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Novel Role for Amphiregulin in Protection from Liver Injury*

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Clinically, the Fas and Fas ligand system plays a central role in the development of hepatocyte apoptosis, a process contributing to a broad spectrum of liver diseases. Therefore, the development of therapies aimed at the inhibition of hepatocyte apoptosis is a major issue. Activation of the epidermal growth factor receptor has been shown to convey survival signals to the hepatocyte. To learn about the endogenous response of epidermal growth factor receptor ligands during Fas-mediated liver injury we investigated the expression of epidermal growth factor, transforming growth factor α, heparin-binding epidermal growth factor-like growth factor, betacellulin, epiregulin, and amphiregulin in the liver of mice challenged with Fas-agonist antibody. Amphiregulin expression, barely detectable in healthy liver, was significantly up-regulated. Amphiregulin administration abrogated Fas-mediated liver injury in mice and showed direct anti-apoptotic effects in primary hepatocytes. Amphiregulin activated the Akt and signal transducer and activator of transcription-3 survival pathways, and up-regulated Bcl-xL expression. Amphiregulin knock-out mice showed signs of chronic liver damage in the absence of any noxious treatment, and died faster than wild type mice in response to lethal doses of Fas-agonist antibody. In contrast, these mice were more resistant against sublethal liver damage, supporting the hypothesis that chronic liver injury can precondition hepatocytes inducing resistance to subsequent cell death. These results show that amphiregulin is a protective factor induced in response to liver damage and that it may be therapeutic in liver diseases.

Hepatocyte apoptosis is a common mechanism to many forms of liver disease. It has been recognized to contribute to the pathogenesis of alcoholic liver disease, viral hepatitis, cholestatic liver disease, and ischemia/reperfusion injury (1–3). More recently hepatocyte apoptosis has been reported to occur in the liver of patients with nonalcoholic steatohepatitis, correlating with the severity of the disease (4). The consequences of hepatocellular apoptosis may extend beyond the mere loss of functional liver mass, because moderate and persistent apoptosis may contribute to the development of liver inflammation and fibrosis (5, 6). Apoptosis can be triggered by the activation of two molecular pathways: an intrinsic mitochondrially mediated cascade and a death receptor pathway. In the liver, the death receptor pathway appears to be predominant and involves the activation of cytokine receptors such as Fas, tumor necrosis factor receptor-1 and tumor necrosis factor-related apoptosis-inducing ligand receptors-1 and -2 (1, 2, 6). Upon activation by their respective ligands these receptors initiate intracellular signaling cascades that ultimately lead to the activation of death-inducing proteolytic enzymes (1, 2). Fas is abundantly expressed in the liver, and Fas-mediated apoptosis has been shown to significantly contribute to toxic or viral damage and acute liver failure (7–9). Interestingly, hepatocyte apoptosis observed in cholestasis is initiated by the ligand-independent activation of Fas by hydrophobic bile salts (1, 10). In addition, lack of Fas expression or RNA interference targeting of Fas results in significant protection from fulminant hepatitis (7, 11). Altogether these observations underscore the clinical relevance of the Fas pathway in liver disease.

The identification of agents that reduce hepatocellular apoptosis is thus of special significance for the development of hepatoprotective therapeutics (12, 13). Intensive research in this area has shown that cytokines such as interleukin-6 (IL-6),[1] growth factors like hepatocyte growth factor, or the hepatocyte-derived insulin-like growth factor binding protein-1 display potent hepatoprotective effects, including the attenuation of apoptosis induced by Fas ligation (14–17). Activation of the epidermal growth factor receptor (EGF-R), a transmembrane protein endowed with tyrosine kinase activity, has been also recognized to convey survival and proliferative signals for the hepatocyte (18–24). Mature hepatocytes express the highest levels of EGF-R of any normal cell type, suggesting an important role for EGF-R signaling in liver function (25, 26). This receptor can be bound and activated by a broad family of ligands that, besides EGF, include transforming growth factor

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[1] The abbreviations used are: IL-6, interleukin-6; ALT, alanine aminotransferase; AR, amphiregulin; AST, aspartate aminotransferase; BTC, betacellulin; EGF, epidermal growth factor; EPR, epiregulin; ERK, extracellular regulated kinase; HB-EGF, heparin-binding EGF-like growth factor; MEK1, extracellular regulated kinase kinase-1; JNK, Jun N-terminal kinase; PI3K, phosphatidylinositol 3-kinase; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor, TUNEL, terminal deoxynucleotide transferase-mediated dUDP nick-end labeling.

α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPR), and amphiregulin (AR) (27, 28). Treatment of isolated murine hepatocytes with EGF, or transgenic overexpression of TGFα in mice, have demonstrated to induce hepatocyte resistance toward Fas-mediated apoptosis (18, 20, 21). It is known that the expression of TGFα and HB-EGF in liver tissue is markedly increased on liver injury induced by CCl_4 (29, 30). Additionally, we have recently reported that the expression of AR is induced in chronic liver damage and is necessary for efficient hepatocyte DNA synthesis after partial hepatectomy (31). However, to our knowledge, the expression of these ligands in the liver during Fas-mediated apoptosis has not been examined. Information on this issue can shed light on the endogenous protective mechanisms triggered in this clinically relevant model and help to design therapeutic interventions. This was the original aim of this study that led us to uncover the hepatoprotective potential of AR.

EXPERIMENTAL PROCEDURES

Animal Experiments—Experiments were performed in conformity with our Institution's guidelines for the use of laboratory animals. Acute liver damage was induced in male C57/BL6 mice (20 g) (Harlan, Barcelona, Spain) and male *AR*+/+ and *AR*−/− littermate mice (20 g) (32) (n = 3–5 per condition and time point), with a single intraperitoneal injection of the agonistic anti-Fas monoclonal antibody Jo2 (4 μg/mouse in saline) (BD Pharmingen, San Diego, CA) (15). Controls received the equivalent volume of saline. For survival experiments male *AR*+/+ and *AR*−/− littermate mice (20 g, n = 11 mice per genotype) received a single intraperitoneal injection of Jo2 antibody (0.3 mg/kg of body weight). The mice were monitored for up to 24 h, and the time of death was recorded. Where indicated mice were injected intraperitoneally with human recombinant AR (9.5 μg/mouse, Sigma) 6 and 0.5 h prior and 3 h after anti-Fas antibody. At the indicated time points mice were bled, and serum was analyzed for alanine and aspartate aminotransferases (ALT and AST). Mice (C57/BL6) were also treated with *Salmonella typhimurium* lipopolysaccharide (15 mg/kg of body weight in saline) or CCl_4 (1 μl/g of body weight in olive oil) (Sigma), intraperitoneally administered. Mice were sacrificed at the indicated time points by cervical dislocation, and livers were snap frozen in liquid nitrogen or formalin-fixed and paraffin-embedded for hematoxylin and eosin staining.

RNA Isolation and Gene Expression Analyses—Total RNA was extracted using the TRI reagent (Sigma). We treated 2 μg of RNA with DNase I (Invitrogen) prior to reverse transcription with Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of RNaseOUT (Invitrogen) and used 1/10 of cDNA preparation for each PCR. PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and quantitated using the Molecular Analyst software (Bio-Rad). Data were normalized to β-actin mRNA levels. Only samples with a comparable amplification of β-actin mRNA were included in the study. We designed all primers to distinguish between genomic and cDNA amplification and sequenced all PCR products to confirm the specificity.

Real-time PCR was performed using an iCycler (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad). To monitor the specificity final PCR products were analyzed by melting curves and electrophoresis. The amount of each transcript was expressed as the n-fold difference relative to the control gene β-actin ($2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the control and target genes), as previously described (33).

TUNEL Assay—Frozen liver tissue sections were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4). The TUNEL assay was performed according to the manufacturer's instructions (Roche Applied Science).

Isolation, Culture, and Treatment of Mouse Hepatocytes—Hepatocytes were isolated from male C57/BL6 mice (20 g) by collagenase (Invitrogen) perfusion as described previously (34). Cells were plated onto collagen-coated 6-well dishes (type I collagen, Collaborative Biomedical, Bedford, MA) 5×10^5 cells per well. Cultures were maintained in minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, 2 mM glutamine, and antibiotics (all from Invitrogen). After 2-h incubation medium was removed, and cells were refed the same medium with 0.5% fetal calf serum.

Apoptosis was induced in cultured mouse hepatocytes by treatment with 0.5 μg/ml anti-Fas antibody and 0.05 μg/ml actinomycin D as described before (15). Where indicated hepatocytes were treated with AR 3 h prior to addition of anti-Fas antibody and actinomycin D. Apoptosis was estimated by the determination of soluble histone-DNA complexes using the Cell Death Detection Assay (Roche Applied Science). Cell death enzyme-linked immunosorbent assays were performed according to the manufacturer's instructions. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor) was calculated as the ratio between the absorbance values of the samples obtained from treated and control cells. The effect of AR on Fas-mediated apoptosis was also tested in the presence of the extracellular-regulated kinase kinase-1 (MEK1) inhibitor PD98059, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002, and the EGF-R inhibitor PD153035, all from Calbiochem. The protective effect of AR (20 nM) on Jo2- and actinomycin D-induced apoptosis in isolated mouse hepatocytes was also assessed by TUNEL staining. Treatments were performed as described above; subsequently, cells were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) for 30 min. The TUNEL assay was performed as recommended by the manufacturer (Roche Applied Science).

Measurement of Caspase-3 Activity—Caspase-3 activity was measured in mouse hepatocytes and liver tissue lysates using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Palo Alto, CA). Cells (5×10^5) were scraped in culture medium, pelleted, and resuspended in lysis buffer. Liver tissue was Dounce-homogenized in lysis buffer and centrifuged at 15,000 rpm for 10 min. Cell lysates and supernatants from liver homogenates (200 μg in 50 μl) were used to measure caspase-3 activity following the manufacturer's instructions.

Western Blot Analysis—Homogenates from liver samples and isolated hepatocytes were subjected to Western blot analysis as described (31). Antibodies used were: affinity-purified biotinylated anti-mouse AR polyclonal antibody (BAF989) (RD Systems), active caspase-3 p17 subunit (9664S), phosphorylated Akt (Ser^{473}) (9271S), and phosphorylated STAT3 (Tyr^{705}) (9131S) and phospho-JNK (9251) (Cell Signaling, Beverly, MA); ERK1/2 (06–182) and STAT3 (06–596) (Upstate Biotechnology, Charlottesville, VA). All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA): $Bclx_L$ (sc8392), phosphorylated ERK1/2 (Tyr^{204}) (sc7383), Akt (sc5298), and Fas (sc1023).

Statistical Analysis—Data are the means ± S.E. Statistical significance was estimated with the Mann-Whitney test. A p value of <0.05 was considered significant.

RESULTS

Expression of EGF-R Ligands in Mouse Liver after the Administration of the Fas-agonistic Antibody Jo2—The expression of the EGF-R ligands EGF, TGFα, HB-EGF, BTC, EPR, and AR was studied by real-time PCR in the liver of control mice and in mice that received a dose of Jo2 antibody previously shown to cause significant liver damage and apoptosis (15). Liver samples were taken for analysis 2, 5, and 10 h after Jo2 administration. As depicted in Fig. 1*A*, serum levels of transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were still normal 2 h after Jo2 injection, increased remarkably between 5 and 10 h of treatment, and decreased thereafter. As shown in Fig. 1*B* we observed different patterns of response to Jo2 treatment among EGF-R ligands. HB-EGF gene expression showed a slight reduction early after Jo2 injection, but remained close to control values for the rest of the study. TGFα gene expression showed a peak 2 h after Jo2 administration and was rapidly down-regulated, reaching values below control levels by 10 h of treatment. EGF gene expression did not change significantly early after Jo2 injection, but dropped below control values after 5 h of treatment. Similarly, BTC gene expression was significantly down-regulated between 2 and 5 h after Jo2 injection and remained so at the last time point tested. The two other EGF-R ligands examined, namely AR and EPR, showed a clear up-regulation in Jo2-treated mice. EPR gene expression was progressively induced, being maximal 10 h post-Jo2 administration, the latest time point tested. Interestingly, in agreement with other reports and our previous observations we noticed that AR gene expression was barely detectable in normal liver (35, 36); however, it was potently induced between 2 and 5 h after Jo2

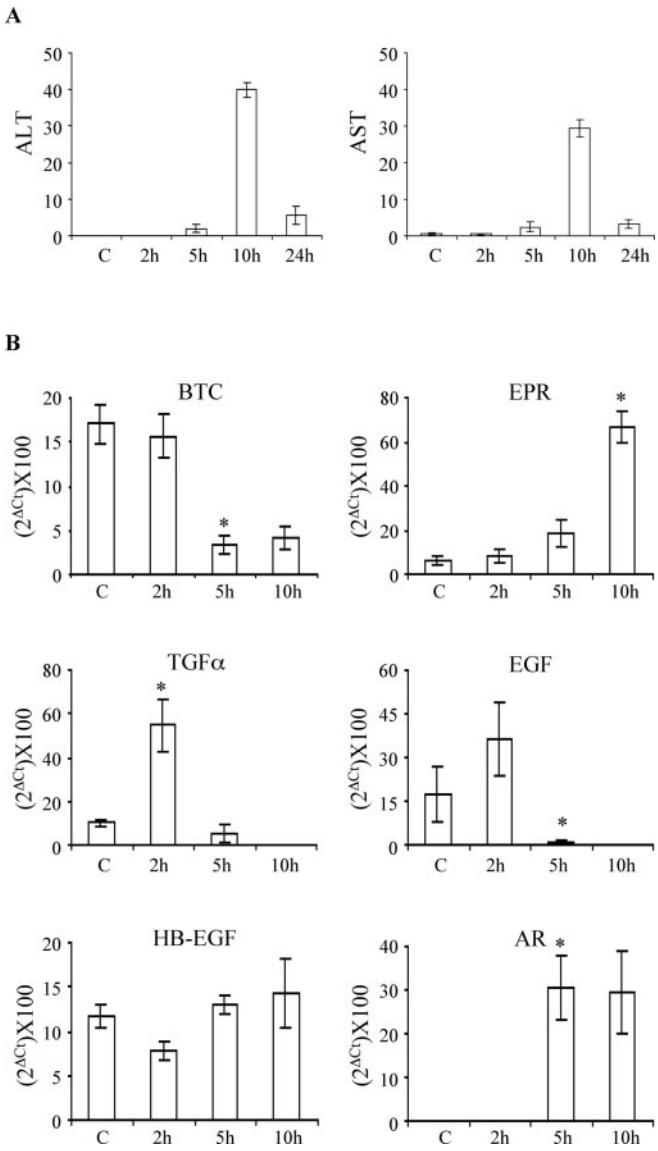

FIG. 1. *A*, serum levels of ALT and AST (10^{-2} IU/liter) at different time points after Jo2 antibody injection in mice. *B*, gene expression profiles of different EGF-R ligands in the liver of control mice (*C*), and at various times after Jo2 antibody injection as determined by real-time PCR ($n = 4$ animals per point). *BTC*, betacellulin; *EPR*, epiregulin; *TGFα*, transforming growth factor α; *EGF*, epidermal growth factor; *HB-EGF*, heparin-binding EGF; *AR*, amphiregulin. *Asterisks* indicate $p < 0.05$ *versus* control mice.

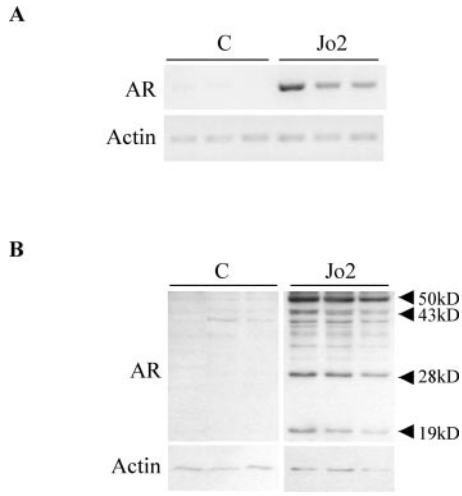

FIG. 2. *A*, *AR* gene expression was assessed by RT-PCR in the liver of control mice and in the liver of mice treated with Jo2 antibody for 5 h. *B*, Western blot analysis of liver extracts from control and Jo2-treated mice performed with a biotinylated affinity purified anti-AR antibody. Samples were obtained after 10 h of treatment with Jo2 antibody. *Arrowheads* indicate the different AR forms. Three representative samples are shown per group.

injection, preceding that of EPR. In concordance with the up-regulation of AR mRNA levels, Western blot analyses performed with a biotinylated affinity-purified anti-mouse AR antibody on liver samples obtained 10 after Jo2 antibody administration allowed us to detect a set of proteins that were present in the liver of treated mice (Fig. 2). Four bands of ~50, 43, 28, and 19 kDa are consistent with the different forms of AR described in epithelial cells (36). The 50- and 28-kDa bands likely represent membrane-anchored forms of AR, whereas the 43- and 19-kDa bands may be proteolytically processed soluble forms of AR (36). The fact that AR gene expression becomes induced during the early time period following Jo2 treatment suggested that AR may be protective against Fas-mediated apoptosis. This observation led us to evaluate the *in vivo* hepatoprotective effects of this EGF-R ligand.

AR Administration Attenuates Fas-mediated Acute Liver Injury in Mice—To test if AR can limit the extent of liver injury we examined the effect of AR administration in mice challenged with Jo2 antibody. Serum levels of ALT and AST were greatly elevated 10 h after Jo2 injection (Fig. 3*A*). AR treatment potently suppressed liver damage, as indicated by the reduced levels of serum transaminases and histopathological analysis of liver tissue sections from AR-treated mice, showing how this growth factor prevented the destruction of the parenchymal architecture induced by Fas ligation (Fig. 3*A*). As mentioned above, apoptotic cell death is a major determinant in Fas-mediated liver damage. To confirm that the hepatoprotective effects of AR against Fas-mediated liver injury derived from an anti-apoptotic action, we performed TUNEL staining and measured caspase-3 cleavage and activity in mouse liver extracts. TUNEL staining showed profuse apoptotic injury in Jo2-treated mice (Fig. 3*B*), which was confirmed by cleavage and activation of caspase-3 (Fig. 3, *C* and *D*). All these apoptosis-related events were significantly prevented by AR treatment (Fig. 3, *B–D*). Prevention of caspase-3 cleavage by AR suggests the specific blockade of Fas-induced apoptotic pathway by this growth factor (Fig. 3*D*). Bcl-2 family proteins inhibit apoptosis induced by variety of stimuli, including Fas-mediated apoptosis (37–39). We assessed the expression of the antiapoptotic protein Bcl-xL by Western blotting 10 h after the injection of anti-Fas antibody. Bcl-xL proteins levels were decreased in the liver of mice treated with Jo2; however, AR administration helped to maintain the Bcl-xL protein levels found in control mouse liver (Fig. 3*D*).

Interestingly, AR mRNA levels are also rapidly induced (between 2 and 3 h) in other models of hepatic injury such as acute CCl_4 or bacterial lipopolysaccharide administration (data not shown). CCl_4 causes necrosis and apoptosis in liver cells through the promotion of membrane lipid peroxidation and the production of high levels of tumor necrosis factor α (40, 41). The administration of AR 0.5 h prior and 12 h after CCl_4, significantly prevented the development of tissue injury and the rise in serum transaminases: from 9193 ± 908 IU/ml ALT in CCl_4-treated mice to 4688 ± 735 IU/ml ALT in CCl_4 plus AR-treated mice, $p < 0.05$. In the same animals AST levels were reduced from 8579 ± 684 to 4475 ± 773 IU/ml in AR-treated mice, $p < 0.05$.

Direct Antiapoptotic Effect of AR on Primary Cultured Hepatocytes—To determine whether the *in vivo* antiapoptotic effects of AR could be mediated by a direct action of AR on the liver

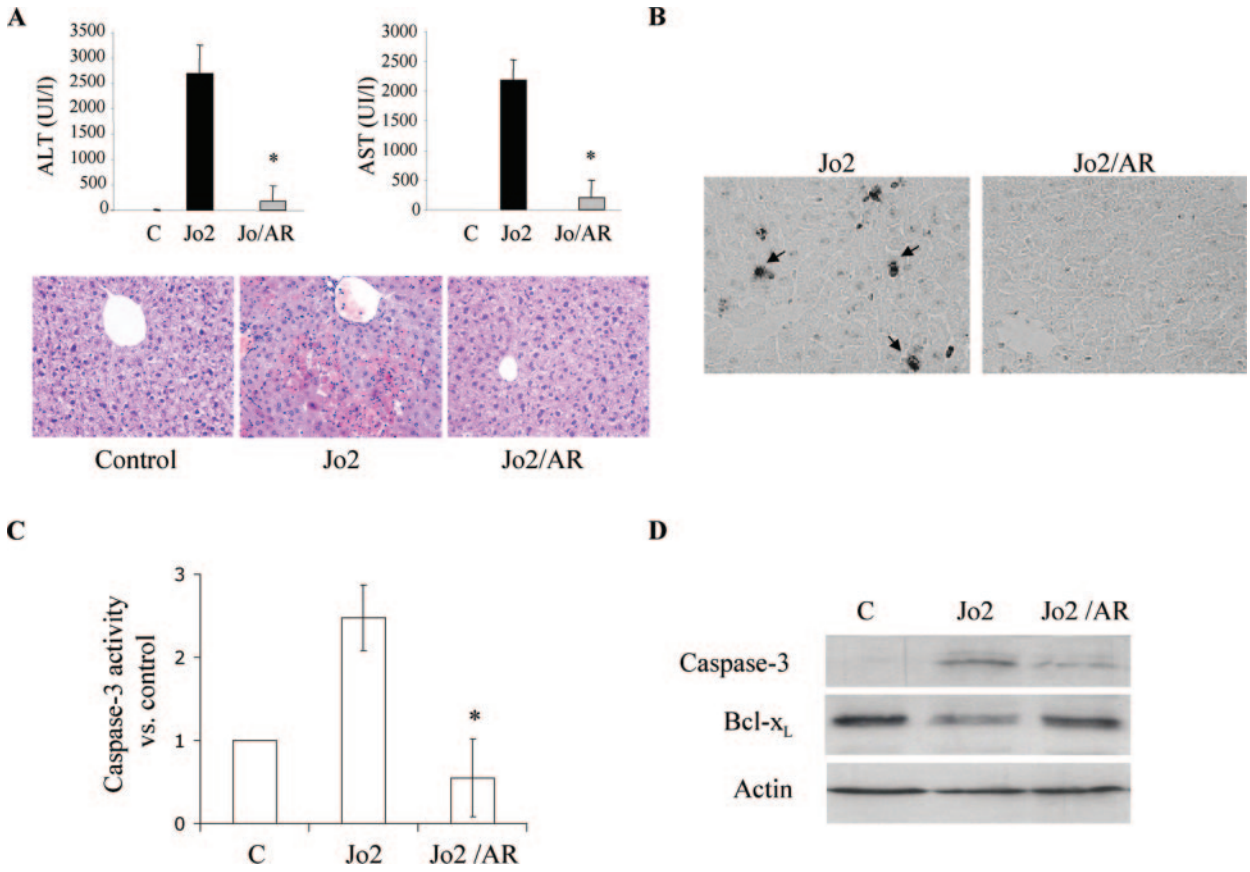

FIG. 3. **AR administration prevents Fas-induced acute liver damage.** *A*, effect of AR pretreatment on serum transaminases and liver histology in mice treated with Jo2 antibody (hematoxylin and eosin staining, original magnification, ×200). Sera and tissue samples were taken 10 h after Jo2 administration. Values are means ± S.E. of three experiments performed in triplicate. *, $p < 0.01$ *versus* Jo2 alone. *B*, TUNEL staining (*arrows*) of liver sections from mice treated with Jo2 or Jo2 plus AR as described in *panel A*. Representative samples are shown; original magnification, ×200. *C*, caspase-3 activity in mouse liver. Liver extracts were prepared 12 h after Jo2 injection. Where indicated mice were pretreated with AR. Values are means ± S.E. of three experiments performed in triplicate. *, $p < 0.01$ *versus* Jo2 alone. *D*, Western blot analysis of active caspase-3 p17 subunit and Bcl-xL proteins in control mouse liver ("*C* ") and in liver extracts obtained 10 h after Jo2 injection. Where indicated mice were pretreated with AR. Membranes were probed for actin as loading control. Representative blots are shown.

parenchymal cells, we used mouse hepatocytes in primary culture. It has been described that hepatocytes exposed to anti-Fas antibodies (Jo2) efficiently undergo apoptosis in the presence of actinomycin D (15, 18, 42). Hepatocytes were pretreated with different concentrations of AR for 3 h prior to actinomycin D and Jo2 antibody addition. Apoptosis, and apoptosis-related molecular events, were measured 18 h later. As shown in Fig. 4*A*, AR dose-dependently protected hepatocytes from apoptosis indicating a direct cytoprotective effect of AR in the prevention of Fas-mediated liver apoptosis. The antiapoptotic effect of AR was further demonstrated by TUNEL staining of isolated hepatocytes treated with Jo2 plus actinomycin D. As shown in Fig. 4*B* the number of TUNEL-positive cells was significantly reduced in AR-treated cultures. Interestingly, the cytoprotective activity of AR was also observed in apoptosis induced by other agents such as okadaic acid, TGFβ, and tumor necrosis factor α plus galactosamine (data not shown). In concordance with the antiapoptotic effect of AR, we observed that caspase-3 cleavage and activation induced by anti-Fas antibody were significantly inhibited by AR (Fig. 4*C*). In agreement with our previous observations performed *in vivo*, we found that, although the levels of the antiapoptotic protein Bcl-xL were reduced in hepatocytes undergoing apoptosis they were maintained in the presence of AR (Fig. 4*C*). On the other hand, Bcl-2 protein was not detected in any sample (data not shown).

To learn about the antiapoptotic signaling of AR, we examined the PI3K/Akt and ERK1/2 (extracellular-regulated kinase 1/2) pathways, general mediators of cell survival (24, 43–46). Mouse hepatocytes in culture treated with AR showed increased phosphorylation of Akt and ERK1/2 (Fig. 5*A*). Another key signaling molecule involved in the protection from apoptosis, including Fas-mediated apoptosis of murine hepatocytes, is signal transducer and activator of transcription 3 (STAT3) (24, 47). We observed that AR stimulated STAT3 phosphorylation (Tyr^{705}), an indicator of STAT3 activation (Fig. 5*A*).

The activation of the EGF-R by AR seems to be essential in the mediation of the antiapoptotic effect of this growth factor on Fas-induced cell death. This became evident when mouse hepatocytes were pretreated for 1 h with the EGF-R inhibitor PD153035 before addition of AR, and the protection afforded by AR was lost (Fig. 5*B*). Downstream of EGF-R, the activation of the PI3K/Akt pathway by AR was also necessary to prevent apoptosis, as demonstrated by the marked inhibitory effect of the PI3K inhibitor LY294002 (Fig. 5*B*). However, the MEK1 inhibitor PD98059 did not interfere with the antiapoptotic effect of AR (Fig. 5*B*).

Response of AR−/− Mice to Anti-Fas Antibody Administration—Once established the antiapoptotic effects of AR on liver parenchymal cells and to further define the role of AR expression in liver damage, we examined the response of *AR*−/− mice to Fas ligation. Unexpectedly, serum transaminases measured at different time points after Jo2 antibody administration were significantly higher in wild type (*AR*+/+) mice when compared with the knock-out littermates (*AR*−/−) (Fig. 6*A*). In agreement with the higher degree of liver injury found in *AR*+/+

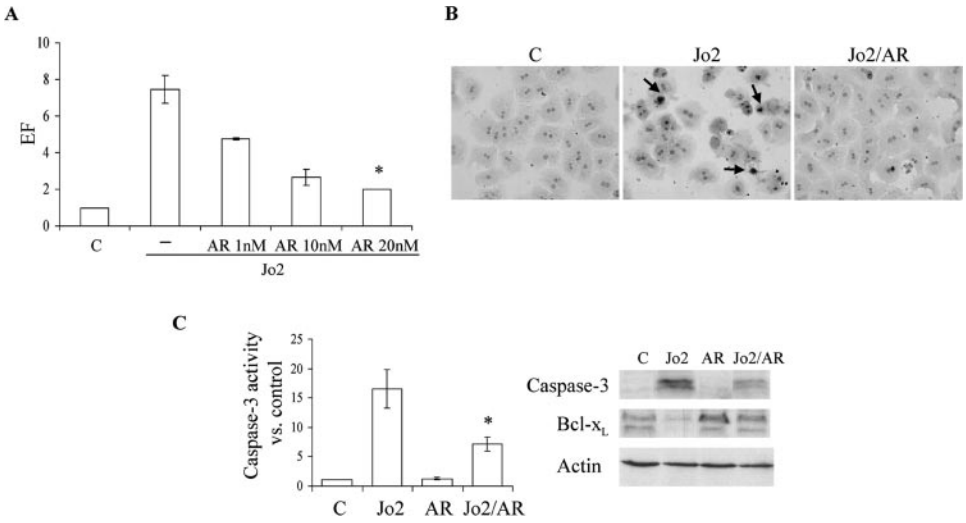

FIG. 4. **Antiapoptotic effect of AR on primary mouse hepatocytes.** *A*, apoptosis was induced in primary cultured mouse hepatocytes by actinomycin D and Jo2 treatment in the presence of increasing concentrations of AR. Apoptosis was assessed by measuring specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor (*EF*)), and was calculated as the ratio between the absorbance values obtained in treated samples and controls ("*C*"). Values are means ± S.E. of three experiments performed in triplicate. *, $p < 0.05$ *versus* Jo2. *B*, TUNEL staining (*arrows*) of isolated and cultured mouse hepatocytes in which apoptosis was induced by actinomycin D and Jo2 treatment in the presence or absence of AR (20 nM). Representative fields are shown; original magnification, ×200. *C*, effect of AR (20 nM) on caspase-3 activity in cultured mouse hepatocytes treated with actinomycin D and Jo2. Values are means ± S.E. of three experiments performed in triplicate. *, $p < 0.05$ *versus* cells treated with actinomycin D and Jo2. Representative Western blot analyses of active caspase-3 p17 subunit and Bcl-xL proteins are shown.

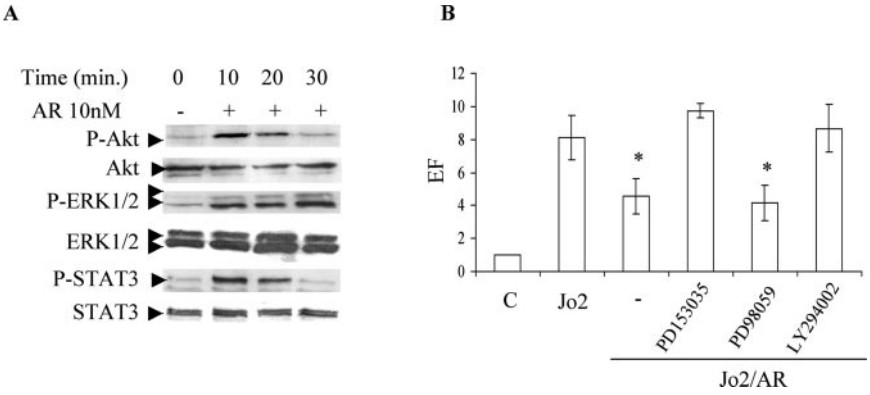

FIG. 5. *A*, activation of antiapoptotic signaling pathways by AR in cultured mouse hepatocytes. The phosphorylation state of Akt, ERK1/2, and STAT3 was assessed by Western blotting in extracts of mouse hepatocytes at different times after AR (10 nM) addition. Representative blots of three experiments performed in duplicate are shown. *B*, effect of AR (20 nM) on actinomycin D and Jo2-induced apoptosis in cultured mouse hepatocytes in the presence of the EGF-R inhibitor PD153035 (1 μM), the MEK1 inhibitor PD98059 (10 μM), or the PI3K inhibitor LY294002 (30 μM). Values are means ± S.E. of three experiments performed in triplicate. *, $p < 0.05$ *versus* Jo2.

mice, we also observed that the extent of caspase-3 activation 6 h after Jo2 administration, as determined by the appearance of its p17 cleavage product, was more pronounced in *AR*+/+ mice than in the *AR*−/− counterparts (Fig. 6*B*). In addition, the induction of stress-activated pathways, such as that triggered by Jun N-terminal kinase (JNK) in response to Fas ligation (48), was markedly blunted in *AR*−/− mice (Fig. 6*C*). The increased resistance to Jo2 cell death in these knock-out mice was not due to a decreased expression of Fas receptor in liver tissue (Fig. 6*D*). Interestingly, the reduced vulnerability to liver injury of *AR*−/− mice was not restricted to Fas-mediated liver damage, because it was also observed for other hepatotoxins such as CCl_4 (data not shown).

Although to a lesser extent than wild type mice, *AR*−/− mice are also vulnerable to liver damage-inducing agents. To further demonstrate the protective effects of AR in Fas-mediated liver injury, *AR*−/− mice were treated with AR and challenged with Jo2 antibody. As shown in Fig. 6*E*, AR treatment attenuated liver injury as judged by serum AST levels measured at 12 h after Jo2 administration. In concordance with the reduced AST levels, prevention of caspase-3 cleavage by AR was also observed (Fig. 6*F*). These observations suggest that exogenous AR can enhance the protective mechanisms that apparently operate in *AR*−/− mice.

Finally, we tested the response of *AR*+/+ and *AR*−/− mice to a lethal dose of Jo2 antibody (0.3 mg/kg, intraperitoneal) (n = 11 mice per genotype). Although we observed similar lethality for both strains as assessed 24 h after antibody injection (8/11 for *AR*+/+ and 9/11 for *AR*−/−), *AR*+/+ mice showed a survival advantage over *AR*−/− animals. Most (8/11) of the *AR*−/− mice that died in response to Jo2 challenge did so in the first 6 h post antibody injection, whereas by that time only one wild type mouse had died. These data suggest that the persistent liver injury observed in *AR*−/− mice, which can confer a certain degree of resistance to sublethal liver damage, does not afford enhanced survival in life-threatening liver injury.

Lack of AR Results in Chronic Liver Damage: Potential Mechanisms for Cell Death Resistance—Our primary observations showing the hepatoprotective potential of AR on Fas-mediated liver damage seemed to be at odds with the resistance

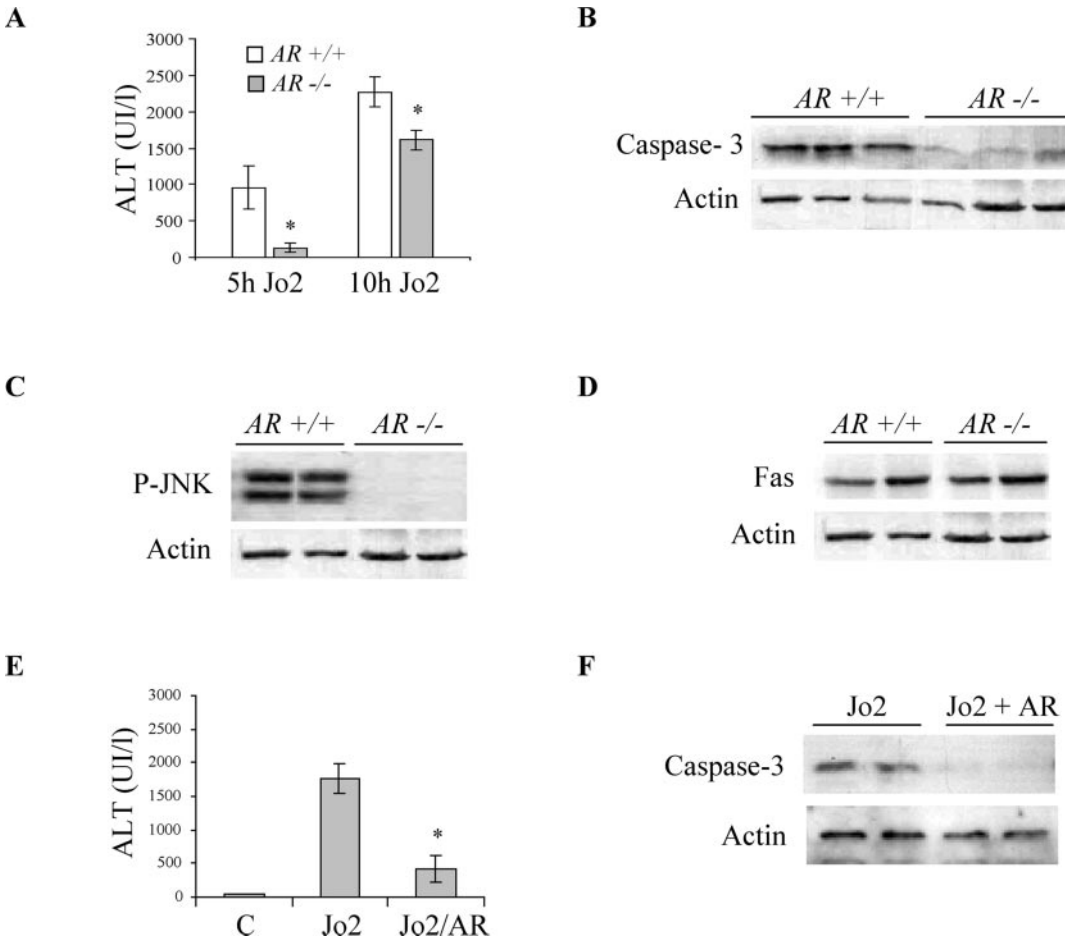

FIG. 6. **Response of *AR*−/− mice to acute liver damage induced by Fas ligation.** *A*, serum levels of ALT in wild type and *AR* knock-out mice at 5 and 10 h after Jo2 injection. *, $p < 0.05$ *versus* wild type mice. *B*, representative Western blot analysis of active caspase-3 p17 subunit in wild type and *AR* knock-out mice 5 h after Jo2 injection. *C*, phosphorylation of JNK in the liver of wild type and *AR* knock-out mice 5 h after Jo2 injection. *D*, expression levels of Fas receptor in wild type and *AR* knock-out mice. Representative blots are shown. *E*, effect of AR pretreatment on serum transaminases in *AR*−/− mice 12 h after Jo2 injection. Values are means ± S.E. of three experiments performed in triplicate. *, $p < 0.05$ *versus* Jo2 alone. *F*, Western blot detection of caspase-3 p17 cleavage product in the liver of *AR* knock-out mice 12 h after Jo2 injection and the effect of AR pretreatment. Representative blots are shown.

to apoptosis and necrosis-like cell death observed in *AR*−/− mice challenged with anti-Fas antibody or CCl_4. However, there is accumulating evidence showing that a pre-existing liver damage can precondition hepatocytes and result in a certain degree of cell death resistance. This phenomenon is widely recognized in the field of liver surgery and transplantation, where acute cell stress or injury induced by ischemic preconditioning can protect against subsequent reperfusion injury (49, 50). More recently, the protective effects of a pre-existing chronic liver disease have also been identified in a murine model of hereditary tyrosinemia (48). Considering this hypothesis, we went on to examine whether *AR*−/− mice displayed signs of chronic liver damage that might precondition hepatocytes against cell death. As shown in Fig. 7*A*, the basal levels of serum transaminases were significantly higher in *AR*−/− mice as compared with wild type animals. We also observed that the hepatic mRNA levels of cistathionine β-synthase and the modifier subunit of γ-glutamylcysteine synthetase, two enzymes that are induced under oxidative stress (34, 51), were up-regulated in AR null mice (Fig. 7*B*). Furthermore, analysis of caspase-3 activation, by detection of its p17 cleavage product, showed that mice lacking AR, but not wild type mice, displayed a basal activation of this apoptosis-related event (Fig. 7*C*). As previously mentioned, the STAT3 pathway is a major hepatoprotective effector activated in the liver as defense mechanism against apoptotic cell death (24, 47, 52). We observed that *AR*−/− mice showed enhanced levels of STAT3 phosphorylation (Tyr^{705}) in the liver (Fig. 7*D*), suggesting the existence of an endogenous protective response against the ongoing liver damage. Cytokines and growth factors with hepatoprotective activity such as IL-6, cardiotrophin-1, and EGF, are known to activate and likely mediate their survival effects through the STAT3 pathway (52–55). Because AR is a member of the EGF-R family of ligands, we first tested the expression of other EGF-R ligands in the liver of *AR*−/− mice that could compensate for the lack of AR and play a role in the resistance to cell death. We did not observe any significant differences in the basal expression levels of EGF, TGFα, EPR, HB-EGF, or BTC mRNAs between the two strains of mice (data not shown). Similarly the expression of the IL-6 family member cardiotrophin-1 was not different between AR wild type and knock-out mice (data not shown). However, the basal mRNA levels of IL-6 in the liver of *AR*−/− mice were significantly higher (2.5-fold) than in wild type mice (Fig. 8). IL-6 is a critical proregenerative factor and a major regulator of the acute phase response (52), its up-regulation in *AR*−/− mice may be indicative of an ongoing stress condition and may play an important role in the enhanced resistance of these mice to acute liver damage. In line with the essential functions of IL-6 we observed that its expression was rapidly and markedly induced in the liver of wild type mice shortly after the injection of Jo2 antibodies (Fig. 8). Interestingly, the up-regulation of IL-6 gene expression in the

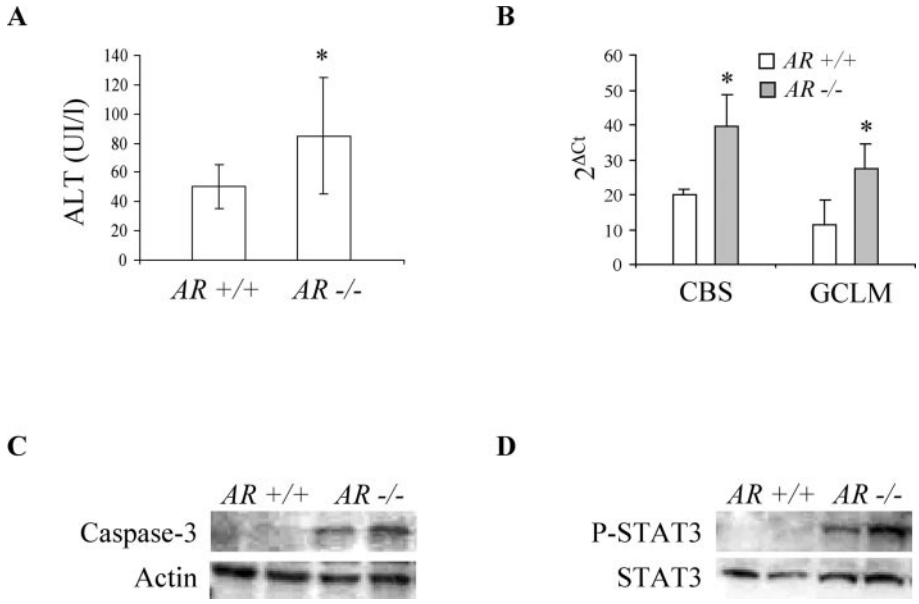

FIG. 7. ***AR−/− mice show signs of chronic liver injury.*** *A*, basal levels of ALT were elevated in AR null mice. *, $p < 0.05$. *B*, expression of cystathionine β-synthase and γ-glutamylcysteine synthetase genes in the liver of wild type and AR knock-out mice determined by quantitative real-time PCR. *, $p < 0.05$ with respect to *AR*+/+ mice. *C*, Western blot detection of caspase-3 p17 cleavage product in the liver of *AR* knock-out mice. *D*, basal levels of STAT3 Tyr^{705} phosphorylation in the livers of wild type and AR knock-out mice. Representative blots are shown.

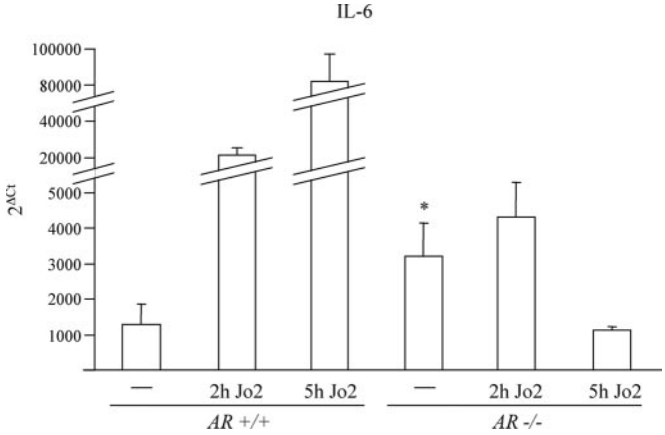

FIG. 8. **Expression of IL-6 mRNA in the liver of *AR*+/+ and *AR*−/− mice before and after the intraperitoneal injection of Jo2 antibody.** Expression levels were determined by quantitative real-time PCR. *, indicates $p < 0.05$ with respect to untreated *AR*+/+ mice.

liver of *AR*−/− mice after Jo2 administration was markedly blunted (Fig. 8). The attenuation of this protective response may be a consequence of the pre-existing hepatoprotective mechanisms triggered in AR knock-out mice.

DISCUSSION

The identification of endogenous protective mechanisms triggered upon liver injury is important not only for the study of the pathophysiology of this organ, but also for the design of more effective therapies that potentiate the natural defensive responses (13, 24, 56). The EGF-R is highly expressed in the hepatocyte and seems to be a relevant mediator of survival and proliferative responses (18–20, 25–27). Activation of the EGF-R by EGF administration or the overexpression of TGFα in transgenic mice has been shown to efficiently protect hepatocytes from apoptotic cell death (19, 21). However, the expression profiles of these and other EGF-R ligands during acute liver damage induced by Fas ligation have not been defined. In the present work we observed that the mRNA levels of TGFα and AR were up-regulated early after Fas-agonist antibody administration, suggesting that these factors may be protective against Fas-mediated apoptosis. Particularly noteworthy was the marked up-regulation of mRNA and protein levels of AR, a growth factor that in contrast to the other EGF-R ligands is barely detectable in the healthy normal human and rodent liver (35). Recently we have shown that the expression of AR is induced in chronic experimental liver damage and in human liver cirrhosis and is readily detected in rodent liver after partial hepatectomy (31). Moreover, AR seems necessary for adequate DNA synthesis during mouse liver regeneration (31). Now we observe that AR is rapidly induced in the mouse liver upon Fas-mediated acute liver damage.

The biological significance of AR up-regulation under these circumstances can be inferred from our experiments in which treatment with recombinant AR significantly prevented Fas-mediated hepatocellular death *in vivo*. Furthermore, the hepatoprotective effect of AR could be extended to acute liver damage mediated by CCl_4. We observed that AR administration blocked the Fas-mediated signal transduction pathway upstream of caspase-3 activation. This effect could be mediated in part by its ability to preserve the expression of the antiapoptotic protein Bcl-xL, which has been involved in the suppression of liver apoptosis driven by Fas antibody injection (14). Experiments conducted in isolated mouse hepatocytes evidenced that AR can directly interact with the parenchymal liver cells and trigger survival signals. We have recently shown that AR can induce the synthesis of DNA in isolated hepatocytes through the activation of the EGF-R (31), and our current *in vitro* experiments show that the activation of the EGF-R by AR is also necessary to convey its antiapoptotic effect. The ability to induce cellular proliferation is often correlated with the promotion of survival (24), and the downstream PI3K, ERK1/2, and STAT3 pathways have been shown to be major regulators of cell proliferation and survival in response to growth factors (24, 43–47, 57). Our data indicate that the antiapoptotic activity of AR on Fas-mediated cell death is independent of the ERK1/2 pathway but dependent of PI3K. Additionally, we also demonstrate that AR activates STAT3, a factor that might also be involved in the hepatoprotective effects of AR.

To gain further insight into the role of AR production during liver damage we made use of AR null mice (32). Unexpectedly, we found that *AR*−/− mice were clearly more resistant to liver damage induced by Fas ligation, and CCl_4 administration, than their wild type counterparts. These observations seemed to be at variance with our previous evidences showing that AR administration abrogated Fas-mediated cell death both *in vivo* and *in vitro* and prevented CCl_4-mediated liver damage. Enhanced resistance to liver injury has been described in animals

that undergo different forms of acute or chronic liver damage. Probably the best-characterized example of this response is the ischemic preconditioning in the prevention of ischemia-reperfusion liver injury (49, 50). The protective effect of sublethal doses of anti-Fas antibodies toward the lethal effects of higher doses of such antibodies has also been recognized (58). More recently the enhanced resistance of the chronically injured liver to subsequent damage has been substantiated in an experimental model of hereditary tyrosinemia (fumarylacetoacetate hydrolase-deficient, *FAH*−/−, mice) (48). Pre-existing chronic liver damage in these mice resulted in protection from ethanol, acetaminophen, and Fas-mediated liver injury and apoptosis (48). Interestingly, similar to what has been observed in the apoptosis-resistant *FAH*−/− mice (48), AR null mice displayed blunted activation of the apoptosis-associated kinase JNK upon Fas ligation. In light of these observations, we were prompted to consider the possibility that lack of AR could contribute to the generation of chronic liver damage that would precondition *AR*−/− mice against subsequent liver injury. The examination of different parameters indicative of liver damage such as serum transaminases and the expression of genes involved in adaptation to oxidative stress and caspase-3 activation suggested indeed that lack of AR may result in the development of mild chronic liver injury. This situation entails the triggering of a protective response in the liver of *AR*−/− mice, phenotypically manifested as the enhanced resistance to Fas- and CCl_4-mediated hepatotoxicity. Interestingly, this compensatory response seemed not to be mediated by other members of the EGF-R family of ligands, because their expression was not induced in the liver of *AR*−/− mice. Instead we found that the basal levels of IL-6 were significantly up-regulated in the liver of these animals and were not further induced upon Fas challenge as happened in wild type mice. The blunted induction of IL-6 expression in *AR*−/− mice after anti-Fas antibody administration may be explained by the pre-existing cell death resistance state. This condition would limit the extent of tissue damage and the concomitant endogenous protective response mediated in part by IL-6. Consistent with this we observed that the levels of STAT3 (Tyr^{705}) phosphorylation, implicated in the signaling of the hepatoprotective effects of IL-6 (52, 53), were enhanced in *AR*−/− mice. The activation of these protective mechanisms may also explain the blunted response of stress-activated pathways such as JNK upon Fas challenge in AR null mice (59, 60). Although at the present time we cannot exclude other mechanisms implicated in the enhanced resistance of *AR*−/− mice to hepatocellular damage besides IL-6 up-regulation, our current observations support the hypothesis formulated by Vogel *et al.* (48) that persistent liver injury may induce resistance to subsequent cell death.

Altogether our findings show that *AR* gene expression is rapidly and consistently induced in different models of liver injury, including the administration of bacterial lipopolysaccharide, suggesting that AR may be part of the primary defensive response of this organ. The response of AR to lipopolysaccharide is of particular relevance, because the liver is continuously exposed to endogenous endotoxins from the colonic Gram-negative flora that reach the portal circulation (61). It is thus possible that, among the potent defensive mechanisms that the liver has developed to resist intestine-derived noxious stimuli, AR might be one of them. In this context, an impaired induction of AR, as occurs in AR knock-out mice, may contribute to the development of the chronic liver damage observed in these animals. Interestingly, although the mild chronic liver injury that spontaneously develops in AR-deficient mice affords protection toward sublethal liver damage, these animals seem to be at a disadvantage when facing life-threatening Fas stimulation. These observations, together with the hepatoprotective effect of exogenous AR in *AR*−/− mice, further underscore the essential role of AR in the protection from liver injury. In summary, our present observations reveal a novel hepatoprotective role of AR and suggest that this factor may be of therapeutic value for patients with liver disease.

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