Interferon β (IFN-β) Production during the Double-stranded RNA (dsRNA) Response in Hepatocytes Involves Coordinated and Feedforward Signaling through Toll-like Receptor 3 (TLR3), RNA-dependent Protein Kinase (PKR), Inducible Nitric Oxide Synthase (iNOS), and Src Protein

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The sensing of double-stranded RNA (dsRNA) in the liver is important for antiviral defenses but can also contribute to sterile inflammation during liver injury. Hepatocytes are often the target of viral infection and are easily injured by inflammatory insults. Here we sought to establish the pathways involved in the production of type I interferons (IFN-I) in response to extracellular poly(I:C), a dsRNA mimic, in hepatocytes. This was of interest because hepatocytes are long-lived and, unlike most immune cells that readily die after activation with dsRNA, are not viewed as cells with robust antimicrobial capacity. We found that poly(I:C) leads to rapid up-regulation of inducible nitric oxide synthase (iNOS), double-stranded RNA-dependent protein kinase (PKR), and Src. The production of IFN-β was dependent on iNOS, PKR, and Src and partially dependent on TLR3/Trif. iNOS and Src up-regulation was partially dependent on TLR3/Trif but entirely dependent on PKR. The phosphorylation of TLR3 on tyrosine 759 was shown to increase in parallel to TLR3/Trif but entirely dependent on PKR. The phosphorylation that is dependent on the up-regulation of iNOS, which activates Src (7). The double-stranded RNA-dependent protein kinase (PKR) is also activated by dsRNA, leading to NF-κB activation (1–5). Currently, there are three classes of pattern recognition receptors involved in the recognition of pathogen-associated molecular pattern and damage-associated molecular pattern molecules, including toll-like receptors (TLRs), RIG-I-like receptors, and NOD-like receptors (2). Among these receptors, TLRs and RIG-I-like receptors are important for the production of type I interferons (IFN-I) (2, 6). IFN-I includes the IFN-α family and IFN-β, which exert a vast spectrum of biological functions. One of the well characterized functions is inhibition of the replication of viruses (7), but IFN-I can also contribute to the pathogenesis of sterile liver injury (8).

Toll-like receptors recognize pathogen-associated molecular patterns and damage-associated molecular patterns and elicit proinflammatory signals, which lead to the activation of immune responses (9). For example, TLR3 recognizes double-stranded RNA (dsRNA) and its analog poly(I:C) and induces the expression of antiviral genes and production of NO via the inducible NO synthase (iNOS, NO2) (9–11). Phosphorylation of five tyrosine residues on TLR3 at Tyr733, Tyr756, Tyr759, Tyr764, and Tyr758 has been shown to participate in dsRNA-induced gene expression (12, 13). Recently, the importance of the tyrosine kinases Bruton’s tyrosine kinase (14) and Src (15) has been implicated in dsRNA-mediated TLR3 Tyr759 phosphorylation in different types of cells. In macrophages, dsRNA induces TLR3 Tyr759 phosphorylation to promote IFN-β secretion that is dependent on the up-regulation of iNOS, which activates Src (7). The double-stranded RNA-dependent protein kinase (PKR) is also activated by dsRNA, leading to NFκB activation (16). Upon dsRNA binding to the TLR3 receptor, PKR is recruited by a TAK1-containing complex in transfected 293 cell lines to engage TLR3 signaling (16).

The innate immune system responds rapidly to molecular patterns from invading micro-organisms or damaged tissue through pattern recognition receptors expressed by both immune cells and non-immune cell types (1–5). Currently, there are three classes of pattern recognition receptors involved in the recognition of pathogen-associated molecular pattern and damage-associated molecular pattern molecules, including toll-like receptors (TLRs), RIG-I-like receptors, and NOD-like receptors (2). Among these receptors, TLRs and RIG-I-like receptors are important for the production of type I interferons (IFN-I) (2, 6). IFN-I includes the IFN-α family and IFN-β, which exert a vast spectrum of biological functions. One of the well characterized functions is inhibition of the replication of viruses (7), but IFN-I can also contribute to the pathogenesis of sterile liver injury (8).

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Hepatocytes express TLR3 and rapidly up-regulate iNOS under a number of conditions, including TLR stimulation (5, 17–21). These cells are the target of several viruses (22, 23), and we recently provided evidence for a robust response to exogenous and endogenous dsRNA in hepatocytes for IFN- I production (6, 24). Here we sought to define the relationship between the iNOS/cGMP/PKG, Src/TLR3/Trif, and PKR pathways in the hepatocyte response to dsRNA. We show that IFN-β production in response to poly(I:C) is entirely iNOS-dependent and that NO exerts a feedforward up-regulation on iNOS expression in a cGMP/PKG-dependent manner. iNOS/NO promotes TLR3 Tyr759 phosphorylation through an Src-dependent mechanism associated with a direct interaction of Src with TLR3 localized to the endosomal compartment. Both iNOS and Src are markedly up-regulated in a PKR-dependent mechanism that itself is dramatically induced in response to poly(I:C). TLR3 signaling through Trif synergistically interacts with PKR to up-regulate iNOS expression upon dsRNA stimulation. These findings demonstrate a robust production of IFN-I in hepatocytes exposed to dsRNA through synergistic signaling pathways dependent on iNOS/NO.

**Experimental Procedures**

**Reagents and Antibodies**—Low molecular weight poly(I:C) (poly(I:C)LMW, 0.2–1 kb), high molecular weight poly(I:C) (poly(I:C)HMW, 1.5–8 kb), and ultrapure LPS (Escherichia coli 0111: B4) were purchased from InvivoGen (San Diego, CA). Williams’ medium E, penicillin, streptomycin, L-glutamine, and HEPES were purchased from Invitrogen. Insulin (Humulin) was acquired from Eli Lilly and Co. (Indianapolis, IN), and fetal calf serum was purchased from Hyclone Laboratories (Logan, UT). Protein A/G plus agarose beads (sc-2003), Src kinase inhibitor (sc-204303), Src siRNA, PKR siRNA, PKGI siRNA, and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The selective imidazolo-oxindole PKR inhibitor C16 (19785) was from Sigma-Aldrich (St. Louis, MO). The transfection reagent GeneJammer was from Agilent Technologies (Santa Clara, CA).

The following specific primary antibodies were used: iNOS (sc-651, NBPI–61319, and 610333), IRF-1 (sc-13041), IRF-3 (sc-9082), IRF-7 (sc-9083), PKR (sc-6282), TLR4 (sc-16240), FAK (sc-558), FAK-Tyr(P)861 (sc-16663), and proliferating cell nuclear antigen (sc-56) from Santa Cruz Biotechnology; TRIF (sc-651, NBP1–62139, and 610333), IRF-1 (sc-13041), IRF-3 (sc-9082), and PKR (sc-6282) from Cell Signaling Technology (Lake Placid, NY); and Src (LS-C96491) from LifeSpan Biotechnologies (Santa Clara, CA).

Animals—C57BL/6 WT 8- to 12-week-old mice were purchased from Charles River Laboratories (Wilmington, MA). TLR4 knockout (TLR4−/−), TLR4-KO, TRIF knockout (TRIF−/−, TRIF-KO), iNOS knockout (iNOS−/−, iNOS-KO), and PKR knockout (PKR−/−, PKR-KO) mice were bred in our facility. C57BL/6J TLR3 knockout (TLR3−/−), TLR3-KO, and wild-type mice of the same age and sex were from The Jackson Laboratory (Bar Harbor, ME). All experimental procedures involving animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Hepatocyte Culture and Poly(I:C) Treatment**—Hepatocytes were isolated from mice as described previously (6, 19, 24). The purity exceeded 99% as measured by flow cytometric assay, and viability was typically measured over 85% using trypan blue exclusion. Hepatocytes (1.5 × 10⁵ cells/ml) were plated on gelatin-coated culture plates or seeded onto coverslips precoated with collagen I (BD Biosciences) in Williams’ medium E with 10% calf serum, 15 mM HEPES, 1 x 10⁻⁶ M insulin, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Hepatocytes were allowed to attach to plates overnight. Prior to treatments, cell culture medium was changed to medium containing 5% calf serum. After washing with PBS, hepatocytes were treated with 20 μg/ml poly(I:C) for stimulation for various durations. Culture medium and cell lysates were collected for further analysis.

**RNA Interference**—The Src siRNA and PKR siRNA were transiently transfected into hepatocytes using GeneJammer transfection reagent according to the instructions of the manufacturer’s instructions. Twenty-four hours later, hepatocytes were transfected with 20 μg/ml poly(I:C) for various durations. Culture medium and cell lysates collected at different time points were subject to Western blotting analysis.

For hepatocytes transfected with PKGIβ siRNA, cells were treated with cytokines after washing with PBS and replenishment with complete William’s E medium. Cells were harvested for detection of iNOS and NF-κB activity.

**Inhibitor Treatment**—Hepatocytes were pretreated with 50 nm Src kinase inhibitor or 250 nm imidazolo-oxindole PKR inhibitor C16 for 2 h after PBS washing. Media containing inhibitor and 20 μg/ml poly(I:C) were replenished after removing the media. Culture medium and cell lysates collected at various durations were subject to further analysis.

**Virus Infection**—Adenoviral vectors carrying bacterial β-galactosidase (Ad-LacZ) and iNOS (Ad-iNOS) were prepared as described previously (25). After overnight culture, hepatocytes were washed twice with PBS prior to infection at a multiplicity of infection of 3 in a volume of 1.5 ml of Opti-MEM (Life Technologies) using 6-well plates. Following a 2-h infection, the normal culture medium was changed for an incubation of 24 h. Cells were treated as designated in the figure legends. Samples were prepared at the indicated time points.

**Preparation of Total Lysates and Nuclear Extracts**—Hepatocytes were washed twice in PBS and harvested with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice for 10 min. The supernatants were collected as whole cell extracts after centrifugation at 12,000 g for 30 min. The lysates from culture media were concentrated by adding less lysis buffer after centrifugation to remove the medium. The nuclear extracts were prepared as described previously (26, 27). The protein concentrations were determined using the BCA protein assay kit (Pierce).

**Measurement of Nitrite Production**—The levels of nitrite (NO₂⁻) in culture supernatants and cell lysates were assessed to...
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determine the amount of NO production according to the instructions of the Griess reagent system (G2930, Promega).

**IFN-β ELISA**—IFN-β levels in culture supernatants and cell lysates were detected using the IFN-β ELISA kit (R&D Systems) according to the instructions of the manufacturer.

**Western Blotting and Co-immunoprecipitation Analysis**—Equal amounts of protein lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes, followed by incubation with optimized dilutions of primary antibodies at 4 °C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Proteins were detected with the ECL kit (Pierce). β-Actin, β-tubulin (Sigma), or proliferating cell nuclear antigen was used as a loading control.

For co-immunoprecipitation, equal amounts of whole cell lysates were incubated with equal amounts of primary antibody rotating at 4 °C overnight, and immune complexes were then precipitated with protein A/G-agarose beads after rotating for 2 h at 4 °C. Electrophoresis loading buffer was added to the beads after washing with wash buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1× protein inhibitor mixture) five times. After denaturing at 95 °C for 5 min, the supernatants were subjected to Western blotting.

**Immunofluorescent Staining**—Hepatocytes grown on cover-slips were briefly washed in PBS and fixed in 2% paraformaldehyde at room temperature for 15 min and stained as described previously (28). Hepatocytes were incubated with 2% BSA in PBS for 1 h, followed by five washes with PBS + 0.5% BSA (PBB). The samples were then incubated with antibodies diluted in PBB against iNOS, Src, and Tyr(P)759-TLR3 and organelle-specific markers, including Rab5 (an early endosome marker) and Rab7 (a late endosome marker) as described above. Samples were washed five times with PBB, followed by incubation in the appropriate Alexa Fluor 488 (1:500, Invitrogen) and Cy3 (1:1000, Jackson ImmunoResearch Laboratories) secondary antibodies diluted in PBB. Samples were washed three times with PBB, followed by a single wash with PBS before 30 s of incubation with Hoechst nuclear stain. The nuclear stain was removed, and samples were washed with PBS before covering the coverslip with Gelvatol (23 g of poly(vinyl alcohol 2000), 50 ml of glycerol, and 0.1% sodium azide to 100 ml of PBS). Positively stained cells in six random fields were imaged on a Fluoview 1000 confocal scanning microscope (Olympus, Center Valley, PA). Imaging conditions were maintained at identical settings within each antibody labeling experiment, with original gating performed using the negative control.

**RT-PCR**—Total RNAs were extracted from hepatocytes using the RNeasy extraction kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. The quality of the RNA was assessed by 1% denaturing agarose gel electrophoresis and spectrophotometry. One microgram of total RNAs of each sample was reverse-transcribed to the first strand of cDNA with oligo(dT)12-18 primers by the Omniscript™ reverse transcriptase kit (Qiagen). Real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was performed using forward and reverse primer pairs prevalidated and specific for iNOS, β-actin, and PKG (Qiagen). All samples were run in triplicates. The expression of the housekeeping gene β-actin was used as an internal control.

**EMSA**—For cell fractionation procedures, protease inhibitors (0.2 mg/ml Pefabloc, 0.01 mg/ml aprotinin, 0.01 mg/ml pepstatin, and 0.01 mg/ml leupeptin) were supplemented to all buffers. Briefly, cells were rinsed twice with ice-cold PBS and harvested in buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, and 0.5% Nonidet P-40) and then incubated on ice for 10 min. Nuclei were pelleted at 5000 × g for 5 min at 4 °C. The pellets were resuspended in buffer A and washed twice. The pellets after the final wash were collected as nuclear extract and lysed in C+D buffer (20 mM HEPES, 1.5 mM MgCl₂, 0.45 mM KCl, 0.02 mM EDTA, and 25% Glycerol) for 1 h on ice. The supernatants were collected at 15,000 × g for 15 min and classified as the nuclear fraction. Equal amounts of nuclear extracts were incubated with 32P end-labeled, 45-mer, double-stranded NF-κB oligonucleotide for 30 min at room temperature. The DNA-protein complexes were separated from free oligonucleotide on 6.5% native polyacrylamide gels. Radioactivity in the gel was exposed to x-ray film (Eastman Kodak Co., Rochester, NY).

**Luciferase Activity Assay**—The mouse iNOS promoter-reporter plasmid (mouesiNOS (1.7) Luc) and the mouse iNOS promoter NF-κB specific mutant site-reporter plasmid (mouesiNOS-NF-κB Luc), which harbors 1.7 kb of upstream 5’-flanking DNA linked to the luciferase reporter gene, were constructed. Cells were transfected in Opti-MEM (Life Technologies) containing 1 µg of DNA and 10 µl of liposomes for 6 h. After washing with PBS, the cells were replenished with complete William’s E medium. To control the transfection efficiency between groups, 0.2 µg of Renilla vector-pGL4.75 (hRluc/CMV) (Promega) was added to each well. Cells were treated with SNAP (100 µM), 8-pCPTcGMP (800 µM), and SNAP + 8-pCPTcGMP for 8 h. After treatment, cells were harvested and lysed in passive lysis buffer (Promega). Luciferase activity was detected with 20 µl of lysate in a Synergy Mx multimode reader (Biotek, Winooski, VT) using the Dual-Luciferase® reporter assay kit (Promega). Luciferase activity was normalized to Renilla activity.

**Statistical Analysis**—Each experiment was performed at least three times. The results were analyzed using analysis of variance in SigmaStat (Systat Software, San Jose, CA). Unless indicated, the results from a representative triplicate experiment are presented. Data are shown as mean ± S.D. p < 0.05 was considered statistically significant.

**Results**

**Poly(I:C) Stimulates TLR3-Tyr759 Phosphorylation and IFN-β Production and Up-regulates Expression of Src and PKR in Hepatocytes**—It has been shown previously in macrophages that the iNOS/Src axis is involved in dsRNA-induced TLR3-Tyr759 phosphorylation and IFN-I up-regulation (7, 29). Hepatocytes also up-regulate IFNs when infected by a number of RNA and DNA viruses and also avidly express iNOS (30–32). Here we show that hepatocytes exposed to high (Fig. 1A) or low (supplemental Fig. S1) molecular weight preparations of the dsRNA mimic poly(I:C) exhibited a time-dependent increase in phosphorylation of TLR3-Tyr759 (Fig. 1A). TLR3-Tyr(P)759 first
appeared at 2 h and continued to increase to the 24-h time point. In parallel, iNOS expression and NO production (measured as nitrite accumulation in the medium) increased (Fig. 1, A and B). The enhanced expression of iNOS and TLR3-Tyr(P)759 was also associated with increased PKR and Src expression as well as the appearance of the known Src phosphorylation target FAK-Tyr(P)861 (Fig. 1A). Furthermore, the levels of transcription factors known to be downstream of TLR3 signaling and involved in IFN-β production, including IRF3 and IRF7, increased in both the whole cell lysates and nuclear extracts in poly(I:C)-treated cells, whereas IRF1 levels remained unchanged (Fig. 1C). Both intracellular and medium levels of IFN-β increased in association with the up-regulation of the iNOS/Src/PKR/TLR3-Tyr(P)759 parameters (Fig. 1, C

FIGURE 1. Poly(I:C)HMW induces a time-dependent increase of iNOS, iNOS activity, TLR3-Tyr(P)759, PKR, Src, Src activity, IRF3 and 7 expression, and nuclear accumulation as well as IFN-β production in cells and supernatant levels. A, mouse hepatocytes (WTMH) were stimulated with poly(I:C)HMW for various durations (0, 0.17, 0.5, 1, 2, 6, 12, and 24 h). Whole cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr(P)759, TLR3, iNOS, PKR, Src, FAK-Tyr(P)861, and FAK. β-Tubulin was used as a loading control. B, mouse hepatocytes were stimulated with poly(I:C)HMW for 24 h. The nitrite concentrations in culture media and whole cell lysates were determined by Griess assay. Data are presented as individual data points and mean. *, p < 0.05. C, mouse hepatocytes were treated with poly(I:C)HMW for various durations (0, 0.17, 0.5, 1, 2, 6, 12, and 24 h). Whole cell lysates, nuclear extracts, and culture media were separated by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr(P)759, TLR3, iNOS, PKR, Src, FAK-Tyr(P)861, and FAK. β-Tubulin was used as a loading control. D, mouse hepatocytes were exposed to poly(I:C)HMW for 24 h. IFN-β levels in culture media and whole cell lysates were examined by ELISA. Values came from at least three independent experiments and are presented as individual data points and mean. *, p < 0.05. E, human hepatocytes (HHC) were treated with poly(I:C)HMW for various durations (0, 1, 2, 6, 12, and 24 h). Whole cell lysates and culture media were resolved by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr(P)759, TLR3, iNOS, PKR, Src, and IFN-β. β-Tubulin or β-actin was used as a loading control. F, HepG2 cells were stimulated with poly(I:C)HMW for various durations (0, 1, 2, 6, 12, and 24 h). Whole cell lysates and culture media were fractionated by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr(P)759, TLR3, iNOS, Src, and IFN-β. β-Tubulin or β-actin was used as a loading control.
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and D). The up-regulation of TLR3-Tyr(P)759, iNOS, Src, and IFN-β was observed in primary human hepatocytes (Fig. 1E) and a human hepatoma cell line (HepG2) following poly(I:C) treatment (Fig. 1F), indicating the conservation of the dsRNA response pathway in human liver cells.

Involvement of TLR3/iNOS/NO/Src in Poly(I:C)-induced IFN-β Production in Hepatocytes—Endosomal sensing of dsRNA occurs through TLR3 in immune cells (33). To determine whether hepatocytes also utilize TLR3 to react to poly(I:C) applied to the outside of the cell, hepatocytes isolated from wild-type or TLR3-/- mice were exposed to poly(I:C) for various durations (0, 0.5, 1, 2, 6, 12, and 24 h). Whole cell lysates and culture media were separated by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr(P)759, PKR, Src, and IFN-β. C, mouse hepatocytes were stimulated with poly(I:C)HMW for 24 h. IFN-β levels in culture media and whole cell lysates were examined by ELISA. Data are presented as individual data points and mean. *, p < 0.05.
FIGURE 4. Phosphorylation of TLR3 Tyr759 depends on iNOS, whereas NO amplifies iNOS expression and induces a feedforward mechanism through cGMP. A, iNOS KO mouse hepatocytes (MHC) were stimulated with poly(I:C)HMW for various durations (0, 0.5, 1, 2, 6, 12, and 24 h). Whole cell lysates and culture media were resolved by SDS-PAGE and immunoblotted with antibodies against iNOS, TLR3-Tyr(P)759, TLR3, PKR, Src, and IFN-β. β-Tubulin or β-actin was used as a loading control. B, mouse hepatocytes were stimulated with poly(I:C)HMW for 24 h. IFN-β levels in whole cell lysates were examined by ELISA. Data are presented as individual data points and mean. *, p < 0.05. C, iNOS KO mouse hepatocytes were infected with Ad-LacZ or Ad-iNOS. After 2 h of infection, normal culture medium with SNAP was added back for 24-h recovery. Poly(I:C)HMW and SNAP were added for 12-h treatment after two PBS washes. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against iNOS, TLR3-Tyr(P)759, and TLR3. β-Tubulin was used as a loading control. D, iNOS KO mouse hepatocytes were infected with Ad-LacZ or Ad-iNOS. After 2 h of infection, normal culture medium with L-N5-(1-iminoethyl)ornithine (L-NIO) was added back for 24-h recovery. Poly(I:C) with L-N5-(1-iminoethyl)ornithine was added for 12-h treatment after two PBS washes. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with specific antibodies that recognize iNOS, TLR3-Tyr(P)759, and TLR3. β-Tubulin was used as a loading control. E, mouse hepatocytes were stimulated with poly(I:C)HMW for 24 h. The nitrite in whole cell lysates was determined by Griess assay. Data are presented as individual data points and mean. *, p < 0.05. F, mouse hepatocytes were stimulated with poly(I:C)HMW for 24 h. The IFN-β levels in whole cell lysates were determined by ELISA. Data are presented as individual data points and mean. *, p < 0.05. G, wild-type mouse hepatocytes (WTMH) were treated with the iNOS inhibitor L-N5-(1-iminoethyl)ornithine for 1, 6, or 12 h. *, p < 0.05. β-Tubulin was used as a loading control. H, wild-type mouse hepatocytes were treated with the cGMP inhibitor ODQ for 1, 6, or 12 h. Whole cell lysates were resolved by SDS-PAGE and immunoblotted by antibodies against iNOS, TLR3-Tyr(P)759, and TLR3. β-Tubulin was used as a loading control. I, lysates from whole liver and isolated mouse hepatocytes were immunoblotted with an antibody against the β1 subunit of soluble guanylyl cyclase.

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We next confirmed that Src expression was required for TLR3 phosphorylation in response to poly(I:C). Both Src knockdown (Fig. 3A) and Src inhibition (Fig. 3B) blocked the formation of TLR3-Tyr(P)759 in response to poly(I:C) without altering total TLR3 levels. Src inhibition also blocked iNOS up-regulation and the up-regulation of IFN-β expression in hepatocytes. To seek evidence that Src had a direct interaction with TLR3-Tyr(P)759, immunoprecipitation experiments were performed (Fig. 3C). Immunoprecipitation of Src effectively pulled down TLR3, but only in the absence of Src inhibition or suppression. Immunoprecipitation of TLR3 led to the pulldown of Src, and this association increased over time in poly(I:C)-treated cells (Fig. 3D). Thus, Src is required for enhanced iNOS expression and TLR3-Tyr759 phosphorylation as well as IFN-β up-regulation in hepatocytes exposed to dsRNA.

To establish the importance of iNOS to this signaling cascade, iNOS−/− hepatocytes or wild-type hepatocytes treated with an NO synthase inhibitor (L-N5-(1-iminoethyl)ornithine) were exposed to poly(I:C). Deletion of iNOS completely prevented TLR3-Tyr(P)759 phosphorylation and the up-regulation of IFN-β (Fig. 4, A and B) but only partially suppressed PKR and Src up-regulation following poly(I:C) treatment. Overexpression of iNOS using an adenoviral vector (AdiNOS, Fig. 4, C–E) increased iNOS expression and NO production but enhanced TLR3-Tyr(P)759 phosphorylation and IFN-β formation only when the cells were also exposed to poly(I:C). The effect of AdiNOS was blocked by inhibiting iNOS activity with the NO synthase inhibitor. An NO donor (SNAP) also overcame the impact of iNOS deletion on poly(I:C)-induced IFN-β production (Fig. 4E). Somewhat unexpected was the observation that iNOS expression was enhanced by NO donor exposure and blocked by NOS inhibitors (Fig. 4, C, D, and F). Hepatocytes up-regulate cGMP levels in response to iNOS or NO (63). We confirmed that hepatocytes express the 1 subunit of soluble guanylyl cyclase (Fig. 4I). The iNOS/NO-dependent increase in iNOS expression appeared to be at least partially cGMP-dependent because the soluble guanylate cyclase inhibitor ODQ also suppressed poly(I:C)-induced iNOS expression (Fig. 4G). This potential feedforward system for iNOS up-regulation is further addressed below.

**Src Co-localizes with TLR3-Tyr(P)759 in Endosomes following Poly(I:C) Stimulation in Hepatocytes**—Our findings show that Src interacts directly with TLR3 following poly(I:C) stimulation (Fig. 5, A and B). TLR3 localizes to endosomes in unstimulated immune cells, such as dendritic cells (33), and translocates to dsRNA-containing endosomes following the uptake of dsRNA into cells (33). Confocal imaging following immunofluorescence staining was carried out to determine where Src and TLR3-Tyr(P)759 interact. As shown in Fig. 5, A and B, Src co-localized with TLR3-Tyr(P)759 in both early (Rab5-expressing) and late (Rab7-expressing) endosomes at 12 and 24 h following poly(I:C) exposure. Although it is not clear whether phosphorylation takes place in the endosome, the appearance of Src with its phosphorylation target suggests that this could be the case.

**FIGURE 5.** Phosphorylated Tyr759-TLR3 associates with Src on endosomes. Wild-type mouse hepatocytes were stimulated with poly(I:C)HMW for 0, 12, and 24 h and fixed for immunofluorescent staining. A, antibodies against Src, iNOS, and TLR3-Tyr(P)759 proteins. Red, Src; green, Rab5; blue, TLR3-Tyr(P)759; white, DAPI. B, red, Src; green, Rab7; blue, TLR3-Tyr(P)759; white, DAPI.
dsRNA Stimulation Leads to IFN-β Expression through Both Trif and PKR in Hepatocytes—TLR3 is known to signal through the adaptor protein Trif (34–37). Downstream signaling can also include the up-regulation of the serine and threonine kinase PKR via IFN (38–41). However, PKR can also respond directly to dsRNA for NFκB activation (40). We next assessed the role of Trif in hepatocytes exposed to poly(I:C). As shown in Fig. 6, A–C, hepatocytes from Trif−/− mice had lower iNOS expression and less TLR3-Tyr759 phosphorylation compared with wild-type hepatocytes following poly(I:C) treatment. Src up-regulation was only slightly lower in Trif−/− hepatocytes relative to wild-type cells exposed to poly(I:C). Nitrite and IFN-β accumulation in cells was significantly suppressed but not completely inhibited in Trif−/− hepatocytes. Interestingly,
PKR deletion or suppression by siRNA almost completely blocked poly(I:C)-induced iNOS expression, Src up-regulation, and IFN-β expression in hepatocytes (Fig. 6, D and E). These data, combined with data from Fig. 2, suggest that PKR acts synergistically with TLR3 to up-regulate iNOS expression and in parallel with TLR3 to regulate Src expression in response to poly(I:C).

**LPS Also Induces IFN-β Production through an Src-dependent Manner in Hepatocytes**—LPS is a potent inducer of iNOS expression in mouse hepatocytes (19, 20) and is known to signal through Trif via TLR4 (7, 10). To determine whether the signaling events observed with poly(I:C) also occurred following LPS stimulation, we exposed wild-type mouse hepatocytes to LPS (100 ng/ml LPS, 20 μg/ml poly(I:C)HMW, or a combination of LPS and poly(I:C)HMW for various durations (0, 1, and 12 h). Whole cell lysates, and culture media were resolved by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr759, TLR3, iNOS, Src, PKR, and IFN-β. β-Tubulin or β-actin was used as a loading control. B, IFN-β levels in whole cell lysates from A were determined by ELISA. Data are presented as individual data points and mean. *, p < 0.05. C, wild-type mouse hepatocytes were stimulated with 100 ng/ml LPS or 20 μg/ml poly(I:C)HMW following transfection with Src siRNA for 24 h or treatment with Src inhibitor for 2 h. Whole cell lysates, nuclear extracts, and culture media were resolved by SDS-PAGE and immunoblotted with antibodies against TLR3-Tyr(P)759, TLR3, Src, IRF3, IRF7, and IFN-β. β-Tubulin, proliferating cell nuclear antigen (PCNA), or β-actin were used as loading controls, respectively. D, IFN-β levels in whole cell lysates from B were determined by ELISA. Data are presented as individual data points and mean. *, p < 0.05.

The iNOS/PKR/Src/TLR3 Axis in TLR-mediated IFN-β Production
As shown in Fig. 8A, SNAP, 8-pCPTcGMP, or IL-1β alone induced iNOS expression. SNAP- and IL-1β-induced iNOS expression was suppressed by the soluble guanylate cyclase inhibitor ODQ whereas 8-pCPTcGMP-induced expression was not. Both SNAP- and 8-pCPTcGMP-induced time- and concentration-dependent increases in iNOS expression (Fig. 8, B–D). The PKG inhibitor KT5823 suppressed both the SNAP- and 8-pCPTcGMP induction of iNOS (Fig. 8E), indicating the stimulation of iNOS involved the cGMP target PKG.

At baseline, hepatocytes expressed the PKG subunits PKGI, PKGII, and PKGIII (Fig. 8F). Remarkably, NO exposure further up-regulated PKGI within 4 h, and the suppression of PKGI expression using siRNA prevented the up-regulation of iNOS induced by SNAP or 8-pCPTcGMP (Fig. 8, F and G). The tran-
scriptional up-regulation of iNOS is known to involve NF-κB (43). To determine whether SNAP or cGMP could up-regulate NF-κB activation in hepatocytes, EMSA was performed. Both SNAP (100 μM) and 8-pCPTcGMP increased NF-κB activation in hepatocytes (Fig. 9, A and C). This could be suppressed by ODQ or KT5823 (Fig. 9, B and D) or by PKGIβ siRNA treatment (Fig. 9D). To implicate regulation at the transcriptional level, hepatocytes were transiently transfected with a 1.7-kb mouse iNOS promoter-luciferase construct or a construct expressing the iNOS promoter with mutated NF-κB binding sites (mutant-pro). As shown in Fig. 9E, both SNAP and 8-pCPTcGMP induced increased luciferase expression in cells transfected with the wild type promoter construct but not the mutant construct. Together, our findings indicate that NO/cGMP/PKG/NF-κB signaling is involved in a feedforward mechanism to promote iNOS expression in hepatocytes.

**Discussion**

Hepatocytes respond to both endogenous and exogenous sources of dsRNA with a robust IFN-I response (44, 45). We have shown previously that this response is restrained in hepatocytes by a rapid up-regulation of adenosine deaminase acting on RNA 1 (ADAR1) (24). Here we show that iNOS/NO are required for IFN-β production in response to poly(I:C) in both murine and human hepatocytes. iNOS is rapidly up-regulated in response to poly(I:C) through a pathway that involves the synergistic actions of TLR3/Trif, Src, and PKR. iNOS/NO promoted Src-dependent phosphorylation of TLR3 at Tyr759, and Src was required for both IFN-β and iNOS expression. Src up-regulation was, in turn, entirely dependent on PKR and partially dependent on TLR3/Trif and iNOS/NO. We also identified a robust feedforward mechanism where NO promotes iNOS expression through a cGMP/PKG-dependent pathway that further enhances NO and IFN-β production. These data provide evidence that iNOS/NO are an integral component of innate immune signaling pathways leading to IFN-I production in hepatocytes.

Both microbial and self-dsRNA can be sensed by receptors of the innate immune system (1, 46). These include TLR3 in the endosomal compartment (33, 38) and PKR as well as Rig-I like receptors, including DExDH box helicases (Rig-I and MDA 5) (16, 41), in the cytosol. Signaling triggered by the interaction of dsRNA with TLR3 or PKR activates the transcriptional expression of a number of genes through NF-κB and the IFN regulatory factor family of transcription factors (IRFs). Among the genes induced by dsRNA are type I IFNs, including IFN-α cytokines, and IFN-β, which sensitizes cells for the detection of invading pathogens, inhibits protein synthesis, and suppresses viral rep-
 Activation of TLR3 leads to signaling through TRIF/TRAF6 and TBK1/IκB-kinase ε for the up-regulation of IRF3 and 7 as well as NFκB (47). PKR has been shown to participate in TLR3 signaling through association with TBK1 (1) and can also activate NFκB following binding to dsRNA (48).

In hepatocytes, TLR3 plays a central role during hepatitis B virus and hepatitis C virus infection (49). Studies in primary human hepatocytes establish that TLR3 triggers antiviral responses (50). Results from experiments using hepatoma cell lines show that PKR activation restrains HCV1a replication through the regulation of NFκB expression (51). TLR3−/− mice have been used to implicate dsRNA responses in the immunopathology associated with concanavalin A-induced hepatitis (53). PKR has been shown to participate in TLR3 signaling through association with TBK1 (1) and can also activate NFκB following binding to dsRNA (48).

In this study, we extend these observations to show that the response to exogenous dsRNA for the production of IFN-β in murine and human hepatocytes requires iNOS/NO, TLR3/TRIF, PKR, and Src. The role of Src-TLR3 interactions as well as iNOS/NO/cGMP in promoting TLR3 phosphorylation has been shown in macrophages (7, 10). Like macrophages, we show that Src-TLR3 interactions in hepatocytes take place in the endosomal compartment and lead to an iNOS/NO-dependent phosphorylation of TLR3 at Tyr759. We show that PKR is expressed at baseline in hepatocytes and that its expression is strongly induced by dsRNA and LPS. Increases in Src and iNOS/NO as well as TLR3 Tyr759 phosphorylation and IFN-β production in response to poly(I:C) were entirely dependent on PKR expression but only partially dependent on TLR3. This suggests that PKR plays roles in promoting TLR3 signaling but also roles independent of TLR3 in the dsRNA response in hepatocytes. A similar Src-dependent up-regulation of TLR3 Tyr759 phosphorylation, IRF3 and 7 levels, and IFN-β production was seen after LPS treatment. Cooperativity between TLR3 and TLR4 has been shown in macrophages (7, 57) and may also take place in hepatocytes. The overlap in the responses to poly(I:C) and LPS is most likely explained by the involvement of Trif in TLR3 and TLR4 signaling shared in common downstream of these receptors.

Hepatocytes are capable of rapid and sustained up-regulation of iNOS across a range of species, including humans and rodents (21, 58, 59). iNOS/NO leads to cGMP-dependent and

FIGURE 10. Schematic of the proposed model for production of IFN-β in response to extracellular dsRNA. In this figure, we incorporate our new findings into a diagram that depicts known pathways for the sensing of extracellular dsRNA. Uptake of dsRNA engages signaling through specific dsDNA receptors, including TLR3, PKR, and RIG-I (1). In this study, we show that iNOS, PKR, Src, and PKGI are all up-regulated at the protein level early after dsRNA addition. We have shown previously that ADAR1 is also rapidly up-regulated and suppresses dsDNA sensing through the RIG-I pathway (24). We now show that TLR3/Trif and PKR act together to up-regulate iNOS and Src to promote TLR3-Tyr(P)759 and IFN-β production. PKR plays a dominant role in the up-regulation of both iNOS and Src, and we propose both TLR3-dependent and -independent roles for PKR based on our findings. iNOS expression is promoted by a feedforward mechanism that depends on cGMP/PKG.
-independent actions in hepatocytes, such as regulation of cell death pathways (60, 61) and activation of TNFα-converting enzyme (TACE/ADAM17) (19, 20). In this study, we show that iNOS/NO is also involved in the production of IFN-β by hepatocytes in response to TLR ligands. We also found that inhibition of iNOS activity or the soluble guanylate cyclase suppressed iNOS expression in response to dsRNA (Fig. 4). This led us to further characterize the roles of NO and cGMP in promoting iNOS expression in hepatocytes as a feedforward mechanism to amplify NO production. NO and cGMP lead to a PKG-dependent up-regulation of iNOS in hepatocytes. Hepatocytes were found to express all three PKG subunits at baseline; however, only the expression of PKGIβ was induced by NO exposure. NO/cGMP-induced increases in iNOS expression were dependent on PKGIβ expression. Transcription of the iNOS gene is NFκB-dependent (62). It is likely that one of the mechanisms for enhancement of iNOS expression by NO/cGMP/PKG is through amplification of iNOS transcription. NO and cGMP induced a sustained increased in NFκB activation and also increased iNOS promoter activation. How the cGMP/PKG pathway promotes NFκB activation is unknown. It is reasonable to speculate that a PKG phosphorylation target regulates NFκB signaling. A number of posttranscriptional pathways are also known to be involved in the regulation of iNOS, and a role for these in iNOS/NO induction of iNOS cannot be excluded.

In summary, this series of experiments provides evidence that iNOS/NO can be rapidly up-regulated by dsRNA through TLR3/PKR/Src-dependent mechanisms and is amplified further through an iNOS/NO/cGMP/PKG feedforward mechanism. Importantly, we establish that iNOS expression is required for IFN-β production in hepatocytes following stimulation with dsRNA. A proposed model for dsRNA sensing leading to IFN-β production by hepatocytes is shown in Fig. 10, which takes into account these new observations as well as recently published findings on the role of ADAR1 in suppressing dsRNA sensing through RIG-I (6, 24). Although gaps in our knowledge of the interactions between these pathways persist, our findings provide insight into the intricate signaling and regulatory pathways controlling the sensing of dsRNA in hepatocytes and thus have important implications for understanding the mechanisms of antiviral immune responses and sterile inflammation in the liver.

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References


The iNOS/PKR/Src/TLR3 Axis in TLR-mediated IFN-β Production


