Early growth response-1 contributes to galactosamine/lipopolysaccharide-induced acute liver injury in mice

Michele T. Pritchard,1 Sanjoy Roychowdhury,1 Megan R. McMullen,1 Luping Guo,3 Gavin E. Arteel,3 and Laura E. Nagy1,2

1Department of Pathobiology and 2Department of Gastroenterology, Cleveland Clinic, Cleveland, Ohio; and 3Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Louisville, Kentucky

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Pritchard MT, Roychowdhury S, McMullen MR, Guo L, Arteel GE, Nagy LE. Early growth response-1 contributes to galactosamine/lipopolysaccharide-induced acute liver injury in mice. Am J Physiol Gastrointest Liver Physiol 293: G1124–G1133, 2007. First published October 4, 2007; doi:10.1152/ajpgi.00325.2007.—Early growth response (Egr)-1 is a transcription factor that regulates genes involved in inflammation, innate and adaptive immunity, coagulation, and wound healing; however, little is known about the role of Egr-1 in acute liver injury. We tested the hypothesis that Egr-1 is involved in acute liver injury induced by galactosamine/lipopolysaccharide (GalN/LPS). GalN/LPS exposure biphasically increased hepatic egr-1 mRNA accumulation at 1 h and again at 4–5.5 h after treatment in wild-type mice. Within 4–5.5 h after GalN/LPS exposure, wild-type mice exhibited histological evidence of hepatic injury, cell death, and extensive areas of hemorrhage, as well as increased plasma alanine aminotransferase activities. In contrast, these parameters were largely attenuated in egr-1−/− mice. The initial expression of tumor necrosis factor-α, macrophage inflammatory protein-2, monocyte chemoattractant protein-1, and intercellular adhesion molecule-1 mRNA or protein was equivalent between genotypes at 1 h after GalN/LPS administration. However, at subsequent time points, hepatic expression of these genes was decreased in egr-1−/− compared with wild-type mice. In addition, neutrophil extravasation from hepatic sinusoids into the liver parenchyma was decreased in egr-1−/− compared with wild-type mice 4 h after GalN/LPS. Whereas caspase-3 activation and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive nuclei were detected in wild-type mice at 4 and 5.5 h after GalN/LPS administration, respectively, these markers of apoptosis were delayed in egr-1−/− mice. Delayed development of apoptosis was associated with an extension of survival by 1 h in egr-1−/− compared with wild-type mice. These data demonstrate that Egr-1 plays an important role in acceleration of hepatic inflammation, apoptosis, and subsequent mortality in GalN/LPS-induced acute liver injury.

inflammation; neutrophils; apoptosis

ACUTE HEPATIC FAILURE, a major contributor to disease-related mortality, often occurs as a consequence of inflammatory disorders such as nonalcoholic and alcoholic hepatitis, sepsis, and drug-induced liver injury (4). The aberrant inflammatory response that characterizes hepatitis is often initiated by lipopolysaccharide (LPS) (18). LPS then triggers increased production of proinflammatory mediators, as well as increased production of chemokines and cell adhesion molecules, leading to the recruitment of several lineages of white blood cells, including neutrophils, to the liver (18). If uncontrolled, these proinflammatory responses contribute to liver injury and acute hepatic failure (5, 24, 38). The commonly used galactosamine (GalN)/LPS model of acute hepatic failure recapitulates the uncontrolled inflammatory responses and recruitment of mononuclear cells to the liver observed in patients with acute hepatic failure (4). GalN is an amino sugar that compromises hepatic function; GalN metabolism by hepatocytes depletes intracellular dUTP, increasing the sensitivity of the hepatocyte to LPS (41). The combined exposure to GalN/LPS results in a combination of tumor necrosis factor (TNF)-α-induced and neutrophil-mediated hepatocyte apoptosis and necrosis (22), characteristic of acute hepatic failure in patients (4).

Early growth response (Egr)-1 (also known as NGFI-A, Zif-268, Krox-24, and TIS8), a zinc finger-containing transcription factor (15), is an immediate early gene. Egr-1 is rapidly and transiently expressed in response to a wide range of stimuli, including growth factors (15), LPS (8), shear stress in blood vessels, hypoxia, and reactive oxygen species (40), and plays an important role in regulation of genes involved in inflammation, innate and adaptive immunity, coagulation, and the wound-healing response (6, 31, 37, 46). Egr-1 directly and indirectly regulates the expression of many genes, including tissue factor, TNF-α, macrophage inflammatory protein (MIP)-2, monocyte chemotactic protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, and platelet-derived growth factor (31, 45). Egr-1 has been characterized as a “master regulator” (45) and is implicated in the progression of a number of diseases involving increased inflammatory responses, including lung ischemia-reperfusion injury, atherosclerosis, and pancreatitis (37). In the liver, Egr-1 is critical for the development of chronic ethanol-induced steatosis (32). However, it is not known whether Egr-1 contributes to hepatic injury during acute inflammation and/or hepatitis. In this study, making use of egr-1−/− mice, we tested the hypothesis that Egr-1 contributes to acute hepatic inflammation and injury in response to GalN/LPS administration.

MATERIALS AND METHODS

Materials. GalN (lot no. 045K0965) and LPS (Escherichia coli serotype 026:B6, lot no. 064K4077) were purchased from Sigma-Aldrich (St. Louis, MO). All primers for real-time reverse transcription PCR were synthesized by Integrated DNA Technologies (Coralville, IA). Antibody pairs were purchased for ELISA detection of TNF-α from BioLegend (San Diego, CA), for MIP-2 (CytoSet) from Biosource (Camarillo, CA), and for MCP-1 (Ready-SET-gol) from eBioscience (San Diego, CA). The active caspase-3 antibody, specific for the p17 fragment of active caspase-3, was purchased from Abcam.

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Address for reprint requests and other correspondence: M. T. Pritchard, Dept. of Pathobiology, Cleveland Clinic, 9500 Euclid Ave. NE40, Cleveland, OH 44195 (e-mail: pritchm@ccf.org).
Liver and reverse-transcribed as previously described (32). Real-time PCR was performed on total plasma protein.

TNF-α/H9251 using the Diagnostic Chemicals enzymatic assay kit (Oxford, CT). Samples were assayed for alanine aminotransferase (ALT) activity.

Animals. Female wild-type (C57BL/6NTac) or egr-1−/− (B6.129-Egr1tm12m N12) mice (8–12 wk) were purchased from Taconic Farms (Germantown, NY). Egr-1−/− mice were backcrossed to C57BL/6NTac for derivation by embryo transfer after 12 generations and maintained by mating homozygous males and heterozygous females. Animals were housed in standard microisoflators and fed standard laboratory chow (rodent diet no. 2918; Harlan-Teklad, Madison, WI). All animal procedures were approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

GalN/LPS administration and sample collection. Mice were weighed and injected with 700 mg/kg GalN and 20 μg/kg LPS by intraperitoneal injections on opposite sides of the abdominal cavity. At select time points after GalN/LPS treatment, mice were anesthetized and euthanized by exsanguination. Livers were removed and weighed, and portions were fixed in formalin, frozen in Optimal Cutting Temperature medium (Sakura Finetek USA, Torrance, CA), and snap-frozen in liquid nitrogen or stored in RNAlater (Ambion, Austin, TX) for future analysis.

Liver histology and terminal deoxynucleotidyl transferase-mediated dUTP nick-end label staining. For histological analysis, formalin-fixed tissue slides were paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin. Slides were coded before examination and viewed by two separate individuals. Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using the ApopTag Plus fluorescence in situ apoptosis detection kit (Chemicon International). Percent TUNEL-positive nuclei of total nuclei was determined 5.5 h after GalN/LPS administration and sample collection.

Liver homogenates, cytokine ELISAs, and Western blot analyses. Liver homogenates were prepared and protein concentrations determined for both ELISA and Western blotting as previously described (36). Twenty to thirty micrograms of protein were used to measure hepatic TNF-α, MIP-2, and MCP-1 by ELISA. Results were normalized to those of total protein concentration. Fifty micrograms of protein were resolved on 15% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were probed with antibodies specific for active caspase-3 and Hsc70, as a loading control.

ICAM-1 immunolocalization. Formalin-fixed paraffin-embedded liver sections (5 μm) were deparaffinized and hydrated consecutively in 100% (twice), 70%, and 30% ethanol, followed by two washes in PBS. Sections were then blocked with PBS containing 2% BSA, 1% fish gelatin, and 0.1% Triton X-100 for 1 h and incubated overnight with polyclonal goat anti mouse ICAM-1 IgG (1:250) at 4°C. Sections were washed in PBS and incubated with Alexa Fluor 594-labeled rabbit anti-goat IgG (1:250) for 2 h at room temperature. Sections were washed in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). No immunostaining was seen in sections incubated with PBS instead of primary antibody.

Neutrophil localization and quantification. Neutrophil accumulation was assessed by localizing chloracetate esterase (CAE), a specific marker for neutrophils, in liver tissue by using the naphthol AS-D chloracetate esterase kit (Sigma) (16) in combination with neutrophil nuclear morphology. CAE staining was carried out on coded formalin-fixed paraffin-embedded liver sections. Assessment of CAE-positive neutrophils in the liver parenchyma and hepatic sinusoids was counted separately in the coded liver sections using the ImagePro Plus image analysis program. CAE-positive cells in the lumen of hepatic sinusoids, and not in direct contact with hepatocytes, were scored as “sinusoidal.” CAE-positive cells in direct contact with hepatocytes, and not in the lumen of hepatic sinusoids, were scored as “parenchymal.” The latter group represents the neutrophils identified as “extravasated.”

Modified survival study. Wild-type and egr-1−/− mice received GalN/LPS and were monitored for signs of a moribund phenotype (e.g., prone posture, shallow and/or weak breathing, and lethargy). Mice were euthanized when they exhibited these signs, and the time to reach the moribund phenotype was recorded as the end point in this study.

Table 1. Real-time PCR primer sequences for genes of interest (GOI)

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<th>GOI</th>
<th>GenBank Accession No.</th>
<th>Primer Bank Accession No.</th>
<th>Primer Sequences</th>
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<td>F: 5'-AGG CAA GAA CCC CAG GAC-3'</td>
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<tr>
<td>MCP-1/CD2</td>
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<td></td>
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<td>ICAM-1/CD54</td>
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<td></td>
<td>R: 5'-AGG TCC AGG TGG CCA TGG GGG-3'</td>
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GOI, genes of interest; Primer Bank, http://pga.mgh.harvard.edu/primerbank/ (see Ref. 44a); na, not applicable; bp, base pairs.

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Statistical analysis. Values are means ± SE. Because of the limited number of age-matched egr-1-/- mice, data were collected from several different experiments. The data were analyzed using a general linear models procedure (SAS, Carey, IN), followed by least square means analysis of differences between groups, blocking for experiment effects when data from more than one experiment were used in any given data set. Data were log-transformed to obtain a normal distribution, if necessary. Student’s t-test was used to analyze data in the neutrophil enumeration study. Kaplan-Meier analysis was used to analyze the results of the survival study.

RESULTS

Egr-1 expression was induced by GalN/LPS administration. Since Egr-1 is considered a master regulator of gene expression in response to proinflammatory signals and cell stress (37), we hypothesized that GalN/LPS administration would increase Egr-1 expression in wild-type mice. Using rtPCR, we found that GalN/LPS rapidly and transiently increased hepatic egr-1 mRNA accumulation twofold over basal at 1 h and returned to basal level by 3 h (Fig. 1). A second, more robust increase in hepatic egr-1 mRNA accumulation began 4 h after GalN/LPS administration, reaching fivefold over baseline egr-1 levels at 5.5 h (Fig. 1).

GalN/LPS-induced liver injury was attenuated in egr-1-/- mice. Liver histology was normal at baseline (basal) (Fig. 2 A) and 1 h (data not shown) after GalN/LPS administration in wild-type and egr-1-/- mice. Four hours after GalN/LPS administration in the wild-type mice, evidence of leukocyte infiltration and cell death, characterized by eosinophilic “blebs” and changes in hepatocyte nuclear morphology that were suggestive of apoptosis and/or oncotic necrosis (39), was observed. These effects were attenuated in the egr-1-/- mice (Fig. 2A). Hepatic architecture in wild-type mice was disrupted with the appearance of extensive areas of hemorrhage and coagulative necrosis 5.5 h after GalN/LPS administration.

Fig. 1. Early growth response (egr)-1 mRNA accumulation in response to galactosamine/lipopoly saccharide (GalN/LPS) administration in wild-type (C57BL/6NTac) mice. Real-time PCR was performed to determine the hepatic accumulation of egr-1 mRNA after GalN/LPS administration. Egr-1 expression was normalized to β-actin, and fold changes in expression were calculated using the $2^{-\Delta\Delta C_{T}}$ method. Data are means ± SE; n = 4–9 mice per experimental condition. a,b,c $P < 0.05$, values with different superscripts are significantly different from each other.

Fig. 2. Liver injury in wild-type and egr-1-/- mice after GalN/LPS administration. A: livers were harvested over time after GalN/LPS administration and stained with hematoxylin and eosin. At 4 h, insets illustrate the infiltration of leukocytes and evidence of hepatocyte injury and/or death. At 5.5 h, hemorrhage was widespread in livers from wild-type mice but rare in egr-1-/- mice. Images are representative of 6–9 mice per experimental condition. Images were taken at ×100 magnification, except for insets (×200). B: plasma alanine aminotransferase (ALT) levels were measured over time after GalN/LPS administration. Data are means ± SE; n = 6–9 mice per experimental condition. a,b,c,d $P < 0.05$, values with different superscripts are significantly different from each other.
However, little hemorrhage and no evidence of necrosis were observed in livers from *egr-1* / mice at this time.

In wild-type and *egr-1* / mice, plasma ALT activities were increased over basal level 4 h after GalN/LPS administration (Fig. 2B). This GalN/LPS-induced increase in ALT was 30% lower in *egr-1* / compared with wild-type mice at 4 h, but this trend did not reach statistical significance (*P* = 0.06). ALTs increased further 5.5 h after GalN/LPS administration in both strains, but this increase was significantly blunted in *egr-1* / compared with wild-type mice.

Egr-1 contributed to TNF-α expression after GalN/LPS administration. Increased TNF-α, produced primarily by Kupffer cells, the liver-resident macrophages, is critical to liver injury in response to GalN/LPS (13). Since Egr-1 is an important contributor to LPS-induced TNF-α expression (47), we hypothesized that *egr-1* / mice would produce less TNF-α in re-

Figure 3. GalN/LPS-induced inflammatory mediator expression in wild-type and *egr-1* / mice. Plasma TNF-α protein was determined using ELISA (A). Hepatic tumor necrosis factor-α (TNF-α; B), macrophage inflammatory protein-2 (MIP-2; D), and monocyte chemoattractant protein-1 (MCP-1; F) mRNA accumulations were determined using real-time reverse transcriptase PCR. Hepatic TNF-α (C), MIP-2 (E), and MCP-1 (G) protein concentrations were determined using ELISA. Data are means ± SE; *n* = 5–9 mice per experimental condition. a,b,c,d,e,f *P* < 0.05, values with different superscripts are significantly different from each other.
response to GalN/LPS in comparison with wild-type mice. Plasma TNF-α peaked at 1 h, then decreased at 4 h, and was modestly increased again 5.5 h after GalN/LPS administration in wild-type mice (Fig. 3A). In egr-1−/− mice, plasma TNF-α also peaked at 1 h and was not different from that in wild-type mice. In contrast, 4 and 5.5 h after GalN/LPS administration, plasma TNF-α in egr-1−/− mice was 65 and 75% less than wild-type mice, respectively. Hepatic TNF-α protein paralleled that of plasma TNF-α protein and peaked 1 h after GalN/LPS administration in both wild-type and egr-1−/− mice, with no difference between genotypes (Fig. 3C). Whereas GalN/LPS-induced TNF-α protein declined in both strains 4 and 5.5 h after GalN/LPS administration, TNF-α was lower in egr-1−/− mice at both these time points in comparison with wild-type mice. In egr-1−/− mice, hepatic TNF-α protein returned to baseline by 5.5 h but remained elevated in wild-type mice.

In contrast to differences in both plasma and hepatic TNF-α protein expression in wild-type and egr-1−/− mice, there were no differences in hepatic TNF-α mRNA accumulation at any time point between the genotypes. Hepatic TNF-α mRNA increased 100-fold over basal level 1 h after GalN/LPS administration in both genotypes (Fig. 3B) and then declined similarly in both genotypes at 4 and 5.5 h posttreatment. Collectively, these data suggest that the contribution of Egr-1 to TNF-α expression, a tightly regulated process at multiple levels, is likely complex.

GalN/LPS-induced hepatic MIP-2 and MCP-1 expression was attenuated in egr-1−/− mice. MIP-2 (or IL-8 in humans) and MCP-1 are two potent chemokines that contribute to liver injury in both humans and animal models (1, 12, 37). Since expression of MIP-2 and MCP-1 is regulated by Egr-1, we hypothesized that GalN/LPS-induced expression of these chemokines would be attenuated in egr-1−/− mice. Hepatic MIP-2 mRNA accumulation 1 h after GalN/LPS administration was robust in both wild-type and egr-1−/− mice (Fig. 3D). In wild-type mice, MIP-2 mRNA levels decreased at 4 h but increased again at 5.5 h. In contrast, in egr-1−/− mice, MIP-2 mRNA accumulation was decreased at 4 h and remained low 5.5 h after GalN/LPS administration, and MIP-2 mRNA was less than in wild-type mice at both time points. Hepatic MIP-2 protein concentration was greatest in wild-type and egr-1−/− mice 1 h after GalN/LPS administration and was not different between the two strains (Fig. 3E). In wild-type mice, hepatic MIP-2 protein was decreased 4 and 5.5 h after GalN/LPS administration but was maintained above basal levels. However, in egr-1−/− mice, MIP-2 protein was lower in egr-1−/− compared with wild-type mice, returning to basal levels at 4 and 5.5 h.

MCP-1 mRNA and peptide accumulation was delayed relative to MIP-2; expression was not induced until 4 and 5.5 h (Fig. 3, F and G). At both 4 and 5.5 h, hepatic MCP-1 mRNA and protein were less in egr-1−/− compared with wild-type mice.

ICAM-1 expression and neutrophil accumulation in hepatic parenchyma were decreased in livers from egr-1−/− mice after GalN/LPS administration. Expression of ICAM-1 is important for the pathophysiology of acute liver injury (11, 18). Because the ICAM-1 promoter contains a functional Egr-1 DNA-binding domain (30), we hypothesized that ICAM-1 expression after GalN/LPS administration would be reduced in egr-1−/− mice. Indeed, in egr-1−/− mice, ICAM-1 mRNA accumulation was different from that in wild-type mice at 4 and 5.5 h after GalN/LPS administration but not at 1 h (Fig. 4A). These data parallel ICAM-1 protein expression by immunostaining. Low baseline expression of ICAM-1 in livers of both wild-type and egr-1−/− mice was localized to central veins and the pericentral region of the liver (Fig. 4B). In wild-type mice, robust ICAM-1 staining was observed in the pericentral and periportal regions of the liver with central veins, portal tracts, and sinusoids brightly stained at 4 h after GalN/LPS administration. In contrast, ICAM-1 immunostaining was less pronounced in egr-1−/− mice (Fig. 4B).

In acute liver injury, ICAM-1 contributes to neutrophil extravasation from hepatic sinusoids into the liver parenchyma (23). Since GalN/LPS-induced ICAM-1 expression was blunted in egr-1−/− mice (Fig. 4), we hypothesized that fewer neutrophils would extravasate from the sinusoids into the liver parenchyma in egr-1−/− mice. The total number of neutrophils localized to the liver and the percentage of those cells that extravasated from the sinusoids into the parenchyma of the liver were determined 4 h after GalN/LPS administration. Although the total number of neutrophils recruited to the liver was equiva-

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Fig. 4. GalN/LPS-induced intercellular adhesion molecule-1 (ICAM-1) expression is reduced in egr-1−/− mice. A: hepatic ICAM-1 mRNA accumulation was determined using real-time PCR. *P < 0.05, values with different superscripts are significantly different from one another. B: ICAM-1 protein localization was determined using immunofluorescence. Images are representative of 2–4 separate, nonoverlapping images from 3 mice per experimental condition.
lent between genotypes (Fig. 5I), there was a 50% decrease in the percentage of neutrophils in the liver parenchyma of egr-1−/− mice compared with wild-type mice (Fig. 5J).

Reduced apoptosis and improved survival were observed in egr-1−/− mice after GalN/LPS administration. Apoptotic hepatocyte cell death is a hallmark of acute liver injury in humans (25, 29) and of GalN/LPS-induced liver toxicity in mice and is dependent on TNF-α signaling through TNF receptor 1 (27, 28). Both caspase-3 activation and TUNEL staining are routinely used to define the induction and progression of apoptosis in both animal models and cell culture systems. Whereas no caspase-3 activation was observed at baseline in wild-type or egr-1−/− mice, caspase-3 activation was observed 4 h after GalN/LPS administration in wild-type but not in egr-1−/− mice (Fig. 6A). However, activation of caspase-3 in egr-1−/− mice did occur 5.5 h after GalN/LPS administration (Fig. 6A). TUNEL staining, a marker for late apoptosis, was undetectable at baseline in wild-type and egr-1−/− mice (Fig. 6B). At 5.5 h after GalN/LPS administration, apoptosis was evident in livers from wild-type mice but not from egr-1−/− mice; 10% of hepatocyte nuclei in livers from wild-type mice were TUNEL positive compared with only 3% of hepatocyte nuclei in egr-1−/− mice (Fig. 6, B and C).

Since multiple mediators and/or markers of hepatic injury were attenuated in egr-1−/− mice after GalN/LPS exposure, we hypothesized that survival would be improved in these animals despite the pathological severity of this model. With the use of a modified survival study, survival was modestly improved in egr-1−/− mice; mean survival time was extended to 417 min after GalN/LPS administration in egr-1−/− mice compared with 358 min in wild-type mice (59-min difference, P = 0.02) (Fig. 7A). Despite improved survival in egr-1−/− mice, liver histology at the time of death in the modified survival study showed extensive hemorrhage, as well as evidence of hepatocyte apoptosis, based on the appearance of condensed nuclei (Fig. 7B). Positive TUNEL staining in the same livers of both wild-type and egr-1−/− mice confirmed these histological findings (Fig. 7C). Collectively, these data suggest that Egr-1 synergizes with other cellular factors to accelerate acute liver injury and hasten time to reach a moribund phenotype in mice after GalN/LPS administration.

**DISCUSSION**

Inflammatory liver injury, characteristic of alcoholic steatohepatitis and other acute and chronic liver diseases, involves LPS and LPS-mediated signaling, Kupffer cells, and complement activation, which leads to hyperexpression of proinflammatory cytokines, chemokines, and adhesion molecules (21, 23). The uncontrolled inflammatory milieu created by these mediators contributes to liver pathology. The transcription factor Egr-1 has been identified as a master regulator of proinflammatory genes, including TNF-α, MIP-2, MCP-1, and ICAM-1, in animal models of lung ischemia-reperfusion injury, atherosclerosis, and pancreatitis (37). However, very little is known about the role of Egr-1 in the pathophysiology of...
Egr-1 AND ACUTE LIVER INJURY

Fig. 6. GalN/LPS-induced hepatic apoptosis is reduced in egr-1−/− animals. A: active caspase-3 (act. casp 3) was measured by Western blot at baseline and 1, 4, and 5.5 h after GalN/LPS administration in wild-type and egr-1−/− mice. Hsc70, loading control. B: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining at baseline and 5.5 h after GalN/LPS administration. Images are representative of 3–6 animals per experimental group. C: quantification of the TUNEL-positive cells. Open bar: wild type, 9.5 ± 1.2% apoptosis (n = 4 mice); filled bar: egr-1−/−, 2.9 ± 0.9% apoptosis (n = 3 mice). *P < 0.05.

Liver disease. Our laboratory (32) has previously demonstrated that egr-1 is required for ethanol-induced fatty liver. However, a role for Egr-1 in acute inflammatory liver injury, such as in hepatitis, is unknown. Making use of egr-1−/− mice, presently we tested the hypothesis that Egr-1 plays a role in acute GalN/LPS-induced liver injury. Our results demonstrated that Egr-1 contributes to GalN/LPS-induced acute liver injury, in part, by enhancing TNF-α, MIP-2, MCP-1, and ICAM-1 expression late in the acute inflammatory response to GalN/LPS.

Decreased expression of these inflammatory mediators in egr-1−/− mice was associated with an attenuation of hepatocyte injury, coagulative necrosis, hepatic apoptosis, neutrophil accumulation in liver parenchyma, and extended survival compared with wild-type mice.

GalN/LPS exposure in wild-type mice increased hepatic egr-1 mRNA accumulation in liver with two distinct peaks of egr-1 mRNA expression at 1 and 4–5.5 h (Fig. 1). It is likely that Kupffer cells, the resident macrophages in the liver, are responsible for the initial increase in egr-1 mRNA expression in response to GalN/LPS, since the hepatocyte-selective toxin GalN will prevent Egr-1 transcription in hepatocytes (14) but not in GalN-insensitive Kupffer cells (19). The second peak in egr-1 mRNA likely involves more complex regulation. Stimulation from secondary mediators, such as TNF-α and ROS (40, 47), that were induced by the initial LPS exposure likely contribute to this second peak in egr-1 mRNA expression. Furthermore, both Kupffer cells and hepatocytes are potential sources of Egr-1 4–5.5 h after GalN/LPS administration, since the inhibitory effect of GalN on transcription in hepatocytes wanes after 3 h (9).

Since expression of the immediate early gene egr-1 must precede expression of the genes it regulates, it is unlikely that Egr-1 makes a substantial contribution to the initial rapid induction (i.e., within 60 min) of inflammatory genes by GalN/LPS. Consistent with this hypothesis, GalN/LPS-stimulated expression of MIP-2, MCP-1, and ICAM-1 mRNAs at 1 h did not differ between wild-type and egr-1−/− mice (Fig. 3, D–G, and Fig. 6). These data suggest that additional, preformed transcription factors, such as NF-κB, contributed to the rapid, early induction of these genes (42). In contrast, the contribution of Egr-1 to gene expression later in the progression of GalN/LPS-induced liver injury was revealed, because hepatic MIP-2, MCP-1, and ICAM-1 mRNA and protein expression were lower in egr-1−/− mice compared with wild-type mice 4 and 5.5 h after GalN/LPS exposure (Figs. 3 and 4).

Egr-1, in combination with NF-κB and AP-1, is required for optimal LPS-induced transcription of the TNF-α promoter in the human monocytic cell line THP-1 (47). In mice, absence of Egr-1 reduces LPS-induced TNF-α expression in liver (32). Although we observed robust induction of TNF-α mRNA accumulation by GalN/LPS, we did not detect a difference in the mRNA accumulation between wild-type and egr-1−/− mice at any time point after GalN/LPS administration. Increased contribution of NF-κB and/or AP-1 in the egr-1−/− mice could be responsible for an apparent lack of contribution of Egr-1 to GalN/LPS-induced TNF-α mRNA accumulation. However, a role for Egr-1 in TNF-α expression was unmasked at the protein level in egr-1−/− mice, because TNF-α protein concentration in plasma and in the liver was reduced at 4 and 5.5 h after GalN/LPS. These data suggest potential points of posttranscriptional TNF-α regulation by Egr-1-mediated gene expression.

This “late-phase” and/or delayed contribution of Egr-1 in GalN/LPS-induced proinflammatory cytokine and/or chemokine expression and acute liver injury is similar to the role of Egr-1 in a model of LPS-induced endotoxemia. In that model,
Neutrophil extravasation from sinusoids into the liver parenchyma (21). Although it has been reported that MIP-2 is dispensable for the recruitment of neutrophils into the liver parenchyma after GalN/LPS exposure (10), MIP-2 may instead contribute to the localization and/or maintenance of neutrophils in the sinusoids of the liver before their stimulus-induced transmigration across the sinusoidal endothelium (3). The total number of neutrophils recruited to the liver was not different between wild-type and egr-1−/− mice. However, the percentage of neutrophils that transmigrated from the sinusoids into the liver parenchyma was decreased in egr-1−/− mice. These data suggest that decreased expression of MIP-2 (Fig. 3, D and E) likely suppressed the generation of primed neutrophils, thus decreasing the number of neutrophils able to extravasate when provided with the appropriate stimulus (Fig. 5).

Once neutrophils are recruited to the hepatic vasculature, they require additional, non-MIP-2-dependent signals from the injured and/or apoptotic hepatocytes (22, 26), as well as appropriate adhesion molecules, to transmigrate from the hepatic sinusoids into the parenchyma (23). Neutrophil transmigration from hepatic sinusoids into the hepatic parenchyma is critical to GalN/LPS-induced hepatocyte necrosis and depends, in part, on ICAM-1 expression and interaction with the β2-integrin Mac-1 (CD11b/CD18) (11). Therefore, the decreased expression of GalN/LPS-induced ICAM-1 expression in egr-1−/− mice (Fig. 4) likely contributed to the decrease in extravasated neutrophils 4 h after GalN/LPS administration (Fig. 5) and improved hepatic architecture 4 and 5.5 h after GalN/LPS in egr-1−/− mice (Fig. 2A). However, extravasation was not completely inhibited in egr-1−/− mice. Low-level ICAM-1 expression in the egr-1−/− mice (Fig. 4) and/or other adhesion molecule-mediated events (e.g., VCAM-1/β1-integrin interactions; for review see Ref. 23) may have supported transmigration of some neutrophils from the sinusoids into the liver parenchyma. These data are similar to the partial attenuation of GalN/LPS-induced liver injury when mice are treated with an ICAM-1 blocking antibody that interferes with ICAM-1/Mac-1 interactions (11).

Although the current data demonstrate a contribution of Egr-1 to acute liver injury via maintenance of chemokines and adhesion molecule expression and subsequent exacerbation of GalN/LPS-induced acute liver injury, Egr-1 also may be involved in additional mechanisms contributing to acute liver injury. For example, Egr-1 regulates the expression of genes involved in extracellular matrix degradation (2, 7, 17), another critical event necessary for white blood cell extravasation from the vasculature into sites of inflammation (43). During acute liver injury induced by GalN/LPS, sinusoidal endothelial cells lose their normal fenestrations and develop large gaps in their cytoplasm in a matrix metalloproteinase (MMP)-2- and MMP-9-dependent process (20). This “gap-formation” phenomenon is associated with the migration of red blood cells, platelets, and neutrophils into the space of Disse (20). Ito et al. (20) hypothesized that the gaps may allow for direct contact between primed neutrophils and hepatocytes and lead to neutrophil-mediated hepatocyte injury and/or death. Because membrane-associated membrane type-1 (MT1)-MMP is important for MMP-2 activation (33) and because MT1-MMP is regulated by Egr-1 (2, 17), it is possible that decreased neutrophil infiltration into the hepatic parenchyma also could be due to
decreased MMP-2-dependent gap-formation in sinusoidal endothelial cells.

Despite the decreased hepatic proinflammatory milieu in egr-1−/− mice and attenuated hepatic pathology at 4 and 5.5 h after GalN/LPS administration, the survival of egr-1−/− mice after GalN/LPS administration was only modestly extended (by 1 h) compared with that of wild-type mice (Fig. 7A). Egr-1−/− mice ultimately succumb to GalN/LPS-induced acute liver injury as do wild-type animals, but with delayed kinetics (Figs. 6 and 7). Eventual mortality in the egr-1−/− mice is consistent with the known, critical role for TNF-α-induced, apoptosis-driven lethal liver injury in the GalN/LPS model (22) and with the equivalent concentration of plasma and hepatic TNF-α 1 h after GalN/LPS administration (Fig. 3, A and C). We hypothesize that the delay in mortality is due to the decrease in plasma and hepatic TNF-α protein concentration observed in egr-1−/− compared with wild-type mice at both 4 and 5.5 h after GalN/LPS administration (Fig. 3, A and C). The delay in apoptosis observed in the egr-1−/− mice may also be related to a protective effect due to the lack of Egr-1 in hepatocytes. Expression of the tumor suppressor/proapoptotic gene phosphatase and tensin (PTEN) homolog is regulated by Egr-1 (34, 44). Therefore, expression of PTEN would likely be attenuated or delayed in egr-1−/− mice and would therefore potentially decrease and/or delay GalN/LPS-induced apoptosis and subsequent mortality.

In conclusion, the data presented demonstrate, for the first time, important roles for Egr-1 in the sustained and/or enhanced expression of TNF-α, MIP-2, MCP-1, and ICAM-1 and subsequent acceleration in the development and progression of GalN/LPS-induced acute liver injury and mortality. In wild-type mice, Egr-1-mediated contributions to liver inflammation and injury occurred after the initial response to GalN/LPS, leading to enhanced and/or persistent proinflammatory gene expression, and correlated with rapid induction of apoptosis and subsequent mortality. Each of these findings was attenuated and/or delayed in egr-1−/− mice. Therefore, Egr-1 functions to enhance and/or prolong the inflammatory milieu in the liver and thus accelerates and/or exacerbates acute liver injury.

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