Supplemental Information

Inhibition of fatty acid oxidation enhances oxidative protein folding and protects hepatocytes from endoplasmic reticulum stress

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Supplemental Experimental Procedures

Cell culture

FaO rat hepatoma cells were grown as described (Rutkowski et al., 2008). Cells were treated with the indicated doses of chemicals or solvent controls. ET and GW6471 were used at various concentrations (20 to 100 μg/ml for ET; 5 to 15 μg/ml for GW6471); the exact concentration used did not qualitatively affect the results obtained. Cells were lysed in 100 mM Tris, pH 8.8, 1% SDS for immunoblotting, or in Trizol for RNA analysis. Lipofectamine 2000 (Invitrogen) was used for transfections.

Molecular analysis

Immunoblot, quantitative real-time RT-PCR, and *Xbp1* RT-PCR methods (including methods for verifying primer specificity and efficiency for qRT-PCR) were carried out as described (Rutkowski et al., 2006). All qRT-PCR expression values were normalized against the average of 2 housekeeping genes (typically *Gapdh* and *Ppia*). Primer sequences not previously published (Rutkowski et al., 2006; Rutkowski et al., 2008; Wu et al., 2007) are given in Table S1. Luciferase assays, including constructs used, were as described (Wu et al., 2007). For knockdown of *Pparα* and *Cpt1a*, 21-mer oligos and an upstream U6 promoter were cloned into pacAd5 mcs IRES eGFP pA using standard methods, as described (Anderson et al., 2000). The control shRNA sequence did not recognize any sequence in the rat genome. Sequences targeted by shRNAs were: *Pparα*: cttcaacatgaacaaggtcaa; *Cpt1a*: ttacaaggacatgggcaagtt; control: tagccgacttgaatcggctga. For luciferase assays, the *Bip -169 luciferase* construct was cotransfected with a β-galactosidase normalization control and the shRNA construct at a ratio of (1:1:6). Detection of protein carbonyls was according to the manufacturer's instructions. For pulse-chase analysis of alpha-1-antitrypsin, cells were transfected with a clone containing human wild-type alpha-1-antitrypsin in a mammalian expression

vector (pcDNA 3.1 Zeo (+)). Two days after transfection, cells were treated for 3 hours with or without 100 μg/ml ET, then washed once in PBS, then provided medium lacking cysteine and methionine, plus or minus ET, for 15 minutes. Cells were then labeled for 10 minutes with met/cys-free medium plus 200 μCi/ml of Tran^{35}SLabel (MP Biomedicals), followed by rinsing once with PBS, and chase in complete medium for the indicated times. Cells were lysed as above. EndoH digestion and immunoprecipitation were as described (Wu et al., 2007).

Supplemental Reference

Anderson, R.D., Haskell, R.E., Xia, H., Roessler, B.J., and Davidson, B.L. (2000). A simple method for the rapid generation of recombinant adenovirus vectors. Gene Therapy *7*, 1034-1038.

Supplemental Figures

Tyra et al., Figure S1

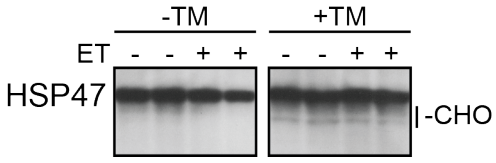

Figure S1. ET does not block the inhibition of N-linked glycosylation by TM. FaO cells were treated in duplicate for 8h in the presence or absence of 250 ng/ml TM and 50 μg/ml ET. Glycosylation status of the ER resident glycoprotein HSP47 was assessed by immunoblot, with underglycosylated chains indicated (-CHO). HSP47 was used because its expression is not regulated by the UPR, thus preventing any confounding effects from attenuation of UPR activation by ET.

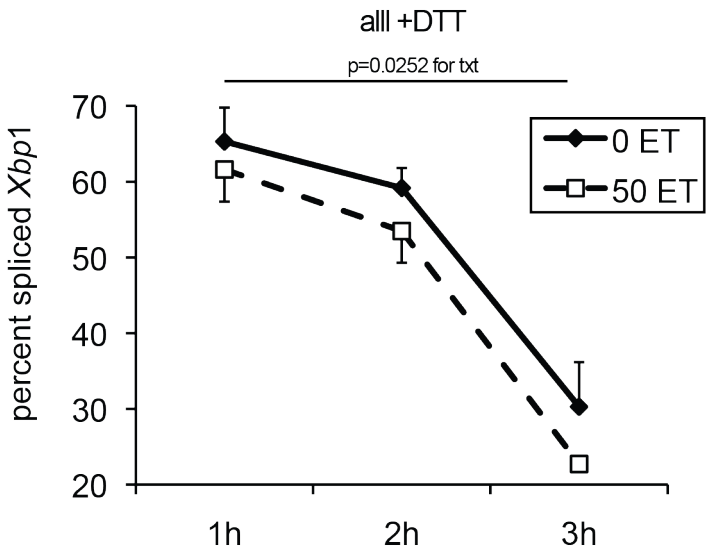

Figure S2. ET inhibits splicing of Xbp1 during DTT treatment.
Cells were pretreated for 3h with 25 or 50 μg/ml ET or vehicle, and then 1 mM DTT was added to all cells. Total RNA was harvested after 1, 2, or 3h of DTT treatment, and *Xbp1* mRNA splicing was assessed by RT-PCR as in Fig. 1C. Densitometric quantitation of splicing at 0 and 50 μg/ml ET is shown (25 μg/ml is not shown for clarity). Two-factor ANOVA was used to assess significance; the effects were not significant relative to time, but were significant for treatment, and that p-value is shown.

Tyra et al., Figure S3

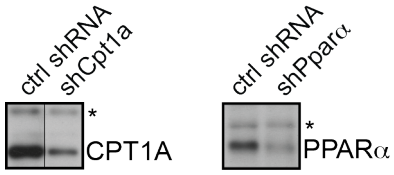

Figure S3. Partial knockdown of CPT1A and PPARα.
Cells were cotransfected with a plasmid encoding either mouse CPT1A-HA (left) or mouse PPARα-HA (right) and a plasmid encoding an shRNA targeting either a control sequence (left lane, each panel) or the indicated specific sequence. The ratio of shRNA plasmid to expression plasmid was 3:1. Cells were harvested 48h after transfection and CPT1A and PPARα were detected by immunoblot with an HA antibody. A nonspecific band is indicated by *, and indicates equal loading. For the CPT1A blot, the two lanes shown were non-adjacent on the gel, and so were electronically spliced from an image of a single exposure.

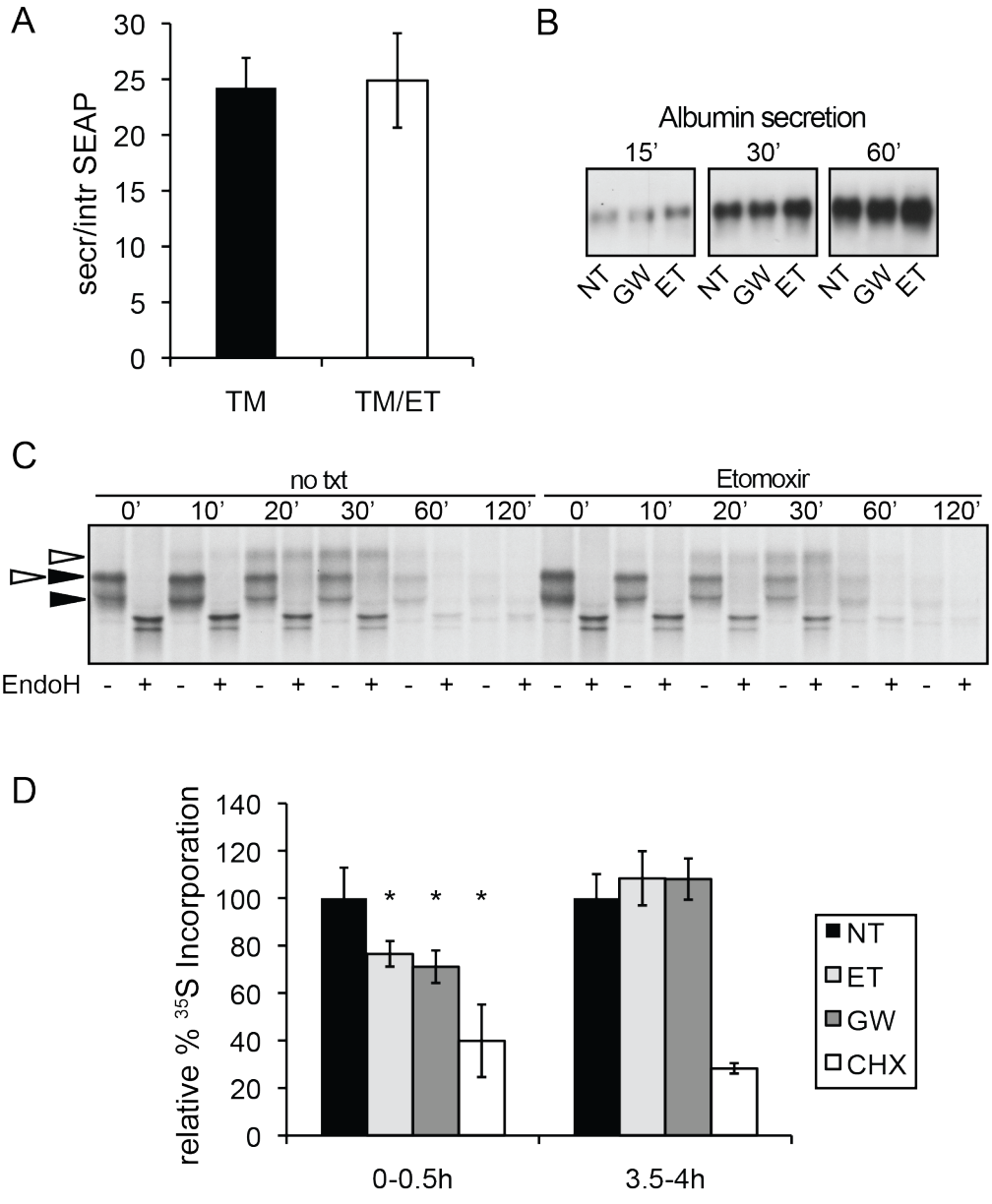
A
secr/intr SEAP
30
25
20
15
10
5
0
TM
TM/ET
B
Albumin secretion
15'
30'
60'
NT
GW
ET
C
no txt
Etomoxir
0'
10'
20'
30'
60'
120'
EndoH
D
relative % 35S Incorporation
140
120
100
80
60
40
20
0
0-0.5h
3.5-4h
NT
ET
GW
CHX

Figure S4. Effects of inhibiting FA oxidation on secretion and protein synthesis. (A) Cells were transfected with a plasmid encoding secreted alkaline phosphatase (SEAP). Cells were treated for 5h with 50 μg/ml ET or vehicle, and then 250 ng/ml TM was added to all cells using fresh media with or without ET as before. Media and lysates were collected after a further 5h, and SEAP activity was quantitated from both as described (Wu et al., 2007). The ratio of secreted to intracellular SEAP is shown here. (B) Cells were pretreated for 2h with 15 μg/ml GW6471, 100 μg/ml ET, or vehicle, and then 2 mM DTT in serum free medium was added to all cells for the indicated times. Secreted albumin was detected in the media by immunoblot. (C) Cells transfected with wild-type alpha-1-antitrypsin were treated with ET or vehicle as indicated, and were pulse labeled with a ^{35}S methionine/cysteine mixture and chased in complete medium for the indicated times. Samples were set aside or treated with Endoglycosidase H (EndoH) to remove immature high-mannose glycans. Both endogenous rat and overexpressed human proteins are visible. EndoH-sensitive forms are indicated by closed arrowheads, and EndoH-resistant mature forms by open arrowheads. (D) Cells were preincubated for 10' with methionine/cysteine-free media, which was then replaced by a mixture containing 10 percent complete media and 90 percent methionine/cysteine-free media, plus 50 μg/ml ET, 5 μg/ml GW6471, 1 μg/ml cycloheximide (CHX), or vehicle. A ^{35}S methionine/cysteine mixture was added either for the first 30' of chemical treatment or for 30' after 3.5h of treatment. Cells were lysed, and equal amounts of protein from the lysates were used to quantitate ^{35}S incorporation by precipitation with trichloroacetic acid as described (Rutkowski et al., 2006). Precipitated material was then quantitated by scintillation counting, and is normalized relative to untreated cells. These data show that ET and GW have a small effect on protein synthesis immediately upon addition, but not at later times.

Table S1. Primer sequences not previously published

Rat Gapdh	*GCTCTCTGCTCCTCCCTGTTCTA* and *AAATCCGTTCACACCGACCTTCAC*
Mouse/Rat Ppia	*AATATGGCGTGTAAAGTCACCA* and *AGCACTGGAGAGAAAGGATT*
Rat Bip	*ATCAACCCAGATGAGGCTGTAGCA* and *GAGGACACACATCAAGCAGAACCA*
Rat Chop	*AATAACAGCCGGAACCTGAGGAGA* and *ACTGTCTCAAAGGCGAAAGGCAGA*
Rat Wars	*GCATCTGGGTCATCTCGTCCCATTTA* and *TGTATGCCTGTTCCAGAGTCAAGTCC*
Mouse/rat Xbp1	*TTGTGGTTGAGAACCAGG* and *TCCATGGGAAGATGTTCTGG*