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RESEARCH ARTICLE

Dietary gossypol suppressed postprandial TOR signaling and elevated ER stress pathways in turbot (*Scophthalmus maximus* L.)

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Bian F, Jiang H, Man M, Mai K, Zhou H, Xu W, He G. Dietary gossypol suppressed postprandial TOR signaling and elevated ER stress pathways in turbot (*Scophthalmus maximus* L.). *Am J Physiol Endocrinol Metab* 312: E37–E47, 2017. First published November 15, 2016; doi:10.1152/ajpendo.00285.2016.—Gossypol is known to be a polyphenolic compound toxic to animals. However, its molecular targets are far from fully characterized. To evaluate the physiological and molecular effects of gossypol, we chose turbot (*Scophthalmus maximus* L.), a carnivorous fish, as our model species. Juvenile turbots (7.83 ± 0.02 g) were fed diets containing gradient levels of gossypol at 0 (G0), 600 (G1), and 1,200 (G2) mg/kg diets for 11 wk. After the feeding trial, fish growth, body protein, and fat contents were significantly reduced in the G2 group compared with those of the G0 group ($P < 0.05$). Gossypol had little impact on digestive enzyme activities and intestine morphology. However, gossypol caused liver fibrosis and stimulated chemokine and proinflammatory cytokine secretions. More importantly, gossypol suppressed target of rapamycin (TOR) signaling and induced endoplasmic reticulum (ER) stress pathway in both the feeding experiment and cell cultures. Our results demonstrated that gossypol inhibited TOR signaling and elevated ER stress pathways both in vivo and in vitro, thus providing new mechanism of action of gossypol in nutritional physiology.

gossypol, turbot (*Scophthalmus maximus* L.); liver fibrosis; TOR; ER stress

COTTONSEED MEAL (CSM) is a plant protein source with high protein content that has been widely used in livestock, poultry, and aquaculture feeds (45, 47, 53, 55). However, the presence of gossypol in CSM as a major antinutritional factor has limited the performance of CSM in animals (38, 55). High levels of free gossypol in diets may be responsible for acute clinical signs in animals (14). The typical negative effects of gossypol in livestock, poultry, and aquatic animals are loss of weight, weakness, appetite decrease, breathing difficulties, reproductive problems, and histological changes in various tissues and organs (38, 46, 47).

Many studies on gossypol have highlighted a wide range of biological activity manifestations. Gossypol exerted its actions through interactions with a spectrum of biomolecules, including enzymes, signaling mediators, and membranes (8). Studies showed that gossypol was highly reactive and bound to metabolic enzymes, including oxidoreductases, transferases, hydrolases, lyases, etc. (8). Gossypol can also bind to a wide range of substances including minerals and amino acids (6). These effects were presumably through the formation of Schiff's bases via the reaction of the aldehyde groups of gossypol with the amino groups of the lysine residues of enzymes, or via H-bond formation with the catechol hydroxyls (8). Furthermore, gossypol has been identified as a BH3-mimetic inhibitor of Bcl-2 family members and therefore induces cell apoptosis through interaction with the mitochondrial caspase pathways (49). Other effects of gossypol included inhibition of cell proliferation through induction of transforming growth factor-β1 (TGFβ1) and downregulation of cyclin D1 (25, 69). Further elucidation of the molecular targets involved in gossypol actions will provide better understanding of the multifaceted biological effects of gossypol and better use of cottonseed as a feed gradient in husbandry.

During recent years, mounting evidence has supported the notion that sensing of nutrition status by signaling pathways is critical for animal growth and homeostasis (21, 22, 43, 65). In particular, nutrient sensing pathways stimulate anabolism when food is abundant, whereas food scarcity shifts toward catabolism (10). Among all of the signaling pathways, target of rapamycin (TOR) signaling is the main mediator of cellular nutrient sensing. It senses and integrates a variety of environmental cues, including growth factors, nutrients, energy, and stress, to regulate organismal growth and homeostasis (33, 64). On the other hand, when cells are exposed to stress, stress response pathways are activated. Stress-induced shutdown of translation is triggered by phosphorylation of the eukaryotic initiation factor 2α (eIF2α), which inhibits the initiation step of protein synthesis (2). Recently, it was demonstrated that the composition of diet, including protein levels (31), amino acid composition (28, 60), lipid content (30), and quality (56) influenced the responses of nutrient sensing pathways. However, few studies were conducted to examine whether antinutritional factors such as gossypol influenced the postprandial responses of nutrient sensing pathways. In this study, we chose turbot (*Scophthalmus maximus* L.), an economically valuable marine carnivorous fish as our experiment model species. The effects of gossypol on phenotypical parameters, histology, and postprandial responses of cell signaling pathways were characterized.

MATERIALS AND METHODS

All animal care and handling procedures in this study were approved by the Animal Care Committee of Ocean University of China.

Diet formulations. The composition of the basal diet is listed in Table 1. Experimental diets were designed by introducing gossypol into the basal diet at 0 (G0), 600 (G1), and 1,200 (G2) mg/kg diets, a range that had been widely used in other studies and represents the gossypol concentration in CSM used in aquafeeds (3, 5, 66). Gossy-

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Table 1. *Composition of basal diet*

Ingredients	g/kg Dry Diets
Fishmeal*	620
Wheat meal*	280
Fish oil	40
Soy lecithin	20
Choline chloride	3
Vitamin premix†	10
Mineral premix‡	20
Calcium propionic acid	1
Ethoxyquinoline	1
Attractants§	5
Proximate composition	
Crude protein	507.2
Crude lipid	125.1
Gross energy/(KJ/g) **	20.66

*Red fishmeal (g/kg dry matter): protein 739.1, crude lipid 98.2; wheat meal (g/kg dry matter): crude protein 175.1, crude lipid 17.9. These ingredients were obtained from Great Seven Bio-Tech (Qingdao, China). †Vitamin premix (mg/kg dry diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 10; vitamin K, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 60; retinol acetate, 32; cholecalciferol, 5; α-tocopherol, 240; ascorbic acid, 2,000; microcrystalline cellulose, 1,473. ‡Mineral premix: (mg/kg dry diet): $CoCl_2$ (1%), 50; $CuSO_4{\cdot}5H_2O$, 10; $FeSO_4{\cdot}H_2O$, 80; $ZnSO_4{\cdot}H_2O$, 50; $MnSO_4{\cdot}H_2O$, 45; $MgSO_4{\cdot}7H_2O$, 1,200; H_2NaOSe(1%), 20; H_2CaIO_4 (1%), 60; Zeolite powder, 8,485. §Attractants (g/kg dry diet): betaine, 2; dimethylpropiothetin, 1; glycine, 1; alanine, 0.5; inosine-5′-diphosphate trisodium salt, 0.5. **Gross energy of experimental diets was calculated according to gross energy values 23.64 KJ/g crude protein, 39.54 KJ/g crude fat, 17.57 KJ/g carbohydrate, respectively.

pol-acetic acid (1 mg = 0.8962 mg gossypol) is the naturally occurring acetic acid form of gossypol and was purchased from Chukang Biotechnology Co. (purity ≥98%). This form contained equal amounts of (+) and (−) isomers of gossypol. Feed ingredients were ground into fine powder through 300-μm meshes. All ingredients were blended thoroughly, added with oil, and mixed with water and pelleted with an experimental feed mill and dried for 16 h in a ventilated oven at 45°C. After drying, the diets were stored at −20°C until used.

Feeding trial and sampling. Juvenile turbots were purchased from a local fish rearing farm (Qingdao, China). Experiments were done at Yihaifeng Aquatic Product Co. (Qingdao, China). All fish were acclimated to laboratory conditions and fed a commercial diet for 2 wk before experiments. After being fasted for 24 h, fish (7.83 ± 0.02 g) were randomly assigned to nine experimental fiberglass tanks (300 liters) with 35 fish per tank and three tanks per treatment. Seawater, continuously pumped from the coast adjacent to the experimental station, passed through sand filters into each tank at ~1.5 l/min. Each diet was randomly assigned to triplicate groups. Fish were manually fed to apparent satiety two times daily at 7:00 AM and 7:00 PM The feeding trial lasted for 11 wk. During the experimental period, the water temperature ranged from 19 to 21°C, salinity from 29 to 32%, and dissolved oxygen was ~6 mg/l, pH from 7.5 to 8.0.

When the feeding trial was completed, fish were left unfed for 48 h to obtain the basal levels of metabolism in fish (56). After they had been starved for 24 h, fish from each tank were weighed and counted. Four fish per tank (12 fish per treatment) were randomly sampled and stored at −20°C for biochemical analysis. Four other fish from each tank (12 fish per treatment) were anesthetized with benzocaine (30 mg/l), and individual body weight, body length, visceral weight, and liver weight were examined to calculate condition factor (CF), viscerosomatic index (VSI), and hepatosomatic index (HSI).

At the end of the 48-h fasting period, the sampled fish was designated as the 0-h sample (fasted fish). Fish were then fed a single meal until apparent satiation. Samples were collected at 2 and 8 h after refeeding, the time period reflecting symbolic postprandial responses in turbot (65). At each time interval, four fish from each tank were collected. The fish were visually checked whether the belly was bulging or not to ensure that the fish had effectively consumed the diet. Blood was taken from the caudal vein using heparinized syringes to heparin anticoagulation tubes and centrifuged at 3,000 *g* for 10 min at 4°C to obtain plasma samples and stored at −80°C until analysis. After the blood was taken, liver and intestine were immediately dissected, frozen in liquid nitrogen, and kept at −80°C for later analysis.

Cell culture and treatment. The turbot primary cell culture was conducted as described before (62). Specifically, turbot (~15 g) was immersed in 75% alcohol for 30 s and then washed with sterile Dulbecco's phosphate-buffered saline (DPBS) with antibiotics (400 U/ml penicillin and 400 μg/ml streptomycin, GIBCO: 15240–112). White dorsal muscle was excised under sterile conditions and collected in cold Leibovitz's L-15 medium (Sigma: L5520) with antibiotics. The tissue was cut into 1.0-mm^3 pieces using stainless steel eye scissors and forceps. After centrifugation at 300 *g* for 10 min, the pellets were digested with 0.05% trypsin solution (GIBCO: 25200–056) for 30 min at room temperature with gentle agitation. The reaction was stopped with L-15 medium containing 10% fetal bovine serum (GIBCO: 10099–141). The cell suspension was filtered through a 70-μm nylon cell strainer (BD Falcon) and centrifuged (300 *g* for 10 min). The cell pellets were then resuspended in cold L-15 medium supplemented with 20 mM HEPES, 10% FBS, 1% antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml), 2 mM GlutaMAX (GIBCO: 35050–061), and basic fibroblast growth factor (2.5 ng/ml; GIBCO: PHG 0024). Cells were diluted to give 2×10^6 cells/ml medium and cultured at 24°C.

Cells of the zebrafish liver cell line (ZFL; ATCC CRL-2643) were cultured in ZFL medium consisting of Leibovitz's L-15, Dulbecco's modified Eagle's medium (DMEM; GIBCO: 12100-046), and Ham's F-12 (GIBCO: 21700-075; 50:35:15) supplemented with 0.15 g/l sodium bicarbonate (GIBCO: 25080-094), 15 mM HEPES, 0.01 mg/ml insulin, 50 ng/ml epidermal growth factor (EGF; GIBCO: PHG0311), 2 mM GlutaMAX, and 5% heat-inactivated fetal bovine serum at 28°C as recommended by ATCC.

For experiment, cells were treated with gossypol (Sigma: G8761) at 0, 5, 10, 20, or 30 μM for 4 h (turbot primary muscle cells) or for 1 h (ZFL). DMSO was used as vehicle. All cell culture experiments were repeated at least three times.

Cytotoxicity assay for gossypol. The cytotoxicity of gossypol was measured by a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide) assay (13, 70). Turbot primary muscle cells were plated at a density of 2.5×10^5 cells/ml into 96-well plates containing 100 μl of L-15 complete medium. After 24-h incubation, cells were treated with various concentrations of gossypol at 0 (control), 5, 10, 20, or 30 μM for 4 h, whereas for ZFL cells were plated at a density of 1.5×10^5 cells/ml into 96-well plates containing 100 μl of ZFL medium. After 24-h incubation, cells were treated with various concentrations of gossypol at 0 (control), 5, 10, 20, or 30 μM for 1 h. Then, 11 μl of MTT (5 mg/ml) was added to each well and incubated for another 4 h. Subsequently, the solution was removed, and 150 μl/well DMSO was added. The resulting MTT-formazan product was quantitated by absorbance at 570 nm using a microplate reader (Synergy HT, BioTek). Each treatment was duplicated in eight wells, and the experiment was repeated three times. The results are expressed as the average optical density ratio of the gossypol treatment group to the control group.

Biochemical analysis. Moisture, crude protein, crude lipid, and ash of ingredients, experimental diets, and fish samples were analyzed using standard methods as described before (65). For enzyme assays, intestine samples were homogenized in ice-cold phosphate-buffered saline and centrifuged at 4,000 *g* for 20 min at 4°C to collect the supernatant. Activities of trypsin, lipase and amylase were determined as described before (39, 63) using enzymatic assay kits (Jiancheng Bioengineering Institute, China).

Analysis of gossypol concentration in tissues. The gossypol concentrations in plasma and liver were determined as described before (35). Briefly, tissue samples were homogenized with extraction reagent (2% 2-amino-1-propanol and 10% glacial acetic acid in *N*,*N*-dimethylformamide) for 45 s. The mixture was heated at 95°C for 30 min, cooled on ice, and then centrifuged at 1,500 *g* for 5 min at 4°C. Subsequently, an aliquot of the supernatant was diluted with the mobile phase (8 vol of acetonitrile and 2 vol of 10 mM KH_2PO_3 adjusted to pH 3.0 with H_3PO_3) and filtered through a syringe filter (0.45 μm) before HPLC analysis. An HP1100 HPLC system (Agilent, Germany) equipped with a 4.6 × 250-mm Zorbax Eclipse C_{18} column (Agilent) was used. The samples were detected at 254 nm, with a flow rate of 1.0 ml/min; (+)- and (−)-gossypol-acetic acid (Sigma: G4382) was used as a standard.

Histological analysis. Four fish from each tank were anesthetized. The intestine and liver were dissected out and placed in Bouin's fixative solution (picric acid saturated solution: formalin: glacial acetic acid = 15:5:1) and transferred to 70% ethanol after 24 h. The fixed samples were then dehydrated in a series of alcohol solutions and embedded in paraffin. The tissues were further dissected into 5-μm sections and mounted onto albumin-coated slides. The intestinal slices were stained with hematoxylin and eosin (H&E), while the liver slices were processed for Masson trichrome staining. The morphological structures of these tissues were observed using an imaging microscope (Olympus, DP72, Nikon, Japan).

All digital images were analyzed using ImageJ v. 1.36. At least 12 slides from each treatment were analyzed. Intestine histology was analyzed to determine the ratios of villi height to gut lumen diameter and (microvilli height to gut lumen diameter. Villus height and microvillus height were measured from the base to the top of each segment (8 measurements per image) as described before (51).

Quantitative real-time PCR. Total RNA was extracted from liver (~50 mg) using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and quantified using a Nanodrop 2000 spectrophotometer (Thermo). The integrity of the RNA was examined using 1.5% agarose gel. cDNA preparation and qRT-PCR analysis were conducted as described (71). All primer sequences of target genes are listed in Table 2, and some sequences were published previously (15, 52, 65). To calculate the expression levels of target genes, results were normalized to elongation factor 1α *(ef1α)*, as no expression changes of *ef1α* were observed in liver among different treatments (data not shown). The gene expression levels were calculated by $2^{-\Delta\Delta CT}$ method (41). The data were reported as fold increase of the control (0 h sample of G0).

Table 2. *Primer sequences used for real-time quantitative PCR*

Gene*	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size (bp)
tnfα†	CCCTTATCATTATGGCCCTT	TCCGAGTACCGCCATATCCT	424
il1b†	TACCTGTCGTGCCAACAGGAA	TGATGTACCAGTTGGGGAA	181
TLR22	ATATTCACACGGAGGCCATT	CTTTGTTTGTGCAACCTGGA	110
MyD88	CCCAATGGTAGCCCTGAGAT	CATCTCGGTCGAACACACAC	153
socs1	AGAAGGTGGGGAAGTGAG	TAGGATGGTAGCCGACAGCA	230
socs3	CACTTTGACTGCGTCCTGA	CCATTGACCGTTTTCCTG	179
TGFβ†	ACAAGCCGACGGGCTACCATG	GGAGAGTGGCTTCAGTTTTTC	173
atf3	TCGCCATCCAGACCAAGC	ACTCCTTCTGCAAATCCTCC	234
chop	ACATGCACCGAGAAAGAGCC	CCTGCCGAACTATTTCCACT	245
redd1	TGGAGCACATCGGACAGGAG	GACGAGGTAGGGGTCCACAG	140
ef1α†	TCATTGGCCATGTCGACTCC	ACGTAGTACTTGGCGGTCTC	226

Partial sequences of some target genes in turbot were obtained through a degenerate PCR strategy in this study, including *MyD88*, myeloid differentiation primary response 88; *socs1*, suppressor of cytokine signaling-1; *atf3*, activating transcription factor 3; *chop*, C/EBP homology protein; *redd1*, regulated in development and DNA damage responses 1. *Abbreviations and GenBank accession nos.: *tnfα*, tumor necrosis factor-α, FJ654645.1; *il1b*, interleukin1β, AJ295836.2; *TLR22*, Toll-like receptor 22, KJ606345.1; *socs3*, suppressor of cytokine signaling-1, HM640022.1; *TGFβ*, transforming growth factor-β, AJ276709.1; *ef1α*, elongation factor-1α, AF467776.1. †Previously published primer sequence.

Western blot analysis. Tissues were homogenized with glass Tenbroeck tissue grinders (Kimble Chase) on ice and lysed with 50 mM Tris·HCl, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, and 1 mM EDTA, pH 7.5, with protease and phosphatase inhibitor cocktails (Roche) at 4°C for 1 h and cleared by centrifugation at 12,000 *g* for 20 min. Protein concentrations were determined with a BCA protein assay kit (Beyotime Biotechnology) using bovine serum albumin as standard. Protein samples (30 μg protein per lane) were separated by SDS-PAGE and transferred to 0.45-μm PVDF membrane (Millipore) for Western analysis. The membrane was blocked with 5% nonfat milk in TBST buffer (20 mM Tris·HCl, 500 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with primary antibody overnight at 4°C before horseradish peroxidase (HRP)-conjugated secondary antibodies were added and visualized using ECL reagents (GoodHere, China). The following antibodies were used: antibodies against AMPKα (2532), phospho-AMPKα (Thr^{172}, 2531), protein kinase B (Akt, 9272), phospho-Akt (Ser^{473}, 9271), TOR (2972), phospho-TOR (Ser^{2448}, 2971), p70 ribosomal protein S6 kinase (p70S6K, 9202), phospho-p70S6K (Thr^{389}, 9205), phospho-p70S6K (Thr^{421}/Ser^{424}, 9204), phospho-p70S6K (Ser^{371}, 9208), ribosomal protein S6 (S6, 2217), phospho-S6 ($Ser^{235/236}$, 4856), eukaryotic initiation factor 4E binding protein-1 (4E-BP1,), phospho-4E-BP1 ($Thr^{37/46}$, 9459), RNA-dependent protein kinase-like ER kinase (PERK, 3192), phospho-PERK (Thr^{980}, 3179), glucose-regulated protein-78 (GRP78, 3183), eIF2α (9722), phospho-eIF2α (Ser^{51}, 3597), and β-tubulin (2146) were purchased from Cell Signaling Technology. Antibodies against activating transcription factor 6α (ATF6α, sc-166659), X-box-binding protein (XBP-1, sc-7160), ATF4 (sc-200), small mothers against decapentaplegic 2/3 (Smad2/3, sc-133098), phospho-Smad2/3 ($Ser^{423/425}$, sc-11769), signal transducers and activators of transcription 1 (STAT1, sc-346), and phospho-STAT1 (Tyr^{701}, sc-7988) were purchased from Santa Cruz Biotechnology. All these antibodies were developed using antigenic regions completely conserved in turbot, and many had been successfully used in turbot and published before (65). The Western bands were quantified using NIH Image 1.63 software.

Statistical analyses. All statistical evaluations were analyzed using the software SPSS 19.0. The growth parameters, intestine index, and cell treatment assay were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests. The effects of time and diets and their interactions were analyzed by two-way ANOVA. Tukey's multiple range tests were used to examine treatment differences among the interactions. When the interaction was significant, the results were further analyzed using one-way ANOVA and Turkey's multiple range test. In case unequal variance was determined by Levene's test, the data were square root-transformed before statistical analysis. Differences were regarded as significant when $P < 0.05$. Data are expressed as means ± SE.

RESULTS

Dietary gossypol reduced growth and nutrient retentions in turbot. As shown in Table 3, the initial body weight in each group was 7.83 ± 0.02 g. However, after an 11-wk feeding trial, fish fed the G2 diet gained less body weight (63.1 ± 0.97 g) than those fed the G0 (72.3 ± 0.93 g) and G1 (71.5 ± 2.57 g) diets ($P = 0.015$; Table 3). Compared with the G0 (15.44 ± 0.12%) group, G1 (14.95 ± 0.17%) and G2 (14.77 ± 0.12%) groups had decreased whole body protein content ($P = 0.03$). Whole body lipid content was also lower in G1 (3.8 ± 0.42%) and G2 (3.32 ± 0.09%) groups compared with that of the G0 group (4.55 ± 0.18%, $P = 0.04$). No signif-

Table 3. *Growth parameters and feed utilization of juvenile turbots (Scophthalmus maximus L.) fed experimental diets for 11 wk*

	G0	G1	G2	*P* Value
Initial body weight (g)	7.82 ± 0.02	7.82 ± 0.02	7.86 ± 0.03	0.535
Final body weight (g)	72.3 ± 0.93[a]	71.5 ± 2.57[a]	63.1 ± 0.97[b]	0.015
Weight gain rate (%)*	825 ± 11.7[a]	814 ± 31.5[a]	703 ± 15.0b	0.012
Specific growth rate (%/day)[†]	2.89 ± 0.02[a]	2.87 ± 0.04[a]	2.70 ± 0.02[b]	0.008
Survival rate (%)[‡]	99.05 ± 0.95	100 ± 0.00	99.05 ± 0.95	0.63
Feed efficiency (g/g)[§]	1.39 ± 0.03	1.39 ± 0.01	1.31 ± 0.02	0.068
Feed intake (%/day) [**]	1.50 ± 0.03	1.50 ± 0.01	1.54 ± 0.01	0.293
Whole body protein (%)	15.44 ± 0.12[a]	14.95 ± 0.17[a,b]	14.77 ± 0.12[b]	0.03
Whole body lipid (%)	4.55 ± 0.18[a]	3.80 ± 0.42[a,b]	3.32 ± 0.09[b]	0.04
Whole body ash (%)	3.10 ± 0.19	3.03 ± 0.23	3.30 ± 0.07	0.57
Condition factor (%)[††]	3.43 ± 0.06	3.48 ± 0.13	3.44 ± 0.08	0.932
Viscerosomatic index (%)[‡‡]	4.27 ± 0.40	4.20 ± 0.12	4.45 ± 0.14	0.783
Hepatosomatic index (%)[§§]	1.05 ± 0.15	1.09 ± 0.09	1.07 ± 0.07	0.965

Values show means ± SE (n = 12); Significance was evaluated by one-way ANOVA followed by Tukey's multiple range tests. G0, control diet; G1, 600 mg gossypol/kg diet; G2, 1,200 mg gossypol/kg diet. [a,b] Mean values in the same row with different superscripted letters were significantly different ($P < 0.05$). *Weight gain rate (%) = (final body weight – initial body weight)/initial body weight × 100%. [†]Specific growth rate (%/day) = (Ln final body weight – Ln initial body weight)/days × 100%. [‡]Survial rate (%) = (final fish number/initial fish number) × 100%. [§]Feed efficiency = wet weight gain (g)/dry feed intake (g). **Feed intake (%/day) = dry feed intake/[(final body weight + initial body weight)/2]/days × 100%. [††]Condition factor (%) = final body weight (g)/body length $(cm)^3$ × 100%. [‡‡]Viscerosomatic index (%) = viscera weight (g)/whole body weight (g) × 100%. [§§]Hepatosomatic index (%) = liver weight (g)/whole body weight (g) × 100%.

icant differences were found for survival rates, feed efficiency ratio, or feed intake as well as CF, VSI, and HSI ($P > 0.05$).

Dietary gossypol accumulations in plasma and liver. The postprandial kinetics of gossypol concentrations in plasma and liver was assessed. As shown in Table 4, plasma gossypol levels remained high at 2 h and 8 h after refeeding, with basal levels (0 h) at 38.97 ± 0.81 μg/ml for G1 and 49.95 ± 1.30 μg/ml for G2, respectively. The liver concentration of gossypol peaked at 2 h after feeding with basal levels (0 h) at 111.2 ± 6.82 μg/g (G1) and 280.91 ± 8.57 μg/g (G2), respectively.

Table 4. *Changes of (+)-, (−)-isomers and total gossypol concentrations in plasma and liver of juvenile turbots (Scophthalmus maximus L.) fed experimental diets for 11 wk*

		Plasma (μg/ml)			Liver (μg/g dry matter)		
Diets	Time (h)	(+)-Isomer	(−)-Isomer	total	(+)-Isomer	(−)-Isomer	total
		Individual treatment means					
G1	0	2.07	36.91	38.97	37.54	73.66[a]	111.20
G1	2	2.89	47.74	50.77	83.79	90.46[a]	174.25
G1	8	3.07	48.59	51.66	66.75	81.67[a]	148.42
G2	0	2.99	46.96	49.95	129.23	151.69[b]	280.91
G2	2	3.15	54.97	58.12	164.67	208.33[c]	373.00
G2	8	3.41	54.55	57.96	135.00	200.00[c]	335.00
Pooled SE		0.07	0.48	0.50	2.58	1.82	2.51
		Means of main effect					
G1		2.68[x]	44.41[x]	47.14[x]	62.69[x]	81.93	144.62[x]
G2		3.18[y]	52.16[y]	55.34[y]	142.96[y]	186.67	329.64[y]
	0	2.53[A]	41.93[A]	44.46[A]	83.38[A]	112.67	196.06[A]
	2	3.02[B]	51.36[B]	54.45[B]	124.23[C]	149.39	273.62[C]
	8	3.24[B]	51.57[B]	54.81[B]	100.88[B]	140.83	241.7[B]
		Two-way ANOVA: P values					
Diet		0.004	<0.001	<0.001	<0.001	<0.001	<0.001
Time		0.005	<0.001	<0.001	<0.001	<0.001	<0.001
Diet × Time		0.164	0.249	0.173	0.22	0.001	0.099

Treatment means represent the average values for 3 tanks per treatment and were analyzed by two-way ANOVA (n = 12). Tukey's test was conducted for individual means only if there was a significant interaction ($P < 0.05$). [a,b,c]Mean values among all treatments within a row with different superscript letters were significantly different when the interaction was significant ($P < 0.05$). [A,B,C]Mean values among 3 time points with different superscript letters were significantly different ($P < 0.05$). [x,y]Mean values among 2 diets with different letters were significantly different ($P < 0.05$).

Gossypol influenced little on intestinal morphology and functions but caused liver fibrosis. As shown in Fig. 1*A*, the intestine morphology did not show significant changes in fish fed different diets. The ratios of villi height to gut lumen diameter and microvilli height to gut lumen diameter were not significantly different among groups. Furthermore, the activities of digestive enzymes, namely trypsin, lipase, and amylase, showed no significant differences among groups ($P > 0.05$, Fig. 1*B*). However, Masson's trichrome staining revealed overdeposition of blue-stained collagen fibers in livers of both G1 and G2 groups (Fig. 1*C*), suggesting the occurrence of liver fibrosis (23, 58). The postprandial mRNA expressions and proteins involved in liver fibrosis were also examined. These included the expression levels of *socs1*, *socs3*, *tnfα*, *il1b*, *TGFβ*, *TLR22*, and *MyD88* and phosphorylation of Smad2/3 and STAT1 (1, 4, 20, 48). The expression levels of *tnfα, il1b, TLR22, MyD88*, and *TGFβ* were upregulated, and *socs1* and *socs3* were downregulated in gossypol-supplemented groups (Fig. 1*D*). Furthermore, dietary gossypol increased the phosphorylation level of Smad2/3 and decreased that of STAT1 (Fig. 1*D*).

Gossypol inhibited postprandial TOR signaling and induced ER stress. The postprandial activities of TOR signaling were examined in liver (Fig. 2*A*). Indicated by their corresponding phosphorylation levels, the postprandial activation of Akt, TOR, S6, and 4E-BP1 were all decreased in both G1 and G2 groups compared with the G0 group. On the other hand, the phosphorylation level of AMPK was increased in both G1 and G2 groups compared with the G0 group. Compared with G0 diet, G1 and G2 diets significantly increased eIF2α phosphorylation and ATF4 levels (Fig. 2*B*). We also examined the mRNA expression of ER stress-related genes, which were activating transcription factor 3 (*atf3*), C/EBP homology protein (*chop*), and regulated in development and DNA damage responses 1 (*redd1*) (26, 59). The mRNA expression levels of these stress-induced genes were all upregulated more in the G2 group than those in the G0 group (Fig. 2*C*).

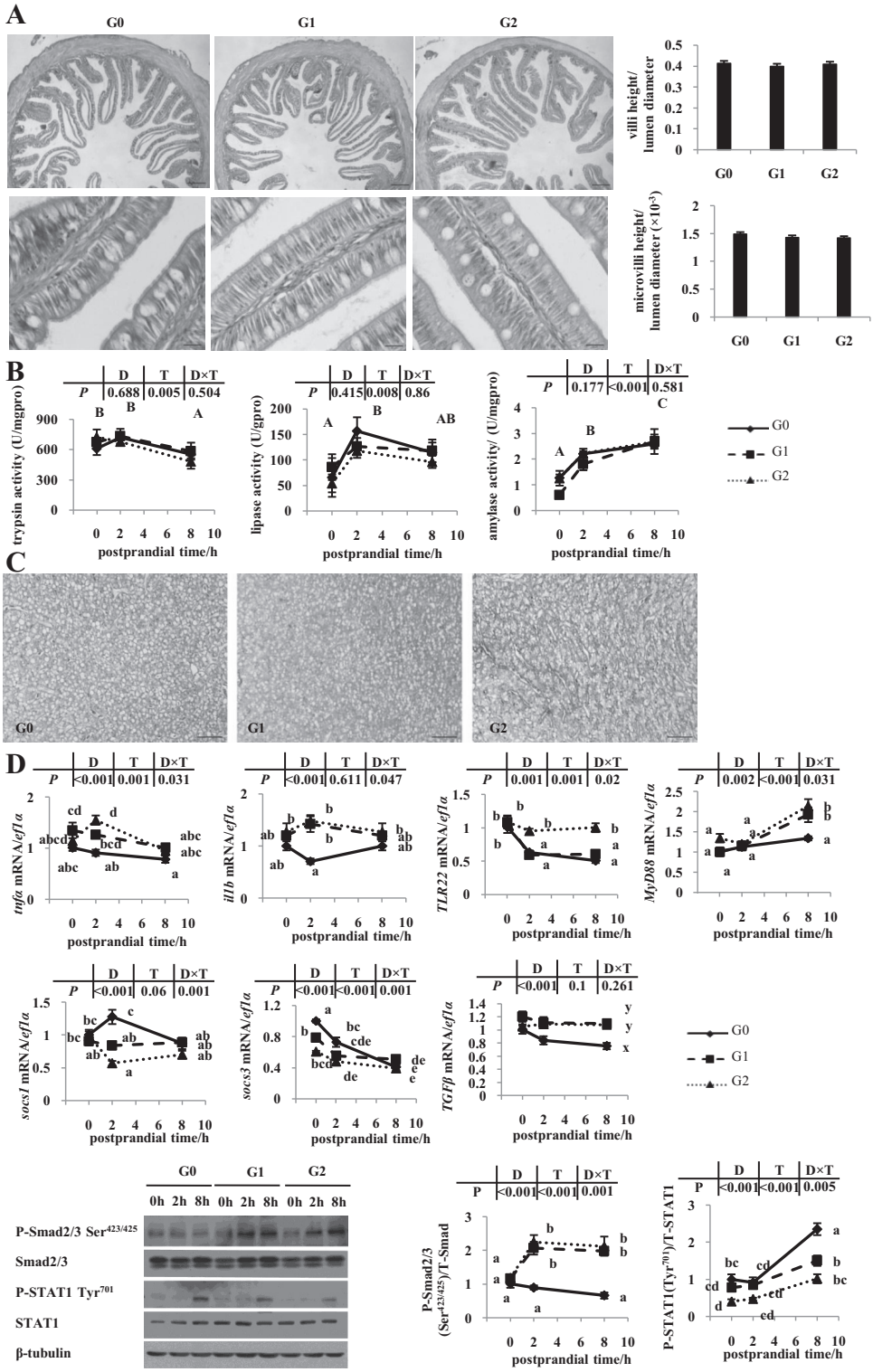

Fig. 1. Gossypol did not affect intestine morphology (*A*) and digestive enzymes activities (*B*) but caused liver fibrosis (*C*) after 11 wk of feeding trial. Expressions of genes related to liver fibrosis were analyzed (*D*). Intestine sections were stained by hematoxylin and eosin. Scale bars, 200 μm (*top*) and 20 μm (*bottom*), respectively. Liver sections were processed for Masson trichrome staining; scale bar, 50 μm. Results are represented as means ± SE ($n = 12$). Significance was evaluated by one-way ANOVA followed by Tukey's multiple range tests in *A*. Statistical analysis for genes related to liver fibrosis were analyzed by two-way ANOVA followed by Tukey's multiple range tests. D, diets; T, time points; T × D, interaction between T and D. G0, control diet (♦); G1, 600 mg gossypol/kg diet (■); G2, 1,200 mg gossypol/kg diet (▲). *tnfa*, tumor necrosis factor-α; *il1b*, interleukin-1β; *TLR22*, Toll like receptor 22; *MyD88*, myeloid differentiation primary response 88; *socs*, suppressor of cytokine signaling; *TGFβ*, transforming growth factor-β; Smad2/3, small mothers against decapentaplegic 2/3; STAT1, signal transducers and activators of transcription 1. [a,b,c,d]Mean values among all treatments with different letters were significantly different when the interaction was significant ($P < 0.05$). [A,B,C]Mean values among 3 time points with different letters were significantly different ($P < 0.05$).

Gossypol inhibited TOR signaling and activated ER stress pathway in cells. To exclude possible indirect and nonspecific pathological effects, we further examined the effect of gossypol in cell cultures. Turbot primary muscle cells were treated with a series of levels of gossypol (0, 5, 10, 20, 30 μM) for 4 h. Under these conditions, no apparent cytotoxicity of gossypol was observed by MTT assay (Fig. 3*A*). However, gossypol activated the phosphorylation of AMPK, and reduced the phosphorylation of Akt, TOR, p70S6K, S6, and 4E-BP1 in a dose-dependent manner (Fig. 3*B*). On the other hand, the levels of eIF2α phosphorylation, PERK, Grp78, ATF6α, XBP-1, and ATF4 were increased (Fig. 3*C*). These effects were further confirmed in ZFL cells and obtained the same results as described above (Fig. 4).

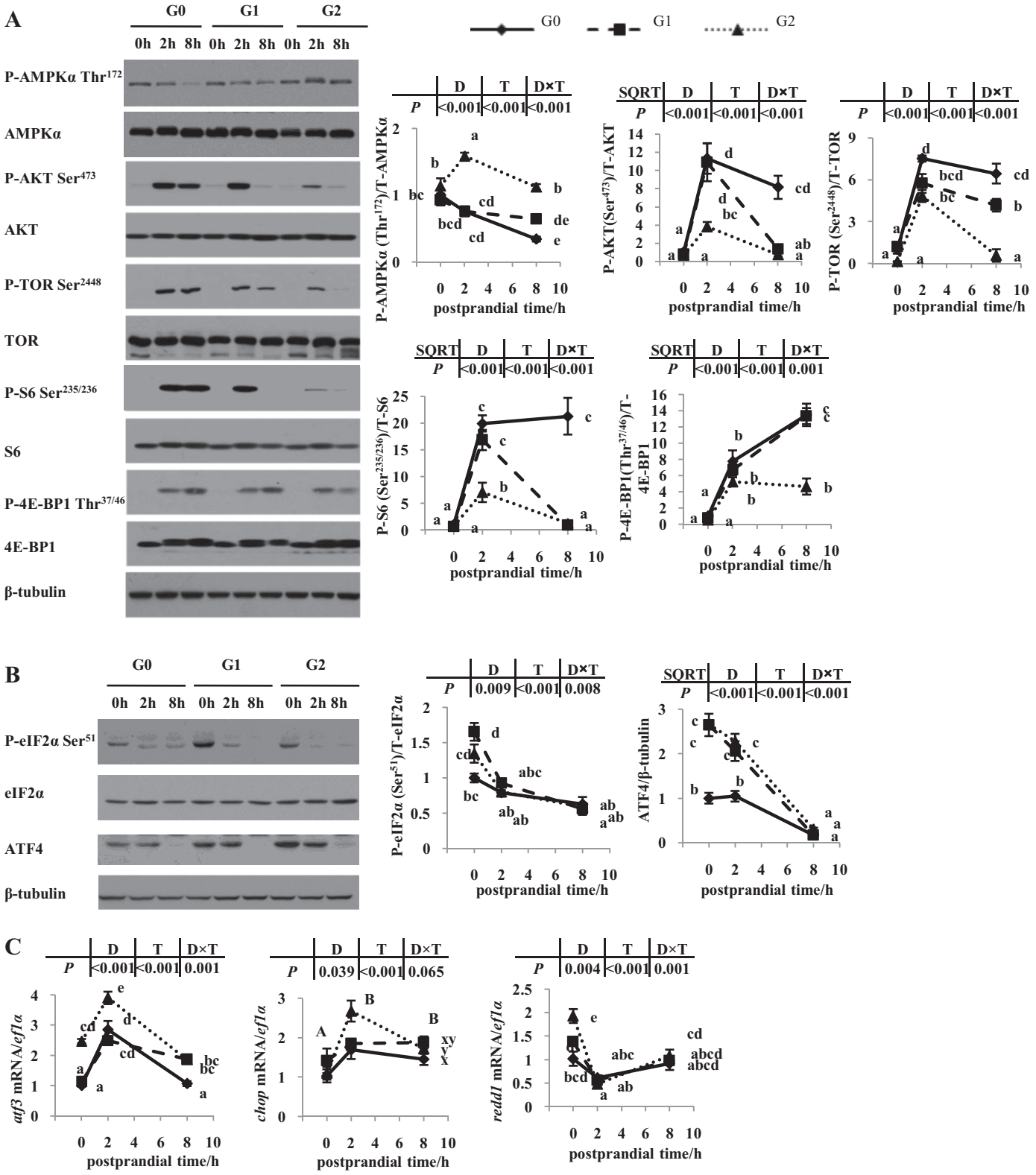

Fig. 2. Dietary modulations of nutrient sensing responses in target of rapamycin (TOR) signaling pathway (*A*), endoplasmic reticulum (ER) stress response pathway (*B*), and expression of related genes (*C*). Results are represented as means ± SE ($n = 12$) and were analyzed using two-way ANOVA followed by Tukey's multiple range test. G0, control diet (♦); G1, 600 mg gossypol/kg diet (■); G2, 1,200 mg gossypol/kg diet (▲). SQRT indicates data were transformed and statistically analyzed with square roots. AMPK, AMP-activated protein kinase; Akt, protein kinase B; S6, ribosomal protein S6; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; *atf3*, activating transcription factor 3; *chop*, C/EBP homology protein; *redd1*, regulated in development and DNA damage responses 1. ^{a,b,c,d,e}Mean values among all treatments with different letters were significantly different when the interaction was significant ($P < 0.05$). ^{A,B}Mean values among 3 time points with different letters were significantly different ($P < 0.05$). ^{x,y}Mean values among three diets with different letters are significantly different ($P < 0.05$).

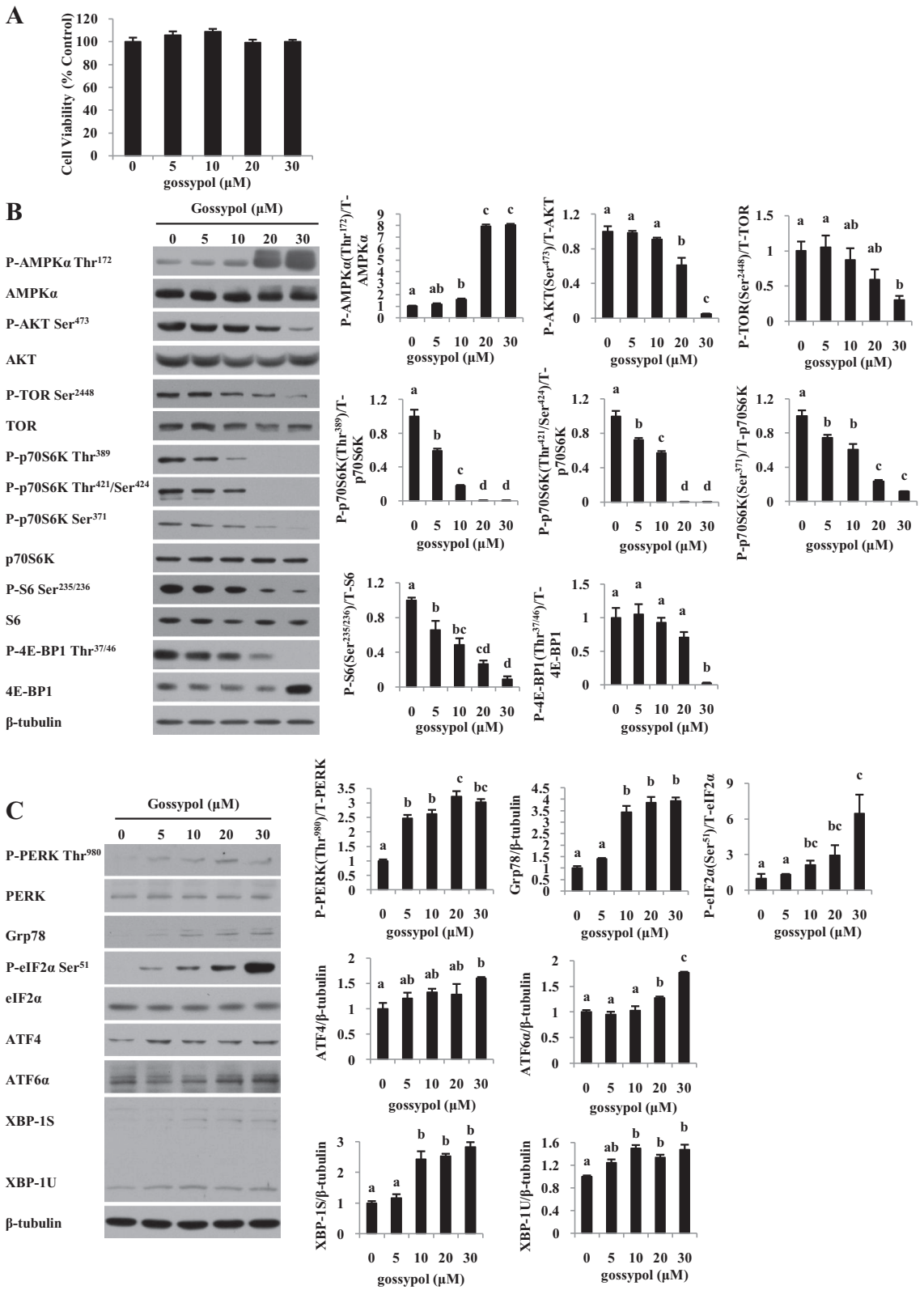

Fig. 3. Gossypol levels below cytotoxicity doses (*A*) inhibited TOR signaling (*B*), and stimulated ER stress pathway (*C*) in turbot primary muscle cells. Cells were treated with gossypol at 0, 5, 10, 20, or 30 μM for 4 h. Values are represented as means ± SE ($n = 3$). Significance was evaluated by one-way ANOVA followed by Tukey's multiple range tests. PERK, RNA-dependent protein kinase-like ER kinase; Grp78, glucose-regulated protein-78; XBP1, X-box-binding protein-1. [a,b,c,d]Mean values with different letters were significantly different ($P < 0.05$).

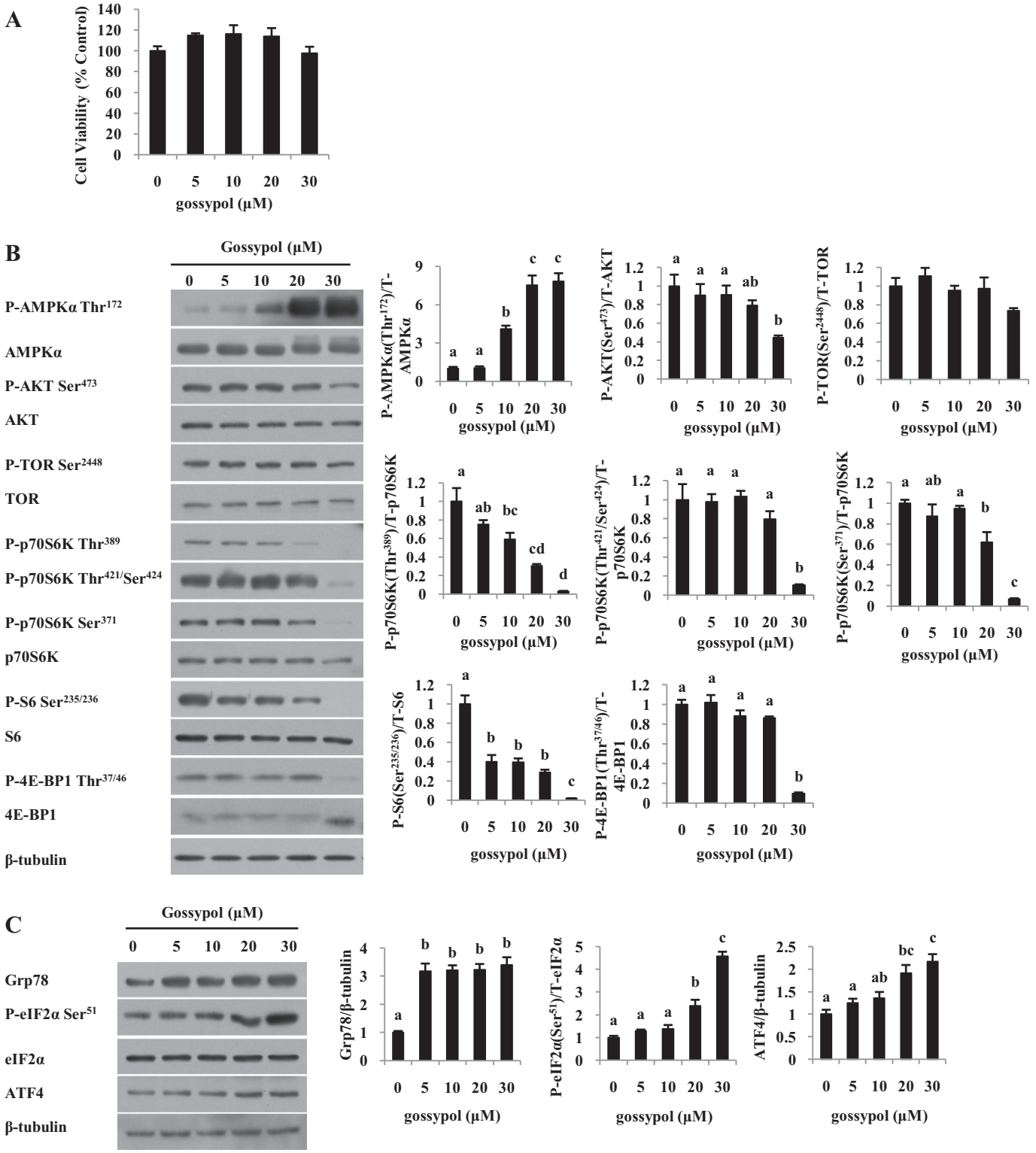

Fig. 4. Gossypol levels below cytotoxicity doses (*A*) inhibited TOR signaling (*B*), and stimulated ER stress pathway (*C*) in zebrafish liver cell line (ZFL). Cells were treated with gossypol at 0, 5, 10, 20, or 30 μM for 1 h. Values are represented as means ± SE ($n = 3$). Significance was evaluated by one-way ANOVA followed by Tukey's multiple range tests. [a,b,c,d]Mean values with different letters were significantly different ($P < 0.05$).

DISCUSSION

Gossypol is reported to inhibit growth in terrestrial (46, 47) and aquatic animals (38). In channel catfish (*Ictalurus punctatus*), growth retardation was observed when dietary gossypol levels were higher than a 1,400 mg/kg diet in one study (9) but only a 300 mg/kg diet in another report (66). Allogynogenetic silver crucian carp (*Carassius auratus gibelio*♀ × *Cyprinus carpio*♂) was reported to tolerate at least a 642 mg free gossypol/kg diet (7). On the other hand, *Tilapia aurea* could tolerate up to an 1,800 mg gossypol/kg diet (54). In the present study, growth performance was not influenced at a 600 mg gossypol/kg diet but significantly reduced at a 1,200 mg gossypol/kg diet. Fish species, diet composition, and environmental conditions in various studies could account for these differences. Reductions of body protein and lipid content further indicated the negative effects of gossypol on fish physiology.

In our experiment, turbot intestine structure showed no significant differences among groups (Fig. 1*A*). In addition, minimal effects of gossypol were found on intestine digestive enzyme activities (Fig. 1*B*). Similar results were also reported in other animals. Allogynogenetic silver crucian carp fed diets containing 642 mg/kg free gossypol showed no significant changes in mid gut histology (7). No significant differences in severity scores were reported in channel catfish fed diets containing up to 1,500 mg gossypol/kg diets (12). Similarly, no clear negative effects were found on small intestine morphology in male broilers fed up to 186 mg gossypol/kg diets (50). All these results suggested that gossypol had minimal influences on digestive tract.

The present study, along with previous studies, showed that gossypol accumulated mainly in the liver (24, 29, 34, 36). Gossypol caused liver damage in a wide range of species. The symptoms included individual cell necrosis, cellular infiltration, and areas of necrosis in male rats (11), mild perivascular lymphoid aggregate formations and bilary hyperplasia in chicks (19), and high pigment deposition in livers from channel catfish (12). In the present study, dietary gossypol caused liver fibrosis in turbot after 11 wk of feeding trial (Fig. 1*C*). Similar symptoms were found in calves fed a diet containing 33% cottonseed meal (68) and dogs that ingested cottonseed bedding contained 1,600 mg/kg gossypol (61). Liver fibrosis is known to result from excessive deposition of extracellular matrix (ECM) proteins and be regulated by integrated signaling networks (4, 37). Persistent inflammation almost always precedes fibrosis, and related signaling molecules are the key mediators of this process. Genes regulating the inflammatory responses (e.g., *il1b*, *tnfα, socs*, and *TLRs*) determine the fibrogenic response to injury (4). Among them, *il1b* and *tnfα* are proinflammatory cytokines that provoke the activation of hepatic satellite cells, which produce ECM proteins and contribute to hepatic injury (1, 40). Recent evidence also demonstrated the importance of TLRs in the activation of hepatic immune and stellate cells during liver fibrosis (1). On the other hand, *socs1* and *socs3* are negative regulators of cytokine signaling and protect against hepatic injury and fibrosis (48, 67). In addition, TGFβ stimulates the expression of many ECM proteins and acts as the major fibrogenic cytokine. Once activated, TGFβ signals via its cognate receptors to Smad proteins, which leads to induction of collagen production (37). STAT1 has been proposed to negatively regulate liver fibrosis through inhibition of TGFβ and satellite cell proliferation (20). Our results showed that gossypol downregulated *socs1* and *socs3* and upregulated *tnfα*, *il1b*, *TLR22*, and *MyD88* levels in turbot (Fig. 1*D*). Furthermore, gossypol activated TGFβ/Smad signaling and inhibited the activation of STAT1 (Fig. 1*D*). These results suggested that gossypol in the diets induced inflammatory responses and led to liver fibrosis.

Recently, increasing evidence has shown that nutrient sensing pathways play a key role in cell growth and proliferation (21, 22, 43). The main mediator of cellular nutrient sensing is the TOR signaling pathway. It regulates cellular and organismal growth and homeostasis by coordinating anabolic and catabolic processes with nutrient, energy, oxygen, and growth factors (57). AMPK is a cellular energy sensor (16). Activated upon energy deprivation, it functions as a negative regulator upstream of TOR (44). To our knowledge, none of the previous studies examined the effect of gossypol on postprandial responses of AMPK and TOR signaling. In the present study, dietary gossypol was found to increase AMPK activation and decrease postprandial activation of major TOR signaling molecules, including TOR, Akt, S6, and 4E-BP1 in turbot (Fig. 2*A*). Mechanistically, suppression of TOR signaling led to reduced protein synthesis and lipid accumulation (17, 32, 42), evidenced by decreased body protein and lipid contents in turbots after an 11-wk feeding trial with gossypol diets. To rule out possible indirect and nonspecific pathological effects, we further confirmed this effect in cell cultures. Cells were treated with gossypol at doses without apparent cytotoxicity, thus ensuring its physiological specificity. Dose-dependent increases in phosphorylation of AMPK and decreases in phosphorylation of Akt, TOR, p70S6K, S6, and 4E-BP1 by gossypol were observed in both turbot primary muscle cell (Fig. 3*B*) and ZFL cultures (Fig. 4*B*). Therefore, our data suggested that gossypol activated AMPK and inhibited TOR signaling both in vivo and in vitro.

Endoplasmic reticulum (ER) has an important role in protein processing. This process is often perturbed when cells are exposed to toxins, hypoxia, infections, or deprivation of essential nutrients and is believed to be mediated by increased phosphorylation of eIF2α (18, 22). In our study, G1 and G2 diets induced higher eIF2α phosphorylation and ATF4 levels, as well as *atf3, chop*, and *redd1* mRNA expressions (Fig. 2, *B* and *C*). These effects were also confirmed in both turbot primary muscle cell (Fig. 3*C*) and ZFL cell cultures (Fig. 4*C*), evidenced by the concentration-dependent increases in eIF2α phosphorylation, PERK, Grp78, ATF6α, XBP-1, and ATF4 levels. Previous reports showed that gossypol activated ER stress in leukemia cell lines (59). High eIF2α phosphorylation could shut down general protein synthesis while promoting translation of certain mRNAs selectively (e.g., ATF4) (27). The combination of suppressed TOR signaling and elevated ER stress pathway would synergistically lead to reduced animal nutrient retentions, as observed in the phenotypic parameters in this study (Table 3).

The pathological effects of gossypol have been well characterized in animals (38, 55). However, much fewer studies have provided mechanistic explanations of the actions of gossypol. In particular, there have been no previous studies on the molecular mechanism of actions of gossypol in fish. The present study demonstrates that gossypol influences the activ-

ities of TOR signaling and ER stress pathways both in vivo and in vitro, thus providing new molecular mechanisms on the actions of gossypol.

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DISCLOSURES

There are no conflicts of interest, financial or otherwise, for any of the authors.

AUTHOR CONTRIBUTIONS

G. H. and K. M. designed the research; F. B., H. J. and M. M conducted the research; F. B., H. Z. and W. X. analyzed the data; G. H. and F. B. wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. **Aoyama T, Paik Y-H, Seki E.** Toll-like receptor signaling and liver fibrosis. *Gastroenterol Res Pract* 2010: 192543, 2010. doi:10.1155/2010/192543.
2. **Bánhegyi G, Baumeister P, Benedetti A, Dong D, Fu Y, Lee AS, Li J, Mao C, Margittai E, Ni M, Paschen W, Piccirella S, Senesi S, Sitia R, Wang M, Yang W.** Endoplasmic reticulum stress. *Ann N Y Acad Sci* 1113: 58–71, 2007. doi:10.1196/annals.1391.007.
3. **Barros MM, Lim C, Klesius PH.** Effect of soybean meal replacement by cottonseed meal and iron supplementation on growth, immune response and resistance of Channel Catfish (Ictalurus puctatus) to Edwardsiella ictaluri challenge. *Aquaculture* 207: 263–279, 2002. doi:10.1016/S0044-8486(01)00740-2.
4. **Bataller R, Brenner DA.** Liver fibrosis. *J Clin Invest* 115: 209–218, 2005. doi:10.1172/JCI24282.
5. **Blom JH, Lee KJ, Rinchard J, Dabrowski K, Ottobre J.** Reproductive efficiency and maternal-offspring transfer of gossypol in rainbow trout (Oncorhynchus mykiss) fed diets containing cottonseed meal. *J Anim Sci* 79: 1533–1539, 2001. doi:10.2527/2001.7961533x.
6. **Braga A, MacIel M, Guerra D, Maia I, Oloris S, Soto-Blanco B.** Extruded-expelled cottonseed meal decreases lymphocyte counts in male sheep. *Rev Med Vet (Toulouse)* 163: 147–152, 2012.
7. **Cai C, Li E, Ye Y, Krogdahl A, Jiang G, Wang Y, Chen L.** Effect of dietary graded levels of cottonseed meal and gossypol on growth performance, body composition and health aspects of allogynogenetic silver crucian carp, Carassius auratus gibelio♀×Cyprinus carpio♂. *Aquacult Nutr* 17: 353–360, 2011. doi:10.1111/j.1365-2095.2010.00801.x.
8. **Dodou K, Anderson RJ, Small DA, Groundwater PW.** Investigations on gossypol: past and present developments. *Expert Opin Investig Drugs* 14: 1419–1434, 2005. doi:10.1517/13543784.14.11.1419.
9. **Dorsa WJ, Robinette HR, Robinson EH, Poe W.** Effects of dietary cottonseed meal and gossypol on growth of young channel catfish. *Trans Am Fish Soc* 111: 651–655, 1982. doi:10.1577/1548-8659(1982)111<651:EODCMA>2.0.CO;2.
10. **Efeyan A, Comb WC, Sabatini DM.** Nutrient-sensing mechanisms and pathways. *Nature* 517: 302–310, 2015. doi:10.1038/nature14190.
11. **El-Sharaky AS, Newairy AA, Elguindy NM, Elwafa AA.** Spermatotoxicity, biochemical changes and histological alteration induced by gossypol in testicular and hepatic tissues of male rats. *Food Chem Toxicol* 48: 3354–3361, 2010. doi:10.1016/j.fct.2010.09.004.
12. **Evans J, Pasnik D, Yildirim-Aksoy M, Lim C, Klesius P.** Histologic changes in channel catfish, Ictalurus punctatus Rafinesque, fed diets containing graded levels of gossypol-acetic acid. *Aquacult Nutr* 16: 385–391, 2010. doi:10.1111/j.1365-2095.2009.00676.x.
13. **Fotakis G, Timbrell JA.** In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 160: 171–177, 2006. doi:10.1016/j.toxlet.2005.07.001.
14. **Gadelha ICN, Fonseca NBS, Oloris SCS, Melo MM, Soto-Blanco B.** Gossypol toxicity from cottonseed products. *ScientificWorldJournal* 2014: 231635, 2014. doi:10.1155/2014/231635.
15. **Gu M, Bai N, Zhang Y, Krogdahl Å.** Soybean meal induces enteritis in turbot Scophthalmus maximus at high supplementation levels. *Aquaculture* 464: 286–295, 2016. doi:10.1016/j.aquaculture.2016.06.035.
16. **Hardie DG.** AMPK–sensing energy while talking to other signaling pathways. *Cell Metab* 20: 939–952, 2014. doi:10.1016/j.cmet.2014.09.013.
17. **Hardie DG, Ross FA, Hawley SA.** AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* 13: 251–262, 2012. doi:10.1038/nrm3311.
18. **Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D.** Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* 5: 897–904, 2000. doi:10.1016/S1097-2765(00)80330-5.
19. **Henry MH, Pesti GM, Brown TP.** Pathology and histopathology of gossypol toxicity in broiler chicks. *Avian Dis* 45: 598–604, 2001. doi:10.2307/1592900.
20. **Hernandez-Gea V, Friedman SL.** Pathogenesis of liver fibrosis. *Annu Rev Pathol* 6: 425–456, 2011. doi:10.1146/annurev-pathol-011110-130246.
21. **Hietakangas V, Cohen SM.** Regulation of tissue growth through nutrient sensing. *Annu Rev Genet* 43: 389–410, 2009. doi:10.1146/annurev-genet-102108-134815.
22. **Hotamisligil GS, Erbay E.** Nutrient sensing and inflammation in metabolic diseases. *Nat Rev Immunol* 8: 923–934, 2008. doi:10.1038/nri2449.
23. **In Jeong SI, Kim SY, Cho SK, Chong MS, Kim KS, Kim H, Lee SB, Lee YM.** Tissue-engineered vascular grafts composed of marine collagen and PLGA fibers using pulsatile perfusion bioreactors. *Biomaterials* 28: 1115–1122, 2007. doi:10.1016/j.biomaterials.2006.10.025.
24. **Jensen DR, Tone JN, Sorensen RH, Bozek SA.** Deposition pattern of the antifertility agent, gossypol, in selected organs of male rats. *Toxicology* 24: 65–72, 1982. doi:10.1016/0300-483X(82)90063-4.
25. **Jiang J, Ye W, Lin YC.** Gossypol inhibits the growth of MAT-LyLu prostate cancer cells by modulation of TGFbeta/Akt signaling. *Int J Mol Med* 24: 69–75, 2009. doi:10.3892/ijmm_00000208.
26. **Jin H-O, Seo S-K, Woo S-H, Kim E-S, Lee H-C, Yoo D-H, An S, Choe T-B, Lee S-J, Hong S-I, Rhee CH, Kim JI, Park IC.** Activating transcription factor 4 and CCAAT/enhancer-binding protein-β negatively regulate the mammalian target of rapamycin via Redd1 expression in response to oxidative and endoplasmic reticulum stress. *Free Radic Biol Med* 46: 1158–1167, 2009. doi:10.1016/j.freeradbiomed.2009.01.015.
27. **Kaplowitz N, Than TA, Shinohara M, Ji C.** Endoplasmic reticulum stress and liver injury. *Semin Liver Dis* 27: 367–377, 2007. doi:10.1055/s-2007-991513.
28. **Kilberg MS, Shan J, Su N.** ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol Metab* 20: 436–443, 2009. doi:10.1016/j.tem.2009.05.008.
29. **Kim HL, Calhoun MC, Stipanovic RD.** Accumulation of gossypol enantiomers in ovine tissues. *Comp Biochem Physiol B Biochem Mol Biol* 113: 417–420, 1996. doi:10.1016/0305-0491(95)02061-6.
30. **Kimball SR, Ravi S, Gordon BS, Dennis MD, Jefferson LS.** Amino Acid-Induced Activation of mTORC1 in Rat Liver Is Attenuated by Short-Term Consumption of a High-Fat Diet. *J Nutr* 145: 2496–2502, 2015. doi:10.3945/jn.115.215491.
31. **Lamming DW, Cummings NE, Rastelli AL, Gao F, Cava E, Bertozzi B, Spelta F, Pili R, Fontana L.** Restriction of dietary protein decreases mTORC1 in tumors and somatic tissues of a tumor-bearing mouse xenograft model. *Oncotarget* 6: 31233–31240, 2015. doi:10.18632/oncotarget.5180.
32. **Laplante M, Sabatini DM.** An emerging role of mTOR in lipid biosynthesis. *Curr Biol* 19: R1046–R1052, 2009. doi:10.1016/j.cub.2009.09.058.
33. **Laplante M, Sabatini DM.** mTOR signaling in growth control and disease. *Cell* 149: 274–293, 2012. doi:10.1016/j.cell.2012.03.017.
34. **Lee K-J, Dabrowski K.** Gossypol and gossypolone enantiomers in tissues of rainbow trout fed low and high levels of dietary cottonseed meal. *J Agric Food Chem* 50: 3056–3061, 2002. doi:10.1021/jf0115387.
35. **Lee K-J, Dabrowski K.** High-performance liquid chromatographic determination of gossypol and gossypolone enantiomers in fish tissues using simultaneous electrochemical and ultraviolet detectors. *J Chromatogr B Analyt Technol Biomed Life Sci* 779: 313–319, 2002. doi:10.1016/S1570-0232(02)00402-6.
36. **Lee KJ, Dabrowski K, Blom JH, Bai SC, Stromberg PC.** A mixture of cottonseed meal, soybean meal and animal byproduct mixture as a fish

meal substitute: growth and tissue gossypol enantiomer in juvenile rainbow trout (Oncorhynchus mykiss). *J Anim Physiol Anim Nutr (Berl)* 86: 201–213, 2002. doi:10.1046/j.1439-0396.2002.00375.x.
37. **Lee UE, Friedman SL.** Mechanisms of hepatic fibrogenesis. *Best Pract Res Clin Gastroenterol* 25: 195–206, 2011. doi:10.1016/j.bpg.2011.02.005.
38. **Li MH, Robinson EH.** Use of cottonseed meal in aquatic animal diets: a review. *N Am J Aquaculture* 68: 14–22, 2006. doi:10.1577/A05-028.1.
39. **Li Z-H, Li P, Shi Z-C.** Molecular responses in digestive tract of juvenile common carp after chronic exposure to sublethal tributyltin. *Ecotoxicol Environ Saf* 109: 10–14, 2014. doi:10.1016/j.ecoenv.2014.07.031.
40. **Liu C, Tao Q, Sun M, Wu JZ, Yang W, Jian P, Peng J, Hu Y, Liu C, Liu P.** Kupffer cells are associated with apoptosis, inflammation and fibrotic effects in hepatic fibrosis in rats. *Lab Invest* 90: 1805–1816, 2010. doi:10.1038/labinvest.2010.123.
41. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402–408, 2001. doi:10.1006/meth.2001.1262.
42. **Ma XM, Blenis J.** Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10: 307–318, 2009. doi:10.1038/nrm2672.
43. **Marshall S.** Role of insulin, adipocyte hormones, and nutrient-sensing pathways in regulating fuel metabolism and energy homeostasis: a nutritional perspective of diabetes, obesity, and cancer. *Sci STKE* 2006: re7, 2006. doi:10.1126/stke.3462006re7.
44. **Martin DE, Hall MN.** The expanding TOR signaling network. *Curr Opin Cell Biol* 17: 158–166, 2005. doi:10.1016/j.ceb.2005.02.008.
45. **Mbahinzireki GB, Dabrowski K, Lee K-J, El-Saidy D, Wisner ER.** Growth, feed utilization and body composition of tilapia (Oreochromis sp.) fed cottonseed meal-based diets in a recirculating system. *Aquacult Nutr* 7: 189–200, 2001. doi:10.1046/j.1365-2095.2001.00172.x.
46. **Morgan SG.** *Gossypol Toxicity in Livestock.* Stillwater, OK: Oklahoma State Univ Div Agric Sci Natural Resources, 2006.
47. **Nagalakshmi D, Rao SVR, Panda AK, Sastry VR.** Cottonseed meal in poultry diets: a review. *Jpn Poult Sci* 44: 119–134, 2007. doi:10.2141/jpsa.44.119.
48. **Ogata H, Chinen T, Yoshida T, Kinjyo I, Takaesu G, Shiraishi H, Iida M, Kobayashi T, Yoshimura A.** Loss of SOCS3 in the liver promotes fibrosis by enhancing STAT3-mediated TGF-β1 production. *Oncogene* 25: 2520–2530, 2006. doi:10.1038/sj.onc.1209281.
49. **Oliver CL, Miranda MB, Shangary S, Land S, Wang S, Johnson DE.** (-)-Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-X(L)-mediated apoptosis resistance. *Mol Cancer Ther* 4: 23–31, 2005. doi:10.1186/1476-4598-4-23.
50. **Özdoğan M, Wellmann K, Paksuz E.** Effect of gossypol on blood serum parameters and small intestinal morphology of male broilers. *J Anim Physiol Anim Nutr (Berl)* 96: 95–101, 2012. doi:10.1111/j.1439-0396.2010.01126.x.
51. **Peng M, Xu W, Ai Q, Mai K, Liufu Z, Zhang K.** Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (Scophthalmus maximus L.). *Aquaculture* 392–395: 51–58, 2013. doi:10.1016/j.aquaculture.2013.02.002.
52. **Piazzon MC, Wiegertjes GF, Leiro J, Lamas J.** Turbot resistance to Philasterides dicentrarchi is more dependent on humoral than on cellular immune responses. *Fish Shellfish Immunol* 30: 1339–1347, 2011. doi:10.1016/j.fsi.2011.02.026.
53. **Robinson EH.** Improvement of cottonseed meal protein with supplemental lysine in feeds for channel catfish. *J Appl Aquacult* 1: 1–14, 1991. doi:10.1300/J028v01n02_01.
54. **Robinson EH, Rawles SD, Oldenburg PW, Stickney RR.** Effects of feeding glandless or glanded cottonseed products and gossypol to Tilapia aurea. *Aquaculture* 38: 145–154, 1984. doi:10.1016/0044-8486(84)90226-6.
55. **Santos JE, Villasenor M, Robinson PH, DePeters EJ, Holmberg CA.** Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. *J Dairy Sci* 86: 892–905, 2003. doi:10.3168/jds.S0022-0302(03)73672-8.
56. **Seiliez I, Panserat S, Lansard M, Polakof S, Plagnes-Juan E, Surget A, Dias K, Larquier M, Kaushik S, Skiba-Cassy S.** Dietary carbohydrate-to-protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. *Am J Physiol Regul Integr Comp Physiol* 300: R733–R743, 2011. doi:10.1152/ajpregu.00579.2010.
57. **Sengupta S, Peterson TR, Sabatini DM.** Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* 40: 310–322, 2010. doi:10.1016/j.molcel.2010.09.026.
58. **Shimamura T, Fujisawa T, Husain SR, Kioi M, Nakajima A, Puri RK.** Novel role of IL-13 in fibrosis induced by nonalcoholic steatohepatitis and its amelioration by IL-13R-directed cytotoxin in a rat model. *J Immunol* 181: 4656–4665, 2008. doi:10.4049/jimmunol.181.7.4656.
59. **Soderquist RS, Danilov AV, Eastman A.** Gossypol increases expression of the pro-apoptotic BH3-only protein NOXA through a novel mechanism involving phospholipase A2, cytoplasmic calcium, and endoplasmic reticulum stress. *J Biol Chem* 289: 16190–16199, 2014. doi:10.1074/jbc.M114.562900.
60. **Tan B, Yin Y, Kong X, Li P, Li X, Gao H, Li X, Huang R, Wu G.** L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. *Amino Acids* 38: 1227–1235, 2010. doi:10.1007/s00726-009-0334-8.
61. **Uzal FA, Puschner B, Tahara JM, Nordhausen RW.** Gossypol toxicosis in a dog consequent to ingestion of cottonseed bedding. *J Vet Diagn Invest* 17: 626–629, 2005. doi:10.1177/104063870501700622.
62. **Wang X, He G, Mai K, Xu W, Zhou H.** Molecular cloning and characterization of taurine transporter from turbot (*Psetta maxima*) and its expression analysis regulated by taurine in vitro. *Aquac Res*, Epub 2016. doi:10.1111/are.13009.
63. **Wen B, Gao Q-F, Dong S-L, Hou Y-R, Yu H-B, Li W-D.** Effects of dietary inclusion of benthic matter on feed utilization, digestive and immune enzyme activities of sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* 458: 1–7, 2016. doi:10.1016/j.aquaculture.2016.01.028.
64. **Wullschleger S, Loewith R, Hall MN.** TOR signaling in growth and metabolism. *Cell* 124: 471–484, 2006. doi:10.1016/j.cell.2006.01.016.
65. **Xu D, He G, Mai K, Zhou H, Xu W, Song F.** Postprandial nutrient-sensing and metabolic responses after partial dietary fishmeal replacement by soyabean meal in turbot (*Scophthalmus maximus* L.). *Br J Nutr* 115: 379–388, 2016. doi:10.1017/S0007114515004535.
66. **Yildirim M, Lim C, Wan PJ, Klesius PH.** Growth performance and immune response of channel catfish (*Ictalurus puctatus*) fed diets containing graded levels of gossypol–acetic acid. *Aquaculture* 219: 751–768, 2003. doi:10.1016/S0044-8486(03)00062-0.
67. **Yoshida T, Ogata H, Kamio M, Joo A, Shiraishi H, Tokunaga Y, Sata M, Nagai H, Yoshimura A.** SOCS1 is a suppressor of liver fibrosis and hepatitis-induced carcinogenesis. *J Exp Med* 199: 1701–1707, 2004. doi:10.1084/jem.20031675.
68. **Zelski RZ, Rothwell JT, Moore RE, Kennedy DJ.** Gossypol toxicity in preruminant calves. *Aust Vet J* 72: 394–398, 1995. doi:10.1111/j.1751-0813.1995.tb06180.x.
69. **Zhang M, Liu H, Guo R, Ling Y, Wu X, Li B, Roller PP, Wang S, Yang D.** Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells. *Biochem Pharmacol* 66: 93–103, 2003. doi:10.1016/S0006-2952(03)00248-X.
70. **Zhang X-Q, Huang X-F, Mu S-J, An Q-X, Xia A-J, Chen R, Wu D-C.** Inhibition of proliferation of prostate cancer cell line, PC-3, in vitro and in vivo using (−)-gossypol. *Asian J Androl* 12: 390–399, 2010. doi:10.1038/aja.2009.87.
71. **Zuo R, Ai Q, Mai K, Xu W, Wang J, Xu H, Liufu Z, Zhang Y.** Effects of dietary n-3 highly unsaturated fatty acids on growth, nonspecific immunity, expression of some immune related genes and disease resistance of large yellow croaker (*Larmichthys crocea*) following natural infestation of parasites (*Cryptocaryon irritans*). *Fish Shellfish Immunol* 32: 249–258, 2012. doi:10.1016/j.fsi.2011.11.005.