Impaired endothelial autophagy promotes liver fibrosis by aggravating the oxidative stress response during acute liver injury

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Supplementary materials

Antibodies

Name	Supplier	Cat no.	Clone no.
SQSTM1/p62	Cell Signalling	#5114	
LC3B	Cell Signalling	#2775	
ATG7	Cell signaling	#2631	
α-SMA	Sigma	#A2547	1A4
PDGFR-β	Santa Cruz	#sc-1627	
total eNOS	BD Bioscience	#610297	Clone 3
phosphorylated eNOS (Ser1177)	Cell signalling	#9571	
HO-1	Enzo Life Sciences	#ADI-SPA-896	
NQO1	Abcam	#ab2346	
GAPDH	Santa Cruz	#sc-32233	6C5
β-actin	Sigma	#A2228	AC-74
αSMA	Abcam	#ab5694	
Von Willebrand Factor (VWF)	Dako	#A0082	
Desmin	Dako	#M0760	D33
LAMP-2	Santa Cruz	#sc-34245	

Cell lines

Name	Citation	Supplier	Cat no.	Passage no.
Human Umbilical Vein Endothelial Cells (HUVEC)		Lonza	CC-2517	Below 12
Mouse liver sinusoidal endothelial cells TSEC	Huebert, R. C. et al. Immortalized liver endothelial cells: a cell culture model for studies of motility and angiogenesis. Lab. Investig. 90, 1770–1781 (2010).	Dr. V Shah		Below 20

Organisms

Name	Supplier	Strain	Sex	Age	Overall n number
Mice	The Jackson Laboratory	C57BL/6	Male & female	10-14 weeks	81
Rats	Charles River	Sprague-Dawley (SD	Male	250-300 g	36

Sequence based reagents

Taqman Atg7	Mm00512209_m1	

Taqman VEGFR2	Mm01222421_m1	Rn00564986_m1
Taqman ET-1	Mm00438656_m1	Rn00561129_m1
Taqman GAPDH	Mm99999915_g1	Rn01775763_g1
Acta2	ACTA2_2_Fw	CTTCGTGACTACTGCCGAGC
	ACTA2_2_Rv	AGGTGGTTTCGTGGATGCC
P62	p62-Fw	TTCGGAAGTCAGCAAACCTGA
	p62-Rv	CCGACTCCATCTGTTCCTCTG
GPx1	GPx1_Fw	TCGGTTTCCCGTGCAATCA
	GPx1_Rv	GTCGGACGTACTTGAGGGAA
GPx4	GPx4_Fw	TTACGAATCCTGGCCTTCCC
	GPx4_Rv	TAGCCGGCTGCAAACTCC
SOD1	SOD1_Fw	CTCACTCTCAGGAGAGCATTCC
	SOD1_Rv	TTCCACCTTTGCCCAAGTCA
SOD2	SOD2_Fw	AAGGAGCAAGGTCGCTTACA
	SOD2_Rv	AATCCCCAGCAGCGGAATAA
CAT	CAT_Fw	GGGATCTTGTGGGAAACAACAC
	CAT_Rv	CTGTGGGTTTCTCTTCTGGCTA
Nrf2	Nrf2_Fw	AGTGGATCCGCCAGCTAC
	Nrf2_ Rv	CTCTGCCAAAAGCTGCATACA
Srxn1	Srxn1_Fw	GCACAACGTACCAATCGCC
	Srxn1_Rv	CAGGGTCCGCCAGGATCG
Nqo1	Nqo1_Fw	TCTCTGGCCGATTCAGAGTG
	Nqo1_Rv	CCAGACGGTTTCCAGACGTT
Gclc	Gclc_Fw	CTGCTGTCCCAAGGCTCG
	Gclc_Rv	TGTACTCCACCTCGTCACCC
Gclm	Gclm_Fw	TGGGCACAGGTAAAACCCAA
	Gclm_Rv	CTGGGCTTCAATGTCAGGGA
Gstm1	Gstm1_Fw	CCGTGCAGACATTGTGGAGA
	Gstm1_Rv	CTGCTTCTCAAAGTCAGGGTTG
Gstm2	Gstm2_Fw	CAGCCCTGACTTTGAGAAAAAGA
	Gstm2_Rv	GACCTTGTTCCCTGCAAACCA
Gapdh	GAPDH_Fw	AGACGGCCGCATCTTCTT
	GAPDH_Rv	TTCACACCGACCTTCACCAT

Software

Software name	Manufacturer	Version
SPSS for Windows	IBM	23.0
Prism	GraphPad	5.01
ImageJ	US National Institute of Health	1.51j8
SDS	Applied Biosystems	2.3
MultiGauge	Fujifilm	2.1

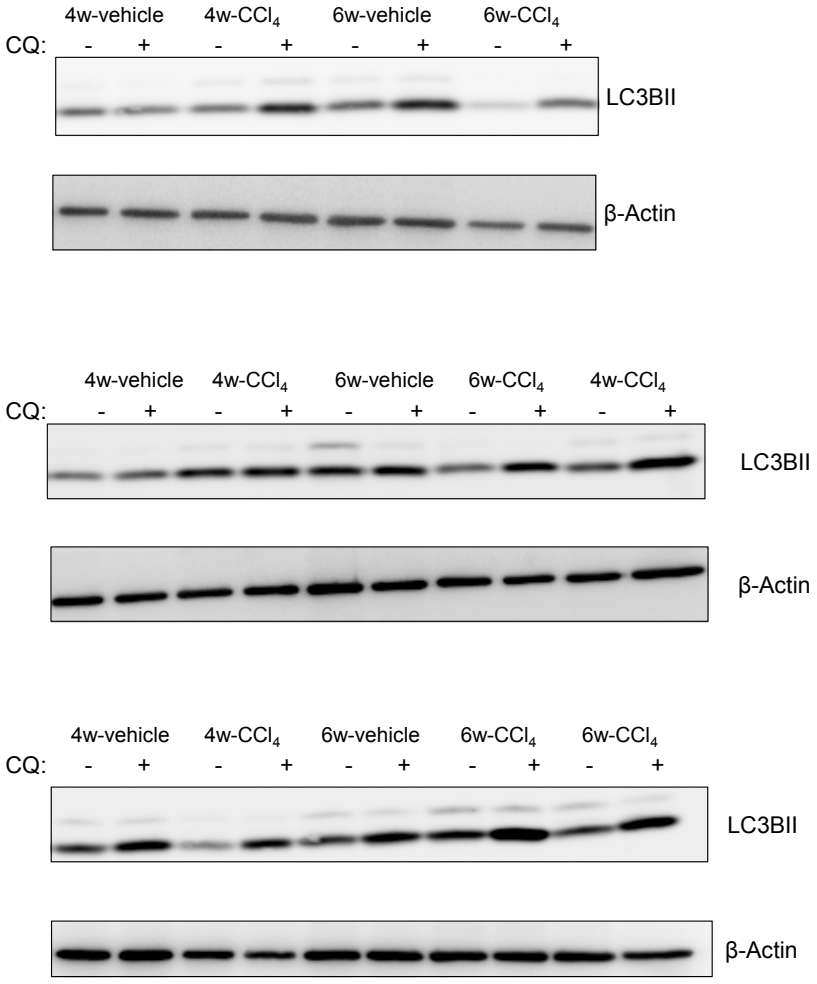

Fig. S1. Autophagy is upregulated during *in vivo* capillarization: Primary LSEC isolated from rats treated with CCl_4 or vehicle for 4 and 6 weeks, directly plated and stabilized for 2h. For autophagy flux assay by western blot cells were treated with CQ during 2h and collected thereafter. All membranes of LC3B II immunoblotting with and without addition of CQ showing autophagy flux displaying an increase at 4 weeks and incapability of further increase at 6 weeks. Protein expressions are expressed as fold change relative to control (*P≤0.05, **P≤0.01, ***P≤0.001, Student's t-test or analysis of variance (ANOVA)).

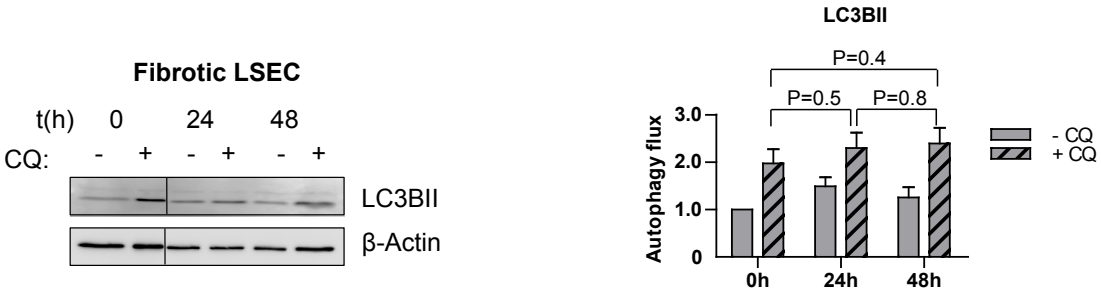

Fig. S2. Fibrotic LSEC are unable to further increase autophagy levels during *in vitro* capillarization. Primary LSEC isolated from rats treated with CCl_4 for 4 weeks and culture in plastic during 24 and 48 h. LC3B II immunoblotting with and without addition of CQ showing inability to increase autophagy flux during plastic culture. Data shows mean value ± SEM of at least 3 experiments. Protein expression is expressed as fold change relative to control (*P≤0.05, **P≤0.01, ***P≤0.001, Student's t-test).

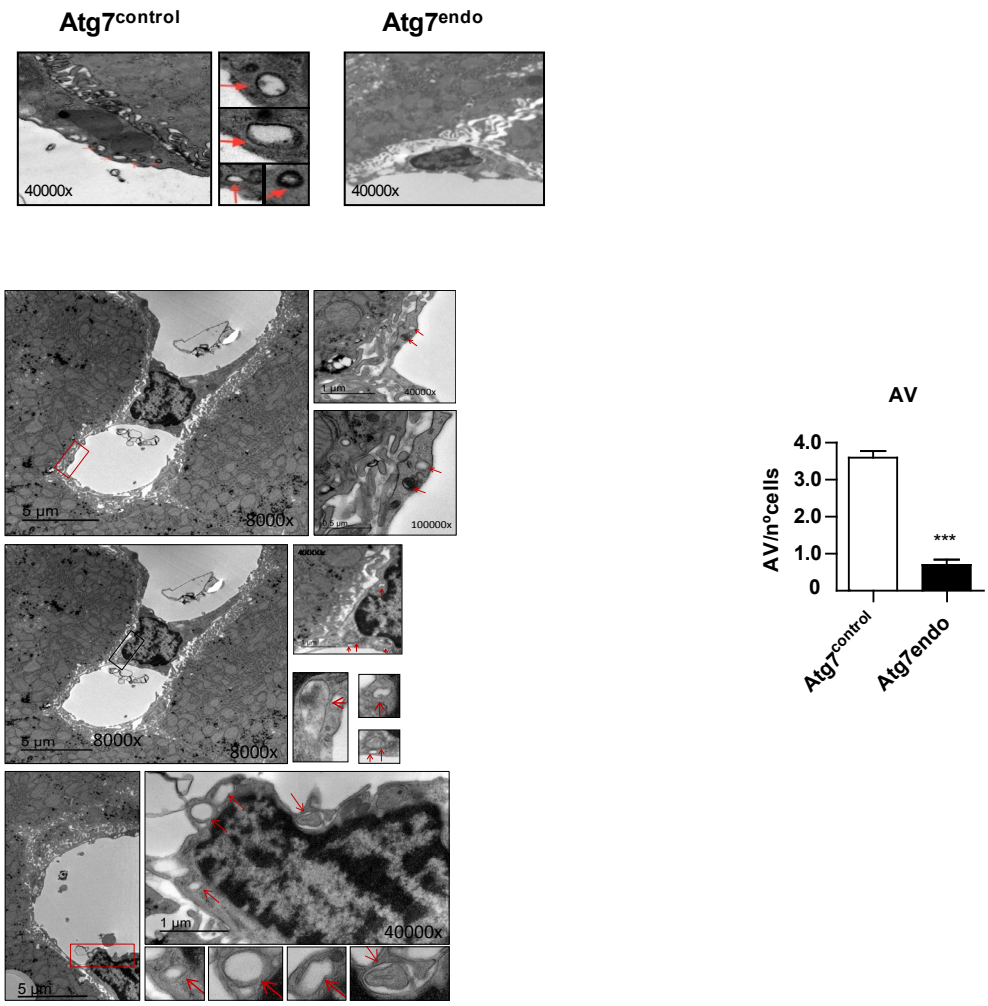

Fig. S3. Generation of Atg7^{endo} mice. Representative whole liver electron micrographs from Atg7^{endo} and ATG7^{control} mice showing autophagic vacuoles (AV) in LSEC and its quantification, illustrating a significant decrease of AV in transgenic animal.

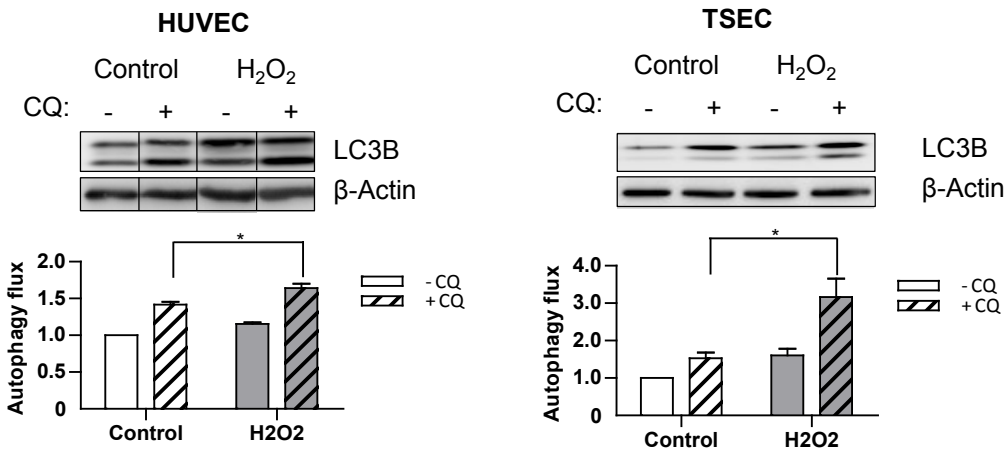

Fig. S4. Oxidative stress triggers autophagy. LC3B II immunoblotting with and without addition of CQ showing autophagy flux in HUVEC (left) and in TSEC (right) after H_2O_2 treatment during 72 h. Data shows mean value ± SEM of at least 3 experiments. Protein expression is expressed as fold change relative to control (*P≤0.05, **P≤0.01, ***P≤0.001, Student's t-test).

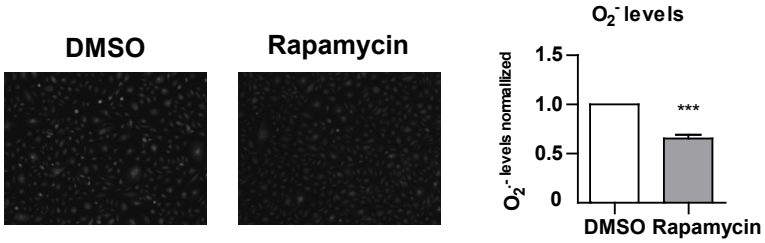

Fig. S5. Increasing endothelial autophagy alleviates oxidative stress: HUVEC cells were treated with Rapamycin for 24 h (autophagic enhancer) and H_2O_2 was added later during 15 h. Cellular superoxide content was then measured by dihydroethidium showing a significant decrease in O_2^- levels. Data shows mean value ± SEM of at least 3 experiments (*P≤0.05, **P≤0.01, ***P≤0.001, Student's t-test).

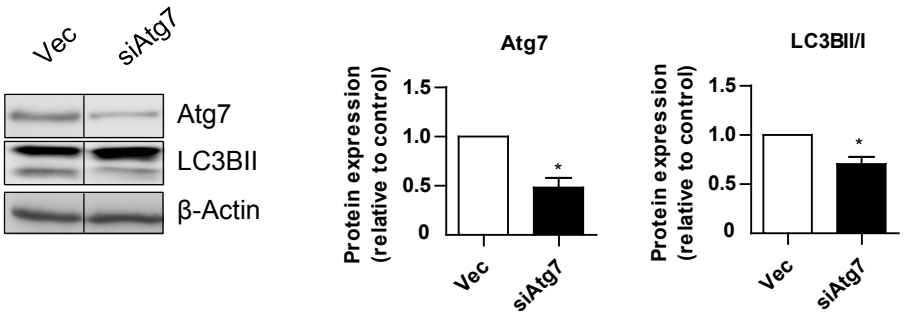

Fig. S6. Knockdown of Atg7 in TSEC. TSEC were transduced with siAtg7 or empty vector (VEC). Immunoblots for Atg7 and LC3B II and their quantification, showing a decrease expression in ATG7 protein and autophagy levels measured as the LC3II/I ratio. Data shows mean value ± SEM of at least 3 experiments. Protein expression is expressed as fold change relative to control (*P≤0.05, **P≤0.01, ***P≤0.001, Student's t-test).

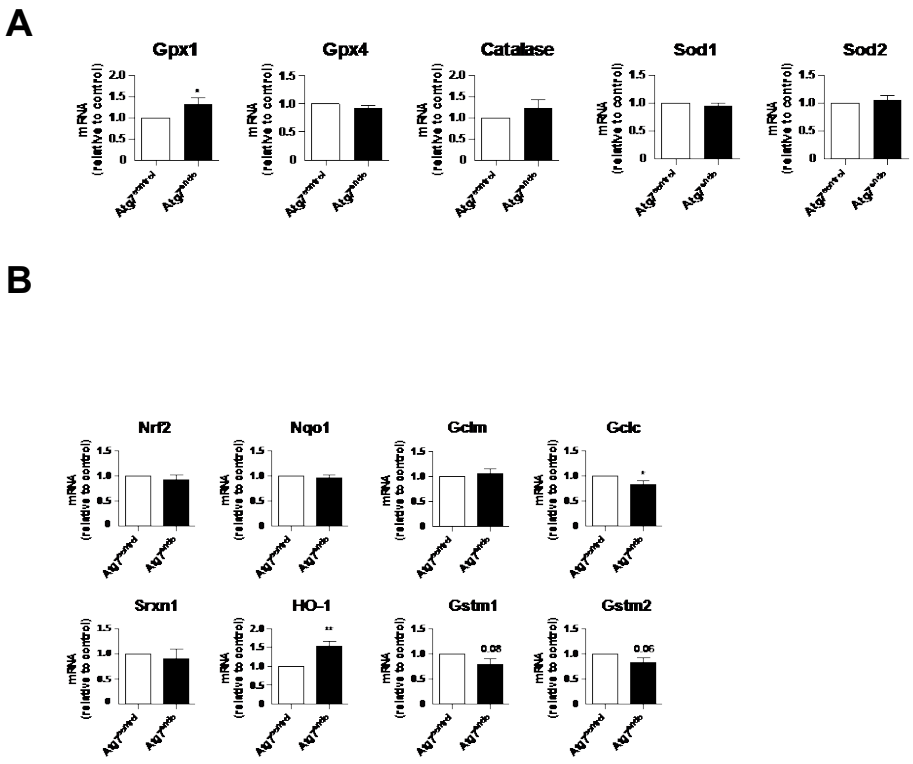

Fig. S7. The classical antioxidant response in whole liver is insufficient to alleviate the accumulation of oxidative stress in Atg7^{endo} mice. Atg7^{endo} and Atg7^{control} mice were treated every other day with CCl4 i.p. for 1 week to induce mild acute liver injury. (A) mRNA changes (qRT-PCR analysis) showing not significant changes in the classical genes that protect against oxidative stress Gpx1, Gpx4, Sod1, Sod2 and catalase and (B) mRNA changes (qRT-PCR analysis) of the Nrf2-dependent antioxidative stress genes Srxn, Nqo1, Gclc, Gclm, and Gstm2. Data shows mean value ± SEM of at least 3 experiments. mRNA expression is expressed as fold change relative to control (*P≤0.05, **P≤0.01, ***P≤0.001, Student's t-test).