Supporting Information

Kumashiro et al. 10.1073/pnas.1113359108

Supporting Information Corrected November 23, 2011

SI Materials and Methods

Hepatic Lipid Metabolites Assay. After purification, long-chain fatty acyl-CoAs, diacylglycerol (DAG), and ceramide fractions were separately dissolved in methanol/H_2O (1:1 vol/vol) and subjected to liquid chromatography-MS/MS analysis. A turboionspray ionization source was interfaced with an API 3000 tandem mass spectrometer (Applied Biosystems). Total long-chain fatty acyl-CoA, DAG, and ceramide contents were obtained from the sum of individual species, respectively. DAG fractionation into the membrane and cytosolic lipid droplet compartments was done as previously reported (1). Briefly, 50 mg tissue were homogenized with 400 μL cold Tris·EDTA-sucrose buffer (10 mM Tris·Base, pH 7.4, 0.5 mM EDTA, 250 mM sucrose) containing a protease inhibitor mixture using a Polytron homogenizer. Homogenates were centrifuged at 35,000 rpm in a Beckman Coulter 40.5Ti rotor (~150,000 × *g*) for 1 h (4 °C). Supernatant (liquid phase, which contains cytosolic lipid droplet) was kept at −20 °C for later use. The pellets were resuspended in 700 μL Tris·EDTA-sucrose buffer, which contain plasma membrane that was transferred into glass vials and used for the DAG assay as described above. Perilipin, which is localized at the periphery of lipid droplets and serves as a protective coating against lipases (2), was detected by Western blotting to confirm the lipid droplet compartment. Protein was extracted from lipid droplet fraction in cytosol and applied for Western blotting similar to the other proteins. Perilipin antibody was purchased from Cell Signaling. Lipid droplet fraction was also confirmed with EM. Top lipid layer after the centrifugation of homogenates was submitted for EM.

PKC Membrane Translocation Assay. First, protein was compartmentalized into membrane and cytosol compartments; 50 mg tissue were homogenized in 300 μL buffer A [20 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose and protease inhibitor (Roche Diagnostics)] and centrifuged at 100,000 × *g* for 1 h (4 °C). The supernatants containing the cytosolic fraction were collected. Pellets were resuspended in 300 μL buffer B (250 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 2% Triton X 100, protease inhibitor mixture) and centrifuged at 100,000 × *g* for 1 h (4 °C) to obtain the plasma membrane fraction. For protein assays, the plasma membrane fraction was diluted with buffer A 40 times to bring the Triton X 100 down to 0.05%; 130 μg crude membrane and cytosol protein extracts were used for Western blotting, resolved by SDS/PAGE using gradient gel, and electroblotted onto polyvinylidene difluoride membrane (DuPont) using a wet-transfer cell (Bio-Rad). The membrane was then blocked for 60 min at room temperature in PBS-Tween (10 mmol/L NaH_2PO_4, 80 mmol/L Na_2HPO_4, 0.145 mol/L NaCl, 0.1% Tween-20, pH 7.4) containing 5% (wt/vol) nonfat dried milk, and then, it was incubated overnight with the primary antibodies against PKC-α, -β, -ε, -δ, -θ, -η, -λ, or -ι (BD Transduction Laboratories) or PKC-ζ or -γ (Abcam). Antibodies were diluted as 1:500–1,000 in rinsing solution. After additional washings, membranes were incubated with HRP-conjugated IgG fraction of goat anti-mouse (for PKC-α, -β, -ε, -δ, -θ, -η, -λ, and -ι) or rabbit (for PKC-ζ and -γ) IgG (Bio-Rad) diluted 1:2,000 in PBS-T containing 5% (wt/vol) nonfat dried milk for 90 min.

Liver Protein Fractionation for ER Stress-Marker Western Blotting. Livers were homogenized in 300 μL ice-cold homogenation buffer [10 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA, protease inhibitor and phosphatase inhibitor (Roche Diagnostics)] with a Dounce homogenizer. The homogenate was centrifuged at 650 × *g* for 10 min (4 °C). The supernatant was transferred into the new tubes and centrifuged at 100,000 × *g* for 20 min (4 °C). The pellet (mitochondria plus nucleus) was resuspended with homogenization buffer and preserved. The supernatant was centrifuged at 110,000 × *g* for 1 h (4 °C). After the centrifuge, the supernatant (cytoplasm) was preserved in new tubes, and the pellet (microsomes) was resuspended with homogenization buffer. Protein assay was done for every fraction at the same time. Western blotting was done with a similar method as the method for PKCs.

1. Bogan JS, McKee AE, Lodish HF (2001) Insulin-responsive compartments containing GLUT4 in 3T3-L1 and CHO cells: Regulation by amino acid concentrations. *Mol Cell Biol* 21:4785–4806.

2. Brasaemle DL (2007) Thematic review series: Adipocyte biology. The perilipin family of structural lipid droplet proteins: Stabilization of lipid droplets and control of lipolysis. *J Lipid Res* 48:2547–2559.

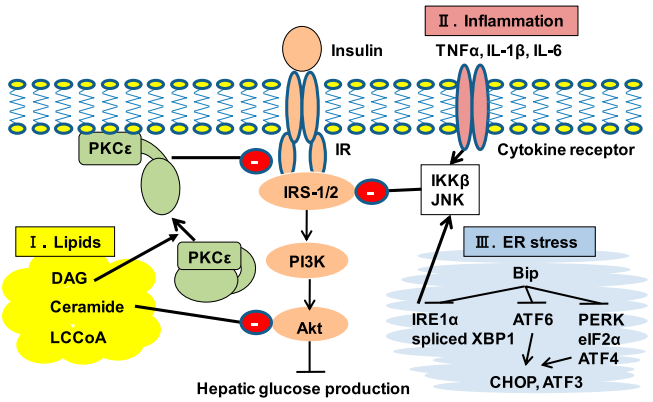

Fig. S1. Putative mechanisms of nonalcoholic fatty liver disease-associated hepatic insulin resistance.

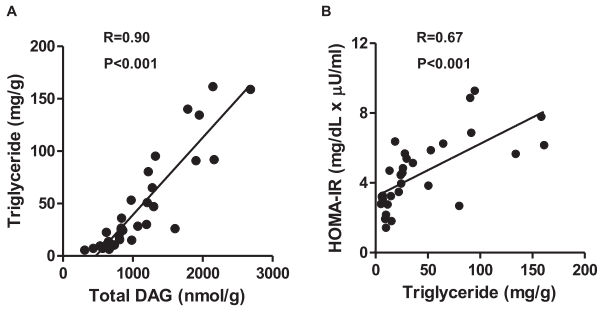

Fig. S2. Correlation between (*A*) hepatic total DAG content and triglyceride content and between (*B*) hepatic triglyceride content and homeostatic model assessment of insulin resistance index (HOMA-IR).

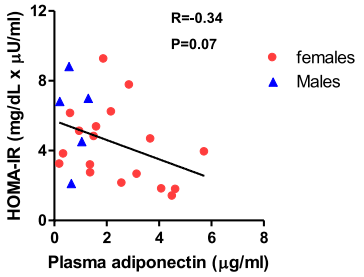

Fig. S3. Correlation between plasma adiponectin and HOMA-IR.

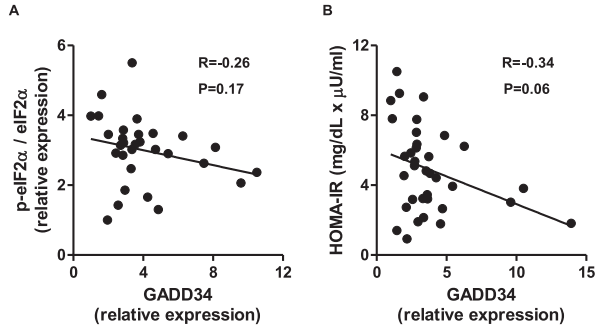

Fig. S4. Correlation between hepatic growth arrest and DNA damage-inducible protein 34 (GADD34) mRNA expression and phospho-eukaryotic translation initiation factor 2α (p-eIF2α)/eIF2α (*A*) or HOMA-IR (*B*).

Table S1. Correlation between DAG species and HOMA-IR (*n* = 35)

Species	*R* value	*P* value	Mean (SD; nmol/g)
C18:1–C16:0	0.54	<0.001	112.6 (110.5)
C20:4–C20:5	−0.50	0.002	239.3 (117.5)
C16:0–C20:4	0.05	0.79	4.2 (1.4)
C18:0–C20:4	−0.33	0.05	7.7 (4.9)
C18:0–C18:0	0.47	0.005	31.2 (29.6)
C18:2–C18:0	0.54	<0.001	14.4 (7.1)
C18:1–C18:0	0.56	<0.001	15.3 (13.6)
C16:0–C16:0	0.45	0.006	36.7 (48.1)
C18:0–C16:0	0.49	0.003	13.5 (12.5)
C18:1–C18:1	0.58	<0.001	186.3 (153.2)
C18:1–C18:2	0.55	<0.001	100.4 (58.4)
C18:2–C18:2	0.41	0.02	35.7 (25.2)
C16:0–C18:2	0.55	<0.001	134.7 (85.0)

Shaded rows connote the most abundant lipid species (light grey, positively related; dark grey, negatively related with HOMA-IR).

Table S2. Correlation between PKC activation and DAG in lipid droplet or HOMA-IR

	DAG in lipid droplet		HOMA-IR	
	R	*P* value	*R*	*P* value
Novel PKC				
ε	0.67	<0.001	0.55	<0.001
δ	0.30	0.13	0.07	0.71
Conventional PKC				
α	0.32	0.09	0.18	0.32
β	0.06	0.77	0.15	0.40
Atypical PKC				
ζ	0.45	0.02	0.21	0.23
ι	0.01	0.97	0.13	0.45

The other PKC isoforms (η, θ, and γ) were undetectable; $n = 30$ for DAG in lipid droplet, and $n = 36$ for HOMA-IR. The shaded column separates the data relating PKC activation and lipid droplet DAG from PKC activation and HOMA-IR.

Table S3. Correlation between ER stress markers and HOMA-IR

	R	*P* value
IRE1α pathway		
spliced XBP1 mRNA	0.04	0.84
JNK1 phosphorylation	0.04	0.83
JNK2,3 phosphorylation	−0.06	0.78
PERK pathway		
eIF2α phosphorylation	0.43	0.02
ATF4 mRNA	0.28	0.11
ATF3 mRNA	−0.30	0.09
ATF6 pathway		
Cleaved ATF6 protein	−0.33	0.08
Chaperones		
BiP mRNA	0.30	0.10
BiP protein	0.13	0.53
CHOP mRNA	0.08	0.67
CHOP protein	0.49	0.02

$n = 28$–32 for proteins or their phosphorylations, and $n = 36$ for mRNAs. ATF, activating transcription factor; BiP, Ig heavy-chain binding protein; CHOP, CCAAT/enhancer binding protein homologous protein; eIF, eukaryotic translation initiation factor; IRE, inositol requiring ER to nucleus signal kinase; JNK, c-Jun N-terminal kinase; PERK, (dsRNA-activated kinase) -like ER kinase; XBP1, X-box binding protein-1. Shaded rows separate individual ER stress pathways and markers.

Table S4. Correlation between inflammation parameters and HOMA-IR

	R	*P* value
Hepatic mRNA expressions		
TNFα	−0.30	0.10
IL-1β	−0.15	0.40
IL-6	−0.22	0.27
CRP	0.16	0.38
Plasma concentrations		
TNFα	−0.14	0.54
IL-1β	−0.16	0.49
IL-6	−0.02	0.94
hsCRP	−0.09	0.68

$n = 36$ for hepatic mRNAs, and $n = 24$ for plasma concentrations. hsCRP, high-sensitive C-reactive protein; IL, interleukin; TNF, tumor necrosis factor. Shaded rows separate hepatic mRNA expression of cytokines from and the plasma concentrations of cytokines.

Table S5. Primer sequences

Primer sequences	Forward	Reverse
ATF3	CTGCCCGCCTTTCATCTG	CAGACACTGCTGCCTGAATCC
ATF4	TCTTACAACCTCTTCCCCTTTCC	CACTTCACTGCCCAGCTCTAAA
BiP	CGCCAAGCGGCTCATC	AACCACCTTGAACGGCAAGA
CHOP	AGAACCAGGAAACGGAAACAGA	TCTCCTTCATGCGCTGCTTT
GADD34	GCCATTGTGTTCGTTGCTCTT	TCGTGCAGCGTCTTGCA
IL-1β	GCACGATGCACCTGTACGAT	AGACATCACCAAGCTTTTTTGCT
IL-6	CTGCGCAGCTTTAAGGAGTTC	TGAGGTGCCCATGCTACATTT
TATA box binding protein	CACAGTGAATCTTGGTTGTAAACTTGA	AAACCGCTTGGGATTATATTCG
TNFα	CCCAGGCAGTCAGATCATCTTC	AGCTTGAGGGTTTGCTACAACAT
Spliced XBP1	GTGAAGGAAGAACCTGTAGAAGATGA	TTTGGGCAGTGGCTGGAT

ATF, activating transcription factor; BiP, Ig heavy-chain binding protein; CHOP, CCAAT/enhancer binding protein homologous protein; GADD, growth arrest and DNA damage-inducible protein; IL, interleukin; TNFα, tumor necrosis factor α; XBP1, X-box binding protein-1.

Other Supporting Information Files

Dataset S1 (XLSX)