CCAAT Enhancer-binding Protein α Is Required for Interleukin-6 Receptor α Signaling in Newborn Hepatocytes*

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Stephanie L. Mackey‡ and Gretchen J. Darlington§§

From the Interdepartmental Program in Cell and Molecular Biology and §Huffington Center on Aging and Department of Pathology, Baylor College of Medicine, Houston, Texas 77030

The acute phase response is an evolutionarily conserved response of the liver to inflammatory stimuli, which aids the body in host defense and homeostasis. We have previously reported that CCAAT enhancer-binding protein α (C/EBPα) is required for the induction of acute phase protein (APP) genes in newborn mice in response to lipopolysaccharide. In this paper, we describe a mechanism by which C/EBPα knock-out mice are unable to induce APP gene expression in response to inflammatory stimuli. We demonstrate that the lack of acute phase response in C/EBPα knock-out mice is because of a hepatocyte autonomous defect. C/EBPα knock-out hepatocytes do not activate STAT3 in response to recombiant interleukin (IL)-6, indicating a defect in the IL-6 pathway. C/EBPα knock-out hepatocytes also do not show activation of other gp130 signaling cytokine promoters such as Janus kinase substrates, gp130, SHP-2, and Tyk2. Further examination of the IL-6 pathway demonstrated that C/EBPα knock-out hepatocytes have decreased IL-6Rα protein levels caused, in part, by reduced protein stability. However, other components of the IL-6 pathway are intact, as demonstrated by rescue of STAT3 activation and APP gene induction with recombinant-soluble IL-6R linked to IL-6 cytokine (Hyper-IL-6) or with another gp130 signaling cytokine, Oncostatin M. In conclusion, C/EBPα is required for the proper regulation of IL-6Rα protein in hepatocytes resulting in a lack of acute phase protein gene induction in newborn C/EBPα null mice in response to lipopolysaccharide or cytokines.

The acute phase response (APR) is the rapid systemic response by the body to inflammatory stimuli such as tissue damage or infection. At the site of damage, macrophages and other surrounding cells detect injury and respond by secreting pro-inflammatory cytokines including IL-1, IL-6, and TNF-α into the blood stream to elicit a systemic response. The liver is highly responsive to these cytokines because of the high density of cytokine receptors on hepatocytes. These cytokines evoke changes in expression of acute phase protein (APP) genes, such as positively regulated APP genes like serum amyloid A (SAA), γ-fibrinogen, and haptoglobin and negatively regulated APP genes like albumin and transferrin. These acute phase proteins are made by hepatocytes and then secreted into the bloodstream where they play a variety of roles in homeostasis, host defense, and minimizing tissue damage (see Refs. 1, 2).

IL-1, IL-6, and TNF-α signal through known receptors to activate three major transcription factor families: NFkB, C/EBPs, and STATs. These factors regulate APP gene expression at the transcriptional level. IL-1 and TNF-α activate NFκB p50 and p65, which recognize the consensus sites in the promoters of many APP genes such as the serum amyloid A gene family (3). IL-6 induces the tyrosine phosphorylation and DNA binding activity of STAT3, which bind fibrogin and α2-macroglobulin promoters among others (4, 5). IL-1 and IL-6 induce C/EBPβ and C/EBPα at both the transcriptional and protein activation levels, whereas C/EBPβ is slightly down-regulated (6, 7). This leads to the replacement of C/EBPα DNA binding at C/EBP sites in promoters of APP genes with C/EBPβ and δ. Previously, it was thought that C/EBPα played a passive role in the APR; however, our laboratory has shown that C/EBPα is required for the induction of many APP genes in mice (8). Neonatal C/EBPα null mice do not elevate APP genes but do induce NFκB, C/EBPβ, and C/EBPβ DNA binding in response to LPS injection. In contrast, C/EBPβ knock-out (KO) mice do not activate STAT3 DNA binding in response to LPS, indicating a defect in the IL-6-STAT3 signaling pathway (8).

IL-6 is thought to be the main stimulator of the induction of APP genes during the hepatic acute phase response, whereas IL-1 and TNF-α influence the expression of subgroups of APP genes and induce IL-6 cytokine production (9). IL-6 knock-out mice induce APP genes in response to the systemic APR inducer bacterial LPS; whereas the response to a localized tissue damage by turpentine injection does not induce APP gene expression in these mice (10). Additionally, IL-6 null mice respond to LPS by activation of STAT3; however, STAT3 is not activated in response to turpentine treatment (11). The authors speculate that other STAT3 activating cytokines, such as leukemia inhibitory factor, IL-11, or Oncostatin M (OncM), may be induced in response to LPS but not to turpentine (10). STAT3 activation by an IL-6-like signaling pathway has recently been shown to be the most important regulator of APP gene expression in mice through tissue-specific inactivation of the STAT3 gene in the liver (12). In these tissue-specific STAT3 knock-out mice, most APP genes are not induced in response to LPS treatment. Interestingly, several genes found to be STAT3-responsive do not have STAT3 binding sites identified in their promoters.

IL-6 cytokines signal by binding to an α-receptor subunit,
IL-6Ra, which binds and signals through homodimerization of the common gp130 signaling molecule. The formation of these receptor complexes brings JAKs in close proximity to each other leading to cross-phosphorylation and activation of the JAKs, including Jak1, Jak2, and Tyk2. The JAKs activate the Ras-MAPK pathway through phosphorylation of the phosphatase, SHP-2, leading to downstream activation of C/EBPβ. JAKs also activate STAT3 by tyrosine phosphorylation, permitting dimerization and entry into the nucleus where STAT3 dimers bind to STAT sites in the promoters of many APP genes (15). We previously reported that neonatal C/EBPα null mice do not respond to LPS treatment by elevating APP genes and do not activate STAT3 DNA binding (8). However, it was not known whether the lack of APR in C/EBPβ null mice was the result of a defect in the hepatocytes or in the extrahepatic cellular signaling of the injury. In this paper, we report that C/EBPβ null hepatocytes cultured in vitro are unable to respond to recombinant IL-6 to induce APP gene expression and STAT3 DNA binding. The IL-1 pathway is intact as the predominantly IL-1-responsive gene SAA3 (16) is induced upon IL-1 treatment of C/EBPβ knock-out hepatocytes. Additionally, we show that STAT3 is not activated because of defects in the IL-6Rα signaling pathway. Specifically, IL-6Rα protein levels in C/EBPβ null hepatocytes are decreased, which is attributed in part to decreased protein stability of the receptor. Finally, the addition of the IL-6-like cytokine Oncostatin M or the soluble IL-6Rα linked to IL-6, termed Hyper-IL-6 (17), is capable of activating STAT3 DNA binding and synergistic induction of the acute phase protein gene, SAA1, in primary hepatocytes derived from C/EBPβ null mice. We propose that the dramatically reduced expression of the IL-6 receptor is responsible for the failure of C/EBPβ knock-out mice to respond to inflammatory stimuli.

### EXPERIMENTAL PROCEDURES

**APR Induction in Mice**—Newborn mice were injected with 5 mg/kg body weight of LPS (Sigma) intraperitoneally to induce a generalized inflammatory response, and livers were harvested at time points after LPS injection. All of the neonatal mice were periodically injected with 10% glucose subcutaneously to counteract the perinatal hypoglycemia seen in C/EBPβ knock-out pups.

**Primary Hepatocyte Isolation and Cell Culture**—Livers from newborn C/EBPβ KO and WT mice were harvested and were mechanically dissociated by scalpel. Hepatocytes were further dissociated from the liver pieces by shaking at 37 °C in EDTA-Earl’s balanced salt solution (Invitrogen) for 10 min followed by a brief wash with M/M medium (three-parts Eagle’s minimal essential medium and one-part Waymouth MAB 83/7 (Invitrogen) with 10% fetal calf serum). Hepatocytes were then incubated in M/M medium containing 0.4 mg/ml collagenase (Roche Applied Science) with 10% fetal calf serum). Hepatocytes were plated in M/M medium to remove non-adherent hematopoietic cells. Hepatocytes were plated in M/M medium (Sigma) for 10 min followed by a brief wash with M/M medium balanced salt solution (BD Falcon) to remove cellular aggregates and tissue debris. Hepatocytes were plated in M/M medium to allow for 2 h, and then cells were washed in M/M medium to remove non-adherent hematopoietic cells. Hepatocytes were finally plated in M/M medium containing dexamethasone (10−8 M), epidermal growth factor (50 ng/ml) (Invitrogen), and insulin (5 μg/ml) (Sigma). Cells were fed every other day and split at 80−90% confluency. Experiments were performed on days 7−10 post-isolation of primary hepatocytes.

**Cytokine Treatment**—Primary hepatocytes were treated with all of the cytokines used at a concentration of 50 ng/ml in fresh medium for the time indicated in the figures. IL-1 was obtained from PeproTech (Rocky Hill, NJ). Oncostatin M and IL-6 were obtained from Sigma.

**Hyper-IL-6** was a generous gift from Dr. Rose-John at Christian-Albrechts Universität (Kiel, Germany).

**Northern Analysis**—Northern blot analysis was performed on liver RNA harvested 4 h after LPS injection and on primary hepatocyte RNA after 20 h of cytokine treatment. Total RNA was isolated using RNA STAT60 according to manufacturer’s directions (Tel-Test, Friendswood, TX). Northern blot was performed as described previously (8).

**Protein Extraction**—Preparation of nuclear extracts was performed as described previously (18). Liver tissue or hepatocytes were lysed in a modified RIPA buffer (50 mM Tris HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na3VO4, 1 mM NaF, and 1× complete protease inhibitors (Roche Applied Science)) by Dounce homogenization on ice for 20 min. Lysate was centrifuged for 10 min to remove cellular debris.

**STAT3 EMSA**—Binding reactions were carried out in binding buffer containing 10 nM HEPES, pH 7.6, 50 mM KC1, 0.1 mM EDTA, 5 mM MgCl2, 10% glycerol, 5 mM dithiothreitol, and 2 μg of poly(dI-dC) (Amersham Biosciences) with 15 μg of nuclear extracts and 1×-l antibodies. After precubination on ice for 20 min, radiolabeled m875E oligonucleotide (5′-ATGCATTTCCTCGCTAAATCAT-3′) was added followed by a 20-min incubation at room temperature. Samples were run on a 5% acrylamide, 0.5× Tris borate-EDTA-denaturing gel at 4 °C. The following polyclonal antibodies were used: STAT3(C-20) from Santa Cruz Biotechnology, Inc. and STAT3(C-20X) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Western Analysis**—50 μg of nuclear extract or 100 μg of RIPA whole cell extract were electrophoresed on a 1× SDS-10% polyacrylamide gel, and Western immunoblotting was performed as described previously (18). Phospho-tyrosine 705 STAT3 antibodies (New England Biolabs, Beverly, MA) were used at a 1:1000 dilution. STAT3(C-20), 1,1000 dilution), gp130(M-20, Santa Cruz Biotechnology, Inc. (1:2500). Anti-mouse HRP secondary antibodies were from Jackson Immunoresearch (1:2500) (West Grove, PA). Anti-mouse STAT3 secondary antibodies were from Santa Cruz Biotechnology, Inc. (1:2500).

**Immunoprecipitation of Tyrosine-phosphorylated Proteins**—1 mg of RIPA liver extract was pre-cleared with 100 μl of BioMag protein A/G beads (Qiagen) rocking at 4 °C for 30 min. Tyrosine-phosphorylated proteins were immunoprecipitated using a mixture of several antibodies—PV20 (3 μg, BD Biosciences), PV20 (0.5 μg, BD Biosciences), and rabbit immunooaffinity-purified IgG anti-phosphotyrosine (3 μg, Upstate Biotechnology, Lake Placid, NY) for 2 h at rocking at 4 °C. 100 μl of BioMag protein A/G beads were added to precipitate the protein antibody complexes for 1 h. Immunocomplexes were isolated using magnetic separators (Qiagen), and the non-precipitated supernatant was saved for Western blot analysis. The immunocomplexes were washed six times with modified RIPA buffer and boiled in SDS loading buffer. Pre-cleared extracts, immunoprecipitated proteins, and the supernatant were electrophoresed on 1× SDS-10% polyacrylamide gels for Western blot analysis.

**Ribonuclease Protection Assay**—Ribonuclease protection assay was performed using the RiboQuant Multi-Probe Rnase protection assay system (BD Biosciences) according to manufacturer’s instructions using 10 μg of RNA. mcr-4 multi-probe template set (BD Biosciences) was used to examine IL-6Ra, gp130, and GAPDH RNA levels in primary hepatocytes. Quantitation was performed by phosphorimaging. Statistical significance was determined by paired Student’s t test.

**Pulse-Chase Stability Assay**—Pulse-labeled [35S]methionine was performed as below in pulse-chase experiments with the time points indicated for the pulse with a 1-h pretreatment of the cells with 200 μM chloroquine and 50 μM MG132. C/EBPs WT and KO hepatocytes were washed twice with methionine-cysteine-free minimum Eagle’s medium and starved for 20 min in methionine-cysteine-free minimum Eagle’s medium. At the cells were then pulsed with 70 μCi/ml of [35S]methionine and cysteine ([35S]methionine and cysteine (PerkinElmer Life Sciences) for 45 min. Cells were then washed three times with chase medium (M/M, 10% fetal calf serum, 15 μg/ml cold methionine, 31.3 μg/ml cold cysteine) and then incubated in 3 ml of chase medium for the time points. RIPA cell lysates were made as described earlier. The extract was pre-cleared with protein A/G, and 10 μg of protein A-G was added for 2 h to precipitate the protein antibody complex. Immunocomplexes were washed 4× with modified RIPA buffer,
boiled in SDS-loading buffer, and run on 8% SDS-polyacrylamide gels. Graph represents the averages of two individual experiments.

Cycloheximide Protein Stability Analysis—C/EBPα WT and KO hepatocytes were treated with 20 μg/ml cycloheximide for 0–4 h. RIPA extracts were made at 0-, 0.5-, 1-, 2-, and 4-h time points. IL-6Rα protein levels were analyzed by Western blot analysis and densitometry using a Molecular Dynamics personal densitometer, and ImageQuant software was performed to quantify protein levels normalized to β-actin loading. Graph represents the averages of three experiments. C/EBPα WT and KO hepatocytes were also pre-treated with 200 μM chloroquine or 50 μM MG132 for 2 h prior to cycloheximide treatment for 0 and 4 h.

Real-time RT-PCR—Primary hepatocyte RNA was isolated using the Qiagen RNeasy Mini-kit with the RNase-free DNase kit in accordance with manufacturer’s protocols. 2 μg of total RNA was used for reverse transcription using oligo(dT) and Superscript II RNase H reverse transcriptase (Invitrogen). The RT reactions were diluted to 1 ng/μl by spectrophotometer 280/260 reading, and 3 μl was used for real-time PCR. Real-time PCR was performed using the SYBR Green PCR Master mixture (Applied Biosystems, Foster City, CA) on an ABI Prism 7700 sequence detection system (Applied Biosystems). Primers used for SAA1 were: forward 5′-GGCGACCTGCGCATCAGGAGGGT3′ and reverse 5′-CCCTTTGAAAGCCTCGTGA-3′. β-actin-loading control primers were: forward 5′-CTGCTTGCCTGCTCCATGAGGTT3′ and reverse 5′-GGCTCAGGAGGACATTAGA-3′. Graphic calculations were performed using the comparative C_{T} method with data scaled to the control samples of each genotype.

RESULTS

Lack of IL-6-induced APP Gene Induction during the APR Is a Hepatocyte Autonomous Defect in C/EBPα Null Mice—Newborn C/EBPα null mice are unable to respond to the bacterial endotoxin LPS and do not induce APP genes, such as γ-fibrinogen and SAA1 (Fig. 1A). However, we have observed that IL-1, TNF-α, and IL-6 mRNA levels are induced in both C/EBPα KO and WT livers in response to LPS treatment (data not shown). Therefore, to determine whether the defect in APP gene induction was because of a systemic defect in the C/EBPα knockout mice or a defect in the hepatocytes themselves, primary hepatocytes derived from newborn C/EBPα knockout and wild type mice were cultured for 7–10 days and treated with recombinant cytokines IL-1 and IL-6 to simulate the acute phase response or directly with LPS. APP genes in WT primary hepatocytes were elevated in response to the recombinant cytokine mixture of IL-1 and IL-6 and LPS, whereas C/EBPα KO primary hepatocytes showed no increase in APP expression (Fig. 1, B and C). Basal levels of γ-fibrinogen are decreased in C/EBPα KO hepatocytes (Fig. 1B) but detectable in longer exposures (data not shown). WT primary hepatocytes showed increased expression (~2-fold) of γ-fibrinogen over basal levels and a large induction of SAA1 from the undetectable basal expression, whereas C/EBPα KO hepatocytes do not induce these genes. However, the SAA3 gene, which is predominantly IL-1-responsive (16), is induced in both WT and KO hepatocytes when treated with recombinant IL-1 and IL-6 mixture (Fig. 1B) and with IL-1 alone (data not shown), indicating that the IL-1 pathway is intact in hepatocytes lacking C/EBPα. The fact that the C/EBPα KO hepatocytes do not respond to the addition of recombinant IL-6 to elevate most APP genes indicates that the hepatocytes lack some component of this pro-inflammatory signaling pathway.

C/EBPα Null Primary Hepatocytes Are Unable to Activate STAT3 in Response to IL-6 Treatment—The IL-6 signaling pathway activates STAT3 DNA binding and tyrosine phosphorylation of STAT3. We then asked whether STAT3, the main transcription factor activated by IL-6 and involved in the induction of APP genes, can be activated by LPS or the IL-1/IL-6 cytokine mixture in C/EBPα knock-out mice. STAT3 is activated in liver in response to 4 h of LPS treatment in WT mice (Fig. 2A) as demonstrated by neutralization of most of the binding with STAT3 antibodies (S3). However, STAT3 DNA binding is not induced in C/EBPα knock-out liver in response to LPS treatment (Fig. 2A). In WT primary hepatocytes, STAT3 DNA binding is induced at both 1 and 4 h after IL-1/IL-6 treatment, whereas C/EBPα null primary hepatocytes induce very little or no STAT3 DNA binding at any time point after IL-1/IL-6 treatment (Fig. 2B). The addition of STAT3 antisera to the binding reaction causes neutralization of STAT3 DNA binding. The specificity of STAT3 binding to a STAT3 consensus-binding oligomer (m67SIE) is shown by competition with cold m67SIE competitor (CC). Western blot analysis of liver nuclear extracts using phospho-tyrosine 705-specific STAT3 antibodies show induction of STAT3 tyrosine phosphorylation in WT mice in response to LPS treatment, whereas C/EBPα KO mouse liver shows very little phosphorylated STAT3 induced by LPS (Fig. 2C). In addition, cultured WT hepatocytes induce STAT3 phosphorylation in response to the IL-1 and IL-6 mixture (Fig. 2D). However, C/EBPα KO primary hepatocytes showed no STAT3 tyrosine phosphorylation in response to the IL-1/IL-6 treatment (Fig. 2D). Because the IL-6-STAT3 pathway is the major inducer of the hepatic acute phase response, it is probable that the lack of APP gene induction in C/EBPα KO mice and hepatocytes is because of a defect in the IL-6-STAT3 signaling cascade.

JAK-associated Tyrosine Phosphorylation Is Reduced in C/EBPα Knock-out Mice—Because JAKs are the family of kinases responsible for the tyrosine phosphorylation of STAT3 in the IL-6 signaling pathway (13), we then asked whether JAK kinases were activated in response to LPS in C/EBPα KO mice. We analyzed the activation of JAK by examining the phosphorylation status of targets of JAK kinases, gp130 and SHP-2, and a member of the JAK family of kinases itself, Tyk2. Immunoprecipitation of proteins containing phosphorylated tyrosine residues and Western blot analysis of gp130, SHP-2, and Tyk2, downstream targets of IL-6 receptor signaling, demonstrated reduced phosphorylation of these proteins in C/EBPα KO liver compared with WT liver following LPS treatment (Fig. 3A). Equal levels of gp130 and SHP-2 in the WT and KO extracts are shown in Fig. 3B, whereas Tyk2 is not detectable at 0 h but induced in both WT and KO liver following LPS treatment. The lack of tyrosine phosphorylation of four substrates (STAT3, gp130, SHP-2, and Tyk2) of JAK kinases in C/EBPα KO liver indicates that the entire IL-6 pathway is not activated in response to recombinant IL-6 or LPS.
C/EBPa Regulates IL-6Ra Signaling

KO Hepatocytes  WT Hepatocytes
0h 1h 4h 0h 1h 4h
- S3 - S3 - S3 - S3 - S3 - S3
WB:gp130
WB:Snp2
Tyk2

Fig. 2. Reduced STAT3 DNA binding and STAT3 phosphorylation in C/EBPa KO livers and primary hepatocytes in response to LPS and IL-1/IL-6 treatments, respectively. EMSA of STAT3 DNA binding activity to a STAT consensus oligomer (M67-SIE) using C/EBPa WT and KO liver nuclear extracts (A) and C/EBPa WT and KO derived primary hepatocyte nuclear extracts (B) is shown. Precipitation with either no antiserum (−), antiserum to STAT3 (S3), or antiserum to STAT1 (S1) was used to identify proteins in the complexes. The addition of cold competitor (CC) oligomer is used to demonstrate the specificity of the binding. Western blot analysis of nuclear extracts from C/EBPa KO and WT mouse liver (C) and C/EBPa WT and KO primary hepatocytes (D) probed with antibodies to phospho-tyrosine 705 STAT3 and total STAT3 is shown. Representative blots for at least three WT and KO animals are shown.

C/EBPa KO Liver and Hepatocytes Have Decreased Levels of the IL-6Ra—Because C/EBPa KO hepatocytes do not respond to IL-6 by elevating APP gene expression, activating JAKs, or activating STAT3, expression levels of the IL-6 receptor signaling components were examined. Ribonuclease protection assay analysis of IL-6Ra in C/EBPa KO and WT mouse livers. The graphs in Fig. 4, A and B, show the phosphorimaging quantitation of the mRNA levels of IL-6Ra and gp130 as a ratio to a GAPDH-loading control. IL-6Ra mRNA levels are not statistically different (p = 0.40, 0.25, and 0.62, respectively, for 0, 1, and 4 h) between the genotypes in control or IL-1 and IL-6 cytokine-treated primary hepatocytes. C/EBPa KO and WT hepatocytes also express gp130 mRNA levels (Fig. 4B) that are not statistically significant different (p = 0.78, 0.49, and 0.19, respectively, for 0, 1, and 4 h).

The protein levels of IL-6Ra and gp130 were also analyzed by Western blot analysis. As shown in Fig. 4C, the protein levels of IL-6Ra are greatly reduced in C/EBPa KO primary hepatocytes. Densitometric quantitation of the ratio of IL-6Ra to β-actin protein levels is shown below the panel. This quantification shows IL-6Ra protein levels to be 4–5-fold lower in C/EBPa KO hepatocytes compared with WT hepatocytes, whereas gp130 protein levels are approximately equal between WT and KO primary hepatocytes with β-actin shown as a loading control. Additionally, we examined the protein levels of IL-6Rα in C/EBPa KO and WT mice in C/EBPa expressing tissues, liver, lung, and brown adipose tissue to determine whether C/EBPa regulates IL-6Rα in all of the C/EBPa-expressing tissues. The IL-6Rα protein levels are reduced in C/EBPa KO liver but are not different between other C/EBPa WT and KO tissues, liver, lung and brown fat (Fig. 4D). This indicates that decreased IL-6Rα protein levels in C/EBPa KO liver is a hepatocyte-specific defect. The discrepancy between IL-6Rα mRNA and protein levels in C/EBPa KO and WT primary hepatocytes suggests that the protein is differentially regulated between the genotypes post-transcriptionally, possibly at the level of protein stability.

Decreased Protein Stability of IL-6Rα in C/EBPa KO-derived Primary Hepatocytes—We asked whether the rate of translation of IL-6Rα was changed between C/EBPa KO and WT hepatocytes. Primary hepatocytes were metabolically labeled with [35S]methionine and cysteine in the presence of protease inhibitors, MG132 and chloroquine, over several time points. The rate of [35S] incorporation into IL-6Rα protein is not statistically different between C/EBPa WT and KO hepatocytes with p = 0.26 and p = 0.21 for the 30- and 60-min time points, respectively (Fig. 5A). We then asked whether decreased protein stability is responsible for the reduced protein levels in C/EBPa KO hepatocytes. To measure the half-life of IL-6Rα protein in primary hepatocytes, we performed a pulse-chase experiment. WT and C/EBPa KO hepatocytes were metabolically labeled with [35S]methionine and [35S]cysteine for 45 min and chased with excess unlabeled methionine and cysteine for the times indicated in Fig. 5, B and C. IL-6Rα was immunoprecipitated and resolved by SDS-PAGE. Pulse-chase analysis of IL-6Rα shows the half-life to be approximately two times longer in C/EBPa WT hepatocytes compared with KO hepatocytes. Confirming that C/EBPa WT hepatocytes have a longer IL-6Rα protein half-life, cycloheximide treatment of the hepatocytes was employed to block new protein synthesis. The rate

C/EBPa KO Hepatocytes  WT Hepatocytes
0h 1h 4h 0h 1h 4h
- S3 - S3 - S3 - S3 - S3 - S3
WB:gp130
WB:Snp2
Tyk2

Fig. 3. Decreased JAK-associated tyrosine phosphorylation of gp130 and Snp2 in C/EBPa KO mouse livers. A, RIP analysis was performed using antibodies to gp130 (IP), phospho-tyrosine (P-Y), and total (Sup) in the extracts. Representative blot for three experiments is shown.

KO Liver and Hepatocytes Have Decreased Levels of the IL-6Ra—Because C/EBPa KO hepatocytes do not respond to IL-6 by elevating APP gene expression, activating JAKs, or activating STAT3, expression levels of the IL-6 receptor signaling components were examined. Ribonuclease protection assay analysis of IL-6Ra in C/EBPa KO and WT mouse livers. The graphs in Fig. 4, A and B, show the phosphorimaging quantitation of the mRNA levels of IL-6Ra and gp130 as a ratio to a GAPDH-loading control. IL-6Ra mRNA levels are not statistically different (p = 0.40, 0.25, and 0.62, respectively, for 0, 1, and 4 h) between the genotypes in control or IL-1 and IL-6 cytokine-treated primary hepatocytes. C/EBPa KO and WT hepatocytes also express gp130 mRNA levels (Fig. 4B) that are not statistically significant different (p = 0.78, 0.49, and 0.19, respectively, for 0, 1, and 4 h).

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of protein degradation of IL-6Ra was determined over a 4-h treatment period. Basal levels of IL-6Ra protein are decreased in C/EBPα KO hepatocytes; therefore, two to three times more protein was loaded from the C/EBPα KO hepatocytes to detect IL-6Ra. IL-6Ra levels were quantitated by densitometry and graphed as a percentage of the starting levels to show the difference in protein degradation rates in four independent experiments (Fig. 5D). The half-life of IL-6Ra in C/EBPα WT hepatocytes is ~2-fold longer than in C/EBPα KO hepatocytes. A representative Western blot of IL-6Ra protein levels during the cycloheximide treatment is shown in Fig. 5E. Although it is noted that rates of degradation are slightly longer in the pulse-chase experiment compared with the cycloheximide experiment, degradation differences are probably due to differences in the two techniques because cycloheximide blocks all protein synthesis and can cause alterations in the hepatocytes. To determine the contribution of different degradation pathways on IL-6Ra stability, we pre-treated the hepatocytes with inhibitors of the main pathways of protein degradation, proteosomal and lysosomal. Both chloroquine, an inhibitor of the lysosomal degradation pathway, and MG132, a proteosomal inhibitor, partially stabilized IL-6Ra protein degradation during cycloheximide treatment in C/EBPα WT and KO hepatocytes (Fig. 5F). These data indicate that decreased IL-6Ra protein stability contributes to the decreased IL-6Ra protein seen in C/EBPα KO hepatocytes and that both the lysosomal and proteosomal pathways of protein degradation are involved in IL-6Ra protein degradation in hepatocytes.

Hyper-IL-6 or Oncostatin M Can Rescue STAT3 Activation and SAA1-synergistic Induction in C/EBPα Null Hepatocytes—Other gp130 signaling cytokines besides IL-6 have been reported to activate STAT3 DNA binding and induce APP gene expression (19). We then asked whether gp130 and the other components of the IL-6 pathway are functional in C/EBPα KO hepatocytes, excluding the IL-6Ra. We examined the response of C/EBPα KO and WT primary hepatocytes to Oncostatin M and Hyper-IL-6. OncM is an IL-6-like cytokine that signals through a dimer of OncMR and gp130 (20). Hyper-IL-6 (HYP) is a recombinant protein designed to have the soluble IL-6Ra peptide linked to the IL-6 cytokine, which signals through gp130 in a constitutive manner (17). WT primary hepatocytes respond to IL-6, HYP, and OncM by inducing STAT3 DNA binding (Fig. 6A). C/EBPα KO hepatocytes do not induce STAT3 DNA binding in response to IL-6 treatment as described earlier in this paper (Figs. 2B and 6A). However, C/EBPα KO hepatocytes induce STAT3 DNA binding in response to Hyper-IL-6 and Oncostatin M. Interestingly, hepatocytes of both genotypes induce stronger STAT3 DNA binding in cells treated with Oncostatin M than with IL-6 or Hyper-IL-6. This may reflect differences in the receptor affinities of OncMR and IL-6Ra for the gp130 signaling protein and their ligands.

SAA1 has previously been shown to be induced by IL-1 alone and have synergistic induction with IL-1 and IL-6 together, whereas IL-6 alone is not a very good inducer of SAA1 in hepatocytes (21). The cytokine induction of SAA1 mRNA is a useful response to measure because of its low basal level and very high transcriptional induction in response to cytokines. We examined SAA1 mRNA induction by real-time RT-PCR in WT and C/EBPα KO hepatocytes in response to several combinations of cytokines. WT hepatocytes responded, in agreement with previously published data (21), with very little induction of SAA1 in response to IL-6, ~20-fold induction with IL-1, and ~60-fold induction in cells treated with IL-1 and IL-6 together, displaying a synergistic induction of SAA1 mRNA to both cytokines (Fig. 6B). KO hepatocytes showed very little induction in response to IL-6 and showed a similar fold induction as WT cells in response to IL-1, ~20-fold (Fig. 6B). However, C/EBPα KO cells responded to IL-1 and IL-6 treatment with only the same level of induction as seen with IL-1 alone, indicating that the SAA1 gene was not synergistically induced in response to both cytokines. This finding confirms our previous data that the IL-6 pathway is not functional in C/EBPα KO primary hepatocytes. To determine whether other gp130 signaling cytokines were able to rescue the synergistic induction of SAA1 mRNA, we treated hepatocytes with Oncostatin M or Hyper-IL-6, either alone or with IL-1. Both WT and C/EBPα KO...
hepatocytes showed little induction in response to the gp130 signaling cytokines alone. However, both Oncostatin M and Hyper-IL-6 in conjunction with IL-1 synergistically induced SAA1 mRNA in both C/EBP\(\alpha\)/H\(_{9251}\)KO and WT hepatocytes (Fig. 6B). Hyper-IL-6 with IL-1 induced both WT and KO hepatocytes to similar levels induced by WT hepatocytes treated with IL-1/IL-6 (60-fold), whereas Oncostatin M in combination with IL-1 induced SAA1 to an even greater extent (100–150-fold) than IL-1/IL-6 or Hyper-IL-6 with IL-1. Additionally, KO hepatocytes responded more strongly to Oncostatin M and IL-1 than WT cells, which may indicate a difference in OncMRs between the genotypes. Together these data demonstrate that the extent of the defect in the IL-6 pathway is limited to the IL-6R because other gp130 signaling molecules can activate STAT3 DNA binding and synergistically induce SAA1 mRNA in C/EBP\(\alpha\) KO hepatocytes.

DISCUSSION

The acute phase response is a highly regulated response by the liver to inflammatory stimuli. Our laboratory previously reported that C/EBP\(\alpha\) null mice were unable to mount an APR in response to bacterial LPS (8). This study examines the mechanism of the defect observed in C/EBP\(\alpha\) knock-out mice with primary hepatocytes in culture during the APR. We demonstrate that C/EBP\(\alpha\) is required specifically in hepatocytes for proper elevation of acute phase protein gene expression in response to inflammatory stimuli such as LPS or recombinant IL-1 and IL-6 cytokines. Primary hepatocytes derived from C/EBP\(\alpha\) null livers are unable to activate the IL-6-JAK-STAT3 signaling pathway. We have shown that the lack of IL-6 signaling is due to decreased protein levels of IL-6R. The decreased IL-6R protein levels are due to decreased protein stability in C/EBP\(\alpha\) null hepatocytes. However, gp130 and the other components of the IL-6 pathway are intact in C/EBP\(\alpha\) null hepatocytes as demonstrated by the rescue of STAT3 activation and synergistic SAA1 gene induction by treatment with other gp130 signaling cytokines, such as recombinant Hyper-IL-6 or Oncostatin M in combination with IL-1. The data presented here show the importance of IL-6-STAT3 signaling in the acute phase response.

Fig. 5. IL-6R\(\alpha\) has a shorter protein half-life in C/EBP\(\alpha\) KO hepatocytes compared with WT hepatocytes. A, representative blot of immunoprecipitated IL-6R\(\alpha\) from C/EBP\(\alpha\) KO and WT hepatocytes metabolically labeled with \[^{35}\text{S}\] methionine and cysteine over 60 min in the presence of protease inhibitors, chloroquine, and MG132. B and C, C/EBP\(\alpha\) WT and KO hepatocytes were pulse-labeled with \[^{35}\text{S}\] methionine and cysteine and chased with excess cold methionine and cysteine. IL-6R\(\alpha\) was immunoprecipitated and quantitated by densitometry. B, graphic representation of two experiments with labeled IL-6R\(\alpha\) levels is represented as a percentage of the 0-h time point. C, representative blot of IL-6R\(\alpha\) during the pulse-chase experiment. D and E, C/EBP\(\alpha\) WT and KO hepatocytes were treated with 20 \(\mu\)g/ml cycloheximide (CHX) for 0–4 h. IL-6R\(\alpha\) protein levels were analyzed by Western blot analysis, and densitometry was performed to quantitate protein levels. D, a graphic representation of four experiments. IL-6R\(\alpha\) protein levels were normalized to \(\beta\)-actin for loading and were represented as a percentage of the 0-h time point. E, representative Western blot of IL-6R\(\alpha\) protein levels during cycloheximide experiment for WT and C/EBP\(\alpha\) KO hepatocytes with \(\beta\)-actin shown as loading control. Ratio of IL-6R\(\alpha\) to \(\beta\)-actin is shown below panel. F, hepatocytes were treated with cycloheximide alone, pretreated for 2 h with 200 \(\mu\)M chloroquine, and then with cycloheximide for 4 h or pretreated for 2 h with 200 \(\mu\)M chloroquine, and then with cycloheximide for 4 h. IL-6R\(\alpha\) protein levels were evaluated by Western blot with \(\beta\)-actin shown as loading control. Ratio of IL-6R\(\alpha\) to \(\beta\)-actin is shown below panel.
a localized inflammatory response, whereas LPS induces a systemic response, which may use a different pathway and elevate other gp130 signaling cytokines such as Oncoatin M or IL-11. Additionally, conditional inactivation of STAT3 in adult mouse liver has conclusively shown STAT3 to be a critical transcription factor required for APP gene induction in response to LPS treatment (12).

IL-6Ra is required for IL-6 cytokine binding leukemia inhibitory factor leading to increased affinity for the gp130 signaling subunits of the receptor complex. C/EBPα has been shown to be involved in granulocyte differentiation, and C/EBPα KO mice do not make fully differentiated neutrophils (29). Zhang et al. (34) have shown that mRNA and functional protein levels of the IL-6Ra are highly reduced in day 19 embryo liver and cultured hematopoietic cells from C/EBPα KO mice. Our data show that in addition to the previously observed reduction of IL-6Ra levels in hematopoietic cells from C/EBPα KO mice, C/EBPα KO hepatocytes also have decreased IL-6Ra levels. However, the mechanism of IL-6Ra regulation by C/EBPα appears to be different between hematopoietic cells at the mRNA level and hepatocytes at the protein level.

We have observed 4-fold lower steady-state levels of IL-6Ra protein in C/EBPα null hepatocytes compared with WT hepatocytes. Cycloheximide and pulse-chase studies showed the half-life of IL-6Ra protein in WT hepatocytes to be approximately twice as long as in the KO hepatocytes. Previous studies have shown the protein half-life of IL-6Ra to be 2–3 h in several types of cell lines, similar to our results (25). Protein steady-state levels are determined by a variety of factors including mRNA levels, the rate of translation, the rate of degradation, and the developmental stage at which IL-6Ra begins expression. In our study, we found larger differences in steady-state levels of IL-6Ra than differences in protein half-life. We ruled out differences in mRNA levels and protein translation as being the cause of the difference in steady-state IL-6Ra protein levels. In addition, we found the rate of IL-6Ra protein degradation to be significantly different between WT and C/EBPα KO hepatocytes, contributing to the differences in steady-state IL-6Ra protein levels.

Interestingly, the 4-fold lower levels of IL-6Ra in C/EBPα KO hepatocytes results in almost no IL-6-STAT3 signaling. This finding suggests that the IL-6Ra levels are below a critical threshold required for IL-6Ra-gp130 signaling. The fact that C/EBPα is a transcription factor localized to the nucleus suggests that C/EBPα does not directly regulate the degradation of IL-6Ra protein at the cell membrane. The indirect regulation of IL-6Ra protein by C/EBPα in liver is further substantiated by the fact that other C/EBPα-expressing tissues such as brown fat and lung have normal levels of IL-6Ra. It is probable that C/EBPα transcriptionally regulates a gene whose protein product influences the degradation of IL-6Ra specifically in hepatocytes.

Down-regulation of IL-6Ra by internalization and degradation has been studied in response to IL-6 ligand stimulus; however, little is known regarding the basal regulation of IL-6Ra protein. Upon IL-6 binding, IL-6Ra is rapidly endocytosed, resulting in complete removal of IL-6 binding sites from the cell surface by 30–60 min (26, 27). The ligand-induced endocytosis of IL-6/IL-6Ra has been shown to be mediated by gp130 through a dileucine motif in the cytoplasmic domain of gp130 (28). Additionally, a soluble form of IL-6Ra can be generated by proteolytic cleavage by a metalloproteinase or by alternative RNA splicing (29, 30). However, generation of soluble IL-6Ra does not inhibit IL-6 signaling but rather increases the plasma half-life of IL-6 and activates cells expressing gp130 that might normally not be responsive to IL-6 (31). It is not clear in
C/EBPα KO hepatocytes whether IL-6Rα is being constitutively endocytosed from the cell membrane or degraded in the cytoplasm by an alternative mechanism. It is unlikely that soluble IL-6Rα is being generated in C/EBPα KO hepatocytes because the hepatocytes would still respond to IL-6 cytokine. Further studies of IL-6Rα protein regulation in C/EBPα KO hepatocytes will help elucidate basal mechanisms of IL-6Rα protein turnover.

In this study, we have shown that reduced IL-6Rα levels are responsible for the lack of IL-6 signaling in C/EBPα KO hepatocytes by rescuing IL-6-like signaling with other gp130 signaling cytokines. Hyper-IL-6 is a recombinant molecule engineered to take advantage of the functional design of the IL-6 receptor, which does not require a cytoplasmic domain to signal in cells. Hyper-IL-6 contains the soluble IL-6 receptor peptide and induces SAA1 mRNA. This may be related to gp130 levels because Oncostatin M is a stronger activator of STAT3 and SAA1 than IL-6 or Hyper-IL-6. This may be related to gp130 levels because Oncostatin M and Hyper-IL-6 are capable of stimulating APP gene expression in newborn hepatocytes. Premature infants have been observed to have neonatal hypoglycemia and abnormal lipid metabolism similar to the phenotype of C/EBPα KO knock-out mice (33). Therefore, it has been proposed that C/EBPα null mice may serve as a good model system for better understanding liver complications and the inflammatory responses of premature infants.

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Stephanie L. Mackey and Gretchen J. Darlington

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