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Early Growth Response Factor-1 Is Critical for Cholestatic Liver Injury

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Hepatocyte injury during cholestasis depends in part on the release of proinflammatory mediators that cause neutrophils to accumulate in the liver and become activated to damage hepatocytes. The mechanism by which cholestasis stimulates production of proinflammatory mediators in the liver is not completely understood. The studies presented here tested the hypothesis that the transcription factor early growth response factor-1 (Egr-1) is required for inflammation to occur in the liver during cholestasis. The results of these studies show that Egr-1 was rapidly upregulated, primarily in hepatocytes, in mice subjected to bile duct ligation, an animal model of cholestasis. To determine whether Egr-1 was required for inflammation and hepatocyte injury during cholestasis, bile duct ligation was performed in wild-type and Egr-1 knockout mice. Hepatocyte injury, neutrophil accumulation, and upregulation of macrophage inflammatory protein-2 (MIP-2) and intercellular adhesion molecule-1 (ICAM-1) in the liver were significantly reduced in Egr-1 knockouts. By contrast, levels of tumor necrosis factor-alpha (TNF-α) and collagen (*i.e.*, a biomarker of liver fibrosis) were not different between wild-types and Egr-1 knockouts subjected to bile duct ligation. Because hepatocytes are exposed to elevated concentrations of bile acids during cholestasis, it was determined that bile acids upregulate Egr-1 in primary mouse hepatocytes. Deoxycholic acid dose-dependently increased Egr-1 protein in hepatocytes. Results from these studies suggest a scenario in which elevated concentrations of bile acids during cholestasis increase expression of Egr-1 in hepatocytes. Egr-1 then upregulates proinflammatory mediators that cause neutrophils to accumulate in the liver and become activated to damage hepatocytes.

Key Words: **cholestasis; early growth response factor-1; liver; inflammation; bile acids.**

INTRODUCTION

Cholestatic liver disease arises when excretion of bile acids from the liver is interrupted (Li *et al.*, 2004). This causes concentrations of bile acids to increase in liver and plasma (Lindblad *et al.*, 1977; Setchell *et al.*, 1997). As this disease progresses, hepatic inflammation and injury develop (Gujral *et al.*, 2004*a*). If cholestasis is not corrected and hepatocyte injury persists, biliary fibrosis and eventually cirrhosis ultimately develop (Ramadori and Saile, 2004).

A major consequence of cholestasis is the development of hepatocyte injury (Patel and Gores, 1995). This reduces liver function, and under chronic conditions is thought to be an important stimulus for the development of fibrosis and, ultimately, cirrhosis. Recent studies indicate that hepatocyte injury during cholestasis depends in part on the release of proinflammatory mediators that cause neutrophils to accumulate in the liver and become activated to damage hepatocytes (Gujral *et al.*, 2003, 2004*a*). Mice deficient in CD18, a component of integrins on neutrophils that is important for adhesion-dependent extravasation and activation of neutrophils, showed reduced hepatic neutrophil accumulation and hepatocyte injury when subjected to bile duct ligation, a well-established model of extrahepatic cholestasis (Gujral *et al.*, 2003). Similar results were observed in mice deficient in intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule that is also important for neutrophil extravasation and activation in the liver (Essani *et al.*, 1995; Gujral *et al.*, 2004*a*; Nagendra *et al.*, 1997). These studies have clearly established a critical role for neutrophils in the mechanism of hepatocyte injury during obstructive cholestasis. The mechanism by which cholestasis triggers production of proinflammatory mediators that promote accumulation and activation of neutrophils in the liver, however, remains unknown. Elucidation of this pathway could provide insight into ways to prevent inflammation from occurring in the liver during cholestasis, thereby reducing hepatocyte injury and fibrosis in patients with this disease. One potential regulator of inflammation in the liver during cholestasis may be the transcription factor early growth response factor-1 (Egr-1).

Several lines of evidence indicate that Egr-1 is an important regulator of inflammation (McMahon and Monroe, 1996). Egr-1 response elements are present in the promoters of several proinflammatory genes, including ICAM-1 and TNF-α (Kramer *et al.*, 1994; Maltzman *et al.*, 1996). Furthermore, studies *in vitro* have confirmed that Egr-1 directly regulates expression of these genes. The most compelling evidence that Egr-1 is important for the development of inflammation comes

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from studies *in vivo* examining the pathogenesis of ischemia–reperfusion injury in the lung and atherosclerosis in the peripheral vasculature (Harja *et al.*, 2004; Yan *et al.*, 2000). Ischemia–reperfusion in the lungs of mice causes rapid upregulation of Egr-1 (Yan *et al.*, 2000). Furthermore, macrophage inflammatory protein-2 (MIP-2), a neutrophil chemokine, and ICAM-1 are upregulated, and neutrophils accumulate in the lung during reperfusion. Upregulation of MIP-2 and ICAM-1, neutrophil accumulation, and lung injury were dramatically reduced in Egr-1 knockout mice subjected to ischemia–reperfusion (Yan *et al.*, 2000). Similar results were obtained in a mouse model of atherosclerosis, which showed that ICAM-1 and the atherosclerotic lesion area were substantially reduced in mice deficient in Egr-1. (Harja *et al.*, 2004). Overall these studies clearly indicate that Egr-1 is an important regulator of inflammation in the lung and peripheral vasculature. Whether Egr-1 is an essential regulator of inflammation in the liver during cholestasis, however, remains to be investigated.

The studies presented herein tested the hypothesis that Egr-1 is required for hepatic inflammation and injury during cholestasis. To this end, expression of Egr-1 was evaluated in the livers of mice with cholestasis. Additionally, it was determined whether liver injury, expression of proinflammatory mediators, hepatic neutrophil accumulation, and liver fibrosis were reduced in Egr-1 homozygous knockout mice with cholestasis.

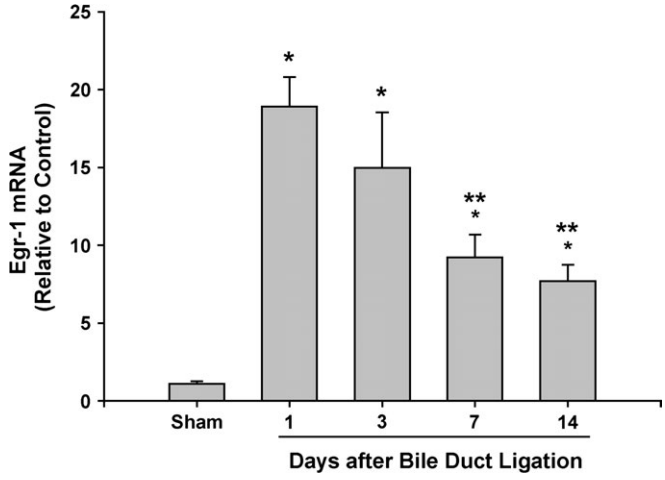

FIG. 1. Upregulation of Egr-1 mRNA in the liver after bile duct ligation. Mice were subjected to bile duct ligation or control sham operation. At the indicated times, the livers were removed and RNA isolated. Egr-1 mRNA was quantified by real-time PCR. Results from sham-operated mice evaluated at different times were combined into one group because no differences were observed among them. Data are expressed as means ± SEM; $n = 5$ mice/group. *Significantly different ($p < 0.05$) from sham-operated mice. **Significantly different ($p < 0.05$) from mice bile duct–ligated 24 h earlier.

MATERIALS AND METHODS

Animals. Male C57BL/6 (Harlan, Indianapolis, IN), C57BL/6NTac (Taconic, Germantown, NY), and Egr-1 knockouts (B6.129-*Egr1*^{tm1Jmi} N12, Taconic) ranging from 8 to 10 weeks of age were used for these studies. Egr-1 knockout mice were backcrossed to C57BL/6NTac mice for 11 generations; therefore, C57BL/6NTac mice were used as controls for Egr-1 knockout studies. C57BL/6 mice from Harlan were used in studies where Egr-1 was detected in the livers of bile duct-ligated animals (Figs. 1 and 2), and for studies utilizing primary hepatocytes (see Fig. 9). Egr-1 knockouts and C57BL/6NTac mice were used for the remaining studies. The animals were maintained on a 12-h light/dark cycle under controlled temperature (18°–21°C) and humidity. Food (Rodent Chow; Harlan-Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

Bile duct ligation. Mice were anesthetized with isoflurane. A midline laparotomy was performed, and the bile duct was ligated with 3–0 surgical silk. The abdominal incision was closed with sutures, and the mice received 0.2 mg/kg Buprenex by subcutaneous injection.

Real-time polymerase chain reaction (PCR). Total liver RNA was isolated using TRI reagent (Chomczynski and Sacchi, 1987) (Sigma Chemical Company, St. Louis, MO), and reverse transcribed into cDNA as described by us previously (Copple *et al.*, 2003*a*). Real-time polymerase chain reaction (PCR) was used to quantify Egr-1, MIP-2, ICAM-1, TNF-α, collagen type III α 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and performed on an Applied Biosystems Prism 7300 Real-time PCR Instrument (Applied Biosystems, Foster City, CA) with the SYBR green DNA PCR kit (Applied Biosystems). The sequences of the primers were as follows: Egr-1 Forward: 5′-GGC AGA GGA AGA CGA TGA AG-3′; Egr-1 Reverse: 5′-GAC GAG TTA TCC CAG CCA AA-3′; MIP-2 Forward: 5′-CTC AGA CAG CGA GGC ACA TC-3′; MIP-2 Reverse: 5′-CCT CAA CGG AAG AAC CAA AGA G-3′; ICAM-1 Forward: 5′-CGA CGC CGC TCA GAA GAA-3′; ICAM-1 Reverse: 5′-GTC TCG GAA GGG AGC CAA GTA-3′; Collagen Type III alpha 1 Forward: 5′-GTC CAC GAG GTG ACA AAG GT-3’; Collagen Type III alpha 1 Reverse: 5‘-CAT CTT TTC CAG GAG GTC CA-3’; TNF-α Forward: 5‘-GAC CCT CAC ACT CAG ATC ATC TTC T-3’; TNF-α Reverse: 5‘-CCT CCA CTT GGT GGT TTG CT-3’; GAPDH Forward: 5‘-GGT CTC GCT CCT GGA AGA TG-3’; GAPDH Reverse: 5‘-GTA TGA CTC CAC TCA CGG CAA A-3’. Stock solutions of primer pairs were made in water that contained each primer at a final concentration of 5 μM. Each Egr-1 and GAPDH real-time PCR reaction contained 5 μl of cDNA diluted 1:10 in water, 0.6 μl of primer pair stock solution, 6.9 μl of water, and 12.5 μl of SYBR green reagent. Each MIP-2, TNF-α, collagen type III α 1, and ICAM-1 real-time PCR reaction contained 5 μl of cDNA diluted 1:10 in water, 3 μl of primer pair stock solution, 4.5 μl of water, and 12.5 μl of SYBR green reagent. C_T values obtained for Egr-1, MIP-2, TNF-α, collagen type III α 1, and ICAM-1 were first normalized with that of GAPDH prior to analysis. Fold change in Egr-1, MIP-2, TNF-α, collagen type III α 1, and ICAM-1 mRNAs was then calculated relative to control (*i.e.*, sham operation) using the comparative C_T method as described in Applied Biosystems User Bulletin #2 (available at the following Web address: www.appliedbiosystems.com.). Melting curve analysis was performed to ensure that the primer pairs amplified a single transcript.

Immunohistochemistry. For Egr-1, type I collagen, and neutrophil immunostaining, livers were frozen in isopentane (Sigma Chemical Company) immersed in liquid nitrogen for 8 min. Sections of frozen liver or cultured hepatocytes were fixed in 4% formalin in phosphate-buffered saline (PBS) for 10 min at room temperature. Cultured hepatocytes were then permeabilized by incubation in acetone at −20°C for 10 min. Sections of liver or hepatocytes were incubated with rabbit anti-mouse Egr-1 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-mouse type I collagen (Chemicon, Temecula, CA) diluted 1:100 in PBS containing 3% goat serum, or with rat anti-mouse neutrophil antibody (Serotec, Oxford, UK) diluted 1:100 in PBS containing 3% goat serum at room temperature for 3 h. For Egr-1 and collagen immunostaining,

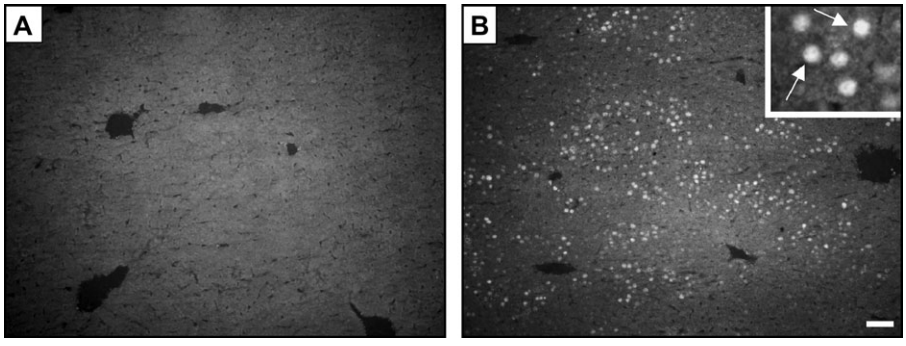

FIG. 2. Increased levels of Egr-1 protein in the liver after bile duct ligation. Mice were subjected to (A) control sham operation or (B) bile duct ligation. Three days after surgery, the livers were removed and Egr-1 was identified in sections of liver using immunohistochemistry. Livers from five mice were evaluated in each group. Egr-1 stains bright white in the photomicrographs. Arrows indicate positive Egr-1 staining. Higher magnification in the inset in B. Bar in B = 100 μm.

the tissues and/or hepatocytes were washed with PBS and then incubated with secondary antibody conjugated to Alexa 488 (green staining; Molecular Probes, Eugene, OR). For neutrophil immunostaining, the Vectastain Elite ABC kit was used according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA).

Quantification of type I collagen in the liver. Type I collagen in the liver was quantified morphometrically by analyzing the area of immunohistochemical staining of type I collagen in a section of liver. An increase in the area of type I collagen staining in the liver is an indicator of fibrosis. Fluorescent staining in sections of liver was visualized on an Olympus BX41 microscope (Olympus, Lake Success, NY). For morphometric analysis of the total area of type I collagen in a liver section, digital images of five randomly chosen, low power (100× magnification) fields per tissue section were captured using an Olympus DP70 camera. Samples were coded such that the evaluator was not aware of the treatment, and the same exposure time was used for all captured images. Scion Image software (Scion Corporation, Frederick, MD) was then used to quantify the total area of type I collagen (number of positive pixels) using methods described in detail previously (Copple *et al.*, 2002*a*).

The staining is expressed as a fraction of the total area. The random fields analyzed for each liver section were averaged and counted as a replicate; *i.e.*, each replicate represents a different mouse.

Assessment of hepatic injury and serum bile acid concentration. Hepatocyte injury was evaluated by measuring the activity of alanine aminotransferase (ALT) in the serum using the Liquid ALT Reagent Set (Pointe Scientific Inc., Brussels, Belgium). In addition, morphometric analysis was performed on hematoxylin and eosin stained sections of liver to estimate the area of hepatocyte necrosis as described in detail previously (Copple *et al.*, 2002*b*). Serum bile acid concentrations were determined by using a commercially available kit (Colorimetric Total Bile Acids Assay Kit; Bioquant, San Diego, CA).

Hepatocyte isolation. Under pentobarbital anesthesia (50 mg/kg i.p.), the abdominal cavity of the mouse was opened, and the inferior vena cava was cannulated with an i.v. catheter (24-gauge × 3/4″; Terumo Medical Corporation, Elkton, MD). Infusion tubing connected to a pump was inserted into the catheter, and the liver was perfused with 50 ml of calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS, Sigma Chemical Company) supplemented with 0.5 mM EGTA, 5.5 mM glucose, and penicillin-streptomycin (Sigma Chemical Company). At this point, the portal vein was cut, and the anterior vena cava between the heart and diaphragm was clamped with a small hemostat. The liver was then perfused with 40 ml of calcium- and magnesium-free HBSS supplemented with 1.5 mM calcium chloride, 5.5 mM glucose, penicillin-streptomycin, and 0.02 g of Type IV Collagenase (Sigma Chemical Company). The liver was removed, and the digested product was centrifuged at 50 × *g* for 2 min to pellet the hepatocytes. The hepatocytes were washed three times with Williams' medium E (Invitrogen, Carlsbad, CA) and then cultured in Williams' medium E containing 10% fetal bovine serum (FBS) and penicillin-streptomycin. After a 3-h attachment period, the medium with unattached cells was removed, and fresh medium was added. Typically, 98% of the cells in the final preparation were hepatocytes, as determined by morphological evaluation (Copple *et al.*, 2003*b*). Hepatocytes are easily distinguished from other nonparenchymal cells types because of their larger size. The viability of the isolated hepatocytes was >90% by the criterion of trypan blue (Sigma Chemical Company) exclusion. Cells were cultured for approximately 16 h before addition of deoxycholic acid.

Statistical analysis. Results are presented as the mean ± SEM. Data were analyzed by analysis of variance (ANOVA). ANOVAs were performed on log X-transformed data in instances in which variances were not homogeneous. Data expressed as a fraction were transformed by arc sine square root prior to analysis. Comparisons among group means were made using the Student-Newman-Keuls test. The criterion for significance was $p < 0.05$ for all studies.

RESULTS

Egr-1 Is Upregulated in the Livers of Mice with Cholestasis

Extrahepatic cholestasis was produced in mice by ligation of the bile duct. At 1, 3, 7, and 14 days after surgery, Egr-1 mRNA levels were analyzed by real-time PCR. As shown in Figure 1, levels of Egr-1 mRNA were increased 19-fold by 1 day after bile duct ligation, and remained significantly elevated thereafter. Although, Egr-1 mRNA levels remained significantly elevated at 7 and 14 days after bile duct ligation, they were significantly reduced compared to Egr-1 mRNA levels at 24 h after bile duct ligation.

Next, immunohistochemical studies were performed on sections of frozen liver to determine whether levels of Egr-1 protein were increased. No Egr-1 was detected in livers from mice that had been subjected to sham operation 3 days earlier (Fig. 2A). Fluorescence in Figure 2A primarily resulted from autofluorescence and not Egr-1 staining. Egr-1 immunostaining was dramatically increased in sections of liver from mice that had been subjected to bile duct ligation 3 days earlier (Fig. 2B). Egr-1 immunostaining was nuclear (confirmed by co-localization with the nuclear stain 4′,6-diamidino-2-phenylindole, DAP-I, data not shown) and was expressed primarily by hepatocytes. Approximately 35% of hepatocytes stained positive

for Egr-1. Similar results were obtained in sections of liver from mice subjected to bile duct ligation 14 days earlier (results not shown).

Egr-1 Is Required for Hepatocyte Injury after Bile Duct Ligation

Because Egr-1 was upregulated in liver after bile duct ligation, we next determined whether it was required for hepatocyte injury. For these studies, wild-type and homozygous Egr-1 knockout mice were subjected to sham operation or bile duct ligation. Fourteen days later, the activity of ALT was measured in the serum as a biomarker of hepatocyte injury. ALT activity was significantly increased in serum from wild-type mice subjected to bile duct ligation (Fig. 3A). ALT activity was markedly lower in serum from Egr-1 knockout mice subjected to bile duct ligation.

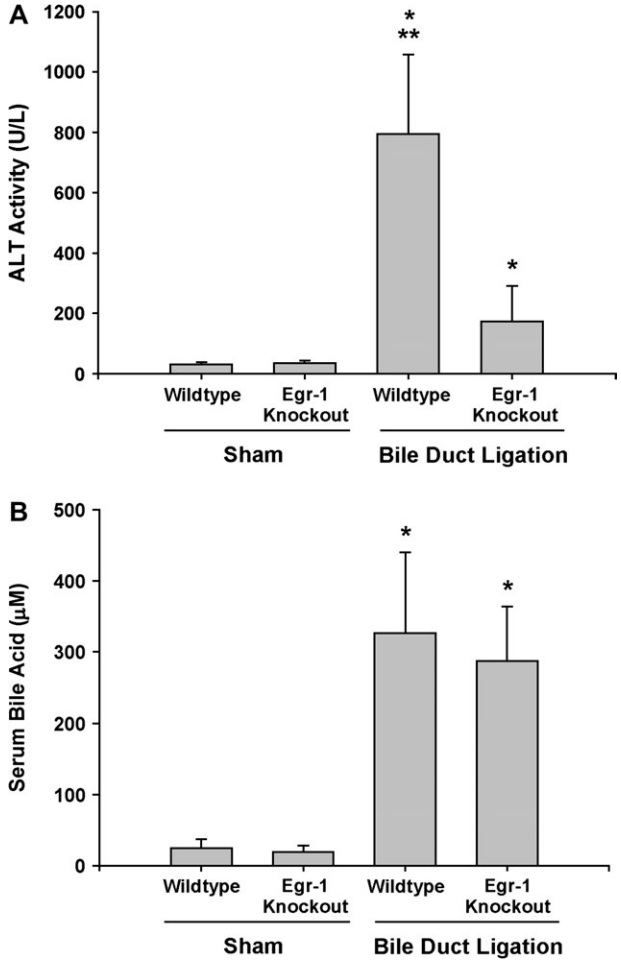

FIG. 3. Hepatocyte injury and serum bile acid concentrations in Egr-1 knockout mice subjected to bile duct ligation. Wild-type and Egr-1 knockout mice were subjected to bile duct ligation or control sham operation. Fourteen days after surgery, (A) alanine aminotransferase (ALT) activity and (B) bile acid concentrations were measured in serum. Data are expressed as means ± SEM; $n = 8$ mice/group. *Significantly different ($p < 0.05$) from sham-operated mice. **Significantly different ($p < 0.05$) from wild-type bile duct–ligated mice.

Next, the concentration of total serum bile acids was measured to determine whether bile acid synthesis or transport was modified in Egr-1 knockout mice with cholestasis. Levels of serum bile acids were significantly increased in wild-type and Egr-1 knockout mice that had been subjected to bile duct ligation 14 days earlier when compared to sham-operated mice (Fig. 3B). There was no significant difference between serum bile acid concentrations in bile duct–ligated wild-type and Egr-1 knockout mice.

In the next step, sections of liver were evaluated histologically. Extensive regions of coagulative hepatocyte necrosis were observed in periportal regions in sections of liver from wild-type mice that had been subjected to bile duct ligation 2 weeks earlier (Fig. 4A). Very few of these regions were observed in liver from bile duct–ligated Egr-1 knockout mice (Fig. 4B). Hepatocyte injury in these sections consisted primarily of scattered, single necrotic hepatocytes localized to periportal regions. No necrotic hepatocytes were observed in liver sections from sham-operated wild-type or Egr-1 knockout mice (data not shown). Next, morphometric analysis was used to estimate the area of hepatocyte necrosis. Hepatocyte necrosis was not detected in either wild-type or Egr-1 knockout mice subjected to sham operation. Approximately 35 ± 7% of the liver in wild-type mice subjected to bile duct ligation contained lesions consisting of necrotic hepatocytes. By contrast, only 5 ± 3% of the liver contained lesions in Egr-1 knockout mice subjected to bile duct ligation (significantly different from wild-type mice subjected to bile duct ligation; $p < 0.05$).

Egr-1 Is Required for Neutrophil Accumulation in the Liver During Cholestasis

As discussed previously, inflammation is important for the development of hepatocyte injury after bile duct ligation (Gujral *et al.*, 2004*a*). Because hepatocyte injury was reduced in Egr-1 knockout mice, and Egr-1 is important for the development of inflammation in other organs, we next determined whether hepatic neutrophil accumulation was reduced in bile duct–ligated Egr-1 knockout mice. For these studies, wild-type and Egr-1 knockout mice were subjected to bile duct ligation or control sham operation. After 14 days, livers were removed and immunohistochemical staining was used to detect neutrophils in sections of liver. Very few neutrophils were detected in liver sections from wild-type (Fig. 5A) or Egr-1 knockout mice (data not shown) subjected to control sham operation. Numerous neutrophils were detected in the livers of wild-type mice subjected to bile duct ligation (Fig. 5B). Neutrophils were evenly distributed throughout the liver lobule. Although, in some periportal regions, neutrophils were more concentrated near proliferating bile ducts. The numbers of hepatic neutrophils were dramatically reduced in bile duct–ligated Egr-1 knockout mice (Fig. 5C). Next, the numbers of neutrophils were

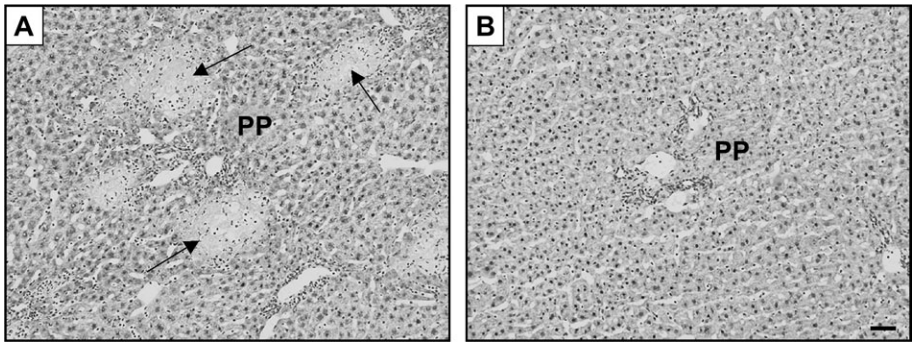

FIG. 4. Photomicrographs of liver sections from wild-type and Egr-1 knockout mice subjected to bile duct ligation. (A) Wild-type and (B) Egr-1 knockout mice were subjected to bile duct ligation. Fourteen days after surgery, the livers were removed and sections of liver were stained with hematoxylin and eosin. Livers from five mice were evaluated in each group. Arrows indicate regions of coagulative hepatocyte necrosis. PP: periportal region. Bar in B = 50 μm.

counted in 20 random 400× fields per tissue section for each treatment group. Neutrophil numbers were significantly increased in the livers of wild-type mice subjected to bile duct ligation (Fig. 6). Significantly fewer neutrophils were detected in the livers of bile duct–ligated Egr-1 knockout mice.

Egr-1 Is Required for Upregulation of MIP-2 and ICAM-1, but not TNF-α, in the Liver During Cholestasis

Previous studies showed that the neutrophil chemokine MIP-2 is upregulated in the liver after bile duct ligation and contributes to hepatic neutrophil accumulation in an animal model of primary biliary cirrhosis (Gujral *et al.*, 2004*b*; Xu *et al.*, 2004). Similarly, ICAM-1 is upregulated in the liver after bile duct ligation and is required for neutrophil accumulation and hepatocyte injury (Gujral *et al.*, 2004*a*). Because studies have shown that Egr-1 is required for upregulation of MIP-2 and ICAM-1 in the lung during ischemia–reperfusion, we next determined whether Egr-1 was required for upregulation of MIP-2 and ICAM-1 in the liver during cholestasis (Yan *et al.*, 2000). MIP-2 mRNA levels were significantly increased in livers of wild-type mice that had been subjected to bile duct ligation 2 weeks earlier (Table 1). Upregulation of MIP-2 mRNA was completely prevented in Egr-1 knockout mice subjected to bile duct ligation. Similarly, ICAM-1 mRNA levels were significantly increased in livers of bile duct–ligated wild-type mice (Table 1). Upregulation of ICAM-1 mRNA was significantly reduced in Egr-1 knockout mice subjected to bile duct ligation.

Studies have shown that Egr-1 regulates TNF-α in Kupffer cells (Kishore *et al.*, 2002). Because TNF-α has been shown to upregulate ICAM-1 in hepatocytes, it is possible that Egr-1 indirectly regulates ICAM-1 by increasing synthesis and release of TNF-α from Kupffer cells (Satoh *et al.*, 1994). To examine this possibility, we determined whether Egr-1 was required for upregulation of TNF-α in the liver during cholestasis. TNF-α mRNA was significantly upregulated in the livers of wild-type mice subjected to bile duct ligation (Table 1). TNF-α was upregulated to a similar extent in Egr-1 knockout mice subjected to bile duct ligation.

Liver Fibrosis Is Not Affected in Egr-1 Knockout Mice with Cholestasis

Since hepatocyte injury and inflammation were reduced in Egr-1 knockout mice with cholestasis, we next determined

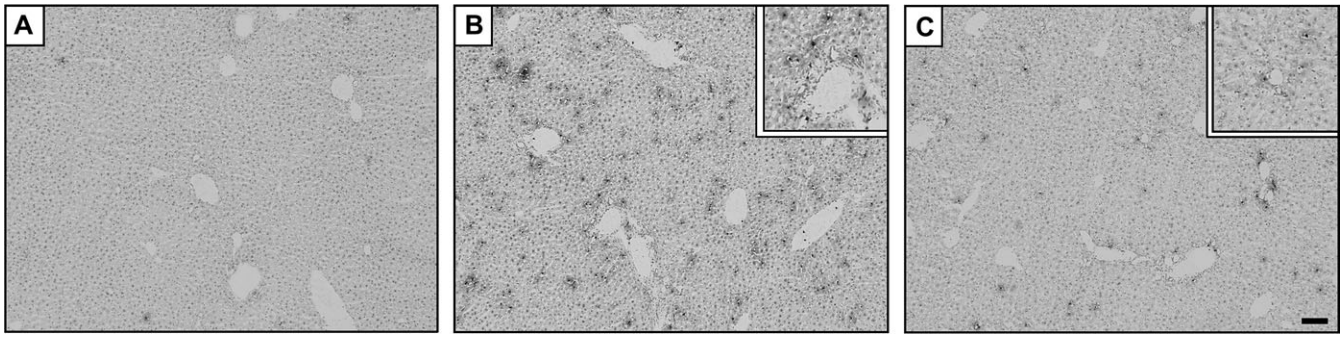

FIG. 5. Photomicrographs of liver sections immunohistochemically stained for neutrophils. Wild-type and Egr-1 knockout mice were subjected to bile duct ligation or control, sham operation. Fourteen days after surgery, the livers were removed and sections of liver were immunohistochemically stained for neutrophils, which appear brown in the photomicrographs. (A) Sham operated wild-type. (B) Bile duct–ligated wild-type. (C) Bile duct–ligated Egr-1 knockout. Higher magnification is shown in the inset in B and C. Sham operated Egr-1 knockout, which is not shown, did not differ from sham-operated wild-type. Bar in B = 100 μm.

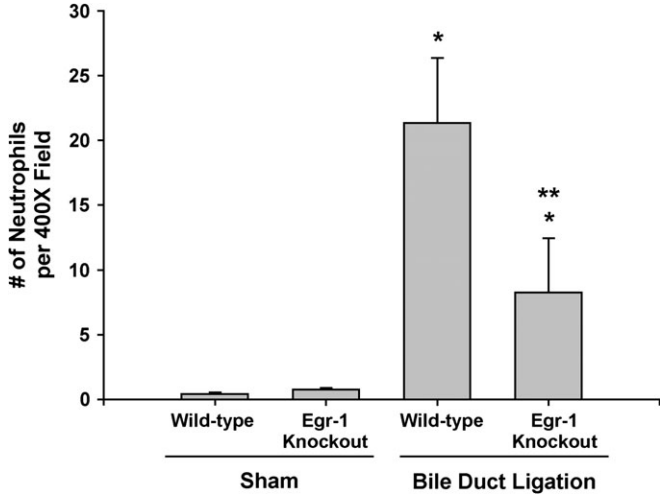

FIG. 6. Neutrophil accumulation in the livers of mice subjected to bile duct ligation. Wild-type and Egr-1 knockout mice were subjected to bile duct ligation or control sham operation. Fourteen days after surgery, the livers were removed and sections of liver were immunohistochemically stained for neutrophils. Neutrophils in 20 random 400× fields were counted in sections of stained livers. Data are expressed as means ± SEM; $n = 8$ mice/group. *Significantly different ($p < 0.05$) from sham-operated mice. **Significantly different ($p < 0.05$) from wild-type bile duct ligated mice.

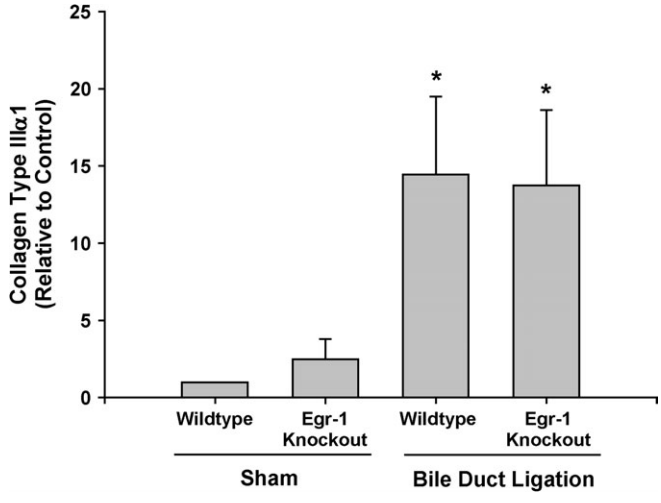

FIG. 7. Upregulation of collagen type III α 1 mRNA in the livers of Egr-1 knockout mice subjected to bile duct ligation. Wild-type and Egr-1 knockout mice were subjected to bile duct ligation or control sham operation. Fourteen days after surgery, RNA was isolated from the liver, and collagen type III α 1 was detected by real-time PCR. Data are expressed as means ± SEM; $n = 8$ mice/group. *Significantly different ($p < 0.05$) from sham-operated mice.

whether liver fibrosis was also reduced. To examine this, we first measured mRNA levels of collagen type III α 1. Collagen type III α 1 mRNA was significantly upregulated in wild-type and Egr-1 knockout mice subjected to bile duct ligation 14 days earlier when compared to sham-operated mice (Fig. 7). There was not a statistically significant difference in collagen type III α 1 mRNA levels between wild-type and Egr-1 knockout mice subjected to bile duct ligation.

Next, type I collagen protein was detected in the liver by immunohistochemistry. Very little type I collagen protein was detected in the livers of wild-type and Egr-1 knockout mice subjected to sham operation (data not shown). By contrast, extensive type I collagen protein was detected primarily in periportal regions around proliferating bile ducts in both wild-type (Fig. 8A) and Egr-1 knockout mice (Fig. 8B) subjected to bile duct ligation 14 days earlier. Next, type I collagen was quantified morphometrically by analyzing the area of immunohistochemical staining. The area of type I collagen immunostaining was significantly increased in wild-type and Egr-1 knockout mice subjected to bile duct ligation when compared to sham-operated mice (Fig. 8C). The level of type I collagen immunostaining in bile duct–ligated wild-type mice was not significantly different from that in bile duct–ligated Egr-1 knockout mice (Fig. 8C).

TABLE 1
Upregulation of Proinflammatory Mediators in the Livers of Egr-1 Knockout Mice Subjected to Bile Duct Ligation

	MIP-2 mRNA (relative to control)	ICAM-1 mRNA (relative to control)	TNF-α mRNA (relative to control)
Wild-type sham	1.00 ± 0.45	1.00 ± 0.47	1.00 ± 0.49
Egr-1 KO sham	1.11 ± 0.33	1.12 ± 0.16	2.06 ± 0.63
Wild-type BDL	13.76 ± 1.05[a]	5.66 ± 0.58[a]	6.18 ± 0.85[a]
Egr-1 KO BDL	3.60 ± 1.04[a,b]	3.56 ± 0.56[a,b]	7.27 ± 0.48[a]

Note. Wild-type and Egr-1 knockout mice were subjected to bile duct ligation or control sham operation. Fourteen days after surgery, RNA was isolated from the liver and MIP-2, ICAM-1, and TNF-α were detected by real-time PCR. Data are expressed as means ± SEM; $n = 8$ mice/group.

[a]Significantly different ($p < 0.05$) from sham-operated mice.

[b]Significantly different ($p < 0.05$) from wild-type bile duct–ligated mice.

Deoxycholic Acid Upregulates Egr-1 in Primary Mouse Hepatocytes

We next performed studies to identify the stimulus for upregulation of Egr-1 during cholestasis. As shown in Figure 1, Egr-1 is rapidly upregulated by 24 h after bile duct ligation. Therefore, the stimulus for upregulation of Egr-1 must also increase rapidly after bile duct ligation. One possibility is bile acids. Concentrations of bile acids are rapidly increased in the liver and serum after bile duct ligation. For example, in our studies, serum concentrations of bile acids were increased by approximately 40-fold by 24 h after bile duct ligation (serum bile acid concentration sham control: 12 ± 4 μM; serum bile acid concentration in bile duct ligated mice: 490 ± 150 μM). Therefore, we determined whether bile acids upregulate Egr-1 in primary hepatocytes. For these studies, primary hepatocytes were isolated from the livers of mice and exposed to various concentrations of deoxycholic acid (DCA) in cell culture for

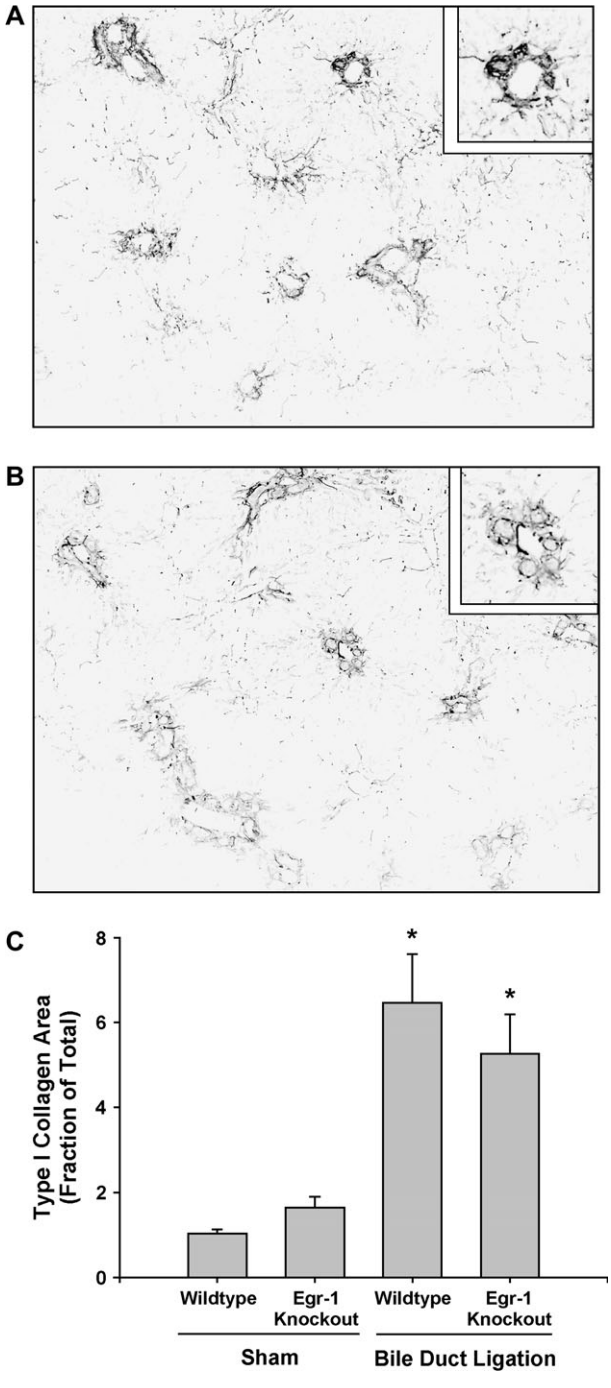

FIG. 8. Deposition of type I collagen in the livers of Egr-1 knockout mice subjected to bile duct ligation. (A) Wild-type and (B) Egr-1 knockout mice were subjected to bile duct ligation or control sham operation. Fourteen days after surgery, type I collagen was detected by immunohistochemistry. (C) Type I collagen was quantified morphometrically by analyzing the area of immunohistochemical staining. Data are expressed as means ± SEM; $n = 8$ mice/group. *Significantly different ($p < 0.05$) from sham-operated mice.

6 h. DCA caused concentration-dependent increases in Egr-1 protein in primary mouse hepatocytes (Fig. 9A). Next, immunohistochemistry was used to detect Egr-1 in primary mouse hepatocytes *in vitro*. Little Egr-1 immunostaining occurred in vehicle-treated hepatocytes (Fig. 9B). By contrast extensive Egr-1 immunostaining was present in the nuclei of DCA-treated hepatocytes (Fig. 9C).

DISCUSSION

Recent studies using transgenic mice have revealed that neutrophils are important for the pathogenesis of hepatocyte injury during obstructive cholestasis. As discussed, mice deficient in either CD18 or ICAM-1 had reduced hepatic neutrophil accumulation and activation and hepatocyte injury after bile duct ligation (Gujral *et al.*, 2003). These studies clearly established a critical role for neutrophils in the mechanism of hepatocyte injury during obstructive cholestasis; however, what remains unclear is the molecular mechanism by which cholestasis triggers production of proinflammatory mediators that promote inflammatory liver injury. Our studies suggest that Egr-1 is vital for this process.

Egr-1 was rapidly upregulated in the liver by 24 h after bile duct ligation. Interestingly, although Egr-1 remained significantly elevated at later times, it was significantly reduced below the 24-h level at 7 and 14 days after bile duct ligation. It is possible that this reduction occurred because the numbers of hepatocytes in the liver were subjected to toxicity. Alternatively, Egr-1 may have been downregulated by negative feedback inhibition of the Egr-1 promoter. Studies have shown that Egr-1 upregulates Nab2 (Kumbrink *et al.*, 2005), a protein that suppresses Egr-1 promoter activity.

Neutrophil accumulation and hepatocyte injury were reduced in Egr-1 knockout mice subjected to bile duct ligation. It is clear that this reduced liver injury was not a result of altered bile acid synthesis or transport, because serum bile acid levels were not significantly different between wild-type mice and Egr-1 knockout mice subjected to bile duct ligation. Egr-1 was required for upregulation of MIP-2 and ICAM-1. As discussed, ICAM-1 is required for accumulation of neutrophils in the livers of bile duct–ligated mice (Gujral *et al.*, 2004*a*). In addition, studies have shown that MIP-2 is important for neutrophil accumulation in the livers of mice treated with α-naphthylisothiocyanate, a hepatotoxicant that produces cholestasis (Xu *et al.*, 2004). Therefore, the mechanism by which Egr-1 promotes neutrophil accumulation in the liver during cholestasis appears to be related to Egr-1–dependent upregulation of ICAM-1 and MIP-2 in the liver.

Egr-1 may directly upregulate ICAM-1 in the liver during cholestasis. An Egr-1 response element has been identified in the ICAM-1 promoter, and studies have shown that Egr-1 binds to and activates this promoter in some cell types (Maltzman *et al.*, 1996). Whether this occurs in hepatocytes, however,

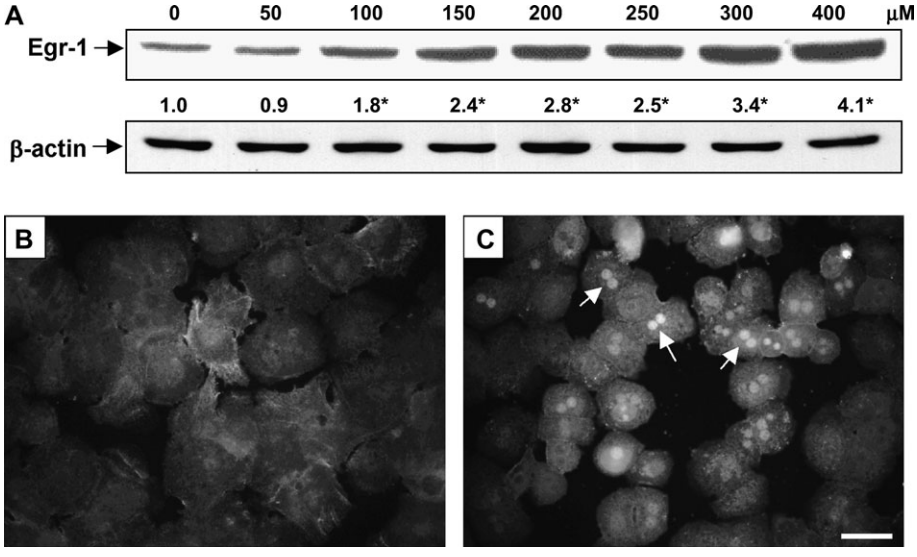

FIG. 9. Upregulation of Egr-1 protein in hepatocytes exposed to DCA. Primary mouse hepatocytes were isolated and treated with various concentrations of DCA; 6 h later, nuclear extracts were prepared for (A) Western blotting to detect Egr-1. The density of each Egr-1 band was measured. The blot was then stripped and reprobed with an anti-β-actin antibody. The density of the bands for Egr-1 were normalized with that of β-actin prior to analysis. Numbers above the Egr-1 bands indicate the concentration of DCA in μM. Numbers below the Egr-1 bands indicate fold difference from vehicle-treated hepatocytes. Representative blot of an $n = 3$, where each n represents hepatocytes isolated from a different mouse. *Significantly different ($p < 0.05$) from untreated hepatocytes. Egr-1 was also detected in hepatocytes treated with either (B) vehicle or (C) 200 μM DCA by immunohistochemistry. Positive staining appears white in the photomicrographs. Arrows indicate positive, nuclear Egr-1 staining. Bar in C = 50 μm.

remains to be investigated. Furthermore, we have detected two putative Egr-1 response elements in the MIP-2 promoter at –572 and –2064 bp upstream of the transcription start site by *in silico* analysis. Whether Egr-1 binds to these sites and activates the MIP-2 promoter remains to be determined.

One possible mechanism by which Egr-1 may regulate expression of MIP-2 and ICAM-1 indirectly is by upregulating TNF-α. Studies have shown that Egr-1 regulates expression of TNF-α (Kishore *et al.*, 2002). Furthermore, studies have shown that TNF-α can regulate expression of MIP-2 and ICAM-1 in some cell types (Iimuro *et al.*, 1997; Satoh *et al.*, 1994). Our results show, however, that the levels of TNF-α were not significantly different between wild-type mice and Egr-1 knockout mice subjected to bile duct ligation, suggesting that this is not the mechanism by which Egr-1 regulates MIP-2 and ICAM-1 in the liver during cholestasis.

Interestingly, although hepatocyte injury primarily occurs in periportal regions during cholestasis, Egr-1 protein and neutrophils were evenly distributed throughout the liver lobule. This suggests that other periportal-specific factors contribute to neutrophil-dependent killing of hepatocytes in this region during cholestasis. One possibility is that bile acid concentrations may be higher in periportal regions, which may sensitize hepatocytes to neutrophil-dependent killing. A second possibility is that elevated concentrations of bile acids activate neutrophils (Dahm *et al.*, 1988). It is possible that during cholestasis Egr-1 promotes neutrophil accumulation throughout the liver lobule; however, high concentrations of bile acids in periportal regions activate neutrophils only in this region. A third possibility is that bile duct epithelial cells produce a neutrophil-activating factor in periportal regions. Studies have shown that under certain conditions bile duct epithelial cells can secrete an unidentified factor that stimulates neutrophils to kill hepatocytes *in vitro* (Hill *et al.*, 1999).

Another interesting observation from these studies is that liver fibrosis was not reduced in Egr-1 knockout mice subjected to bile duct ligation, even though hepatocyte injury and inflammation were reduced. This suggests that hepatocyte injury and neutrophils are not an important stimulus of fibrosis in the liver during cholestasis. These results are similar to a recent report, which showed that neutrophil depletion did not prevent liver fibrosis in bile duct–ligated rats (Saito *et al.*, 2003). It is possible that elevated concentrations of bile acids are an important stimulus for fibrosis during cholestasis. In support of this, it was shown recently that bile acids stimulate hepatic stellate cell proliferation *in vitro* (Svegliati-Baroni *et al.*, 2005).

Our results show further that the stimulus for upregulation of Egr-1 in the liver during cholestasis may be elevated concentrations of bile acids. Egr-1 was rapidly upregulated in the liver by 24 h after bile duct ligation. This suggested that the stimulus for upregulation of Egr-1 must have also increased rapidly after bile duct ligation. One possibility was bile acids, the concentrations of which increased 40-fold by 24 h after ligation of the bile duct. Therefore, the hypothesis was tested that bile acids upregulate Egr-1 in hepatocytes. Our studies showed that deoxycholic acid dose-dependently increased Egr-1 protein levels in primary mouse hepatocytes. These studies suggest that

elevated concentrations of bile acids are an important stimulus for upregulation of Egr-1 in the liver during cholestasis. Although the mechanism by which bile acids upregulate Egr-1 in primary hepatocytes is unclear, this may occur through activation of the epidermal growth factor receptor (EGFR). Studies have shown that various bile acids, including deoxycholic acid, activate the EGFR by mechanisms that are not completely understood (Qiao *et al.*, 2001; Rao *et al.*, 2002). Furthermore, others have shown that treatment of hepatocytes with EGF upregulates Egr-1 (Liu *et al.*, 2000; Tsai *et al.*, 2001). Therefore, it seems possible that the mechanism by which bile acids upregulate Egr-1 in hepatocytes may occur through activation of the EGFR.

Overall, our studies indicate that Egr-1 is a critical regulator of inflammation in the liver during cholestasis and may be the critical link between elevated concentrations of bile acids and the production of proinflammatory mediators. Taken together, our studies suggest that during early stages of cholestasis, elevated concentrations of bile acids upregulate Egr-1 in hepatocytes, which increases expression of proinflammatory mediators that cause neutrophils to accumulate in the liver and become activated to damage hepatocytes.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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