493/503 (D3922; Thermo Fisher Scientific Waltham, MA, USA) staining for
169 livers and hepatocytes were conducted according to the methods as our previously
170 described [14, 24]. For statistics of relative areas for hepatic vacuoles in H&E and

171 lipid droplets in oil-red O staining, we randomly examined 10 fields and each sample
172 were quantified with Image J. The protocols for TEM observation have been
173 described in Wei et al. [24]. For the Bodipy 493/503 staining, hepatocytes were
174 observed with a laser scanning confocal microscope (Leica Microsystems, Wetzlar,
175 Germany) to visualize the intensity of fluorescence. **In those microscopes, scale bar**
176 **was 10 μ m with \times 40 objective and \times 3 zoom, and scale bar was 5 μ m with \times 40**
177 **objective and \times 6 zoom, respectively.** The green dots were defined as lipid droplet,
178 which were quantified by Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD,
179 USA).

180 **Determination of the contents of TG, nonesterified fatty acid (NEFA) and serum** 181 **glucose**

182 TG, NEFA and serum glucose concentrations were determined with commercial
183 kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the
184 manufacturer's instructions. Proteins concentrations were determined with the
185 Bradford Protein Assay Kit of Nanjing Jiancheng Bioengineering Institute (Nanjing,
186 China).

187 **Determination of enzymatic activities and indices of oxidative stress**

188 Activities of several enzymes related to lipid metabolism, such as **G6PD (glucose**
189 **6-phosphate dehydrogenase), 6PGD (6-phosphogluconate dehydrogenase), ME (malic**
190 **enzyme), ICDH (isocitrate dehydrogenase), FAS (fatty acid synthase), and CPT I**
191 **(carnitine palmitoyltransferase 1),** were measured as previously described [24, 26].
192 One unit of enzyme activity, defined as the amount of enzyme that converts 1 μ M of
193 substrate to product per minute at 30°C, was expressed as units per milligram of
194 soluble protein. Protein concentrations were determined with the Bradford Protein
195 Assay Kit (Nanjing, China).

196 We also measured several parameters involved in oxidative stress, such as SOD
197 activity and the levels of MDA, ROS, GSH and GSSG. SOD activity was measured
198 based on aerobic reduction of NBT at 535nm by superoxide radicals following the
199 method described in Pan et al. [14]. MDA level was measured using malondialdehyde
200 (MDA) assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China)
201 base on measuring thiobarbituric acid-malondialdehyde (TBA-MDA) complex
202 absorbance at 532nm. Intracellular ROS level was measured using oxidation-sensitive
203 fluorescent probe DCFH-DA (287810; MilliporeSigma) as described previously [26].
204 Liver and intracellular GSH and GSSG levels were determined according to the

205 studies by Giustarini et al. [27]. All these analyses were conducted in three replicates.

206 **Detection of autophagic vesicles and free Ca²⁺ in the hepatocytes**

207 Detection of autophagic vesicles and free Ca²⁺ in the hepatocytes were performed
208 according to recent studies [24, 28, 29]. Fluo-4 AM (F14201; Thermo Fisher
209 Scientific) was used to detect free Ca²⁺. AO (318337; MilliporeSigma), MDC (30432;
210 MilliporeSigma) were used to detect the autophagic vesicles and Lyso-Tracker Red
211 DND-99(L7528; Thermo Fisher Scientific) was used to detect lysosomal activity
212 based on the published protocols [28]. Briefly, the treated cells were incubated with
213 the corresponding reagents for 30 min, followed by 3 PBS washes, and then observed
214 with the laser scanning confocal microscope (Leica) to visualize the intensity of
215 fluorescence (×40 objective and ×2 zoom, scale bar: 25µm). The fluorescences of the
216 stained cells were quantified on a CytoFlex Flow Cytometer (Beckman Coulter) and
217 data analysis was performed with FlowJo v.10 software. The relative fluorescence
218 intensity of Ca²⁺, and co-localization of lipid droplets and autolysosomes were
219 quantified using Image J analysis software.

220 **RNA isolation and quantitative real-time PCR (qRT-PCR)**

221 Total RNA was isolated with Trizol reagent (Thermo Fisher Scientific) and
222 transcribed into the cDNA with a Reverse Transcription Kit (Thermo Fisher
223 Scientific). qPCR assays were performed based on the methods described in our
224 recent publications [26]. The gene specific primers are given in [Supplementary Table](#)
225 [2](#). Ten housekeeping genes (18S rRNA, β-actin, HPRT, B2M, UBCE, TUBA,
226 GAPDH, RPL7, TBP and ELFA) were selected to test their transcription stability. The
227 relative expression of genes was calculated using the 2^{-ΔΔCt} method when normalizing
228 to the geometric mean of the best combination of 2 genes as analyzed by geNorm
229 (<https://genorm.cmgg.be/>).

230 **Western blotting**

231 Based on the protocol described in our recent publication [24], we used western
232 blot analysis to test protein expression levels, such as LC-3B, Beclin-1, SQSTM1/p62,
233 BiP/GRP78 and PPARγ. Briefly, liver and cell lysates were prepared with RIPA buffer
234 (Thermo Fisher Scientific). Proteins (40µg from each sample) were then separated on
235 10 or 15% SDS-polyacrylamide gel (depending on the molecular size of proteins to be
236 analyzed), transferred to PVDF membranes (Thermo Fisher Scientific), and then
237 blocked with 8% (w/v) skimmed milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 150
238 mM sodium chloride, 0.1% Tween 20) for 1 h and then washed thrice with TBST

239 buffer for 10 min each, followed by incubation with specific primary antibodies
240 including rabbit anti-LC3B (1:1000, ab51520; Abcam, Cambridge, MA, USA),
241 anti-Beclin-1 (1:1000, #3738; Cell Signaling Technology, Danvers, MA, USA),
242 anti-SQSTM1/p62 (1:500, #5114; Cell Signaling Technology), anti-BiP/GRP78
243 (1:500, #3183; Cell Signaling Technology), anti-PPAR γ (1:500, ab59256; Abcam),
244 anti-GAPDH (1:4000, #2118; Cell Signaling Technology), and anti- β -actin(1:2000,
245 #4970;Cell Signaling Technology) for overnight at 4°C, followed by incubating with
246 goat anti-rabbit IRDye 800CW secondary antibody (1:20000, 926-32211; Li-Cor
247 Biosciences, Lincoln, NE, USA) or HRP-conjugated anti-rabbit IgG antibody (1:2000,
248 #7074; Cell Signaling Technology). Immunoreactive bands were visualized via
249 Odyssey Infrared Fluorescent Western Blots Imaging System (Li-Cor Bioscience) or
250 enhanced chemiluminescence (Cell Signaling Technology) and quantified via
251 densitometry using Image J (version 1.42, National Institutes of Health).

252 **Immunofluorescence**

253 We used immunofluorescence to analysis the distribution of LC3B in
254 hepatocytes, based on the protocol of Cai et al. [30]. In brief, after the indicated
255 treatments, cells were washed in PBS and fixed in 4% paraformaldehyde at room
256 temperature for 15 min. Then the cells were blocked for 1h in 5% BSA, and followed
257 by incubation with specific primary antibodies rabbit anti-LC3B (1:500, ab51520;
258 Abcam) overnight at 4 °C. The cells were washed thrice with PBS for 5 minutes each
259 time, followed by incubation with a Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647,
260 1:500, ab150079; Abcam) secondary antibody for 60 min at room temperature in the
261 dark. DAPI was used to stain the nucleus of hepatocytes. The images were captured
262 with the laser scanning confocal microscope (Leica) ($\times 40$ objective and $\times 5$ zoom,
263 scale bar: 7.5 μ m), and fluorescence intensity was quantified by software Image J.

264 **Dual luciferase reporter assay for detecting ChoRE at the PPAR γ promoter** 265 **region**

266 Based on the PPAR γ promoter characterized in our recent study [31], we
267 constructed PPAR γ promoter into the pGL3 basic vector with ClonExpressTM II One
268 Step Cloning Kit (Vazyme, Piscataway, NJ, USA). The carbohydrate response
269 element-binding protein (ChREBP) binding sites [carbohydrate response element
270 (ChoRE), 5'-GGCACGTGTG-3'] in the PPAR γ promoter from yellow catfish were
271 predicted by JASPAR database (<http://jaspar.genereg.net/>). The site mutation of the
272 ChoRE was performed with the Quick-Change Site-Directed Mutagenesis Kit

273 (Vazyme). These mutated constructs were named mutation 1 (5'-ATGCGCGCAT -3')
274 and mutation 2 (5'-TAGGGCGCGA-3'). HEK 293T cells were transfected with
275 different plasmids by using Lipofectamine 2000 Transfection Reagent (Thermo Fisher
276 Scientific) in 24-well plates. Cells were collected to assay luciferase activity by the
277 Dual-Luciferase Reporter Assay System (Promega, Minneapolis, MN, USA)
278 according to the manufacturer's instructions.

279 **EMSA (electrophoretic mobility shift assay) analysis for detecting the direct** 280 **binding of ChoRE at the PPAR γ promoter**

281 EMSA analysis for detecting the direct binding of ChoRE at the PPAR γ promoter
282 followed the protocols described in our recent publications [31, 32]. Nuclear proteins
283 were prepared and determined by the bicinchoninic acid method. Each
284 oligonucleotide duplex of ChoRE (5'-AGTGTGGCACGTGTGTAATA-3') was
285 incubated with 10 μ g nuclear extracts according to the manufacturer's instructions.
286 The reaction mixture was incubated at room temperature for 30 min, and then
287 detected by electrophoresis on 6% native polyacrylamide gels and ultimately
288 transferred to a nylon membrane. The transferred DNA was cross-linked to the
289 membrane and detected by chemiluminescence reaction. Competition analyses were
290 performed by using 100-fold of unlabeled oligonucleotide duplex, with or without the
291 mutation. These mutated constructs were named mutation 1
292 (5'-AGTGTATGCGCGCATTAAATA-3') and mutation 2
293 (5'-AGTGTTAGGGCGCGATAATA-3').

294 **Statistical analysis**

295 Results were expressed as mean \pm S.E.M.. Firstly, the normality of the different
296 treatments was evaluated with the Shapiro-Wilk test. Then, all data were evaluated by
297 one-way analysis of variance and further analyzed by post hoc Duncan's multiple
298 range testing to determine statistical significance. For the comparison between two
299 groups, Student's *t* tests were used (unpaired, two-tailed). The analysis was performed
300 with SPSS 19.0 (IBM, Armonk, NY, USA). *P* < 0.05 was considered significant.

301

302 **Results**

303 **Growth performance, morphological parameters and serum glucose**

304 In the present study, the survival was 100% among the three treatments
305 (Supplementary Table 3). WG, SGR and FI (feed intake) increased with dietary
306 carbohydrate levels. However, the FCR (feed conversion rate) declined with the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

307 increase of dietary carbohydrate supplementation ($P < 0.05$). HSI tended to increase
308 with dietary carbohydrate levels but the differences were not statistically significant
309 among three treatments. VSI and VAI showed no significant differences among three
310 treatments.

311 **HCD increases liver lipid accumulation**

312 The amount of cytoplasmic vacuolation in the liver was highest in HCD group
313 than those in LCD and ICD groups (Supplementary Fig. 1A-C). HCD increased the
314 amount of hepatic lipid droplets (Supplementary Fig. 1D-F) and hepatic TG content
315 (Supplementary Fig. 1G). These observations were further confirmed by the areas
316 quantified for lipid droplets in the H&E and Oil Red O staining (Supplementary Fig.
317 1H and I).

318 **HCD increased lipogenesis and reduced lipolysis and fatty acid β -oxidation**

319 Activities of lipogenic enzymes (G6PD, 6PGD and FAS) increased with dietary
320 carbohydrate levels, but ICDH and ME activities showed no marked differences
321 among the three treatments (Supplementary Fig. 2A). Glucose transporter 2 (GLUT2)
322 is a high capacity transporter expressed in the livers [33]. The hepatic GLUT2 mRNA
323 abundances for fish fed the HCD (Supplementary Fig. 2B) were higher than those in
324 the LCD and ICD groups. The mRNA abundances of lipogenic genes (G6PD, FAS
325 and ACC α), and the transcriptional factors related to lipogenesis (PPAR γ , SREBP-1,
326 LXR α and ChREBP) for fish fed the HCD (Supplementary Fig. 2C) were higher than
327 those in the LCD and ICD groups. 6PGD mRNA levels tended to increase with
328 dietary carbohydrate levels. mRNA abundances of the lipolytic genes CPT1A and
329 HSL for fish fed the HCD were lower than those in the LCD and ICD groups. **The**
330 **mRNA abundances of mitochondria (ACADL, ACAD8) and peroxisomal β -oxidation**
331 **(ACAA2) for fish fed the HCD were lower than those in the LCD group, but the**
332 **mRNA level of ACADVL, ACADSB, HADHAB, ECSH1, ACOX1 and ACAA1**
333 **showed no marked differences among the three treatments (Supplementary Fig. 2D).**

334 **HCD triggers hepatic ER stress, UPR and oxidative stress**

335 To determine whether dietary carbohydrate cause ER stress, UPR and oxidative
336 stress, thereby mediating the change of lipid metabolism, we first examined the
337 ultrastructural changes of the liver. HCD caused the swelling of ER (Fig. 1A),
338 indicating the occurrence of ER stress. Then, we examined if HCD influenced the
339 mRNA expression of marker genes of ER stress and UPR (Fig. 1B). The mRNA
340 expression of liver GRP78/BiP were significantly increased in HCD group, further

1 341 confirming HCD-induced occurrence of ER stress. The mRNA levels of hepatic
2 342 PERK, eIF2 α and ATF6 were significantly higher in the HCD group than those in
3 343 LCD and ICD groups, indicating the HCD-induced activation of PERK–eIF2 α and
4 344 ATF6 pathways. Western blotting analysis indicated that the protein levels of ER
5 345 stress markers (GRP78) were significantly higher in HCD group than those in ICD
6 346 groups (Fig. 1C and D). SOD activities and the ratio of GSH/GSSG decreased with
7 347 dietary carbohydrate levels (Fig. 1E and G), and that the MDA levels increased with
8 348 dietary carbohydrate addition (Fig. 1F), indicating the activation of oxidative stress.

14 349 **HCD increased hepatic autophagosome formation**

16 350 Given the evidence that autophagy regulates lipid metabolism [6], we
17 351 investigated the effect of HCD on autophagy. TEM observations demonstrated that
18 352 HCD increased the number of LDs, which was accompanied by increased
19 353 autophagosome formation (Fig. 2A-C). Some LDs were combined with the
20 354 autolysosome, indicating a direct interaction between autophagy and lipid metabolism
21 355 (autophagy-mediated lipophagy). The mRNA levels of autophagy-related genes
22 356 (ATG1A, ATG6, ATG4A, ATG5, ATG7, ATG8A, ATG8B, ATG9A, ATG9B and p62)
23 357 were significantly higher in HCD group than those in LCD group (Fig. 2D). Moreover,
24 358 the protein levels of autophagy markers (LC3B-II and Beclin-1) were significantly
25 359 higher in HCD group than those in LCD group (Fig. 2E and F). These results
26 360 confirmed that HCD induced autophagy.

36 361 **Lipophagy mediates high glucose (HG)-induced changes of lipid metabolism**

38 362 To gain insight into the mechanisms of lipophagy mediating the high
39 363 glucose-induced change in lipid metabolism, we isolated the primary hepatocytes
40 364 from yellow catfish and conducted several *in vitro* experiments. The MTT assay
41 365 showed that glucose concentrations of 5-25 mM had no significant influence on the
42 366 viability of hepatocytes (Supplementary Fig. 3A). Thus, we choose the dose of 10mM
43 367 glucose as HG (high glucose) group in the *in vitro* experiments and the concentration
44 368 showed optimum response in TG accumulation and also is also a physiologically
45 369 relevant dose in the plasma of yellow catfish fed the HCD. HG incubation increased
46 370 GLUT2 mRNA expression and TG content (Supplementary Fig. 3B and C). HG also
47 371 up-regulated mRNA expression of Plin2 and Plin3 (the LD-specific coat protein)
48 372 (Supplementary Fig. 3D). Bodipy 493/503 staining further confirmed that HG
49 373 incubation led to elevated LD accumulation (Supplementary Fig. 3E and F).

60 374 Then, we used seven methods to confirm the HG-induced autophagosome

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

375 formation. MDC is widely used as a probe to label autophagic vacuoles whereas
376 Lyso-Tracker Red is used for labeling lysosomes specifically. Depending on the
377 acidity of AO, autophagic lysosomes presented as orange/red fluorescent vesicles,
378 whereas nuclei appeared green [28]. AO, MDC, and Lyso-Tracker Red staining
379 demonstrated that HG incubation increase intracellular acidic compartments
380 (Supplementary Fig. 4A-G). Co-staining with MDC and Lyso-Tracker Red
381 demonstrated that HG increased autophagosomes (blue dots), autolysosomal (red dots)
382 formation and autophagic flux (purple dots), indicating HG-induced increase of
383 autophagic flux (Supplementary Fig. 4G). Meanwhile, TEM observation confirmed
384 that the intracellular acidic vesicles correlated with the formation of autophagosomes
385 and that HG incubation increased autophagosomes (Fig. 3A). Immunoblot analysis of
386 Beclin-1 and LC3B-II (autophagosome marker), and p62/SQSTM1 (autophagic flux
387 protein) revealed that HG up-regulated the protein expression of Beclin-1 and
388 LC3B-II, and down-regulated P62 protein expression (Fig. 3B and C), indicating that
389 HG increased autophagosome formation and autophagic flux. Besides, we used
390 immunofluorescence to assess autophagosome marker LC3, and found HG-induced
391 increase of fluorescence intensity (Fig. 3D and E). Overall, these data confirmed that
392 HG increased autophagosome formation.

393 To detect the role autophagy plays in regulating HCD-induced changes of lipid
394 deposition, we treated cells with two autophagy inhibitors which block autophagy via
395 distinct mechanisms: 3-methyladenine (3-MA, suppressing the activity of class III
396 PI3K) and CQ (inhibition of lysosome function). 3-MA or CQ incubation showed no
397 significant effect on cell viability (Supplementary Fig. 5A), and intracellular TG
398 contents (Supplementary Fig. 5B). However, 3-MA and CQ pre-treatment tended to
399 increase the HG-induced TG deposition although the differences did not reach
400 statistical significance in the CQ pre-treated group. Bodipy 493/503 staining
401 confirmed that 3-MA and CQ pre-incubation increased green mean fluorescence
402 intensity and the number and size of LDs induced by HG (Supplementary Fig. 5C and
403 K). Meanwhile, we quantified autophagy by flow cytometric analysis of the red/green
404 fluorescence ratio using AO staining. 3-MA or CQ pretreatment alleviated the
405 HG-induced increase in the red: green fluorescence ratio (Supplementary Fig. 5F and
406 G). Confocal microscopic images of hepatocytes stained with AO indicated that 3-MA
407 or CQ pretreatment alleviated the HG-induced autophagy (Supplementary Fig. 5J).
408 MDC staining showed that 3-MA pretreatment alleviated but CQ pretreatment

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

409 up-regulated the HG-induced increase in the fluorescence density (Supplementary Fig.
410 5D). This is understandable because 3-MA and CQ inhibited different processes of
411 autophagy. 3-MA is an early-stage autophagy inhibitor and suppresses the activity of
412 class III PI3K, and CQ is a late-stage autophagy inhibitor that blocks fusion of
413 autophagosomes with lysosomes. Lyso-Tracker Red staining showed that 3-MA or
414 CQ pretreatment alleviated the HG-induced increase in the fluorescence density
415 (Supplementary Fig. 5E), and alleviated the HG-induced autophagy (Supplementary
416 Fig. 5I). Taken together, all of these observations prove that autophagy mediated
417 HG-induced changes of lipid deposition.

418 **Lipophagy mediated HG-induced changes in lipid metabolism**

419 To determine whether LDs are associated with lipophagy, we performed the
420 co-localization studies of autophagosomes, lysosomes and LDs. The colocalization of
421 the autolysosomes and the LDs was observed in hepatocytes co-stained with
422 Lyso-Tracker Red (red) and Bodipy 493/503 (green). HG induced the increase of
423 LDs' amounts and the colocalization of the autolysosomes (red) and the LDs (green),
424 which indicated the induction of lipophagy (yellow) (Fig. 4A and B). TEM analysis
425 confirmed that LDs integrated with the autolysosomes in HG-treated cells, indicating
426 the occurrence of HG-induced lipophagy (Fig. 4C). To further confirm the role of
427 lipophagy in HG-induced changes of lipid metabolism, we analyzed some parameters
428 involved in lipid metabolism (Fig. 4D-G). HG induced the increases in intracellular
429 NEFA content, indicating an increased flux in TG breakdown from LDs (Fig. 4E). HG
430 incubation also up-regulated the mRNA levels of fatty acid binding protein liver-B,
431 long-chain fatty acyl-CoA synthetases (ACSL1 and -4), acyl-CoA binding proteins
432 (ACBP-4 and -5), consistent with enhanced NEFA release (Fig. 4D). However, HG
433 treatment decreased CPT I activity (lipolytic enzyme) and mRNA expression of
434 CPT1A, and increased the activities of 6PGD, G6PD, ME, and FAS (lipogenic
435 enzymes) (Fig. 4D, F and G). These indicated that HG activated lipogenesis and
436 inhibited lipolysis which in turn increased TG synthesis, consistent with increased
437 flux in TG breakdown from LDs, indicating that lipophagy plays a regulatory role in
438 the dynamic balance of lipogenesis and lipolysis.

439 **Oxidative stress mediated HG-induced lipophagy and lipid accumulation**

440 In an attempt to elucidate the mechanism of oxidative stress mediating
441 HG-induced lipophagy and HG-induced changes of lipid metabolism, we used NAC
442 (ROS scavenger) to block oxidative stress pathway. First, NAC did not significantly

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
443 influence the viability (Fig. 5A) of hepatocytes. NAC pre-treatment abrogated the
444 HG-induced reduction in SOD activity and the ratio of GSH/GSSG, and HG-induced
445 increase in MDA and ROS contents (Fig. 5B-E, and O). Also, NAC pretreatment
446 alleviated the HG-induced increase in the contents of TG and NEFA, activities of
447 lipogenic enzymes (G6PD, 6PGD, and FAS), and alleviated HG-induced reduction in
448 CPT I activity (lipolytic enzyme) (Fig. 5F-M). Meantime, NAC pre-treatment
449 alleviated the HG-induced increase in the mRNA expression of lipogenic genes and
450 transcription factors (G6PD, 6PGD, ACCa, FAS, SREBP-1, PPAR γ , and ChREBP),
451 and alleviated the HG-induced reduction in mRNA expression of lipolytic genes (LPL,
452 and CPT1A) (Supplementary Fig. 6A). Furthermore, NAC pretreatment alleviated the
453 HG-induced increase in the red: green fluorescence ratio (Fig. 5N and P) and in
454 relative mRNA expression of autophagy related genes (Supplementary Fig. 6B).
455 Overall, these data demonstrate that the oxidative stress played an important role in
456 the HG-induced activation of lipophagy and HG-induced changes of lipid
457 metabolism.

458 ER stress was involved in HG-induced lipophagy activation and lipid metabolism

27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
459 Next, we explored the mechanism of ER stress mediating HG-induced changes
460 of lipid metabolism and lipophagy. Firstly, TEM observation revealed that HG
461 treatment induced swelling and altered morphology of ER (Fig. 6A). ER is the main
462 depot for intracellular free Ca²⁺ and Ca²⁺ will be released from ER to cytoplasm when
463 ER stress occurs. We found that HG treatment led to the time-dependent increase in
464 Ca²⁺ fluxes (Fig. 6B and C), and up-regulated the protein expression of GRP78/Bip
465 (ER stress marker protein) (Fig. 6D and E). These results confirmed that HG induced
466 ER stress. As a chemical chaperone, 4-PBA was reported to alleviate ER stress [30].
467 We found that 4-PBA had no marked effect on cell viability (Fig. 6F). However,
468 4-PBA pretreatment significantly suppressed the HG-induced increase in mRNA
469 expression of GRP78, PERK, eIF2 α , ATF4 and IRE1 α (Supplementary Fig. 7A).
470 4-PBA pretreatment reduced HG-induced increase of TG content although the
471 differences were not statistically significant (Fig. 6G). Furthermore, 4-PBA
472 pretreatment markedly abolished the HG-induced increase in activities of lipogenic
473 enzymes (G6PD and FAS) (Fig. 6H-L), but had no significant effect on HG-induced
474 reduction in CPT I activity (lipolytic enzyme) (Fig. 6M). 4-PBA pre-treatment also
475 alleviated the HG-induced increase in mRNA expression of lipogenic genes and
476 transcription factors (6PGD, ACCa, FAS, SREBP-1, PPAR γ , and ChREBP), and

477 HG-induced reduction in mRNA expression of lipolytic PPAR α (Supplementary Fig.
478 7B). In addition, 4-PBA pretreatment alleviated the HG-induced increase in the red:
479 green fluorescence ratio (Fig. 6N and O) and mRNA expression of autophagy related
480 genes (Supplementary Fig. 7C). Taken together, all these results support that ER stress
481 mediated HG-induced lipophagy activation and changes of lipid accumulation.

482 **HG triggers the activation of lipophagy and changes of lipid metabolism through** 483 **PPAR γ pathway**

484 To determine whether HG influenced mRNA expression of lipogenesis-related
485 transcription factors, the mRNA levels of PPAR γ , SREBP-1, PPAR α , and ChREBP
486 were measured (Supplementary Fig. 6A). PPAR γ was the key integrator of lipid
487 metabolism and autophagy [20, 34]. The present study indicated that HG significantly
488 up-regulated the mRNA and protein levels of PPAR γ (Fig. 7A-C). Next, we used
489 T0070907 (PPAR γ specific inhibitor) to explore the effects of PPAR γ signaling on
490 lipid metabolism and autophagy. As expected, T0070907 pretreatment abolished the
491 HG-induced TG accumulation (Fig. 7D) and alleviated HG-induced increase in the
492 mRNA levels of lipogenic genes (G6PD, 6PGD, ACCa, FAS, SREBP-1 and ChREBP)
493 (Supplementary Fig. 8A). T0070907 pretreatment also alleviated HG-induced
494 increase in mRNA expression of genes involved in autophagosome membrane
495 initiation (ATG1B and ATG6), autophagosome membrane expansion (ATG3, ATG4B
496 and ATG5), vesicle recycling (ATG13 and ATG9A) and cargo recruitment (ATG8B
497 and p62) (Supplementary Fig. 8B). Additionally, T0070907 pretreatment alleviated
498 HG-induced increase of the red: green fluorescence ratio (Fig. 7 E and F). All of these
499 results indicated that PPAR γ mediated HG-induced lipophagy activation and changes
500 of lipid metabolism.

501 In an attempt to elucidate the mechanisms of HG activating lipophagy- and lipid
502 metabolism- relevant genes at the transcriptional level, we further explored how HG
503 modulated PPAR γ signaling. ChREBP has emerged as a major mediator of
504 intracellular glucose-sensory transcriptional activator and regulates gene expression
505 by binding to the ChoRE motif of target genes [35]. By analyzing the promoter
506 regions of PPAR γ obtained in our laboratory [31], we found that ChoRE was located
507 at -534 to -515 bp of PPAR γ promoter region of yellow catfish, which consisted of the
508 evolutionarily conserved core sequence CACGTG (Fig. 7G). Thus, we used luciferase
509 reporter system to estimate whether HG could transcriptionally regulate the PPAR γ
510 promoter activity, and site-mutation analysis of ChREBP binding sites on

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
511 pGI3-PPAR γ -784/+63 vectors (mutations 1 and 2) was used to estimate the
512 importance of the putative ChoRE sequences. Interestingly, our results revealed that
513 HG incubation remarkably enhanced the luciferase activity of PPAR γ promoter (Fig.
514 7H), and the mutation of ChoRE significantly reduced the luciferase activity of the
515 PPAR γ promoter. The mutation of site 1 (Mutation-1) did not significantly affect the
516 HG-induced changes of the relative luciferase activity, but the mutation-2 at ChoRE
517 binding site suppressed the HG-induced increase of luciferase activity, suggesting that
518 ChREBP transactivated PPAR γ by binding to the ChoRE motif in the PPAR γ
519 promoter region. Thus, the ChoRE motif was important for the HG-induced mRNA
520 expression of PPAR γ . Next, we used EMSA assay to determine whether ChREBP can
521 directly bind with the promoter region of PPAR γ . Our EMSA analysis indicated that
522 the putative ChoRE sequences of the PPAR γ promoter could directly bind with
523 nuclear extract; the direct interaction can be disrupted by unlabeled wild-type and
524 restored by the mutant probes (Fig. 7I). Moreover, HG incubation can significantly
525 enhance the binding activity of ChREBP to ChoRE (Fig. 7I, lane 6), suggesting that
526 the -534 to -515 bp region of PPAR γ promoter could react with ChREBP. Together,
527 these evidences confirmed that HG-activated lipophagy and lipid metabolism occurs
528 via enhancing ChREBP DNA binding to the PPAR γ promoter region.

529 Discussion

34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
530 In agreement with many other studies [35, 36], our study indicated that high
531 dietary carbohydrate supplementation induced the lipid accumulation. Furthermore,
532 our result indicated that HCD increased hepatic TG accumulation via the upregulation
533 of lipogenesis and the downregulation of lipolysis. Similarly, Postic et al. [35]
534 reported that a diet rich in carbohydrate stimulates lipogenic pathways. Here,
535 increasing dietary carbohydrate levels also up-regulate dietary energy content, which
536 in turn will increase lipid deposition and influence lipid metabolism, as reported in
537 other studies [23,37].

49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
538 Autophagy is important for regulating energy homeostasis and lipid content in
539 hepatocytes [6]. The present study indicated that high carbohydrate induced
540 autophagosome formation and that autophagy mediated HG-induced changes of lipid
541 metabolism. Similarly, Gou et al. [38] showed that the expression of LC3B-II was
542 markedly increased, and the autophagic vacuoles in cytoplasm was markedly
543 accumulated when the HK2 cells were treated with high glucose. Emerging evidence
544 indicates that LC3B-II, an autophagosome protein, is co-localized with lipid droplets;

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

545 lipid droplet-specific autophagy has since been termed lipophagy [6]. The present
546 study found that HG incubation activated lipophagy and increased intracellular NEFA
547 content, indicating an increased flux in TG breakdown from LDs. Thus, our study
548 indicated that lipophagy regulated HG-induced changes in lipid metabolism.

549 In order to explore the mechanism of high carbohydrate addition inducing
550 autophagy, we determine the HG-induced changes of oxidative and ER stress. Our
551 data indicated that ROS and ER stress mediated the HG-induced autophagy. Similarly,
552 Wang et al. [39] pointed out that ER stress triggered autophagy. Other studies pointed
553 out that ER stress was involved in lipid accumulation [29, 40], which was also
554 observed in the present study. Oxidative stress and ROS production together with ER
555 stress caused lipid peroxidation and resulted in autophagy through several distinct
556 mechanisms involving autophagy-related genes and antioxidant enzymes [11]. The
557 decreased GSH/GSSG ratio and SOD activity were hallmarks of oxidative stress and
558 is involved in autophagy [41, 42]. Chen et al. [41] indicated that increasing oxidative
559 stress upregulated autophagy. Zhao et al. [43] pointed out that oxidative stress
560 mediated fructose-induced TG deposition in BRL-3A cells. In the present study,
561 pretreatment with antioxidant NAC impaired the reduction in GSH/GSSG ratio and
562 SOD activity, and significantly abrogated ROS production, and in turn, prevented
563 autophagy activation and lipid accumulation, suggesting the important role of
564 oxidative stress in mediating HG-induced lipophagy and lipid metabolism.

565 Next, we further explored the direct relationship between HG-induced lipophagy
566 activation and the HG-induced changes of lipid metabolism. Studies pointed out that
567 PPAR γ was a key regulator of the transcriptional control of genes involved in lipid
568 metabolism and autophagy [20, 34]. PPAR γ activation can induce autophagy and lipid
569 accumulation [20, 44]. Our present study found that HG upregulated the mRNA and
570 protein expression of PPAR γ ; Moreover, HG-activated lipophagy and lipid
571 accumulation occurs via enhancing ChREBP DNA binding to the PPAR γ promoter
572 region, which in turn induced transcriptional activation of the key autophagy- and
573 lipogenesis-related genes [44, 45]. ChREBP is considered to be a pivotal sensor
574 protein of intracellular glucose, specifically binding to the ChoRE promoter region of
575 its downstream target genes [35]. Postic et al. [35] showed that ChREBP was required
576 for the carbohydrate-induced transcriptional activation of enzymes involved in TG
577 synthesis. Therefore, ChREBP has emerged as a major mediator of glucose action on
578 lipogenic gene expression and appears to act as a central “bridge” which connected

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

579 the glucose and metabolic process. Here, we identified the putative ChoRE motif
580 (5'-GGCACGTGTG-3') in the PPAR γ promoter region at -534 and -515 bp.
581 Moreover, the dual luciferase reporter assay and EMSA assay revealed a direct link
582 between ChREBP and PPAR γ , which indicated that endogenous ChREBP protein was
583 recruited to the putative binding sites of PPAR γ .

584 **Conclusion**

585 Combining our data, we proposed a model, suggesting a novel mechanism of
586 high carbohydrate diets inducing lipid accumulation (Supplementary Fig. 9). HCD
587 induced lipogenesis and suppressed lipolysis, and activated lipophagy, oxidative and
588 ER stress; lipophagy mediated HCD-induced changes of lipid metabolism via
589 oxidative and ER stress pathways. Meanwhile, HG enhanced the ChREBP DNA
590 binding capability at the ChoRE of PPAR γ promoter region, which in turn induced
591 transcriptional activation of the key autophagy- and lipogenesis-related genes. It is
592 noteworthy to point out that high dietary carbohydrate increased energy level, which
593 potentially influences lipid metabolism and lipophagy. The mechanism of dietary
594 energy levels influencing lipid metabolism and lipophagy remained to be investigated.

595 **Acknowledgments**

596 This work was supported by the National Key R&D Program of China
597 (2018YFD0900400) and Fundamental Research Funds for the Central Universities,
598 China (grant nos. 2662018PY089).

599 **Disclosure of potential conflicts of interest**

600 No potential conflicts of interest were disclosed.

601 **Authors' contributions**

602 Z.L. and T.Z. designed the experiments. T.Z. carried out animal and cell
603 experiments and sample analysis with the help of K.W., Y.H.X., G.H.C., and C.C.W.;
604 T.Z., Z.L. and C.H. analyzed data; T.Z. wrote the manuscript, and Z.L. and C.H.
605 revised the manuscript. All the authors read and approved the manuscript.

606 **References**

- 607 1. Fabbrini E, Sullivan S, Klein S (2010) Obesity and nonalcoholic fatty liver
608 disease: biochemical, metabolic, and clinical implications. *Hepatology*
609 51:679–689
- 610 2. Farrell GC, Larter CZ (2006) Non-alcoholic fatty liver: from steatosis to cirrhosis.
611 *Hepatology* 43:S99–S112
- 612 3. Abdelmalek MF, Suzuki A, Guy C, Unalp-Arida A, Colvin R, Johnson RJ, Diehl
613 AM (2010) Increased fructose consumption is associated with fibrosis severity in
614 patients with nonalcoholic fatty liver disease. *Hepatology* 51:1961-1971

- 615 4. Neuschwander-Tetri BA (2013) Carbohydrate intake and nonalcoholic fatty liver
616 disease. *Curr Opin Clin Nutr* 16:446-452
- 617 5. Levine B, Klionsky DJ (2004) Development by self-digestion: molecular
618 mechanisms and biological functions of autophagy. *Dev Cell* 6:463-477
- 619 6. Singh R, Kaushik S, Wang YJ, Xiang YQ, Novak I, Komatsu M, Tanaka K,
620 Cuervo AM, Czaja MJ (2009) Autophagy regulates lipid metabolism. *Nature*
621 458:1131-1135
- 622 7. Bechmann LP, Hannivoort RA, Gerken G, Hotamisligil GS, Trauner M, Canbay A
623 (2012) The interaction of hepatic lipid and glucose metabolism in liver diseases. *J*
624 *Hepatol* 56:952-964
- 625 8. Dong HQ, Czaja MJ (2011) Regulation of lipid droplets by autophagy. *Trends*
626 *Endocrinol Metab* 22:234-240
- 627 9. Singh R, Cuervo AM (2012) Lipophagy: connecting autophagy and lipid
628 metabolism. *Int J Cell Biol* 2012:282041
- 629 10. Lavallard VJ, Gual P (2014) Autophagy and non-alcoholic fatty liver disease.
630 *Biomed Res Int* 2014:120179
- 631 11. Azad MB, Chen YQ, Gibson SB (2009) Regulation of autophagy by reactive
632 oxygen species (ROS): implications for cancer progression and treatment.
633 *Antioxid Redox Signal* 11:777-790
- 634 12. Kawai D, Takaki A, Nakatsuka A, Wada J, Tamaki N, Yasunaka T, Koike K,
635 Tsuzaki R, Matsumoto K, Miyake Y, et al (2012) Hydrogen- rich water prevents
636 progression of nonalcoholic steatohepatitis and accompanying
637 hepatocarcinogenesis in mice. *Hepatology* 56:912-921
- 638 13. Panieri E, Santoro MM (2016) ROS homeostasis and metabolism: a dangerous
639 liason in cancer cells. *Cell Death Dis* 7:e2253
- 640 14. Pan YX, Luo Z, Zhuo MQ, Wei CC, Chen GH, Song YF (2018) Oxidative stress
641 and mitochondrial dysfunction mediated Cd-induced hepatic lipid accumulation
642 in zebrafish *Danio rerio*. *Aquat Toxicol* 199:12-20
- 643 15. Wang H, Sun RQ, Zeng XY, Zhou X, Li SP, Jo E, Molero JC, Ye JM (2015)
644 Restoration of autophagy alleviates hepatic ER stress and impaired insulin
645 signalling transduction in high fructose-fed male mice. *Endocrinology*
646 156:169-181
- 647 16. Madaro L, Marrocco V, Carnio S, Sandri M, Bouché M (2013) Intracellular
648 signaling in ER stress-induced autophagy in skeletal muscle cells. *FASEB J*
649 27:1990-2000
- 650 17. Rutkowski DT, Wu J, Back SH, Callaghan MU, Ferris SP, Iqbal J, Clark R, Miao
651 H, Hassler JR, Fornek J, et al (2008) UPR pathways combine to prevent hepatic
652 steatosis caused by ER stress-mediated suppression of transcriptional master
653 regulators. *Dev Cell* 15:829-840
- 654 18. Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, Maeda
655 N, Krisans SK, Malinow MR, et al (2001) Homocysteine-induced endoplasmic
656 reticulum stress causes dysregulation of the cholesterol and triglyceride
657 biosynthetic pathways. *J Clin Invest* 107:1263-1273
- 658 19. Patel L, Pass I, Coxon P, Downes CP, Smith SA, Macphee CH (2001) Tumor
659 suppressor and anti-inflammatory actions of PPAR γ agonists are mediated via
660 upregulation of PTEN. *Curr Biol* 11:764-768
- 661 20. Zheng JL, Zhuo MQ, Luo Z, Pan YX, Song YF, Huang C, Zhu QL, Hu W, Chen Q
662 L (2015) Peroxisome proliferator-activated receptor gamma (PPAR γ) in yellow
663 catfish *Pelteobagrus fulvidraco*: molecular characterization, mRNA expression
664 and transcriptional regulation by insulin *in vivo* and *in vitro*. *Gen Comp Endocr*

665 212:51-62

- 1 666 21. Meyer A, Van de Peer Y (2005) From 2R to 3R: evidence for a fish-specific
2 667 genome duplication (FSGD). *Bioessays* 27:937–45.
- 3 668 22. Gong G, Dan C, Xiao S, Guo W, Huang P, Xiong Y, Wu J, He Y, Zhang J, Li X, et
4 669 al (2018) Chromosomal-level assembly of yellow catfish genome using
5 670 third-generation DNA sequencing and Hi-C analysis. *GigaScience* 7 doi:
6 671 10.1093/gigascience/giy120.
- 7 672 23. Ye WJ, Tan XY, Chen YD, Luo Z (2009) Effects of dietary protein to carbohydrate
8 673 ratios on growth and body composition of juvenile yellow catfish, *Pelteobagrus*
9 674 *fulvidraco* (Siluriformes, Bagridae, *Pelteobagrus*). *Aquac Res* 40:1410-1418
- 10 675 24. Wei CC, Luo Z, Hogstrand C, Xu YH, Wu LX, Chen GH, Pan YX, Song YF
11 676 (2018) Zinc reduces hepatic lipid deposition and activates lipophagy via
12 677 Zn^{2+} /MTF-1/PPAR α and Ca^{2+} /CaMKK β /AMPK pathways. *FASEB J* 32:
13 678 6666-6680
- 14 679 25. Yang SB, Tan XY, Zhang DG, Cheng J, Luo Z (2018) Identification of ten
15 680 SUMOylation-related genes from yellow catfish *Pelteobagrus fulvidraco*, and
16 681 their transcriptional responses to carbohydrate addition *in vivo* and *in vitro*. *Front*
17 682 *Physiol* 9:1544
- 18 683 26. Wu K, Luo Z, Hogstrand C, Chen GH, Wei CC, Li DD (2018) Zn stimulates the
19 684 phospholipids biosynthesis via the pathways of oxidative and endoplasmic
20 685 reticulum stress in the intestine of freshwater teleost yellow catfish. *Environ Sci*
21 686 *Technol* 52:9206-9214
- 22 687 27. Giustarini D, Dalle-donne I, Milzani A, Fanti P, Rossi R (2013) Analysis of GSH
23 688 and GSSG after derivatization with n-ethylmaleimide. *Nat Protoc* 8:1660-1669
- 24 689 28. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Arozena AA,
25 690 Adachi H, Adams CM, Adams PD, Adeli K, et al (2016) Guidelines for the use
26 691 and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*
27 692 12: 1-222
- 28 693 29. Song YF, Luo Z, Zhang LH, Hogstrand C, Pan YX (2016) Endoplasmic reticulum
29 694 stress and disturbed calcium homeostasis are involved in copper-induced
30 695 alteration in hepatic lipid metabolism in yellow catfish *Pelteobagrus fulvidraco*.
31 696 *Chemosphere* 144:2443-2453
- 32 697 30. Cai XY, Liu YL, Hu YQ, Liu XZ, Jiang HY, Yang SH, Shao Z, Xia Y, Xiong L
33 698 (2018) Ros-mediated lysosomal membrane permeabilization is involved in
34 699 bupivacaine-induced death of rabbit intervertebral disc cells. *Redox Biol*
35 700 18:65-76
- 36 701 31. Wu K, Tan XY, Xu YH, Chen GH, Zhuo MQ (2018) Functional analysis of
37 702 promoters of genes in lipid metabolism and their transcriptional response to
38 703 STAT3 under leptin signals. *Genes* 9:334
- 39 704 32. Xu YH, Luo Z, Wu K, Fan YF, You WJ, Zhang LH (2017) Structure and
40 705 functional analysis of promoters from two liver isoforms of CPT I in grass carp
41 706 *Ctenopharyngodon idella*. *Int J Mol Sci* 18:E2405
- 42 707 33. Thorens B (1996) Glucose transporters in the regulation of intestinal, renal, and
43 708 liver glucose fluxes. *Am J Physiol* 270:G541-G553
- 44 709 34. Assumpção JAF, Magalhães KG, Corrêa JR (2017) The role of ppar γ and
45 710 autophagy in ros production, lipid droplets biogenesis and its involvement with
46 711 colorectal cancer cells modulation. *Cancer Cell Int* 17:82.
- 47 712 35. Postic C, Dentin R, Denechaud PD, Girard J (2007) ChREBP, a transcriptional
48 713 regulator of glucose and lipid metabolism. *Annu Rev Nutr* 27:179-192
- 49 714 36. Chen B, Zheng YM, Zhang JP (2018) Comparative study of different

715 diets-induced NAFLD models of zebrafish. *Front Endocrinol* 9:366

1 716 37. Dias J, Alvarez MJ, Diez A, Arzel J, Corraze G, Bautista JM, Kaushik SJ (1998)

2 717 Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European

3 718 sea bass (*Dicentrarchus labrax*). *Aquaculture* 161: 169-186.

4 719 38. Gou R, Chen JT, Sheng SF, Wang RQ, Fang YD, Yang ZJ, Wang L, Tang L (2016)

5 720 Kim-1 mediates high glucose-induced autophagy and apoptosis in renal tubular

6 721 epithelial cells. *Cell Physiol Biochem* 38:2479-2488

7 722 39. Wang H, Sun RQ, Camera D, Zeng XY, Jo E, Chan SM, Herbert TP, Molero JC,

8 723 Ye JM (2016) Endoplasmic reticulum stress up-regulates Nedd4-2 to induce

9 724 autophagy. *FASEB J* 30:2549-2556

10 725 40. Lebeaupin C, Vallée D, Hazari Y, Hetz C, Chevet E, Bailly-Maitre B (2018)

11 726 Endoplasmic reticulum stress signaling and the pathogenesis of non-alcoholic

12 727 fatty liver disease. *J Hepatol* 69:927-947

13 728 41. Chen Y, Azad MB, Gibson SB (2009) Superoxide is the major reactive oxygen

14 729 species regulating autophagy. *Cell Death Differ* 16:1040-1052

15 730 42. Zhang Z, Guo M, Zhao S, Shao J, Zheng S (2016) ROS-JNK1/2-dependent

16 731 activation of autophagy is required for the induction of anti-inflammatory effect

17 732 of dihydroartemisinin in liver fibrosis. *Free Radic Biol Med* 101:272-283

18 733 43. Zhao, XJ, Yu, HW, Yang, YZ, Wu, WY, Chen, TY, Jia, KK, Kang, LL, Jiao, RQ,

19 734 Kong, LD (2018) Polydatin prevents fructose-induced liver inflammation and

20 735 lipid deposition through increasing miR-200a to regulate Keap1/Nrf2 pathway.

21 736 *Redox Biol* 18:124-137

22 737 44. Rovito D, Giordano C, Vizza D, Plastina P, Barone I, Casaburi I, Lanzino M, De A

23 738 F, Sisci D, Mauro L, et al (2013) Omega-3 PUFA ethanolamides DHEA and EPA

24 739 induce autophagy through PPAR γ activation in MCF-7 breast cancer cells. *J Cell*

25 740 *Physiol* 228:1314-1322

26 741 45. Zhou J, Zhang W, Liang B, Casimiro MC, Whitaker-Menezes D, Wang M, Lisanti

27 742 MP, Lanza-Jacoby S, Pestell RG, Wang C (2009) PPAR γ activation induces

28 743 autophagy in breast cancer cells. *Int J Biochem Cell Biol* 41:2334-2342

29 744

30 745

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure captions:

Fig. 1 Dietary carbohydrate supplementation triggers ER stress, UPR and oxidative stress in the liver of yellow catfish. (A) Liver ultrastructure (TEM, original magnification $\times 10000$, bars, 1 μm). m, mitochondria; nu, hepatocyte nucleus; er, endoplasmic reticulum; sm, swelling and vesiculation of mitochondria; ser, swelling of endoplasmic reticulum. (B) The relative mRNA expression of genes involved in ER stress and UPR. (C) Western blot analysis of ER stress marker GRP78. (D) Relative quantification of protein levels of GRP78 were normalized to GAPDH. (E) Hepatic SOD activity. (F) Hepatic MDA content. (G) The ratio of GSH/GSSG in the liver. Values are mean \pm S.E.M. ($n = 3$ replicate tanks and was used as three biological replicates. At least three fish were sampled for each tank and used as technical replicates). mRNA expression values were normalized to housekeeping genes (β -actin and HPRT) expressed as a ratio of the LCD. *P* value was calculated by one-way ANOVA and further post hoc Duncan's multiple range testing. Values without the same letter indicate significant difference among three treatments ($P < 0.05$).

Fig. 2 Dietary carbohydrate supplementation increases hepatic autophagosome formation of yellow catfish. (A-C) Representative image of liver ultrastructure (TEM, Scale bars, 2 μm). Black arrows represent the autophagosome. (D) Relative mRNA levels of key hepatic autophagy-related genes. (E) Western blot analysis of LC3B and Beclin1. (F) Relative quantification of protein levels of LCB were normalized to β -actin and Beclin1 were normalized to GAPDH. Relative mRNA expression values were normalized to housekeeping genes (β -actin and HPRT) expressed as a ratio of the low carbohydrate diet LCD. All data are expressed as mean \pm S.E.M. ($n = 3$ replicate tanks and was used as three biological replicates. At least three fish were sampled for each tank and used as technical replicates). *P* value was calculated by one-way ANOVA and further post hoc Duncan's multiple range testing. Values without the same letter indicate significant difference among three treatments ($P < 0.05$).

Fig. 3 High glucose concentration activates autophagy of hepatocytes from yellow catfish. The primary hepatocytes from *P. fulvidraco* were incubated in control (5 mM glucose) or HG (10mM glucose) for 48 h in M199 medium. (A) Representative transmission electron microscope image of hepatocytes. Black arrows represent the autophagosome. (B and C) Western blot analysis of LC3B, Beclin1, and P62 protein levels ($n = 3$). (D and E) Representative confocal images and relative red fluorescence

8
9
10
11
12
13
14 784 intensity showing LC3-II protein by immunofluorescence staining. Relative protein
15 785 levels of LC3B, Beclin1, and P62 were normalized to GAPDH. All data are expressed
16 786 as mean \pm S.E.M. (n=3 at least). *P* value was calculated by Student's *t* tests. **P* < 0.05,
17 787 ***P* < 0.01, compared with control.
18
19 788
20 789

21 790 **Fig. 4 Lipophagy, which provided free fatty acids for the synthesis of TG but not**
22 **for mitochondrial β -oxidation, alleviated HG-induced steatosis.** The primary
23 791 hepatocytes from *P. fulvidraco* were incubated in control (5 mM glucose) or HG
24 792 (10mM glucose) for 48 h in M199 medium. (A) The co-localization of the
25 793 autolysosomes and the lipid droplets in hepatocytes co-stained with 50nM
26 794 Lyso-Tracker Red and 5 μ g/ml BODIPY 493/503 (green) indicating the induction of
27 795 lipophagy (yellow). (B) Schematic representation of the colocalization between
28 796 autolysosome and LDs; (C) Representative TEM image of hepatocytes after control or
29 797 HG incubation. Black arrows represent the lipophagy; (D) The mRNA levels of genes
30 798 in the hepatocytes related to the fatty acid metabolism and mitochondria β -oxidation.
31 799 (E) NEFA content in hepatocytes; (F, G) Activities of lipogenic (6PGD, G6PD, ICDH,
32 800 ME and FAS) and lipolytic (CPTI) enzymes in the hepatocytes. Relative mRNA
33 801 expression values were normalized to housekeeping genes (EIFA and RPL7)
34 802 expressed as a ratio of the control. All data are expressed as mean \pm S.E.M. (n=3 at
35 803 least). *P* value was calculated by Student's *t* tests. **P* < 0.05, ***P* < 0.01, compared with
36 804 control.
37 805
38 806

39 807 **Fig. 5 Mitochondrial oxidative stress pathway mediated HG-induced autophagy**
40 808 **and lipid accumulation in the primary hepatocytes of yellow catfish.** The primary
41 809 hepatocytes from *P. fulvidraco* were incubated in control (5mM glucose) or HG
42 810 (10mM glucose) for 48 h in M199 medium with or without 2-h pretreatment with a
43 811 ROS scavenger (0.5mM NAC). (A) Cell viability. (B-D) Activity of T-SOD, MDA
44 812 content, and ratio of GSH/GSSG. (E) The intracellular ROS was quantified by
45 813 calculating FL1 (green) mean fluorescence intensity (DCFH-DA fluorescent staining).
46 814 (F-K) Activities of lipogenic (6PGD, G6PD, ICDH, ME and FAS) and lipolytic (CPTI)
47 815 enzymes. (L) TG content. (M) NEFA content. (N) The autophagy was quantified by
48 816 flow cytometric analysis of red/green (FL2/FL1) fluorescence ratio (acridine orange
49 817 fluorescent staining, 1 μ M). (O) The presence of DCFH-DA-stained intracellular ROS
50 818 was determined by flow cytometry analysis of green fluorescence intensity. (P) The
51 819 presence of acridine orange-stained intracellular autophagic vacuole was determined
52 820 by flow cytometry analysis of red/green (FL2/FL1) fluorescence ratio. All data are
53 821 expressed as mean \pm S.E.M. (n=3 at least). *P* value was calculated by Student's *t* tests.
54
55
56
57
58
59
60
61
62
63
64
65

8
9
10
11
12
13
14 822 * $P < 0.05$, ** $P < 0.01$, compared with control; # $P < 0.05$, ## $P < 0.01$, compared with HG
15 823 group.
16 824

17 825 **Fig. 6 ER stress pathway mediated HG-induced lipid deposition and autophagy**
18 826 **of the primary hepatocytes from yellow catfish.** Hepatocytes were incubated in
19 827 control (5mM glucose) or HG (10mM glucose) for 48 h in M199 medium with or
20 828 without 2-h pretreatment with an ER stress inhibitor (100 μ M 4-PBA). (A)
21 829 Representative TEM images. Black arrow pointing to endoplasmic reticulum. (B)
22 830 Representative confocal microscopy image of hepatocytes stained with Ca²⁺
23 831 fluorescent probe (Fluo-4 AM, 4 μ M), showing a time-dependent changes in green
24 832 fluorescence levels of primary hepatocytes. (C) Schematic represent quantification of
25 833 the Fluo-4 AM staining. (D, E) Western blot analysis of GRP78/Bip protein levels (n
26 834 =3). (F) Cell viability. (G) TG content. (H-M) Activities of lipogenic (6PGD, G6PD,
27 835 ICDH, ME and FAS) and lipolytic (CPTI) enzymes. (N) The autophagy was
28 836 quantified by flow cytometric analysis of red/green (FL2/FL1) fluorescence ratio
29 837 (acridine orange fluorescent staining, 1 μ M). (O) The presence of acridine
30 838 orange-stained intracellular autophagic vacuole was determined by flow cytometry
31 839 analysis of red/green (FL2/FL1) fluorescence ratio. All data are expressed as mean \pm
32 840 S.E.M. (n=3 at least). P value was calculated by Student's t tests. * $P < 0.05$, ** $P < 0.01$,
33 841 compared with control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with HG group.
34 842

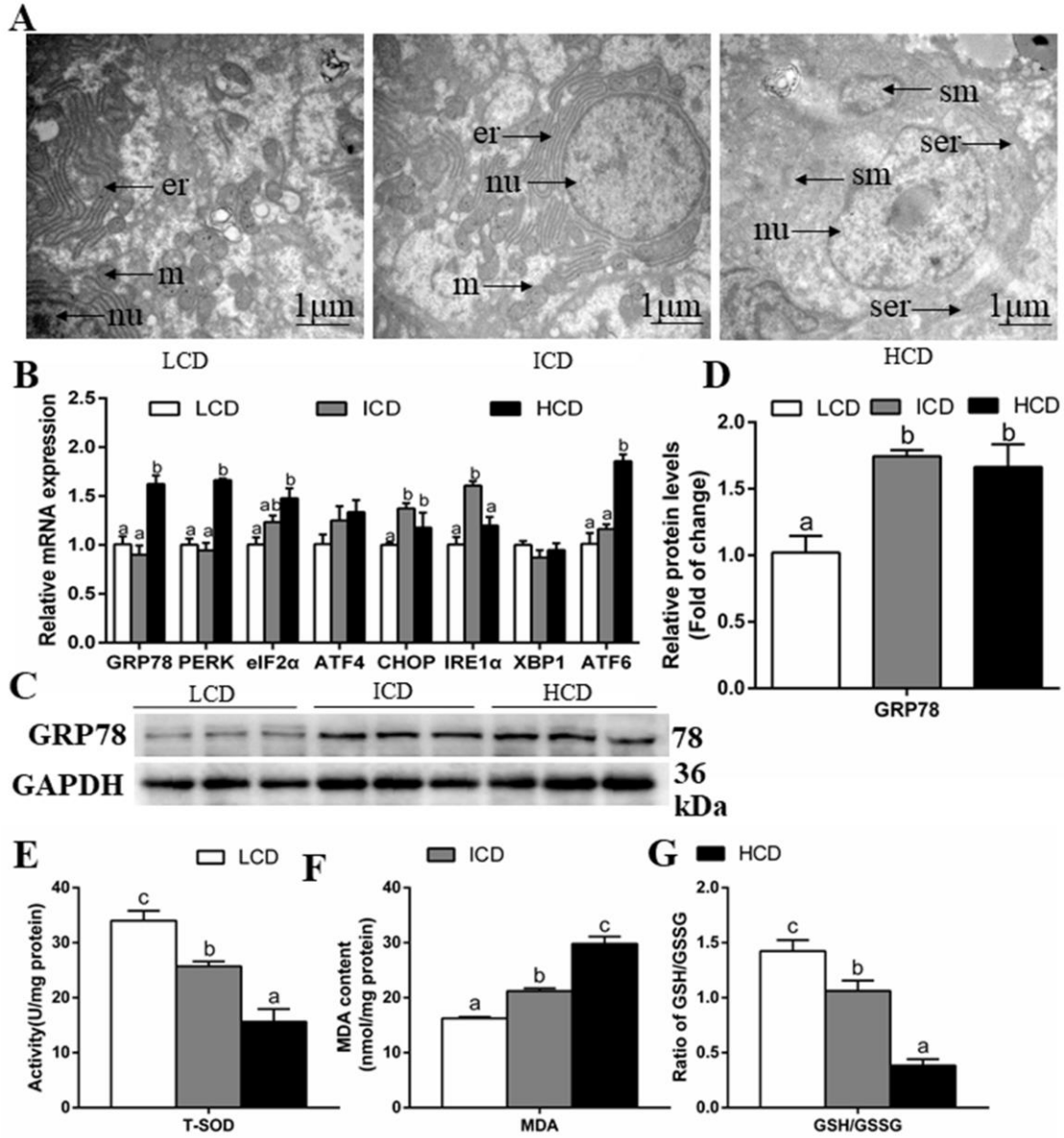
35 843 **Fig. 7 HG activates autophagy and lipid accumulation through PPAR γ pathway**
36 844 **in hepatocytes of yellow catfish.** (A) The mRNA levels of PPAR γ . (B, C) Western
37 845 blot analysis of PPAR γ protein levels. (D) TG content of the primary hepatocytes
38 846 from *P. fulvidraco* were incubated in control (5mM glucose) or HG (10mM glucose)
39 847 for 48 h in M199 medium with or without 2-h pretreatment with 1 μ M T0070907
40 848 (PPAR γ inhibitor). (E) The autophagy was quantified by flow cytometric analysis of
41 849 red/green (FL2/FL1) fluorescence ratio using 1 μ M acridine orange fluorescent
42 850 staining. (F) The presence of acridine orange-stained intracellular autophagic vacuole
43 851 was demonstrated by flow cytometry analysis of red-to-green (FL2/FL1) fluorescence
44 852 ratio. (G) ChREBP binding sequence (ChoRE) located at -534 bp to -515 bp of
45 853 PPAR γ promoter region of yellow catfish. (H) Site-mutation analysis of ChREBP
46 854 binding sites on pG13-PPAR γ -784/+63 vectors. (I) EMSA of putative PPAR γ binding
47 855 sequences (ChoRE). The 5'-biotin labeled double-stranded oligomers were incubated
48 856 with nuclear protein. A 100-fold excess of the competitor and mutative competitor
49 857 oligomers was added to the competition and mutant competition assay, respectively.
50 858 All data were expressed as mean \pm S.E.M. (n=3 at least). P value was calculated by
51 859 Student's t tests. * $P < 0.05$, ** $P < 0.01$, compared with control; # $P < 0.05$, ## $P < 0.01$,

8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

860 compared with HG group. The comparison between other groups was shown in the
861 figure.

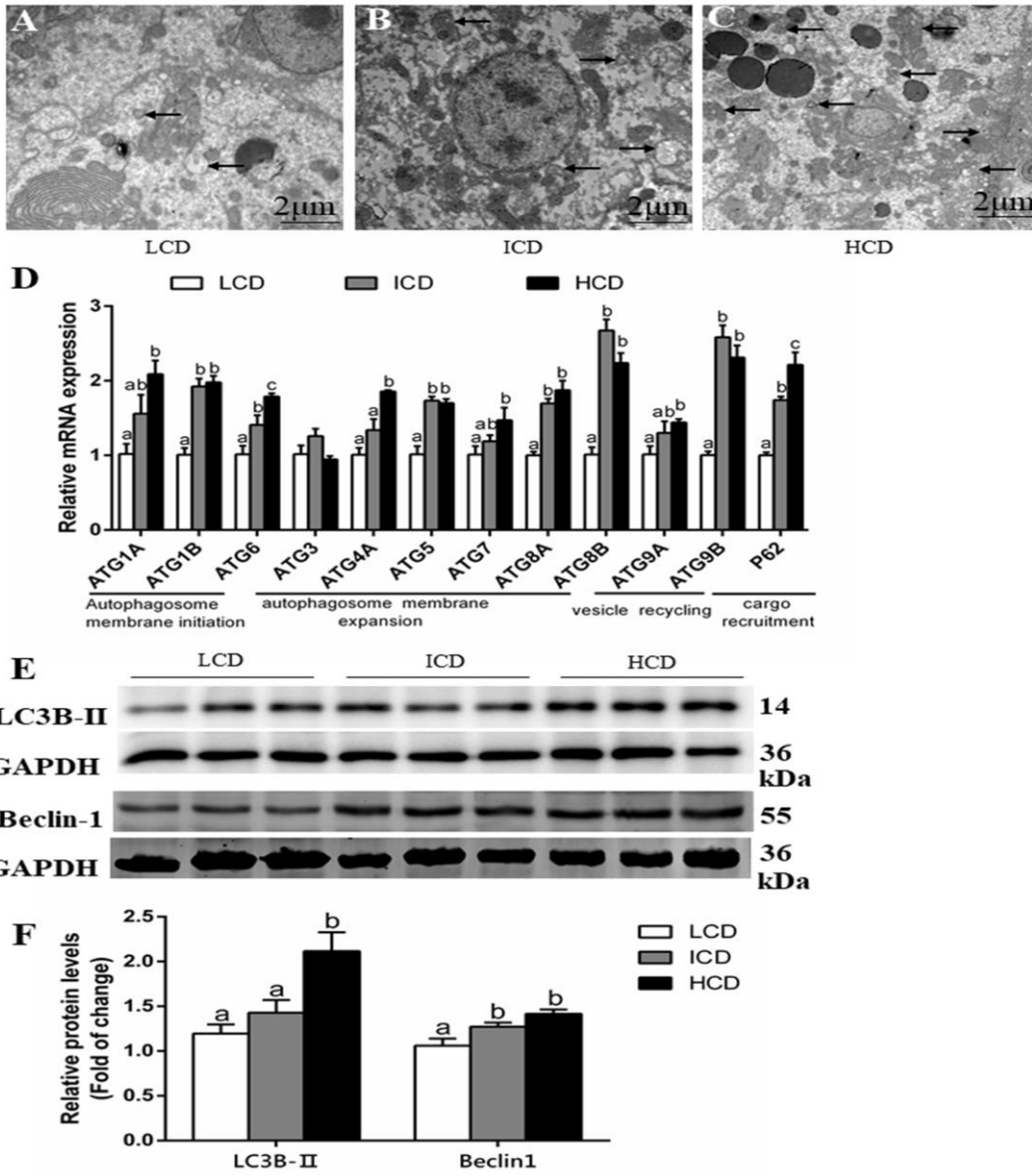
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897

Figure 1



901

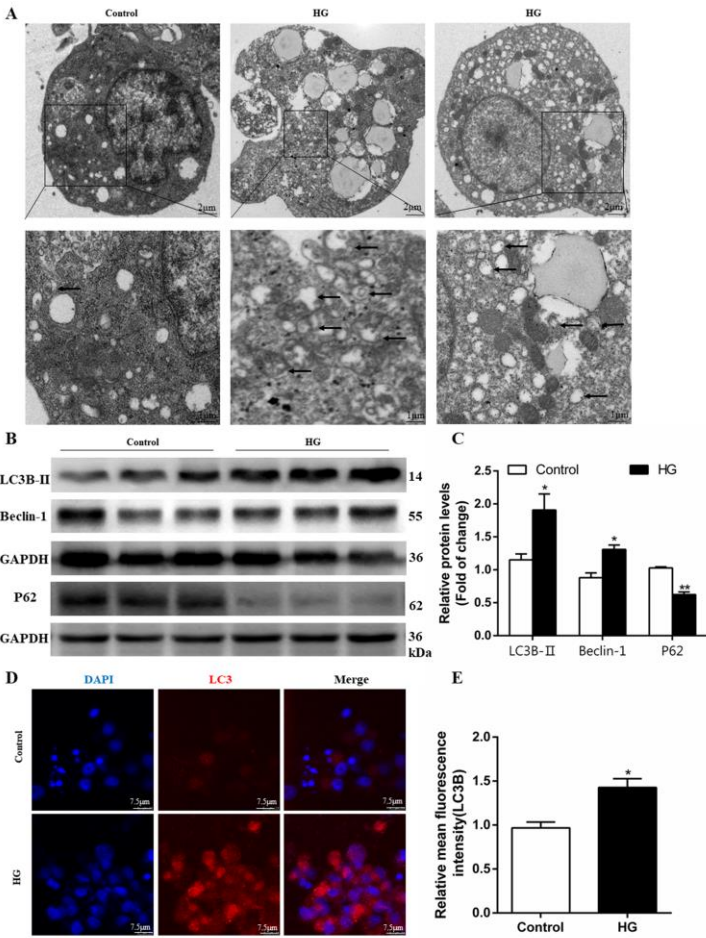
Figure 2



902

903
904

Figure 3

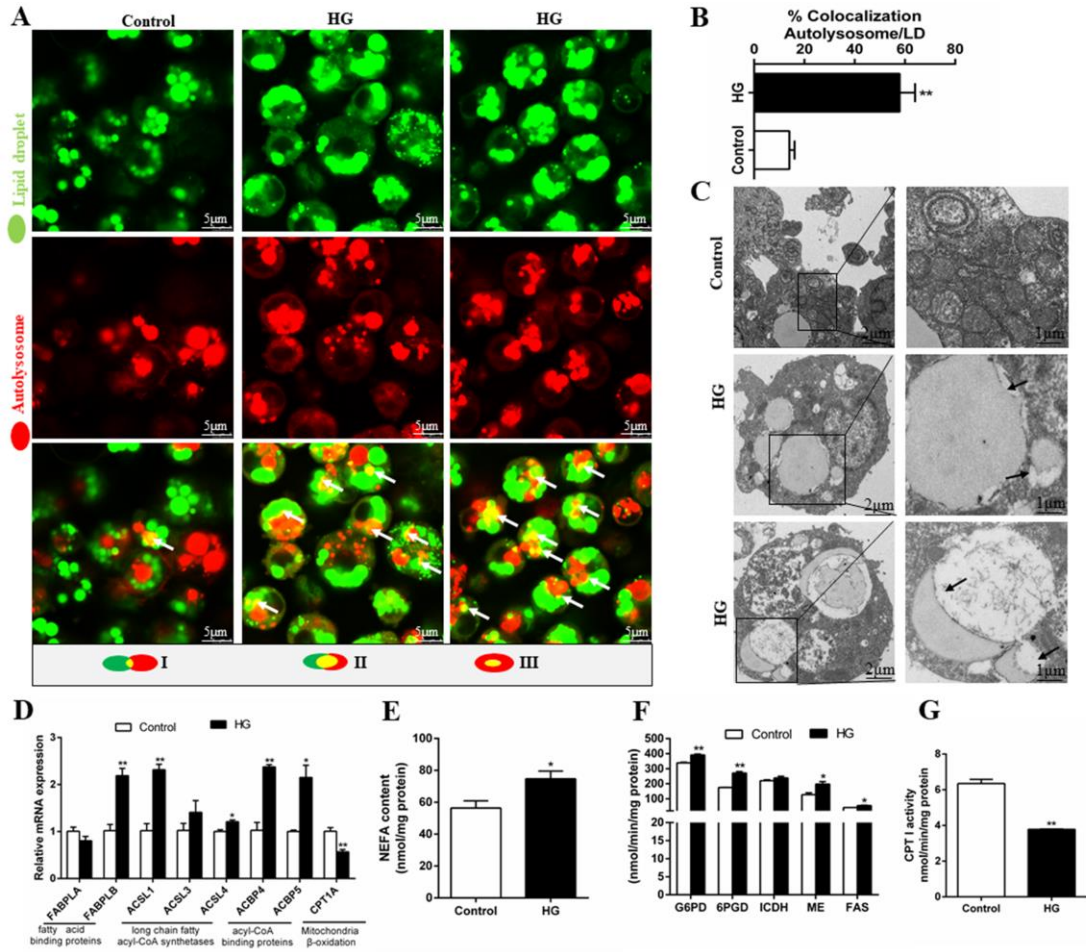


905

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

906
907
908

Figure 4



909

Figure 5

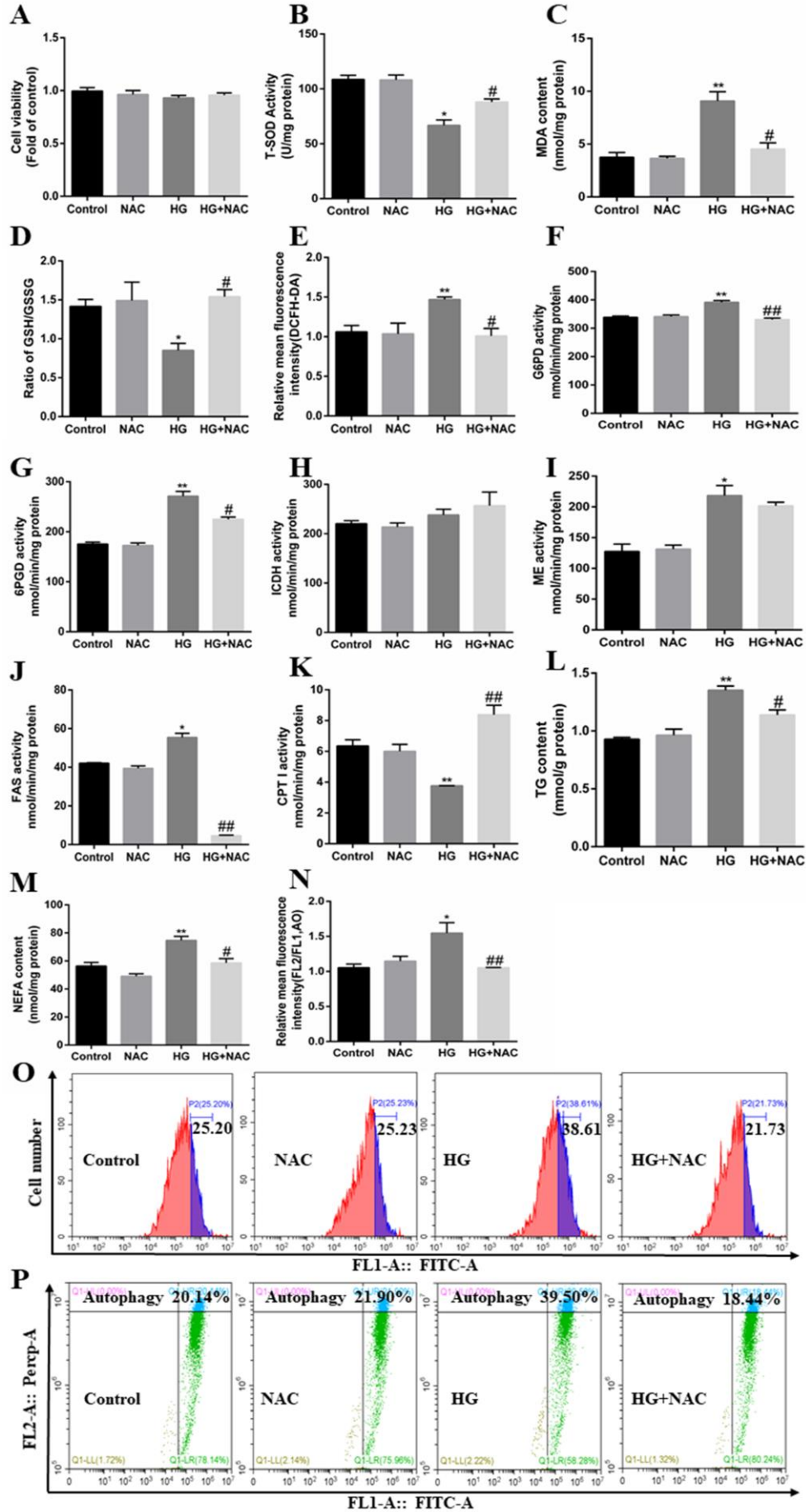


Figure 6

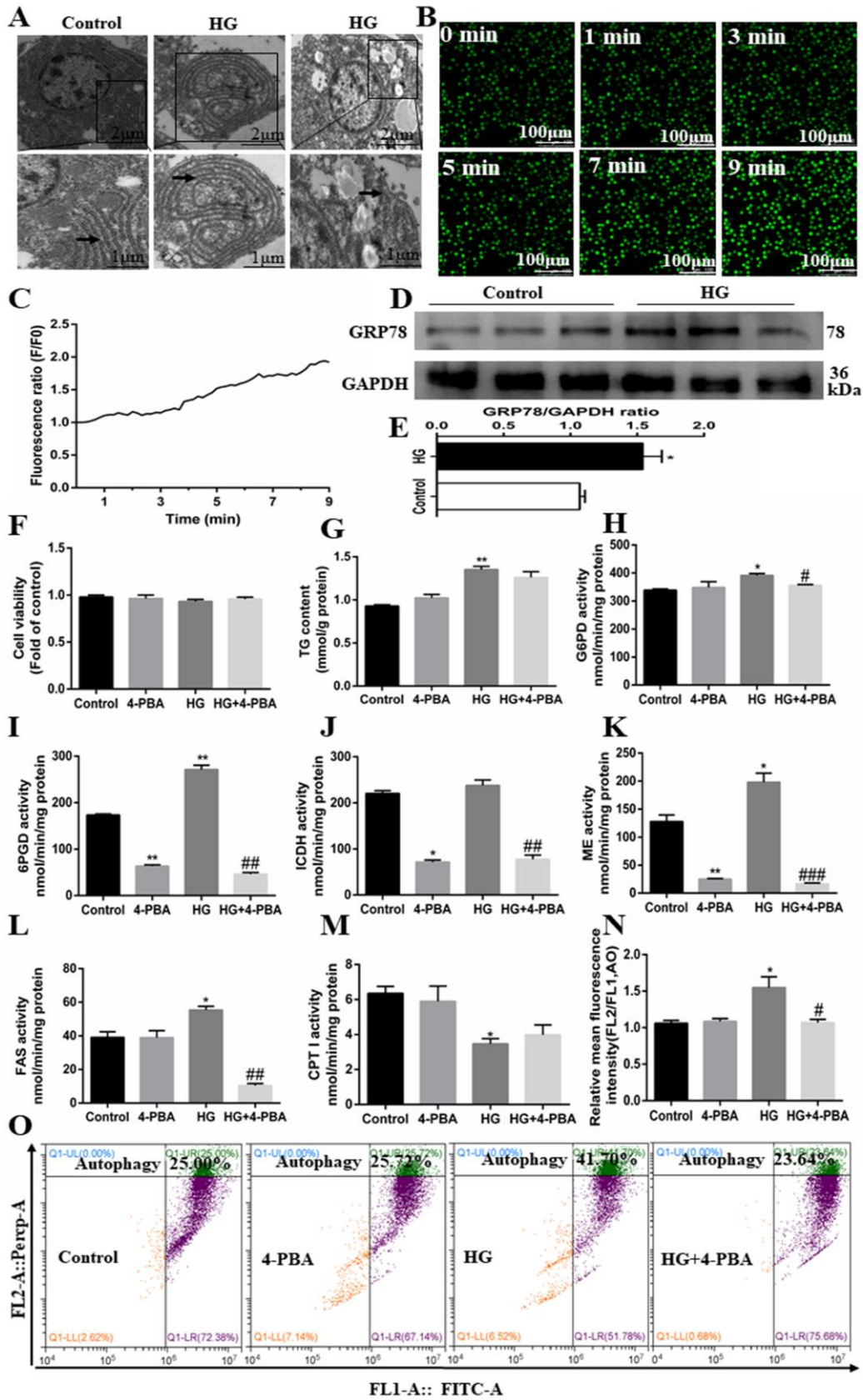
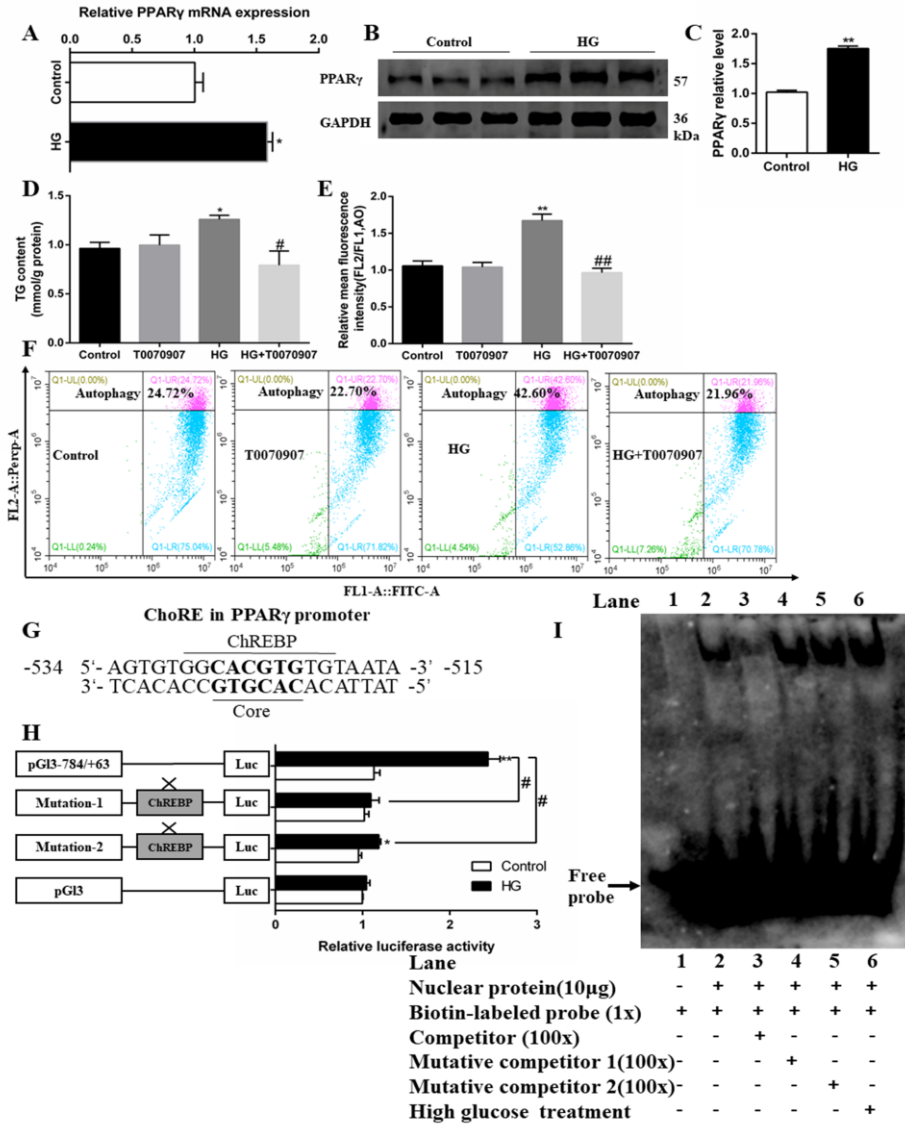
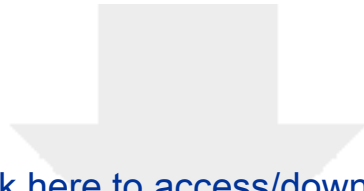


Figure 7





[Click here to access/download](#)

Supplementary Material

Supplementary materials20190705.doc

