Autophagy confers resistance to lipopolysaccharide-induced mouse hepatocyte injury

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1Department of Medicine and the Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York; 2Division of Digestive Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia; 3Department of Biochemistry, Sargodha Medical College, University of Sargodha, Sargodha, Pakistan; and 4Department of Pathology, Albert Einstein College of Medicine, Bronx, New York

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Lalazar G, Ilyas G, Malik SA, Liu K, Zhao E, Amir M, Lin Y, Tanaka KE, Czaja MJ. Autophagy confers resistance to lipopolysaccharide-induced mouse hepatocyte injury. Am J Physiol Gastrointest Liver Physiol 311: G377–G386, 2016. First published July 28, 2016; doi:10.1152/ajpgi.00124.2016.—During sepsis, bacterial products, particularly LPS, trigger injury in organs such as the liver. This common condition remains largely untreatable, in part due to a lack of understanding of how high concentrations of LPS cause cellular injury. In the liver, the lysosomal degradative pathway of autophagy performs essential hepatoprotective functions and is induced by LPS. We, therefore, examined whether hepatocyte autophagy protects against liver injury from septic levels of LPS. Mice with an inducible hepatocyte-specific knockout of the critical autophagy gene Atg7 were examined for their sensitivity to high-dose LPS. Increased liver injury occurred in knockout mice, as determined by significantly increased serum alanine aminotransferase levels, histological evidence of liver injury, terminal deoxynucleotidetransferase-mediated deoxyuridine triphosphate nick end-labeling, and effector caspase-3 and -7 activation. Hepatic inflammation and proinflammatory cytokine induction were unaffected by the decrease in hepatocyte autophagy. Although knockout mice had normal NF-κB signaling, hepatic levels of Akt1 and Akt2 phosphorylation in response to LPS were decreased. Cultured hepatocytes from knockout mice displayed a generalized defect in Akt signaling in response to multiple stimuli, including LPS, TNF, and IL-1β. Akt activation mediates hepatocyte resistance to TNF cytotoxicity, and anti-TNF antibodies significantly decreased LPS-induced liver injury in knockout mice, indicating that the loss of autophagy sensitized to TNF-dependent liver damage. Hepatocyte autophagy, therefore, protects against LPS-induced liver injury. Conditions such as aging and steatosis that impair hepatocyte autophagy may predispose to poor outcomes from sepsis through this mechanism. Akt; apoptosis; liver; sepsis; tumor necrosis factor

NEW & NOTEWORTHY

Autophagy is required for hepatocytes to resist injury from high concentrations of LPS. With a genetic decrease in hepatocyte autophagy, increased liver injury occurred in response to LPS from sensitization to TNF-dependent hepatic cell death in association with an impairment in Akt signaling. Human conditions such as aging and hepatic steatosis may worsen the clinical outcome from sepsis as the result of their concomitant decrease in hepatic levels of autophagy.

SEPSIS REPRESENTS A SYSTEMIC response to bacterial products, prominent among which is endotoxin or LPS (13, 25). Sepsis is a major source of morbidity and mortality that yearly affects over 400,000 individuals in the United States and contributes to one-third of all hospital deaths (2, 18, 20). The availability of a wide spectrum of effective antibiotics has failed to reduce mortality from this condition, which remains over 25% (25). Prominent among the sites of end-organ damage from sepsis is the liver, where LPS-induced injury is mediated through the mechanisms of tissue ischemia, immune cell infiltration, and the direct cytotoxicity of cytokines. An enhanced understanding of the pathophysiological basis of the end-organ damage and dysfunction in sepsis is critical to the development of more effective therapies for this condition.

Macroautophagy is a lysosomal degradation pathway that performs critical cellular functions in the liver, such as maintaining energy homeostasis and degrading aged or damaged cellular organelles and proteins (7). LPS induces autophagy in the liver (1), suggesting that this pathway may be important in modulating the hepatic effects of LPS. The cell type in which autophagy has been linked to immune responsiveness and inflammation is the macrophage. Autophagy in macrophages modulates the clearance of infectious pathogens and inflammasome-dependent production of the proinflammatory cytokine IL-1β (9). Recently, our laboratory demonstrated that macrophage autophagy regulates hepatic immune responsiveness to low-dose LPS through effects on macrophage polarization and inflammasome activation (14, 17).

Little is known about the function of hepatocyte autophagy in the liver’s response to LPS. Our investigations of TNF-dependent liver injury from the hepatotoxic galactosamine have demonstrated that autophagy protects against hepatocyte injury and cell death from TNF (1). Although these studies were conducted in a model in which low-dose LPS injures the liver through sensitization by the toxin galactosamine, these findings suggested that autophagy may modulate hepatic injury and death from the high levels of LPS that occur with sepsis. Cross talk between the autophagic and apoptotic pathways has been demonstrated at multiple levels, and, although autophagy can mediate cell death under certain conditions, this pathway is considered largely an antiapoptotic, prosurvival mechanism (16). Hepatocellular toxicity to TNF is regulated by a number of signaling pathways, including NF-κB, Akt, and the MAPK JNK (26). Autophagy can modulate signaling pathways through the degradation of regulatory proteins, such as IκBα, which allows NF-κB activation (6). In addition, decreased Akt signaling in response to insulin has been reported in hepatocyte autophagy-deficient mice in the setting of obesity (37). The realization that autophagy can regulate signal...
transduction suggested that autophagy may modulate some of these TNF resistance pathways in response to high concentrations of LPS.

In the present study, we examined whether hepatocyte autophagy functions in the liver’s response to high concentrations of LPS alone. We demonstrate that mice with a hepatocyte-specific knockout of Atg7-dependent autophagy are sensitized to LPS-induced liver injury and hepatocyte death. Injury was associated with a defect in hepatic Akt signaling, and primary hepatocytes had impaired Akt activation in response to a number of stimuli, including TNF. The decreased Akt activation in response to LPS resulted in TNF-dependent liver injury, as it was abrogated by treatment with anti-TNF antibodies. Hepatocyte autophagy serves a critical function in the resistance to liver injury from LPS.

MATERIALS AND METHODS

**Animal model.** Mice were maintained in a pathogen-free facility with 12:12-h light-dark cycles and unlimited access to food and water. All studies were performed in 10- to 14-wk-old male mice. Atg7<sup>f/f</sup> (C57BL/6 background. Genotypes were confirmed by PCR with established primers. To activate Cre expression and generate mice with a hepatocyte-specific knockout of Atg7, Atg7<sup>2loxP</sup> mice were crossed with ERT-Alb-Cre mice with a tamoxifen-inducible, albumin promoter-driven Cre recombinase to generate ERT-albumin-Cre-Atg7<sup>2loxP</sup> or Atg7<sup>2cre</sup> mice with a hepatocyte-specific knockout of autophagy, as previously described (1). Both mouse strains are on a C57BL/6 background. Genotypes were confirmed by PCR with established primers. Conditions for the genotyping were approved by the Albert Einstein Institutional Animal Care & Use Committee and followed the NIH guidelines on the care and use of animals.

**ALT assay.** Serum alanine aminotransferases (ALTs) were measured using a commercial kit (TECO Diagnostics, Anaheim, CA).

**Histology.** Livers were fixed in 10% neutral formalin, stained with hematoxylin and eosin, and graded in a blinded fashion by a single pathologist for the degree of liver injury and inflammation. The percentage of hepatic parenchyma with apoptosis/necrosis or inflammation was semiquantitatively graded on a sliding scale as follows: 0, absent; 0.5, minimal; 1, mild; 1.5, mild to moderate; 2, moderate; 2.5, moderate to marked; and 3, marked.

**TUNEL assay.** Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-positive cells in 10 randomly selected fields (400 magnification) were counted per liver section.

**Protein isolation and Western blotting.** Total liver protein was isolated, as previously described (32). Protein concentrations were determined by the Bio-Rad (Hercules, CA) protein assay, and Western blotting was performed as previously described. Membranes were exposed to antibodies that recognized NF-κB p50 (Santa Cruz Biotechnology, Santa Cruz, CA; no. SC-114-G), NF-κB p65 (Santa Cruz Biotechnology; no. SC-109), IkBα (Santa Cruz Biotechnology; no. SC-203), LC3 (Cell Signaling, Beverly, MA; no. 2775), p62 (Cell Signaling; no. 2631), caspase-3 (Cell Signaling; no. 9665), caspase-7 (Cell Signaling; no. 9492), tubulin (Cell Signaling; no. 2148), GAPDH (Cell Signaling; no. 2118), Akt (Cell Signaling; no. 9272), P<sub>38</sub>-Akt (Cell Signaling; no. 9275), P<sup>70</sup>-Akt (Cell Signaling; no. 9278), p<sup>70</sup>-Akt1 (Cell Signaling; no. 9018), p<sup>70</sup>-Akt2 (Cell Signaling; no. 8599), P-GSK-3β (glycogen synthase kinase-3β) (Cell Signaling; no. 9331), cytochrome oxidase (Abcam, Cambridge, MA; no. MS-407), cytochrome c (BD Biosciences, San Jose, CA; no. 565433), β-actin (Sigma Aldrich; no. A5454), NOPP140 (U. Thomas Meier, Albert Einstein College of Medicine, Bronx, NY), and SQSTM1/p62 (p62) (Enzo, Plymouth Meeting, PA; no. BML-PW9860). Western blot signals were quantitated by a FluorChem densitometer (Alpha Innotech, San Leonardo, CA).

**Caspase-3 activity.** Mouse liver caspase-3 activity was determined biochemically by commercial kit (R&D Systems, Minneapolis, MN). Activity is expressed as the level relative to that in untreated control mice.

**Immunofluorescence.** At death, a piece of liver tissue was coated with OCT, frozen in 2-methylbutane for 15 min, and stored at −80°C until sectioning. Frozen sections (5 μm) were cut with a cryostat, air dried (10 min), fixed in methanol (−20°C, 10 min), rehydrated in PBS (10 min), and then incubated with blocking solution (2% normal donkey serum, 1% BSA, and 0.05% Tween 20 for 1 h). After removal of the blocking solution, sections were incubated overnight at 4°C with rat anti-mouse Ly6G antibody (Biolegend, San Diego, CA; no. 127602) or rat anti-mouse CD68 antibody (Abd Serotec, Raleigh, NC; no. MCA1957GA), diluted 1:200 in the blocking solution. Sections were then washed in PBS twice (10 min) and incubated for 1 h with donkey anti-mouse Cy3 antibody (Jackson ImmunoResearch, West Grove, PA; no. 111–165–152) diluted 1:200. Sections were washed twice in PBS and then air dried and mounted in ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Life Technologies, Carlsbad, CA). Positive cells were counted from 10 randomly selected high-power fields per section.

**Quantitative real-time reverse-transcription PCR.** Total liver RNA was isolated using the commercial kit RNasy Plus (QIAGEN, Valencia, CA). Reverse transcription was carried out with 1 μg of RNA in an Eppendorf Mastercycler (Hamburg, Germany) using a high-capacity cDNA reverse transcription kit (ABI, Foster City, CA).

Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>18S</td>
<td>Forward: 5'-TGAGAGGGAAGAATACTGGTTC-3'</td>
<td>Reverse: 5'-GCTGAGGGCCGATGGTCGG-3'</td>
</tr>
<tr>
<td>18S</td>
<td>Forward: 5'-GACCAAGAATCTGGTCAGGAA-3'</td>
<td>Reverse: 5'-ACCTGGACTGCTGCAACGC-3'</td>
</tr>
<tr>
<td>Birc2</td>
<td>Forward: 5'-ACCTGAGATATAGCATTCCC-3'</td>
<td>Reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'</td>
</tr>
<tr>
<td>Birc3</td>
<td>Forward: 5'-ACCAGGATATAGCATTCCC-3'</td>
<td>Reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'</td>
</tr>
<tr>
<td>Cyt c1</td>
<td>Forward: 5'-GCCGCAATAGTGTCGGTCC-3'</td>
<td>Reverse: 5'-GGTACGACTGCTGGTGCTCT-3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward: 5'-AGGCTGCTGAGAACGCTATCT-3'</td>
<td>Reverse: 5'-ATCTTCTGGGCTGCTGAACT-3'</td>
</tr>
<tr>
<td>Ifnγ</td>
<td>Forward: 5'-ATGGGGGACAAGAATCTGGT-3'</td>
<td>Reverse: 5'-AGGCTGCTGAGAACGCTATCT-3'</td>
</tr>
<tr>
<td>Il-1β</td>
<td>Forward: 5'-GACCAAGAATCTGGTCAGGAA-3'</td>
<td>Reverse: 5'-ATCTTCTGGGCTGCTGAACT-3'</td>
</tr>
<tr>
<td>Il-6</td>
<td>Forward: 5'-AGGCTGCTGAGAACGCTATCT-3'</td>
<td>Reverse: 5'-ATCTTCTGGGCTGCTGAACT-3'</td>
</tr>
<tr>
<td>Il-10</td>
<td>Forward: 5'-AGGCTGCTGAGAACGCTATCT-3'</td>
<td>Reverse: 5'-ATCTTCTGGGCTGCTGAACT-3'</td>
</tr>
<tr>
<td>Tnf</td>
<td>Forward: 5'-GCCGCAATAGTGTCGGTCC-3'</td>
<td>Reverse: 5'-GGTACGACTGCTGGTGCTCT-3'</td>
</tr>
</tbody>
</table>
Annealing of primers was done at 25°C for 10 min, followed by elongation at 37°C for 2 h and inactivation of the enzyme at 85°C for 5 min. Negative controls (no added transcriptase) were performed in parallel. PCR for Tnf, Il-6, Il-1/H9252, Ifn/H9253, Il-10, Traf2, Birc2, Birc3, and Gapdh was performed in duplicate in a 7500 Fast Real-Time PCR System (ABI). The primer sequences in Table 1 were purchased from Integrated DNA Technologies (Coralville, IA). PCR was carried out using Power SYBR Green Master Mix (ABI). Taq polymerase was activated at 95°C for 10 min. The cycling parameters were denaturation at 95°C for 30 s and extension at 60°C for 1 min (for 40 cycles). Data analysis was performed using the 2^(-ΔΔCT) method for relative quantification. All samples were normalized to Gapdh.

Mitochondrial DNA content. Total liver DNA was isolated using the commercial kit DNeasy Blood & Tissue (QIAGEN). Real-time PCR for the cytochrome c_1 and 18S rRNA genes was performed as described above using the primers in Fig. 1. Mitochondrial DNA content was quantified by normalizing values for the cytochrome c_1 gene (mitochondrial DNA) to that for the 18S rRNA gene (nuclear DNA).

ATP assay. Liver and mitochondrial ATP content was measured with a commercial kit (Biovision, Milpitas, CA) and normalized to protein content.

GSH assay. The 5,5'-dithiobis (2-nitrobenzoic acid)-GSH disulfide recycling assay was used for the determination of total GSH in whole livers and mitochondrial GSH in isolated mitochondria, as previously described (36). Values were normalized to protein content.

Mitochondrial isolation. Livers were separated into mitochondrial and cytosolic fractions, as previously described (33). Briefly, the liver was dounce homogenized, and the homogenate was centrifuged at
slow speed to pellet membranes, nuclei, and other debris. The supernatant was then centrifuged at high speed to pellet mitochondria. Nuclear separation. Nuclear and cytosolic fractions were isolated from liver, as previously described (29). Briefly, the liver was dounce homogenized, and the homogenate was centrifuged on a sucrose gradient to separate the nuclear and cytosolic fractions.

Primary hepatocytes. Primary hepatocytes were obtained by liver perfusion from tamoxifen injected Atg7Δhep mice and Atg7F/F littermate controls, as previously described (11). Only hepatocyte isolations with a viability of ≥90% were used, and cells were subsequently purified on a Percoll gradient. Hepatocytes were plated in serum-free Williams E medium on collagen-coated dishes. After 3 h, cells were supplied with fresh media and cultured overnight. The medium was replaced 2 h before treatment with LPS 100 ng/ml, TNF 15 ng/ml, or IL-1β 17 ng/ml (R&D Systems, Minneapolis, MN) for various times.

Statistical analysis. Numerical results are reported as means ± SE and are derived from at least three independent experiments, unless otherwise indicated. The unpaired Student’s t-test was used to assess significance between control and treated groups. Statistical significance was defined as \( P < 0.05 \).

![Fig. 3. Inflammatory response to LPS is unaffected by decreased hepatocyte autophagy. A–E: relative mRNA levels for the indicated cytokine genes measured by qRT-PCR in littermate control (Con) and Atg7Δhep knockout (KO) mice (n = 3–8). F: immunofluorescence staining for Ly6G in the livers of Con and KO mice at the indicated times after LPS treatment (×400 magnification). G: quantification of the numbers of Ly6G-positive cells (n = 3–5). H: immunofluorescence staining for CD68 (×200 magnification). I: numbers of CD68-positive cells (n = 3–5). Values are means ± SE. HPF, high-power field.](http://ajpgi.physiology.org/)

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RESULTS

Decreased hepatocyte autophagy sensitizes mice to LPS-induced liver injury. To determine whether autophagy functions in hepatocyte resistance to liver injury from LPS, Atg7/H9004 hep mice with a hepatocyte-specific knockout of Atg7-dependent autophagy were evaluated for their sensitivity to liver injury from high-dose LPS. Initially, tamoxifen-injected littermate control and knockout mice were examined for the effectiveness of the Atg7 knockout. Atg7/H9004 hep mouse livers had decreased levels of Atg7 and an inhibition of autophagy, as demonstrated by immunoblot findings of decreased Atg7 and LC3-II content with a compensatory increase in LC3-I, and elevated p62 levels (Fig. 1). LPS upregulates autophagy in mouse liver (1), but the induction of autophagy by LPS was blocked in knockout mice.

Mild liver injury occurred in response to LPS in littermate control mice, as reflected by increased serum ALT levels that peaked at <60 IU/l within 48 h (Fig. 2A). ALT levels in Atg7/H9004 hep mice peaked within the same time period but were significantly increased compared with control mice at all time points, including a 10-fold increase at 24 h (Fig. 2A). Blinded, semiquantitative assessment of hematoxylin and eosin-stained sections revealed increased levels of hepatic injury in LPS-treated Atg7/H9004 hep mice (Fig. 2B), with increased numbers of apoptotic cells (Fig. 2C). A greater induction of cell death was also demonstrated by findings of significantly higher numbers of TUNEL-positive cells in the livers of knockout mice (Fig. 2, D and E). Finally, increased hepatocyte apoptosis was confirmed by the presence of elevated levels of the active, cleaved protein forms of effector caspase-3 and -7 in Atg7/H9004 hep mouse livers by immunoblotting (Fig. 2F). Knockout mouse livers also had increased levels of caspase-3 biochemical activity (Fig. 2G). Loss of hepatocyte Atg7-dependent autophagy, therefore, sensitized mice to significantly increased liver injury and cell death from LPS.

LPS-induced activation of the inflammatory response is unaffected by decreased hepatocyte autophagy. In response to LPS, neutrophils and macrophages infiltrate the liver parenchyma and promote liver injury through the elaboration of proinflammatory cytokines that trigger cellular damage. Although these studies employed hepatocyte-specific autophagy knockout mice, the possibility existed that greater initial injury in knockout hepatocytes led to the release of factors that amplified the innate immune response to LPS, thereby causing increased liver injury. Assessment of the induction of critical LPS-induced cytokines by quantitative real-time reverse-transcription PCR (qRT-PCR) revealed marked increases in hepatic cytokine gene expression with LPS, but no significant differences in the relative mRNA levels of Tnf, Il-6, Il-1β, Ifnγ, or Il-10 between control and knockout mice (Fig. 3, A–E). Immunofluorescence staining with a neutrophil-specific anti-Ly6G antibody demonstrated a marked infiltration of neutrophils with LPS treatment, but equivalent neutrophil numbers in the livers of control and knockout mice (Fig. 3, F and G).

Fig. 4. Mitochondrial accumulation and dysfunction do not occur in knockout (KO) mice. A: ratios of mitochondrial to nuclear DNA in the livers of control (Con) and Atg7/H9004 hep KO mice untreated and LPS-treated for the indicated number of hours. B: immunoblots of total liver protein from individual Con (Cre−) and KO (Cre+) mice probed for the mitochondrial proteins cytochrome c (Cyt c) and cytochrome oxidase (Cyt ox). C: relative levels of ATP in the livers. D: whole liver GSH levels. E: mitochondrial GSH levels. Values are means ± SE; n = 3–6. *P < 0.05, compared with Con mice at the same time point.
Macrophage numbers as measured by CD68 positivity were not altered by LPS treatment in either control or knockout mice (Fig. 3, H and I). These findings indicate that the increase in LPS-induced liver injury in Atg7<sup>−/−</sup> mice was not the result of a hyperactive innate immune response to LPS.

*Increased liver injury is not mediated by impaired mitophagy.* Previous studies have implicated defects in mitophagy, the selective removal of damaged or dysfunctional mitochondria by autophagy, as a mechanism of cellular injury during sepsis (23). We, therefore, assessed whether decreased hepatocyte autophagy led to the retention of damaged and potentially injury-triggering mitochondria or compromised mitochondrial integrity. Measure of mitochondrial number by means of the ratio of mitochondrial to nuclear DNA demonstrated a similar decrease in mitochondrial number at 12 h with a return to baseline levels by 24 h in both control and knockout mouse livers (Fig. 4A). Also indicative of equal mitochondrial numbers in control and Atg7<sup>−/−</sup> mouse livers was the finding of equivalent hepatic levels of the mitochondrial proteins cytochrome c and cytochrome oxidase by immunoblotting of total hepatic protein (Fig. 4B). Mitochondrial energy homeostasis was maintained in knockout mice as hepatic ATP levels were essentially unchanged with LPS treatment in both control and Atg7<sup>−/−</sup> mice (Fig. 4C). Oxidant stress occurred with LPS treatment, as indicated by decreased total liver (Fig. 4D) and mitochondrial (Fig. 4E) GSH, but decreases were equivalent in control and knockout mice. The failure of the decrease in hepatocyte autophagy to affect hepatic mitochondrial number, liver ATP content, or hepatic and mitochondrial GSH levels in Atg7<sup>−/−</sup> mice indicate that the sensitization to LPS-induced injury was not secondary to impaired mitophagy.

**NF-κB activation is unaffected by the inhibition of hepatocyte autophagy.** Activation of the transcription factor NF-κB is critical for hepatocyte resistance to LPS-induced TNF cytotoxicity (27, 35), and autophagy regulates NF-κB signaling (19). To determine whether decreased NF-κB activation mediated the sensitization of knockout mice to hepatotoxicity from LPS, the effect of autophagy inhibition on LPS-induced NF-κB signaling was examined at several levels. Immunoblots for the cytosolic NF-κB inhibitor IκBα, which is degraded during NF-κB activation, revealed equivalent decreases in IκBα from 30 to 60 min following LPS treatment in the livers of control and knockout mice (Fig. 5, A and C). Nuclear translocation of the active p65 (Fig. 5, B and D) and p50 NF-κB subunits in response to LPS was also equivalent in control and knockout livers (Fig. 5, B and E). The purities of the cytosolic and nuclear protein loadings were confirmed by probing for the nuclear loading control NOPP140 and the cytosolic protein loading control GAPDH (Fig. 5, A and B).

**Fig. 5. NF-κB activation is unaltered by the decrease in hepatocyte autophagy.** A: hepatic cytosolic protein fractions from individual control (Con) (Cre −) and knockout (KO) (Cre +) mice untreated or treated with LPS for the indicated hours and probed for IκBα, the nuclear loading control NOPP140, and the cytosolic protein loading control GAPDH. B: nuclear protein fractions from the same mice probed for the p65 and p50 subunits of NF-κB, NOPP140, and GAPDH. C–E: relative protein levels of IκBα, p65, and p50 proteins, respectively, by densitometric scanning of immunoblots (n = 4). F–H: relative Traf2, Birc2, and Birc3 mRNA levels, respectively, determined by qRT-PCR in Con and KO mice untreated, or treated with LPS for hours indicated. Values are means ± SE.
nuclear preps were demonstrated by the restricted presence of GAPDH in the cytoplasmic fractions and NOPP140 in the nuclear isolates (Fig. 5, A and B). Finally, equal induction of the NF-κB-dependent genes Traf2, Birc2, and Birc3 occurred in control and knockout mice, as assessed by qRT-PCR of whole liver (Fig. 5, F–H). Together these findings prove that loss of hepatocyte autophagy did not alter protective NF-κB signaling in response to LPS.

**Decreased hepatocyte autophagy impairs Akt signaling.** In addition to NF-κB, Akt signaling serves as a central prosurvival pathway in hepatocytes in response to LPS (12, 22). We, therefore, examined whether decreased hepatocyte autophagy altered hepatic Akt signaling from LPS. Immunoblots of whole liver protein demonstrated a significant increase in Akt phosphorylation following LPS administration that peaked at 12 h in control mice (Fig. 6A). Levels of active, phosphorylated Akt were markedly decreased in the livers of knockout mice (Fig. 6, A and B). Decreased phosphorylation occurred at both Ser473 and Thr308 and in both the Akt1 and Akt2 isomers (Fig. 6A).

Akt signaling was functionally impaired as the reduction in Akt phosphorylation was associated with decreased phos-
phosphorylation and, therefore, activation of the Akt downstream substrate GSK-3β (Fig. 6A).

The use of a hepatocyte knockout, and the overall decrease of Akt phosphorylation in whole liver, which is composed mainly of hepatocytes, suggested that decreased phosphorylation occurred in hepatocytes. To confirm this fact, Akt activation was examined in primary hepatocytes isolated from the livers of tamoxifen-injected control and knockout livers and treated in vitro, not only with LPS but also with the proinflammatory cytokines TNF and IL-1β. Primary hepatocytes from Atg7<sup>−/−</sup> mice had decreased Akt phosphorylation in response to LPS (Fig. 6, C and F). TNF (Fig. 6, D and G), and IL-1β (Fig. 6, E and H). Inhibition of autophagy, therefore, leads to a hepatocyte defect in Akt signaling in response to inflammatory factors.

**Inhibition of autophagy sensitizes the liver to LPS-induced TNF toxicity.** The reduction in knockout mice of Akt signaling critical for hepatocyte resistance to TNF toxicity suggested that increased liver injury resulted from sensitization to LPS-induced TNF. The administration of anti-TNF antibodies to Atg7<sup>−/−</sup> mice before LPS injection almost completely abrogated liver injury compared with mice treated with IgG isotype control antibody, as determined by serum ALT levels (Fig. 7A) and TUNEL staining (Fig. 7B). Anti-TNF treatment also significantly decreased the severity of histological liver injury (Fig. 7C). Together these data demonstrate that, with reduced autophagy, high-dose LPS triggers apoptotic hepatocyte death that is mediated to a large extent by sensitization to TNF cytotoxicity.

**DISCUSSION**

Sepsis is a major clinical problem for which the development of an effective therapy has been hindered by an incomplete understanding of how the bacterial product LPS triggers injury in end organs, such as the liver. LPS induces autophagy in mouse liver and hepatocytes (1, 5), and increased numbers of autophagosomes under electron microscopy have been reported in human livers from septic patients, suggesting that hepatic autophagy is increased by LPS in humans as well (34). The induction of autophagy by LPS, and the known hepatoprotective functions of autophagy (8), suggested a potential role for autophagy in preventing liver injury from high levels of LPS. Studies with nonspecific lysosomal inhibitors or a lack of hepatocytes to death from LPS did not occur through a lack of hepatic mitophagy, as the numbers of mitochondria and the levels of mitochondrial ATP and oxidative stress were unaffected by the loss of hepatocyte autophagy. Thus there was no evidence that decreased removal of damaged mitochondria in response to LPS was the mechanism of increased liver injury in the knockout mice. It remains possible that, although mitophagy did not play a role in knockout mouse sensitization to LPS-induced injury, knockout mice still developed increased mitochondrial injury that promoted hepatocyte death. Normal energy and redox status suggest intact mitochondrial function, but do not completely exclude this possibility.

The lack of mechanistic involvement of mitophagy in the sensitization to LPS-induced injury, together with findings that the production of potentially toxic cytokines was unaltered, led to an examination of whether knockout mice had a defect in hepatocyte-protective signaling pathways. NF-κB is critical to protect hepatocytes from LPS-induced TNF, but NF-κB signaling as assessed by IkB degradation, p50/p65 NF-κB nuclear translocation, and the induction of NF-κB-dependent genes was unaltered in knockout mice. When the hepatoprotective Akt signaling pathway was examined, the livers of Atg7<sup>−/−</sup> mice had reduced Akt activation, as reflected in decreased phosphorylation of both Akt1 and Akt2 after LPS injection.

**Fig. 7.** Inhibition of TNF decreases liver injury in knockout (KO) mice. Control (Con) and KO mice were pretreated 4 h before LPS administration with an isotypic IgG control antibody or a monoclonal IgG2a against mouse TNF and killed at 24 h for analysis of serum ALT levels (*P < 0.03, compared with KO mice treated with control antibody; n = 7–9; A); TUNEL staining (*P < 0.001, compared with KO mice treated with control antibody; n = 4–8; B); and histological grade of liver injury (*P < 0.04, compared with KO mice treated with control antibody; n = 4–8; C). Values are means ± SE.
That the decrease in Akt phosphorylation in whole liver was due at least in part to a hepatocyte effect was demonstrated by the finding that cultured hepatocytes from Arg7Δhep mice had decreased Akt phosphorylation in vitro. Akt phosphorylation was reduced in response to both LPS directly and to the LPS-inducible cytokines TNF and IL-1β. The mechanism by which autophagy promotes Akt phosphorylation is unclear. The decrease in Akt activation was not due to a defect in LPS receptor binding, as NF-κB signaling was intact in the knock-out mice. A reduction in autophagy could prolong activation of a signaling pathway by preventing the normal timely degradation of activated pathway proteins (3). The mechanism by which a decrease in autophagy could block activation is less clear.

Akt activation is likely a generalized cellular protective response to LPS toxicity, as Akt protects against cardiac dysfunction, as well through the prevention of apoptosis by an undefined mechanism (10). Our study specifically examined whether liver injury from decreased Akt activation secondary to impaired autophagy was TNF dependent, as Akt signaling is a critical resistance pathway against hepatocyte TNF toxicity (12, 22). Injury was prevented by TNF neutralization, demonstrating the decrease in Akt signaling sensitized the liver to cytoxicity from LPS-induced TNF. These findings, along with our laboratory’s previous study demonstrating the critical function of autophagy in preventing TNF-dependent hepatotoxic liver injury (1), point to the importance of the lysosomal degradative pathway of autophagy in hepatoprotection against inflammatory mediators.

The finding that decreased hepatocyte autophagy sensitizes the liver to increased injury from LPS may explain in part the known susceptibility of certain patient populations to an adverse clinical outcome from sepsis. Over 75% of the deaths from sepsis in the United States occur in individuals over the age of 65 yr (21). Aging is associated with an impairment in autophagic function (24), and the decrease in hepatic autophagy that occurs with aging has been previously implicated in the long term control of tumor necrosis factor-α-induced nuclear factor-κB (NF-κB) activity. J Biol Chem 286: 22886–22893, 2011.


Disclosures
No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

References

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