

Molecular characterization of ER Stress sensor IRE1 α and IRE1 β and their expression analysis in response to different fatty acid in grass carp (*Ctenopharyngodon idellus*)

Zhiguang Chang

Northwest Agriculture and Forestry University

Minghui Yang

Northwest Agriculture and Forestry University

Hong Ji (✉ jihong0405@hotmail.com)

Northwest A&F University <https://orcid.org/0000-0002-6383-776X>

Research Article

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1 Molecular characterization of ER Stress sensor IRE1 α and IRE1 β and their expression analysis in
2 response to different fatty acid in grass carp (*Ctenopharyngodon idellus*)

3 Zhiguang Chang¹, Minghui Yang¹, Hong Ji^{1*}

4 ¹ College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi
5 Province, 712100, China.

6 *Corresponding author: College of Animal Science and Technology, Northwest A&F University,
7 Yangling, Shaanxi Province, 712100, China.

8 Tel/Fax: +86-29- 87092585

9 E-mail: jihong@nwsuaf.edu.cn

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27 Abstract: Inositol-requiring enzyme 1 (IRE1) is the most evolutionarily conserved endoplasmic
28 reticulum (ER) membrane protein. Here, we identified and analyzed two IRE isoforms (IRE1 α and
29 IRE1 β) in grass carp (*Ctenopharyngodon idellus*). The coding sequences of IRE1 α and IRE1 β
30 were 3096 and 2835 nucleotides, and they encoded proteins of 1031 and 944 amino acids,
31 respectively. Each of the two IRE1s proteins had four PPQ domains, a transmembrane helix region,
32 an S_TKc and a PUG domain, which were relatively conserved in comparison to mammals.
33 qRT-PCR revealed that IRE1 α was the highest in liver and brain, whereas the highest expression
34 of IRE1 β expressed was found in the hindgut, which may be closely related with their role.
35 Moreover, we have compared the effect of saturated (palmitic acid, PA) , monounsaturated fatty
36 acid (oleic acid, OA) and polyunsaturated fatty acids (Docosahexaenoic acid, DHA) on ER stress
37 in CIK cells, and found that PA and DHA induced UPR-related gene expression in dose-dependent,
38 and OA increased expression of IRE1 α and decreased expression of IRE1 β , ATF4 and ATF6 at
39 lower doses. Additionally, our studies revealed that blocking IRE1 α pathway using specific
40 inhibitor 4 μ 8c, subsequently reversed PA-induced autophagy and inflammation, indicating the role
41 of IRE1 α in mediating PA-induced CIK cells. Taken together, the study demonstrated the IRE1 α
42 and IRE1 β that likely have an important role in physiological processes induced by fatty acids in
43 grass carp.

44 Keywords: IRE1, fatty acids, autophagy, inflammation, *Ctenopharyngodon idellus*

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61 1. Introduction

62 The endoplasmic reticulum (ER) is a dynamic intracellular organelle and plays vital roles in
63 cellular homeostasis, development, and stress responsiveness (Bravo et al., 2013). Any
64 physiological or pathological perturbation can disrupt ER homeostasis and resulting in ER stress
65 (Kaufman, 1999), such as lipid accumulation, reactive oxidative stress (ROS), protein overload,
66 iron imbalance, hypoxia and viral infections (Ron and Walter, 2007). To combat the deleterious
67 effects of ER stress, cells activate the unfolded protein response (UPR). The UPR includes three
68 major simultaneous response pathways, namely inositol requiring enzyme 1 (IRE1), protein kinase
69 RNA-like ER kinase (PERK), and activating transcription factor 6(ATF6) (Kaufman, 2002; Ron
70 and Walter, 2007). Upon ER stress, activation of these three sensors can trigger pro-inflammatory
71 response or cell apoptosis that is critically involved in the pathogenesis of a variety of diseases,
72 such as obesity, type 2 diabetes, and age-associated pathogenesis (Bhattarai et al., 2020;
73 Hummasti and Hotamisligil, 2010).

74 Inositol-requiring enzyme 1 (IRE1) was originally identified as a primary transducer of
75 Unfolded Protein Response (UPR) (Tirasophon et al., 1998). IRE1 is the most evolutionarily
76 conserved sensor and is found from yeast to metazoans (Acosta-Alvear et al., 2018). On ER stress,
77 IRE1 is activated through trans-autophosphorylation and dimerization/oligomerization (Hetz,
78 2012), and then splices the mRNA encoding X-box binding protein 1 (XBP1) via its RNase
79 activity to generate XBP1s (Shemorry et al., 2019). In turn, XBP1s activates transcriptional
80 programs that regulate a subset of UPR- and non-UPR-associated genes (Wang et al., 2017).
81 Moreover, IRE1 α can recruit TRAF2, leading to activation of c-Jun N-terminal kinase
82 (JNK)-mediated signaling pathway associated with inflammatory and metabolic diseases (Urano
83 et al., 2000). Together, these findings reveal the IRE1 α -XBP1 signaling pathway was involved in
84 regulation of cellular processes including ER-associated degradation, inflammatory signaling, cell
85 proliferation and membrane biogenesis (Stewart et al., 2017).

86 In farmed fish, HFD commonly cause excess fat accumulation, which leading to liver injury
87 and lipid metabolism disorders (Cao et al., 2019). Therefore, focused on the mechanism of lipid
88 metabolism by excessive fatty acid in different tissues of fish would be highly beneficial. In this
89 study, we cloned and identified the IRE1 α and IRE1 β homologs named IRE1 α and IRE1 β from
90 *Ctenopharyngodon idellus*, and detected their expression patterns in response to different fatty
91 acid or thapsigargin (TG) induced ER stress in CIK cells. We also investigated whether inhibition
92 of IRE1 α markedly restored PA-induced autophagy and inflammation-related genes. These
93 preliminary findings help to understand the role of IRE1 in grass carp.

94 2. Materials and methods

95 2.1 Cloning of IRE-1 cDNA

96 Total RNA was extracted from various tissues of healthy grass carp by using Trizol reagent
97 (Invitrogen). First strand cDNA synthesis was carried out using a PrimeScript 1st Strand cDNA
98 Synthesis Kit (TaKaRa) according to the manufacturer's instructions. PCR amplification primers
99 were designed based on the IRE-1 gene from the grass carp transcriptome database (Table 1). The
100 PCR products were gel-extracted and ligated into pMD18-T (Takara). Following transformation
101 into competent *Escherichia coli* DH5 α cells, positive clones were screened using ampicillin
102 selection and colony PCR and then sequenced (Sangon Biotech).

103 2.2 Sequence and Phylogenetic analysis

104 The amino acid sequence was predicted using the translate tool, and the molecular weight,
105 isoelectric point (*pI*), and net charge of the peptides were calculated using the ProtParam tool, both
106 available on the ExpASy molecular biology server (<http://www.expasy.org/tools>). The protein
107 domain features were predicted by SMART (<http://smart.embl-heidelberg.de/>). Multiple sequence
108 alignments were generated using Clustal W (version 1.83). The phylogenetic tree was constructed
109 using the Neighbor-Joining method in MEGA 7.0 software with 1000 bootstrap replicates.

110 2.3 Cells culture

111 *Ctenopharyngodon idellus* kidney (CIK) cells line was obtained from China Center for Type
112 Culture Collection (CCTCC, Wuhan) and maintained in DMEM medium supplemented with 10%
113 fetal bovine serum (Gibco, USA), and in a humid atmosphere of 5% CO₂ at 28°C.

114 2.4 Fatty acid treatment

115 Palmitic acid (PA), Docosahexaenoic acid (DHA) and oleic acid (OA) were purchased from
116 Sigma-Aldrich, and a stock solution was prepared by dissolving 100% ethanol at 50 mM and
117 stored at -80 °C. Cells were treated with the indicated concentrations of fatty acid (50-200 μ M)
118 dissolved in media containing 1% fatty acid-free BSA for 24 hours.

119 2.5 Quantitative real-time PCR (qRT-PCR) analysis

120 Total RNA was extracted from tissue and cell samples, and then reverse transcribed to obtain
121 cDNA. The obtained cDNA were served as template for real-time quantitative PCR (qPCR)
122 analysis to examine the expression level of six RBR genes. Three replicates were conducted for
123 each sample and β -actin as an internal reference gene. Amplification reactions were performed in
124 triplicate in 20 μ l containing 1.0 μ l cDNA sample, 10 μ l 2 \times SYBR premix Ex Taq (TaKaRa), 0.4 μ l
125 of each primer, and 8.2 μ l ddH₂O. The program was performed as follows: 1 cycle of 94 °C/5 min;
126 40 cycles of 94 °C/30 s, 60°C/30 s, and 72 °C/30 s. The obtained data were evaluated using the
127 $2^{-\Delta\Delta C_t}$ method and then statistically analyzed. The primers for Q-PCR were listed in Table 1.

128 2.6 Statistical analyses

129 Each experiment was done independently at least three times with similar results. Results
130 were expressed as mean \pm S.D. All data were expressed as Mean \pm SEM (N=3) and the groups
131 denoted by stars (*) represent a significant difference at $P < 0.05$ from controls (** means $P <$
132 0.01).

133 3. Results

134 3.1 Sequence analysis of IRE1 α and IRE1 β cDNA

135 The full-length of IRE1 α cDNA was 4038 bp (GenBank accession no. MH370854), and the
136 ORF was 3096 bp encoding 1031 amino acids (Supplement Fig.1). The predicted molecular
137 weight and isoelectric point of the deduced IRE1 α protein was 116.3 kDa and 6.13, respectively.
138 The full-length of IRE1 β cDNA was 3504 bp (GenBank accession no. MK616212), and the ORF
139 was 2835 bp encoding 944 amino acids (Supplement Fig.2). The predicted molecular weight and
140 isoelectric point of the deduced IRE1 β protein was 105.2 kDa and 6.24, respectively. The
141 predicted IRE1 α and IRE1 β protein shared the conservative signal peptide domain,
142 pyrrolo-quinoline quinine domains (PPQ), Ser/Thr protein kinase domain (S_TKc) and peptide
143 N-glycanase domain (PUG) (Supplement Fig.3). Moreover, the predicted IRE1 α and IRE1 β
144 protein consist of conserved domains that are critical for their function, that contains the putative
145 signal peptide, ER-luminal domain, transmembrane region, and kinase and RNase subdomains,
146 which displays a very high homology to human IRE1 α and IRE1 β , respectively (Fig.1).

147 3.2 Phylogenetic analysis based on IRE1 α and IRE1 β amino acid sequence

148 To determine the evolutionary status of IRE1 α and IRE1 β , a phylogenetic tree was
149 constructed based on the amino acid sequence of IRE1 α and IRE1 β , respectively (Fig.2).
150 According to the tree, mammals and fish can be separated into two branches. Moreover, multiple
151 sequence alignment and amino acid identity comparisons indicated that IRE1 α and IRE1 β share a
152 sequence identity with other species, exhibiting 48.16–92.43 % and 51.65–92.90 % AA sequence
153 identities, respectively (Supplement Fig.4 and Supplement Fig.5). Moreover, the encoded IRE1 α
154 protein, consisting of 1031 amino acids, displays a very high homology to human IRE1 α (73.46%).
155 The encoded IRE1 β protein, consisting of 944 amino acids, displays a very high homology to
156 human IRE1 β (52.69%). While, the amino acid sequence of IRE1 α and IRE1 β has 48% identity.

157 3.3 Expression of IRE1 α and IRE1 β mRNA in different tissues

158 Expression levels of the IRE1 α and IRE1 β transcript in twelve tissues of grass carp were
159 analyzed to determine its tissue distribution. The IRE1 α mRNA levels was the highest in liver and
160 brain, followed by mid-intestine, kidney, heart, hindgut, gill, spleen, head kidney and lowest in red
161 muscle and white muscle, whereas the highest expression of IRE1 β expressed was found in the
162 hindgut, with a moderate level of expression observed in the heart, mid-intestine, foregut, gill,

163 white muscle, brain, liver, but only weak expression in the spleen, head kidney, kidney and red
164 muscle (Fig.3). indicate that may play different roles in different tissues in grass carp.

165 3.4 Expression patterns of IRE1 α and IRE1 β in response to ER stress in CIK cells

166 In order to determine the expression pattern of IRE1 α and IRE1 β mRNA in response to ER
167 stress, we treated CIK cells with an ER stress inducer, such as tunicamycin (TM) or thapsigargin
168 (TG). First, Figure 2B shows that TM and TG treatment could activate the expression of ER
169 stress-related genes, such as GRP78, ATF6 and CHOP. Moreover, the expression of IRE1 α and
170 IRE1 β were also up-regulated in a dose-dependent manner by TG treatment, but IRE1 β expression
171 was higher than IRE1 α expression in all treatments. In addition, we also found that the levels of
172 TNF α and NF- κ B were all increased after TG stimulation, suggesting that ER-stress related
173 inflammation was activated (Fig.4). These results suggest that IRE1 α and IRE1 β have different
174 expression pattern in response to ER stress and involve in ER-stress induced inflammatory
175 reactions in CIK cells.

176 3.5 Expression patterns of IRE1 α and IRE1 β in response to different fatty acids

177 To compare the effects of different fatty acids on IRE1, we incubated CIK cells for 24 h with
178 OA, PA, or DHA at concentrations ranging from 50 to 200 μ M. The results revealed that different
179 concentrations of DHA and PA could significantly increase the mRNA levels of ER stress-related
180 genes, such as GRP78, ATF6 and CHOP. Whereas OA could decrease the expression of ER related
181 genes, such as GRP78, CHOP and ATF6, except IRE1 α (Fig.5). Moreover, DHA and OA
182 treatment showed a significant increase in the number of lipid droplets, compared with PA (data
183 not shown). These results suggest that IRE1 α and IRE1 β have different expression profile in
184 response to different fatty acids induced metabolism of lipid droplets in CIK cells.

185 3.6 Inhibition of IRE1 α ameliorates PA induced autophagy and inflammation

186 IRE1 α is a multi-functional protein (Riaz et al., 2020), we wanted to examine the importance
187 of IRE1 α in PA induced CIK cell inflammation and autophagy, CIK cells were exposed to PA,
188 with or without 4 μ 8c. As shown in Fig.6, IRE1 α inhibition significantly inhibited the ATG5, ATG7
189 and Beclin-1 expression induced by PA. Moreover, 4 μ 8c successfully reduced the levels of
190 inflammation -related gene expression induced by PA. These results indicated that IRE1 α is
191 involved in PA-induced autophagy and inflammation in CIK cells.

192 4. Discussion

193 IRE1 exists in two isoforms: IRE1 α /ERN1 and IRE1 β /ERN2. To date, the function of IRE1 α
194 was identified from *Larimichthys crocea* (Zhang et al., 2020). In this study, we cloned the grass
195 carp IRE1 α and IRE1 β cDNA. Phylogenetic tree analysis showed that IRE1 protein of grass carp
196 were closely related to that of *Sinocyclocheilus anshuiensi*. Similar to mammalian IRE1, the IRE1

197 protein of grass carp also has conserved domain, including an endoribonuclease (RNase) and a
198 serine/threonine kinase (Fig.1). These results confirmed that IRE1 is highly conserved among
199 different species. Consistent with previous study (Bertolotti et al., 2001; Martino et al., 2013), our
200 results show that IRE1 α was expressed in all tissues examined, with the highest expression level
201 in liver, and IRE1 β was highly expressed in the intestine in grass carp. Some studies indicated that
202 IRE1 β plays a constitutive role in lipid metabolism through MTP mRNA degradation in intestinal
203 epithelial (Iqbal et al., 2008; Tsuru et al., 2013). Recent studies suggest that IRE1 β and IRE1 α
204 appear to have distinct enzymatic activities, and IRE1 β acts as a negative regulator of IRE1 α in
205 intestinal epithelial cells (Grey et al., 2020), but the exact regulatory function remain unknown.
206 Obviously, they have different physiological functions.

207 IRE1 can be activated lipid-dependent in yeast and mammalian (Robblee et al., 2016; Volmer
208 and Ron, 2015). Previous studies also demonstrated that DHA was incorporated into TAG as well
209 as OA, PA was not incorporated into TAG (Caviglia et al., 2011). In this study, we compared the
210 effects of OA, PA and DHA regarding their ability to induce ER stress and lipogenesis. Our results
211 found that OA and DHA can significantly induce the formation of TAG, but they did not
212 significantly induce ER stress. In contrast, PA can induce endoplasmic reticulum stress more
213 effectively, but no obvious lipid droplets were observed in CIK cells. These findings suggest that
214 TAG itself does not appear to be the mediator of fatty acid-induced ER stress (Caviglia et al.,
215 2011), although the exact mechanism remains unclear. Moreover, it has been show that human
216 IRE1 α activation by membrane lipid saturation (Cho et al., 2019). Here, we found that compared
217 to DHA and OA, PA induced IRE1 α and IRE1 β expression more rapidly and dramatically, and the
218 expression of IRE1 β is higher than that of IRE1 α , implying that IRE1 β rather than IRE1 α was
219 closely related with lipid saturation in grass carp.

220 Multiple mechanisms are involved in PA-induced kidney injury in human, including ER
221 stress, autophagy and inflammation (Park et al., 2019). Previous reports have also demonstrated
222 that IRE1 α has emerged as a key regulator of autophagy and inflammation (Bao et al., 2018;
223 Cheng et al., 2014; Junjappa et al., 2018). Here, we found that inhibition of IRE1 α attenuates
224 PA-induced inflammation and autophagy. How IRE1 β affects these interactions and regulatory
225 mechanisms also remain to be determined. Interestingly, treatment of CIK cells with 4 μ 8C, which
226 are IRE1 α -specific inhibitors, reduced the IRE1 β expression, these may be due to they complement
227 each other's role in protein folding homeostasis (Tschurtschenthaler et al., 2017). Taken together,
228 our results demonstrated that IRE1 α inhibition protected from PA-induced autophagy and
229 inflammation in CIK cells.

230 In conclusion, we cloned the full-length sequences of IRE1 α and IRE1 β , and confirmed

231 IRE1 α signaling is activated in response to different fatty acids in CIK cells. Importantly, we
232 found that IRE1 α integrates autophagy and inflammation. However, the mechanisms of IRE1 β
233 remain to be further explored in grass carp.

234 Availability of data and material

235 Data and materials are available upon request.

236 Competing interests

237 The authors disclose no conflicts of interest. All the authors listed have approved to submit to
238 your journal.

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242 Authors' contributions

243 Zhiguang Chang performed the experiments and wrote the manuscript; Minghui Yang
244 collected the literature; Hong Ji primarily revised the manuscript. All authors have read and
245 approved the final manuscript.

246 Ethics declarations

247 All procedures were performed in accordance with the Guide for Care and Use of Laboratory
248 Animals and approved by the Northwest A&F University Institutional Animal Care and Use Com
249 mittee.

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351 Table. 1. PCR primer and interference sequence information in this study.

Primer name	Forward (5'→3')	Reverse (5'→3')	Application
IRE1 α F1	AGTTGATAAACGCATTGAAC	ACCAGTGGAGTGTTCCCTA	Gene cloning of IRE1 α
IRE1 α F2	CACTGTATGTAGGGAAACAC	TCTCTTCTCTGGCTCCATGC	Gene cloning of IRE1 α
IRE1 α F3	CAGATGTTAAGCATGGAGCC	CTGGCATTACTAACA AAAAG	Gene cloning of IRE1 α
IRE1 β F1	TATTTGTTATTTTATTATAGGA	GCACAAGGGAGGTAGAAGCA	Gene cloning of IRE1 β
IRE1 β F2	AGACAGACTCTCATCTGTATG	TTTGTATGCATGTGTACTGGC	Gene cloning of IRE1 β
IRE1 β F3	CCCTGTACGACTGCCAGTACA	GGCTGGAGATTCACGTAATCT	Gene cloning of IRE1 β
IRE1 α	CCTGCATGGTAGACATCTTCTC	TTCTCTGGCTCCATGCTTAAC	Real time RT-PCR
IRE1 β	GTGGCAGGGTTCTACTTATGG	GCTCTCCTTTGTGAACTGGTAG	Real time RT-PCR
GRP78	TTCAAGCCGGAGTTCTGTCC	GTTGTCAGAAGCGGTGGAGA	Real time RT-PCR
CHOP	GCGTGGTATGAAGACCTACAG	CGCTGACCATCGTTTCCA	Real time RT-PCR
ATF6	AGCTGGAGGATCTTGGGGAT	TGATCGAGGGCTACTCCACA	Real time RT-PCR
TNF α	GATTGGAGAGTGAACCAGGAC	CCTGGCTGTAGACGAAGTAAAT	Real time RT-PCR
NF- κ B	GAACATTTAACCCACGCAAGAG	GGCCTTTCCTGCCATCTAATA	Real time RT-PCR
XBP-1s	GGTCTTCTGAGTCCGCAGCAG	AGACTCAGTGTCTGCAGGGCC	Real time RT-PCR
IL-10	ATGCCAACCTCCTGTTCTTC	CACTTCCACCTGCTCCATATC	Real time RT-PCR
TGF β 1	GTGACGCCAGCATTGTATCTA	GTCAGCGTTGCTGGAATTTATC	Real time RT-PCR
ATG5	GGAGGAGATGTGGTTTGAGTATG	GCCCAGAACTGGTCGAATTTA	Real time RT-PCR
Beclin-1	AGCTCGACACATCCTTCAAC	CTGCGACTCAAGTTCTCCATAG	Real time RT-PCR
ATG7	TTTGAAGACTGTCTGAGCGG	GCCATTGTCAACTCGGAAAAG	Real time RT-PCR
β -actin	CGTGACATCAAGGAGAAG	GAGTTGAAGGTGGTCTCAT	Real time RT-PCR

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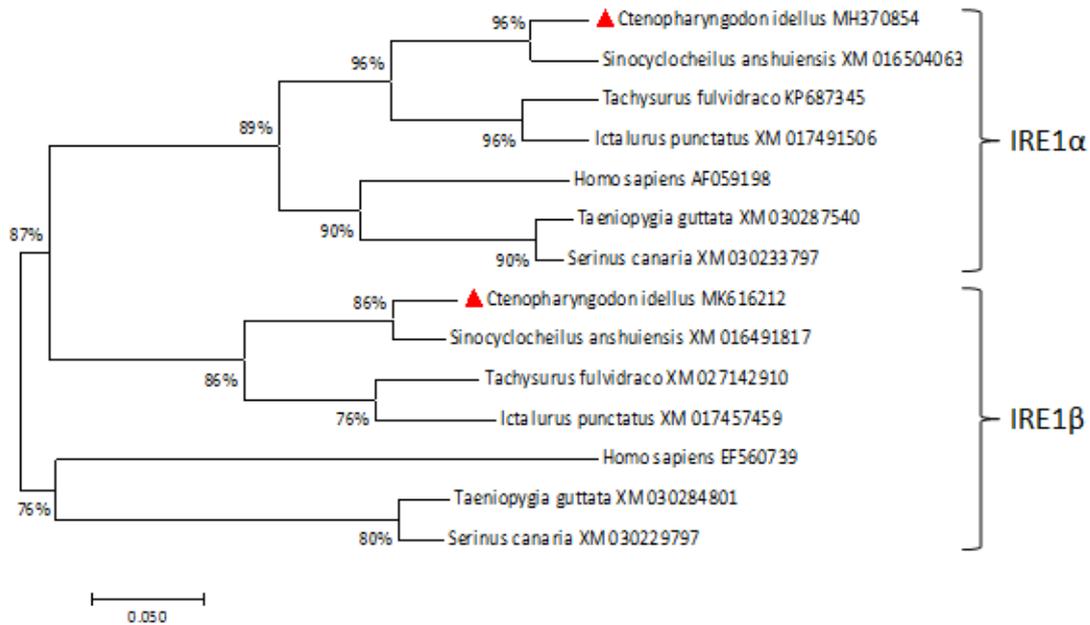
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360 Fig.1 Amino acid sequence alignment of the IRE1 α and IRE1 β in grass carp with homologs from
 361 human, respectively. Identical residues are marked by asterisks underline. Signal peptide and
 362 domains are as indicated.

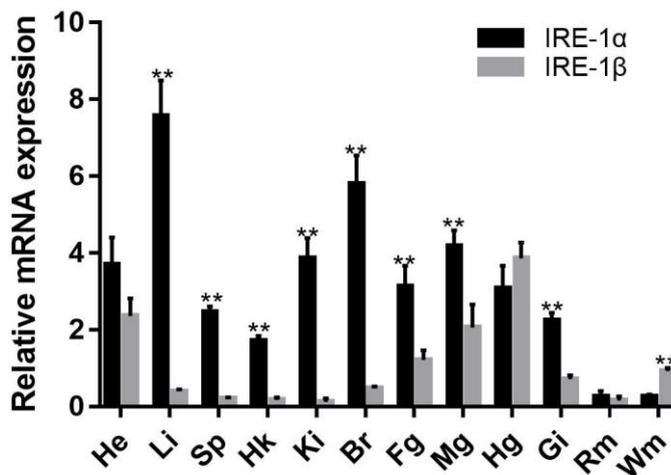
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C. idellus_IRE1 α	MMAWGVCSRALVWIIITAIHWSSHSLSMGFCSSSTVSVFESLLFVSTLDGSLHMAVSRKSTIKRWTLKEDPVLQVPTHTVSEPAFLPDPNDGSLYLGSFNNE	100
C. idellus_IRE1 βMMDWRQMLLLPILLSFTG.IVSCQEGSSVSLPESELLFVSTLDGSLHMAVSRKSTIKRWTLKEDPVLQVPTHTVSEPAFLPDPNDGSLYLGSFNNE	94
Homo_sapiens_IRE1 αMEARLRLLLLTLLLP...LGIFGTSSTVTLPEITLLFVSTLDGSLHMAVSRKSTIKRWTLKEDPVLQVPTHTVSEPAFLPDPNDGSLYLGSFNNE	91
Homo_sapiens_IRE1 β	.MASAVRGSSEWFRPRLSCLQFAALLGTLSPQVHTLREEMLLVSTLDGSLHMAVSRKSTIKRWTLRDDFVIESEPMYVTEPAFLSDPNDGSLYLGSFNNE	99
Consensus	r l l l l s v pe llfvstldgslha sk tg ikwtlkedpv qvpt v epafldpndgslly lg k e	
C. idellus_IRE1 α	GLTKLPFTIPELVQASPCRSSDGLIYMKKQDIWVVDLITGKQCITLSSYAEMLCFSSLLYLGRTEYTIIMYDTKSRRLRNWATYSDYAFTLEDDDD.	199
C. idellus_IRE1 β	GLMKLPFTIPELVQASPCRSSDGLIYMKKQDIWVVDLITGKQCITLSSSSSDICPSAPFLYLGRTEYTIIMYDTKIQELRNWATYNDYSAPFLDDDK	194
Homo_sapiens_IRE1 α	GLTKLPFTIPELVQASPCRSSDGLIYMKKQDIWVVDLITGKQCITLSSAFADSLCPSISLLYLGRTEYTIIMYDTKTRLRNWTYFDYPAASLPEDE.	190
Homo_sapiens_IRE1 β	GLMKLPFTIPELVHASPCCRSSDGLIYMKKQDIWVVDLITGKQCITLSSAFADSLCPSISLLYLGRTEYTIIMYDTKTRLRNWTYFDYPAASLPEDE.	193
Consensus	gl klpftipelvqaspcrssidg ly gkkqd w vvd tgeqk tl cps lly grteytitmydtk elrwnaty dy a l dd	
C. idellus_IRE1 α	THYHMAFFVSNEDGLVVTVDSESGVLMWVNYNSVVAIILVQREGLRQVPHINAVETLRYLTFMS].EVGRITQWYVPEPPEKK.TEDKIMSLIYVVK	297
C. idellus_IRE1 β	YETKMSHFASSEDGLVVTVDSDSGEVLWVQKDFSVVAGFVILWQSDSLRRAPEHMTVAETLRYLTFLAENIQSCTMKWVQVETREHSHSTKIQVPTLYVVK	294
Homo_sapiens_IRE1 α	GDYKMSHFVSNEDGLVVTVDSESGVLMWVNYNSVVAIILVQREGLRQVPHINAVETLRYLTFMS].EVGRITQWYVPEPPEPE.TAKSKLTPFLYVVK	288
Homo_sapiens_IRE1 β	PGKYMSHLSACMGLLLITVDGSSVLMWVNYNSVVAIILVQREGLRQVPHINAVETLRYLTFMS].HIRLPASGPRDITLFTLDTQLMLIYVVK	292
Consensus	y mshf s gdlvvtvd sg vlv q spv y w glr ph va etlryltf g w y f e k l tlyvkg	
C. idellus_IRE1 α	HSTLYASPSLVHGVTVVPRGRITPMLLEGGFNSQESSVEN.EQECVITPSTVVKFSAPLKERNNMVMRNSLLIGHHETPPPAHNRLEKFEESTPRQH	396
C. idellus_IRE1 β	TDSDLYASTSLVHQSVAVVFKGLTLARIEGPHIAGVITNGRPECEITPSTVVKVPPSSSTS....LQNCWLLIGHHETPPVAHTMLRDFEENLQRSG	389
Homo_sapiens_IRE1 α	YSTLYASPSMVHGVAVVPRGSTLPLLEGGFNDGVTIGD.KGECVITPSTVVKFDPLKSKNKNLYLRNMLLIGHHETPLSSTFKMLERFENMLPKHR	387
Homo_sapiens_IRE1 β	DETSEYFKALVHTVEVALVPRGTLAPADGPHITDEVILQV.SGREGSSTAVVYFSSVALP.....SQWLLIGHHETPPVHTMLRVTHTLSSGT	384
Consensus	t lyas slvh gva vprg tl egp t vt ec itpst vk g n wllighhe pp aht ml fp l	
C. idellus_IRE1 α	VNIIPPTATEKITEEKVNESGMEDNSPLPFPVFS.SLQEEPGRLE.RVETVDSMLRDMATHIFCTFLLAGWVAIITVYKSVHRKQQLCHQCFQCCMEEKL	494
C. idellus_IRE1 β	D.VIIPPRGSGSSFRSAS.....HPLFPVTK.....PSSSE...VLEPDLTSDPMTVLVWVLLLSAWIAFLLTHRRPVKISQ....RSEEPVDSNL	466
Homo_sapiens_IRE1 α	ENVIPADSEKKSFEVINLVDQTSENAETVTSRSDVEEKFAHAPARPEAVDSMLRDMATHILSFLLAGWVAIITVYKSLMHQQQLCHQCFQCKELERIQ	487
Homo_sapiens_IRE1 β	AETREPPENQAPAFLELLSLSREKLWDSLHP....EEKTE...DSYLSLGGPQDLAASLTAVALLSWLLFVNRQQCPQVVEK.....QDETPLAPAD	471
Consensus	ipp p p p ds d t t ll gw af t q q	
C. idellus_IRE1 α	QLLQRQLLIFQEPDLPDPTDFLGAARTSESSGHSSENVTPRASNHSNMQSSEMGSAN...EHEDTEDESNIYVIGNTLSESPRDLVGHGAEGTIVY	589
C. idellus_IRE1 β	LPGLTNQ...SAPPEENTPPTSSSYNNSNERTSSVASNQTREFFSSKDS...AMTTAGC.....SQNEHPDVVEVGKISFSEPEVLGHGEGTGFVWF	553
Homo_sapiens_IRE1 α	LLQQCQLLFPFHPGDTAQDGLLDTSGPYSESSSTSSPSTPRASNHSLCGSSASKAGSSPSLEQDDGDETSVIVGKISFQKDLVGHGAEGTIVY	587
Homo_sapiens_IRE1 β	FAHISQL.....AQSLHSGASRRSKRKLQSPSKQQLPDDP.....EAEQLTVGKISFQKDLVGRGAGGTGFVWF	536
Consensus	qq p l s sp p s s e v v gkisf p dvlghgaegt v	
C. idellus_IRE1 α	RQCFDNRVAVKRILPECFVFADEVQLLRESDEHPNVIRYFCTERDRQFYIAIELCSFLQEYVEKDFDRHGLEPITLILQITMSGLSHLSLNIHVHR	689
C. idellus_IRE1 β	RQCFDNRVAVKRILPECFVFADEVQLLRESDEHPNVIRYFCTERDRQFYIAIELCAATLQYVEVDFPSCPYSLNVPSELQITMSGLSHLSLNIHVHR	653
Homo_sapiens_IRE1 α	RQCFDNRVAVKRILPECFVFADEVQLLRESDEHPNVIRYFCTEKDRQFYIAIELCAATLQYVECKDFEAHLGLEPITLILQITMSGLSHLSLNIHVHR	687
Homo_sapiens_IRE1 β	RQCFEFSRVAVKRILPECFVLRREVQLLRESDEHPNVIRYFCTERGFYIAIELCRASFLQEYVENPDLDRGLEPEFVVLQITMSGLSHLSLNIHVHR	636
Consensus	rg fd r vavkrilpecf fa devqllresdehpnviryfcterdrqf yiaielc atlqeyve d glep ll qtmssl hlslnivhr	
C. idellus_IRE1 α	DLKPNILISMENAHGRVEMISDFGLCKKLVGRHSFSRSGVPGTEGWIAPVLSEDAHPNFT].....CMVDIFSAGCVFYVYVSESG	774
C. idellus_IRE1 β	DLKPNILISLFGALGRVEMISDFGLCKKLPDGRHSFSRSGVPGTEGWIAPVLSEDAHPNFT].....YVVDIFSAGCVFYVYVSESG	753
Homo_sapiens_IRE1 α	DLKPNILISMENAHGRVEMISDFGLCKKLVGRHSFSRSGVPGTEGWIAPVLSEDAHPNFT].....YVVDIFSAGCVFYVYVSESG	772
Homo_sapiens_IRE1 β	DLKPNILITGDSQSLGRVEMISDFGLCKKLVGRHSFSRSGVPGTEGWIAPVLSEDAHPNFT].....SAVVDIFSAGCVFYVYVSESG	721
Consensus	dlkp nil s p g a isdfglckkl grhsfs sg pgtewiape l k npt vdifsagcvfyv s g	
C. idellus_IRE1 α	HFFCKSLRCQANILGAVSDHLQPNRHEDIVARNLIEQLMSMEFRPFSADRVLKHPFFWSLQKQLQFFQDVSRIEKEPDLSPVRCLEGRGRVVKG	874
C. idellus_IRE1 β	HFFGDLTRQCANILGAVSYNDLDFHEDIVARNDLIERMIGAEPEFRPSAASILKHPFFWSLQKQLQFFQDVSRIEKEPDLSPVRCLEGRGRVVKG	853
Homo_sapiens_IRE1 α	HFFCKSLRCQANILGAVSDLDFHEKHEDIVARELIEKMIAMDFCRPSANDVLKHPFFWSLQKQLQFFQDVSRIEKEPDLSPVRCLEGRGRVVKM	872
Homo_sapiens_IRE1 β	HFFGSLTRQCANILGAVSDFLHEEVEDIVARDLVGAMSSPLQPRPSAPQVLAHPFFWSRQKQLQFFQDVSVDLEKESLQEPVLRALRGGCAVVRD	821
Consensus	hpfq sl rqanil ga ldhl hedv ar lie m p rpsa vlkhpffws ekqlqffqdvdsrieke d piv le ggr vv	
C. idellus_IRE1 α	DWRHITVPLQIDLRFRFVYKGGSVRDLLRAMRNKHHYRELPEVQETLGSIPDFEVSVFTSRFFLLQHTHLAMRFCAVERPEFLPYHASELLTHHTD	974
C. idellus_IRE1 β	NWRMHISAPLQDLRKFRTYKGGSVRDLLRAMRNKHHYHELPSAQIALGVEVDFGLAVFTSRFFLLHHTHSALSICAFERLFPYHYH.....	944
Homo_sapiens_IRE1 α	DWRHITVPLQIDLRFRFVYKGGSVRDLLRAMRNKHHYRELPEVRETLGLLPDPEVQFTSRFFLLHHTHTRAMELCHERLFPYHYH.....	963
Homo_sapiens_IRE1 β	NWRHISMPQLIDLRKFRSFKGGSVRDLLRAMRNKHHYRELPEVQALGQVDFEVQFTSRFFLLHHTHTRAMELCHERLFPYHYH.....	913
Consensus	wr hi plqtdlrkfr ykg svrdllramrnkhhylrelpev lg pd fv yftrfp ll hth am ca erlf pyy	
C. idellus_IRE1 α	AHCATQTPPTQHSSSEVPSGLSSSSQISAVIPELITTEMHITQTPVPPALCAQETGA	1030
C. idellus_IRE1 β	944
Homo_sapiens_IRE1 αEPPEPCHVTPDAL.....	977
Homo_sapiens_IRE1 βSEARRPCHSATGR.....	926
Consensus	p	

365 Fig.2 Phylogenetic tree based on the protein sequences of IRE1 α and IRE1 β from grass carp and
 366 other species using the neighbor-joining (NJ) method in MEGA 5.0. Branch support values
 367 represent a percentage of 1000 bootstrap replicates.
 368

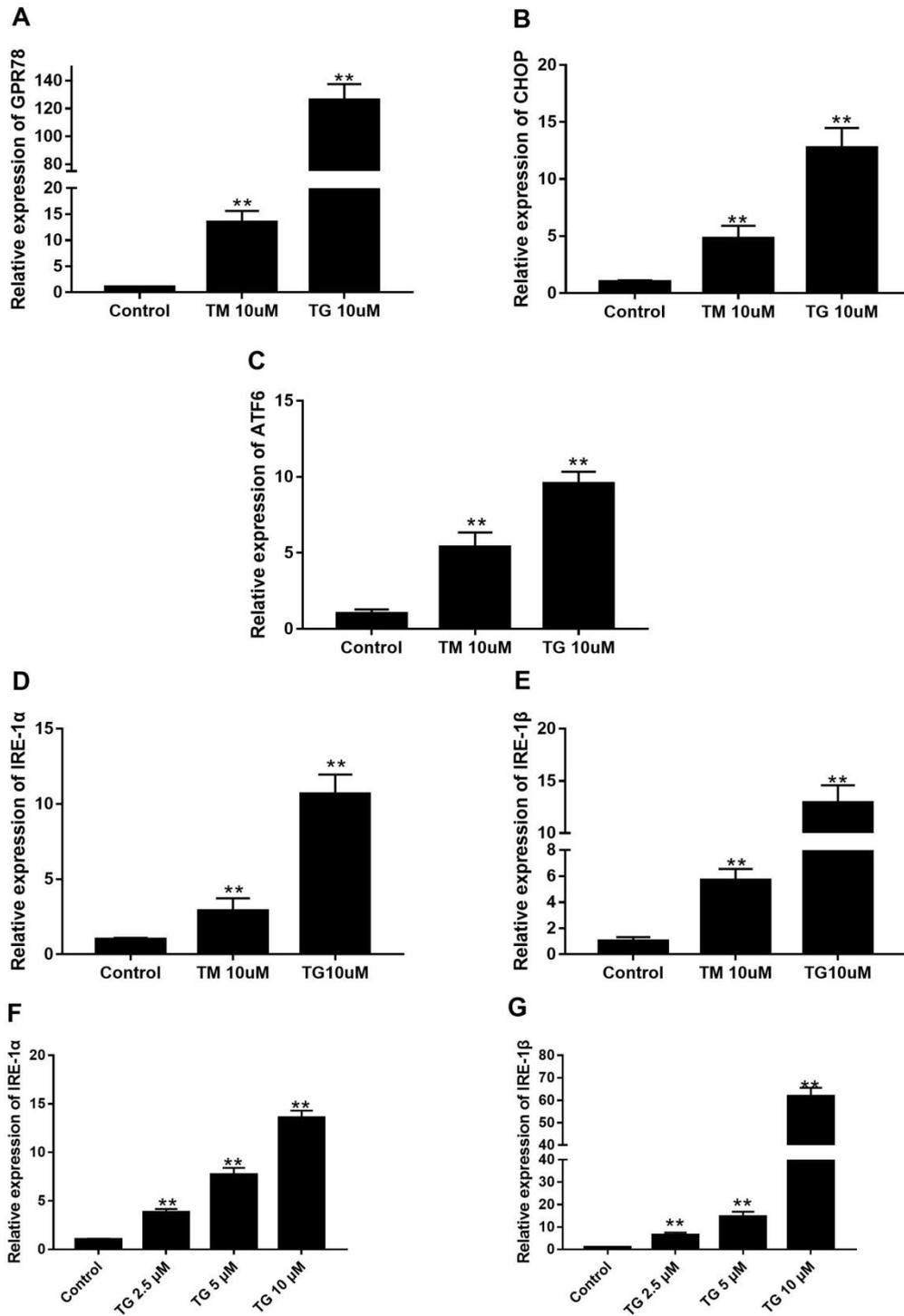


369 Fig.3 Expression profiles of IRE1 α and IRE1 β gene in 12 tissues of grass carp obtained by qPCR.
 370 The 12 tissues were heart (He), liver (Li), spleen (Sp), head kidney (Hk), kidney (Ki), brain (Br),
 371 foregut (Fg), mid-gut (Mg), hindgut (Hg), gill (Gi), red muscle (Rm) and white muscle (Wm).
 372 β -actin served as an internal control to calibrate the cDNA template for all of the samples.



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386 Fig.4 IRE1 α and IRE1 β levels were upregulated in response to ER stress in CIK cells. Cells were
 387 treated with TM (10 μ M) and TG (0, 2.5, 5, or 10 μ M) for 24 hours. The relative mRNA levels were
 388 detected by Real-time PCR.

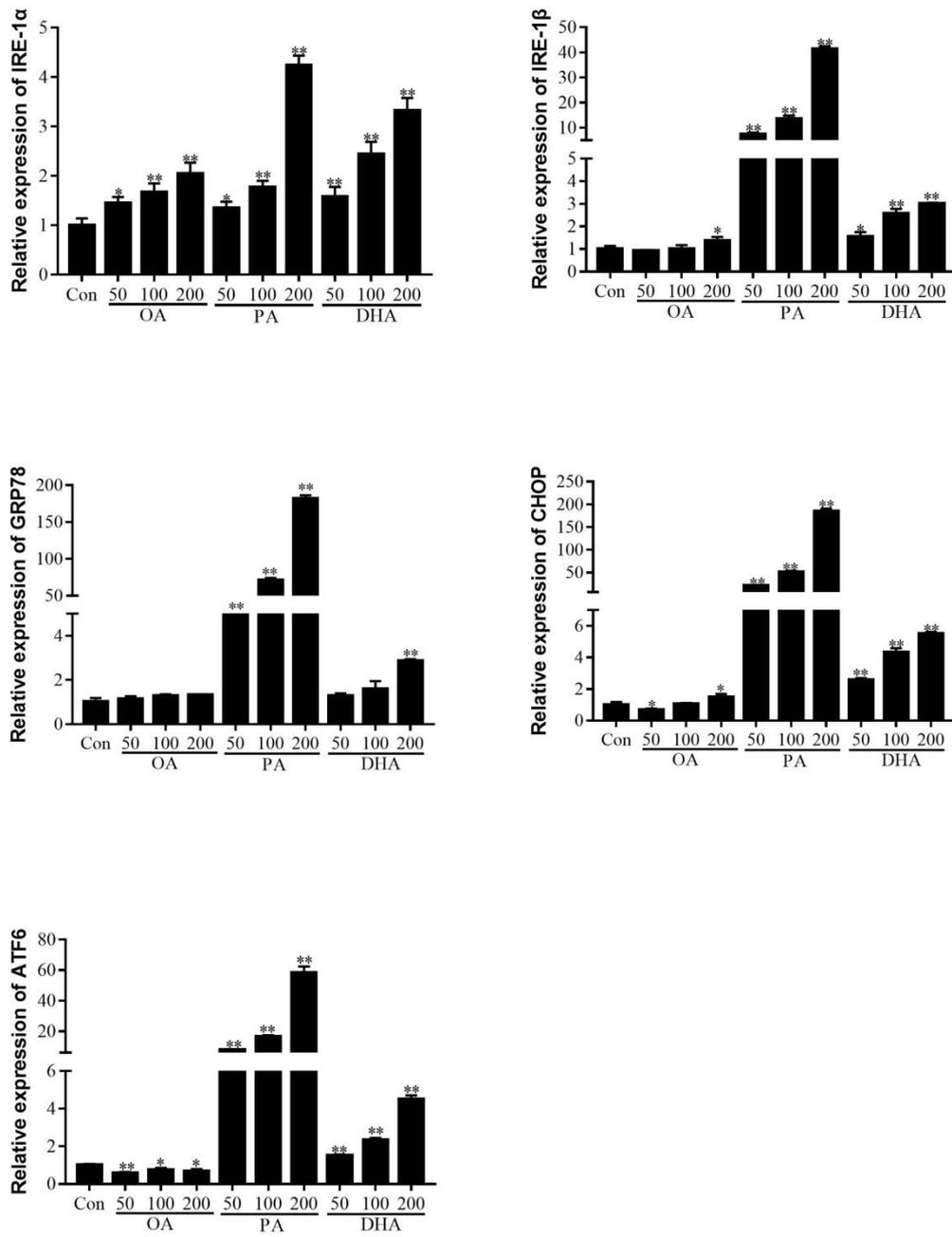


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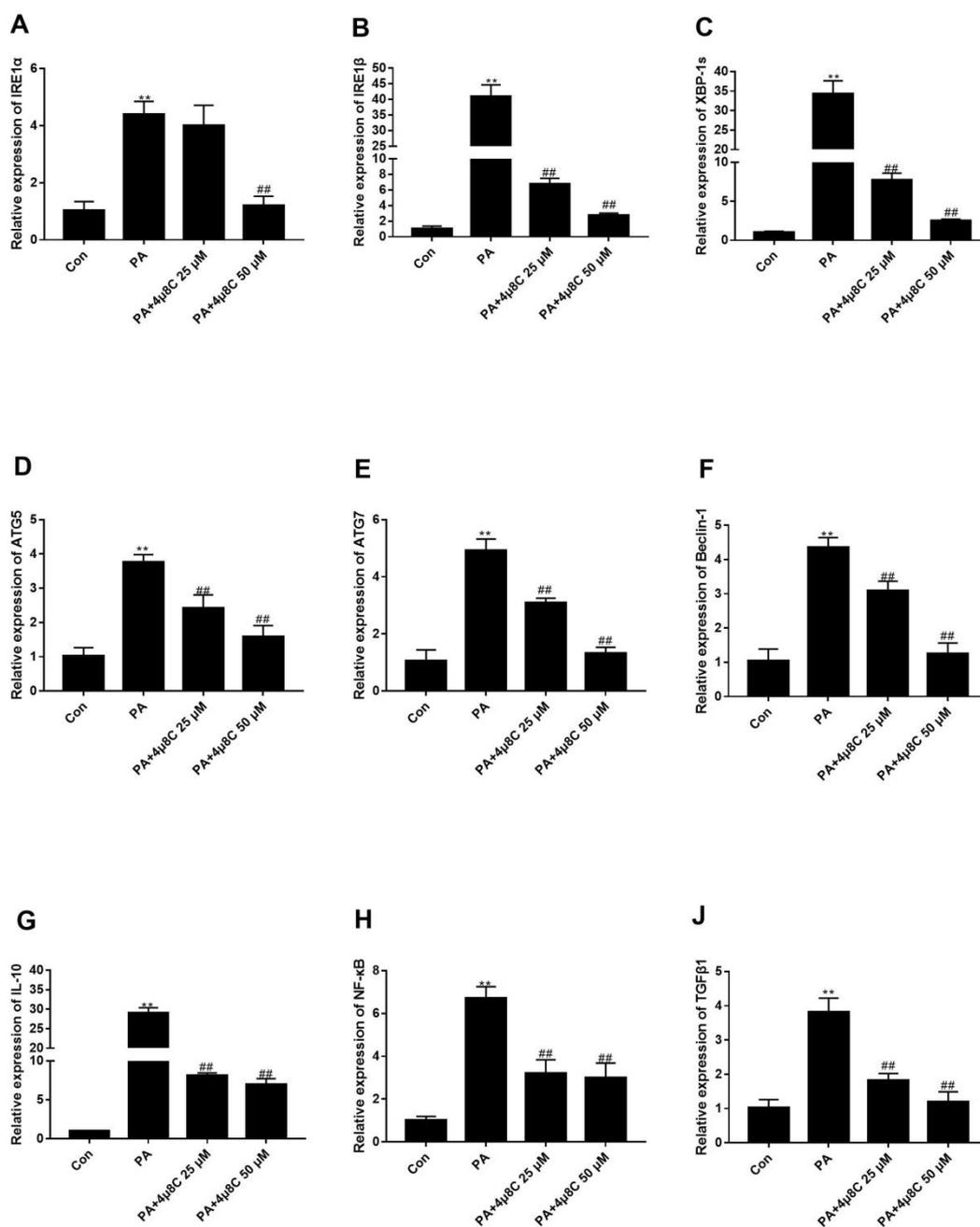
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392 Fig.5 Effects of fatty acids on ER stress in CIK cells. CIK cells were incubated with various
 393 concentrations of fatty acids for 24 hours. Cells treated with BSA as the control, after incubation,
 394 Total RNA was extracted and then quantified using Real-time PCR.



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398 Fig.6 Inhibition of IRE1 α ameliorates PA induced autophagy and inflammation. CIK cells were
 399 later either pretreated with 4 μ 8C for 4 h, followed by treatment with PA (200 μ M) for 24 h. CIK
 400 cells treated with vehicle (DMSO) and BSA were used for control. Total RNA was extracted and
 401 then quantified using Real-time PCR. *significantly different compared with controls (P < 0.05); #
 402 significantly different compared with PA (P < 0.05).
 403



Figures

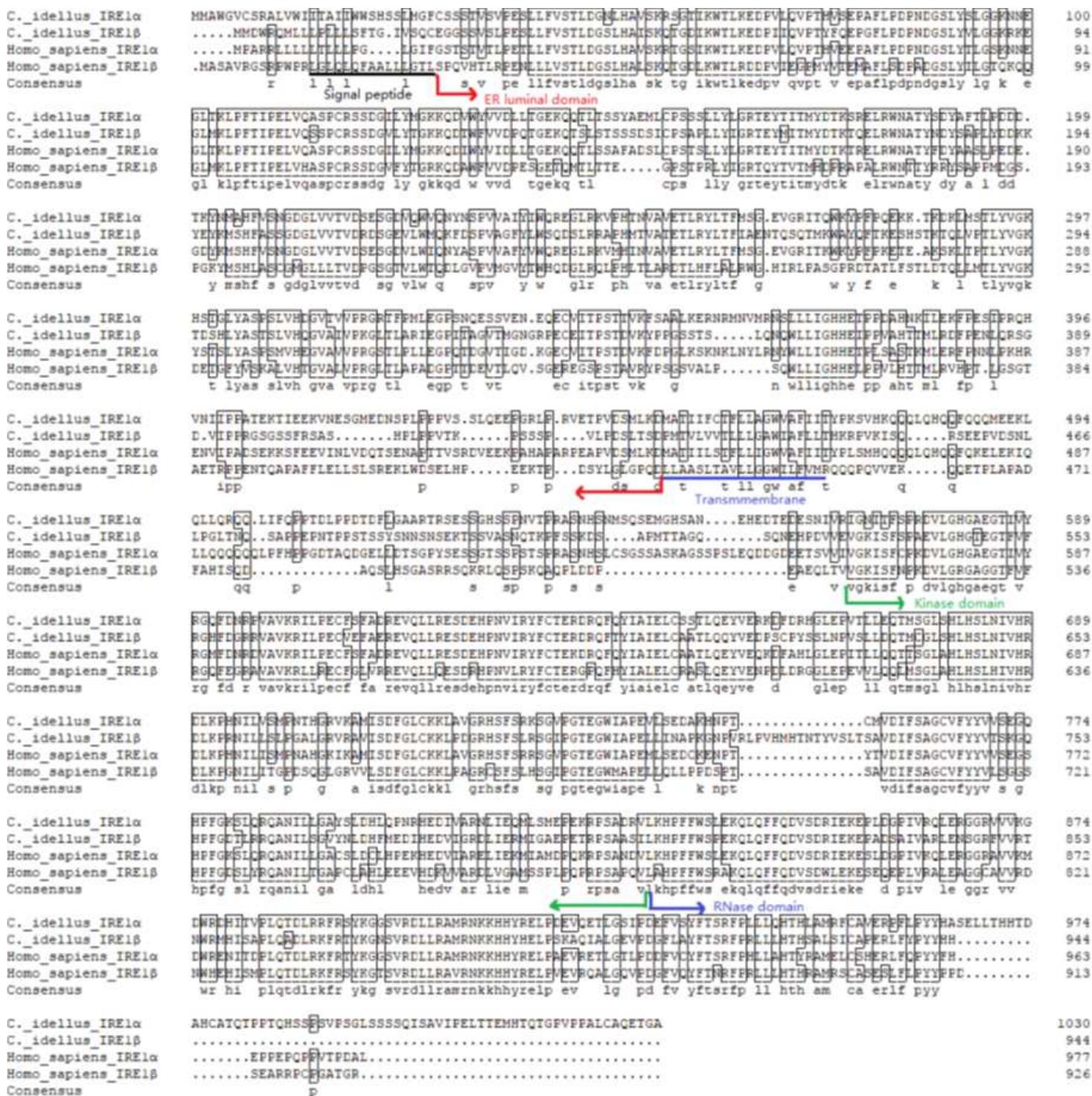


Figure 1

Amino acid sequence alignment of the IRE1α and IRE1β in grass carp with homologs from human, respectively. Identical residues are marked by asterisks underline. Signal peptide and domains are as indicated.

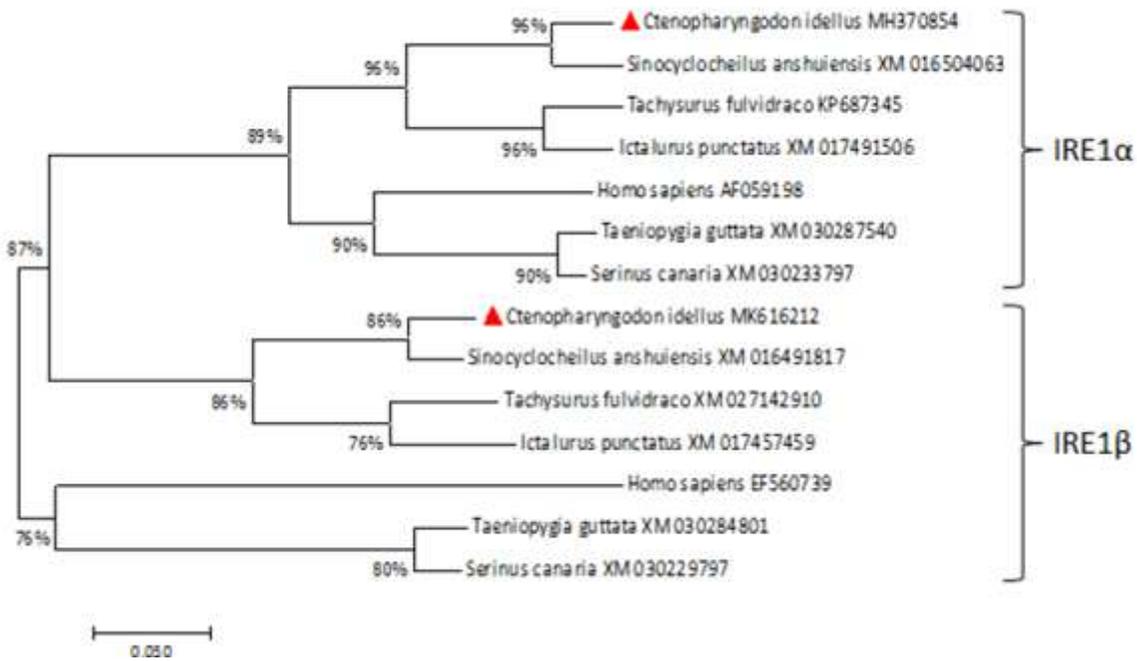


Figure 2

Phylogenetic tree based on the protein sequences of IRE1 α and IRE1 β from grass carp and other species using the neighbor joining (NJ) method in MEGA 5.0. Branch support values represent a percentage of 1000 bootstrap replicates.

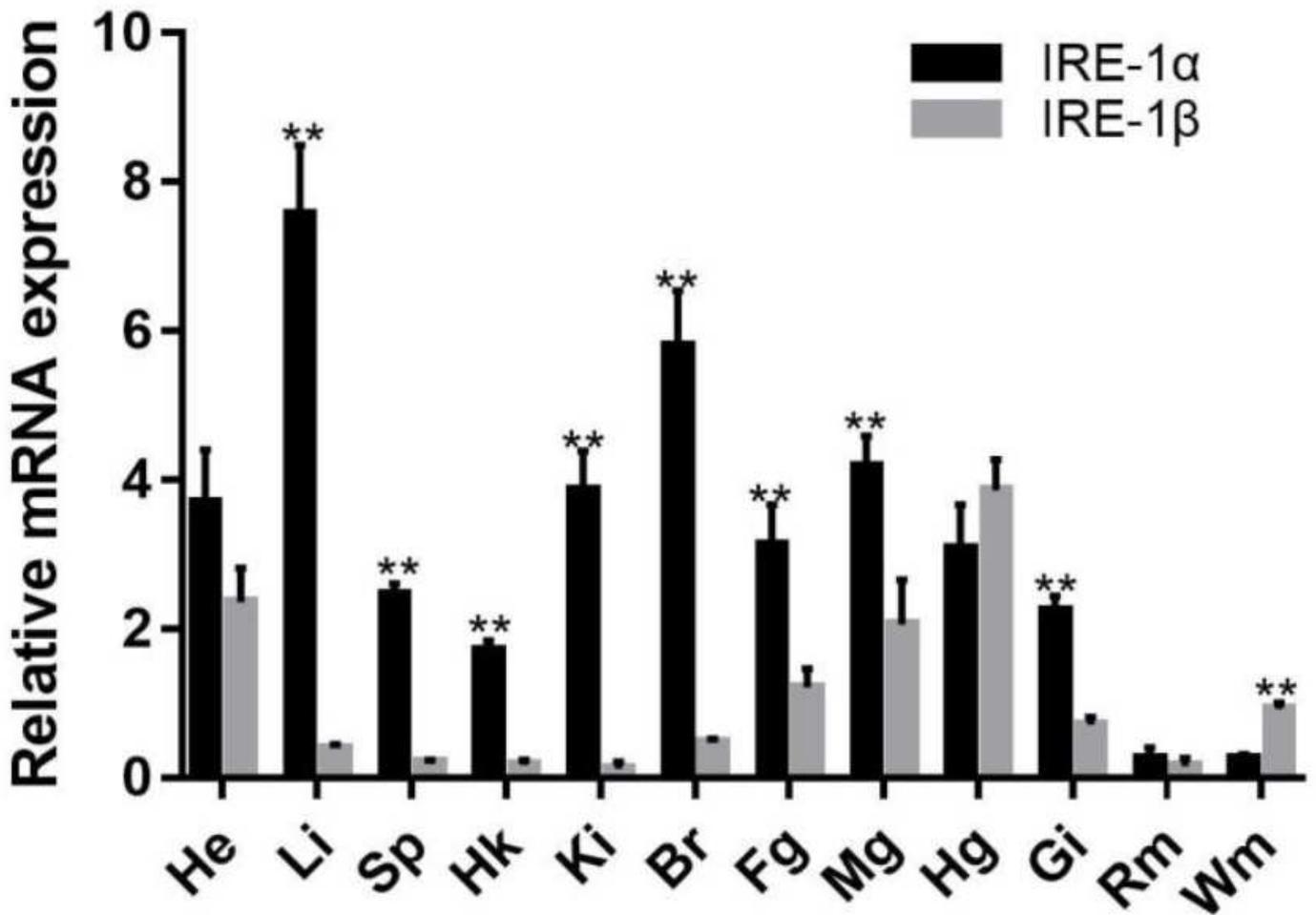


Figure 3

Expression profiles of IRE1 α and IRE1 β gene in 12 tissues of grass carp obtained by qPCR. The 12 tissues were heart (He), liver (Li), spleen (Sp), head kidney (Hk), kidney (Ki), brain (Br), foregut (Fg), mid gut (Mg), hindgut (Hg), gill (Gi), red muscle (Rm) and white muscle (Wm). β actin served as an internal control to calibrate the cDNA template for all of the samples.

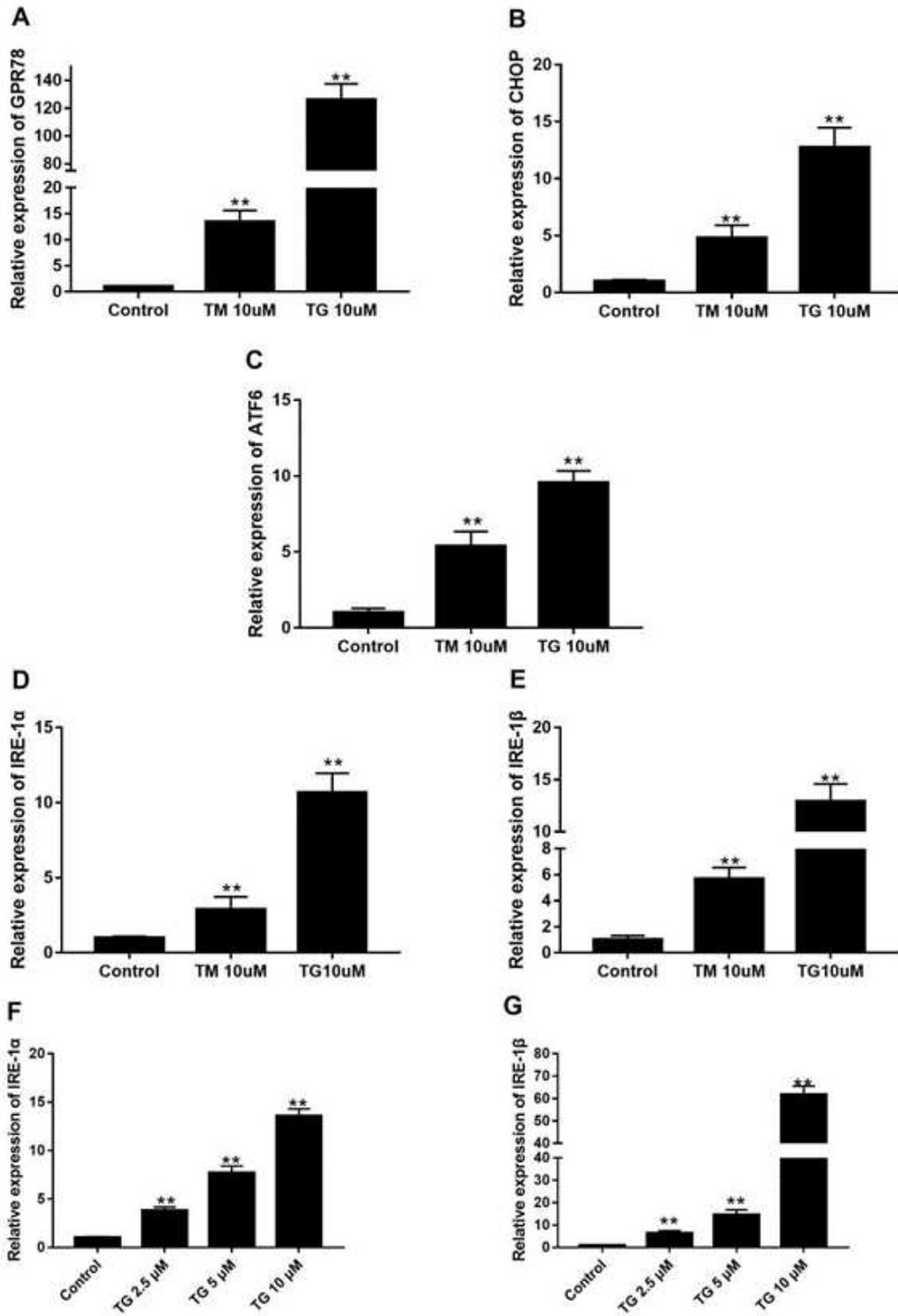


Figure 4

IRE1 α and IRE1 β levels were upregulated in response to ER stress in CIK cells. Cells were treated with TM (10 μ M) and TG (0, 2.5, 5, or 10 μ M) for 24 hours. The relative mRNA levels were detected by Real time PCR.

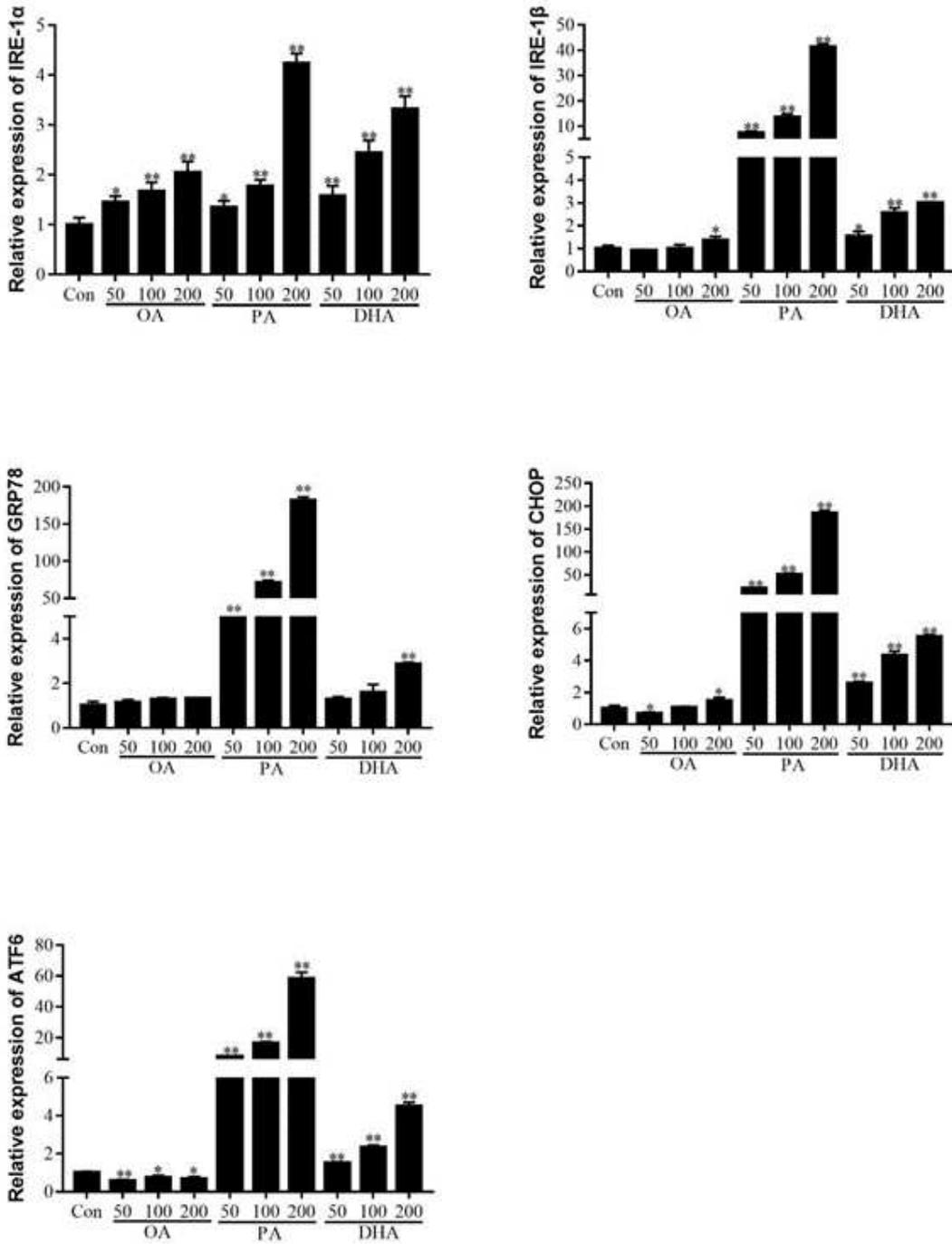


Figure 5

Effects of fatty acids on ER stress in CIK cells CIK cells were incubated with various concentrations of fatty acids for 24 hours C ells treated with BSA as the control, after incubation, Total RNA was extracted and then quantified using Real time PCR.

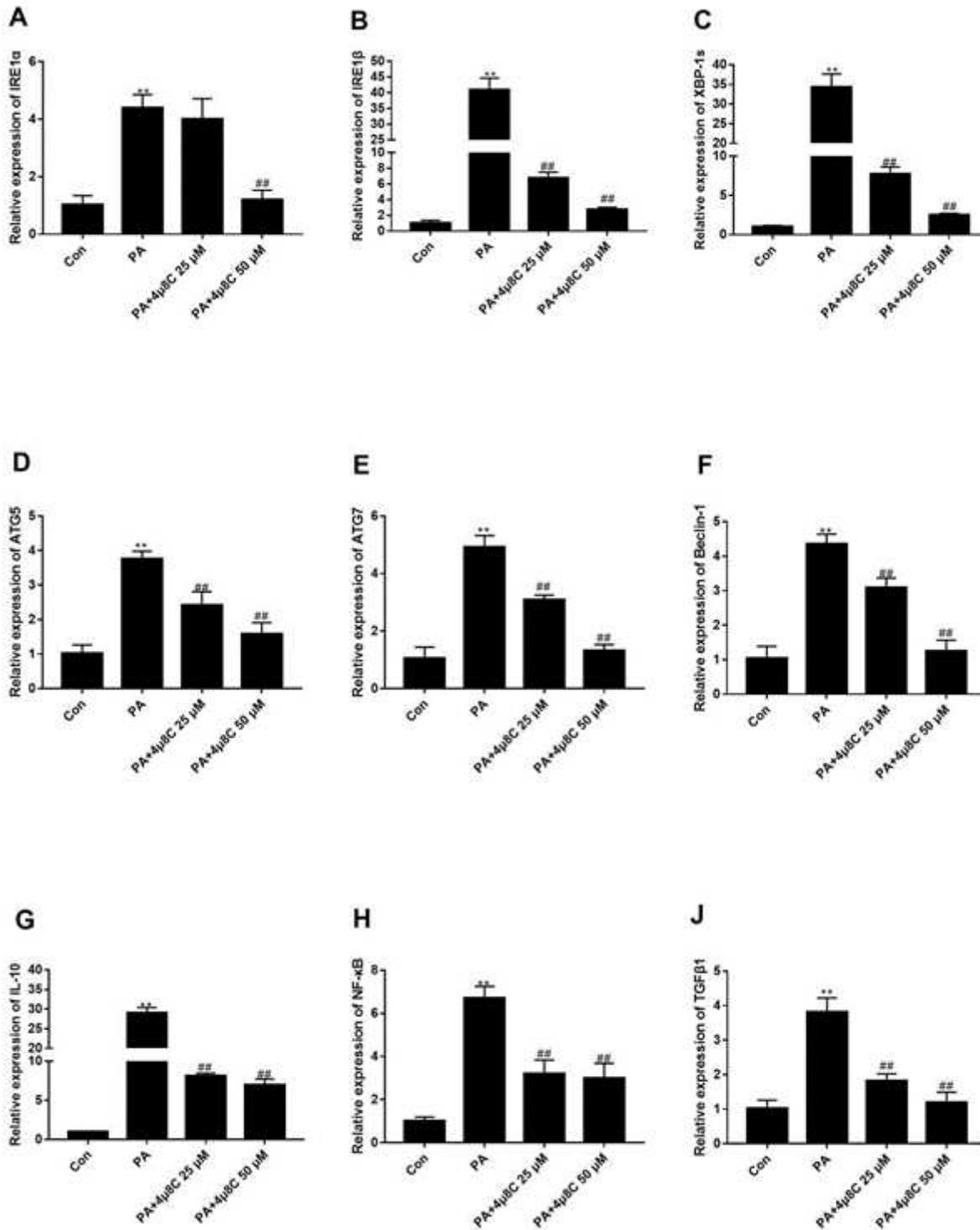


Figure 6

Inhibition of IRE1α ameliorates PA induced autophagy and inflammation CIK cells were later either pretreated with 4μ8C for 4 h, followed by treatment with PA (200 μM) for 24 h. CIK cells treated with vehicle (DMSO) and BSA were used for control. Total RNA was extracted and then quantified using Real time PCR. *significantly different compared with controls (P < 0.05); # 401 significantly different compared with PA (P 0.05).

Supplementary Files

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