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Nrf2 Activates Augmenter of Liver Regeneration (ALR) via Antioxidant Response Element and Links Oxidative Stress to Liver Regeneration

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Liver regeneration can be impaired by permanent oxidative stress and activation of nuclear factor erythroid 2–related factor 2 (Nrf2), known to regulate the cellular antioxidant response, and has been shown to improve the process of liver regeneration. A variety of factors regulate hepatic tissue regeneration, among them augmenter of liver regeneration (ALR), attained great attention as being survival factors for the liver with proproliferative and antiapoptotic properties. Here we determined the Nrf2/ antioxidant response element (ARE) regulated expression of ALR and show ALR as a target gene of Nrf2 *in vitro* and *in vivo*. The ALR promoter comprises an ARE binding site and, therefore, ALR expression can be induced by ARE-activator tertiary butylhydroquinone (tBHQ) in hepatoma cells and primary human hepatocytes (PHH). Promoter activity and expression of ALR were enhanced after cotransfection of Nrf2 compared with control and dominant negative mutant of Nrf2. Performing partial hepatectomy in livers from $Nrf2^{+/+}$ mice compared with $Nrf2^{-/-}$ knock-out (KO) mice, we found increased expression of ALR in addition to known antioxidant ARE-regulated genes. Furthermore, we observed increased ALR expression in hepatitis B virus (HBV) compared with hepatitis C virus (HCV) positive hepatoma cells and PHH. Recently, it was demonstrated that HBV infection activates Nrf2 and, now, we add results showing increased ALR expression in liver samples from patients infected with HBV. ALR is regulated by Nrf2, acts as a liver regeneration and antioxidative protein and, therefore, links oxidative stress to hepatic regeneration to ensure survival of damaged cells.

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INTRODUCTION

The liver is frequently challenged by metabolic overload, lipid accumulation, viruses or toxins which induce the formation of radicals and other reactive oxygen species (ROS) (1,2) and may lead to cellular damage. Crucial players in the defense of oxidative stress are antioxidant proteins and enzymes detoxifying electrophiles and radicals. It is well known that expression of these cytoprotective proteins is controlled by *cis*-acting regulatory elements in the promoters of these genes, the antioxidant responsive elements (AREs) (3). ARE-driven genes include glutathione *S*-transferases (GSTs), NAD(P)H quinone oxidoreductase 1 (NQO1) and glutamate-cysteine ligase catalytic subunit (GCLC) (4,5). Furthermore, it has been shown that ARE-driven gene expression is regulated mainly by the basic leucine zipper transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) (6), an important player in the defense against oxidative stress and a multiorgan protector in oxidative stress-related diseases (7). Nrf2 is a member of the cap "n" collar family of transcription factors. In its inactive state, Nrf2 is associated with the actin-anchored protein Keap1 and localized in the cytoplasm. Activation of Nrf2 by electrophiles and radicals leads to dissociation of Nrf2 from Keap1 and to its translocation into the nucleus binding in partnership with other nuclear proteins to ARE sequences followed by activation of ARE-responsive genes (7).

Recently, Nrf2 has been shown to play a crucial role in liver regeneration after partial hepatectomy, since enhanced oxidative stress caused insulin/IGF resistance in Nrf2-deficient hepatocytes, thereby inhibiting efficient regeneration

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(8). Furthermore, it was found that Nrf2 protects the liver from acute and chronic toxin-mediated damage and therefore ameliorates fibrogenesis, which is in part due to Nrf2-regulated target genes in hepatocytes (9). Moreover, *in vitro* and *in vivo* studies have shown that hepatitis B virus (HBV) induces activation of Nrf2/ARE-regulated genes ensuring protection of HBV-infected cells against oxidative damage and survival of the infected cell (10). Interestingly, infection of hepatocytes with hepatitis C virus does not activate Nrf2 and inhibits ARE-regulated gene expression. (11).

Liver regeneration is a process regulated by a variety of molecules of which ALR gains great attention because hepatotrophic ALR supports the process of liver regeneration after partial hepatectomy (12,13), and exerts beneficial effects in models of hepatic failure (14,15) and liver fibrosis (16). ALR is a member of the ALR/Erv1 protein family with a flavin adenine dinucleotide (FAD)-linked sulfhydryl oxidase activity and is found extracellularly as well as within the cytoplasm and in the mitochondrial inner-membrane system (12,17,18). The protein ALR was identified to augment hepatocyte proliferation under conditions in which the regenerative process has already been primed and was therefore denoted as a comitogen (19,20). The proliferation augmenting the effect of ALR was attributed to its ability to activate epidermal growth factor (EGF) receptor phosphorylation and subsequent stimulation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk) cascade (21) as well as activate protein kinase B (Akt/PKB) through an EGF receptor independent pathway (22). Furthermore, ALR was described to have a functional role of a protective and antiapoptotic/cell survival factor in neuroblastoma (23) and hepatoma cells (24,25) after hydrogen peroxide treatment (23,25) or irradiation-induced oxidative stress (24). This was mainly due to the reduction of mitochondrial-mediated apoptosis characterized by ameliorated mitochondria membrane integrity, reduced cytochrome c release and enhanced ATP levels (23–25). In addition, downregulation of ALR expression using antisense oligonucleotide has demonstrated similar results in rat hepatocytes (26) and hepatoma cells (24) as well as a prosurvival role of ALR in the maintenance of murine embryonic stem cell pluripotency (27). Less is known about the regulation of ALR expression under conditions of oxidative stress. Microarray analyses using chemopreventive compounds indicate that Nrf2 regulates expression of cytoprotective proteins including ALR (gene: Gfer) leading to secondary protection against DNA or protein damage which enhances cell survival (28,29).

In this study, we analyze whether the Nrf2 is involved in the regulation of ALR expression and ALR might therefore be one of the factors causing cell protective properties induced by Nrf2. We examined induction of ALR expression upon activation of Nrf2 and verified the specific role of Nrf2/ARE using *in vitro* Nrf2 overexpression and a Nrf2 $^{-/-}$ mouse model of partial hepatectomy. Finally, we could demonstrate that HBV infection, known to induce Nrf2 activity followed by enhanced ARE-driven gene expression, increases ALR expression *in vitro* and in human HBV-positive liver tissues.

MATERIALS AND METHODS

Liver Samples and Primary Human Hepatocytes

Tissue samples from patients undergoing liver surgery at the University Medical Center Regensburg were used, including primary hepatocellular carcinoma and adjacent nontumorous tissue. Primary human hepatocytes (PHH) were isolated and cultivated as described recently (31). Briefly, nonneoplastic tissue samples from liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. PHHs were isolated using a modified two-step ethylene glycol tetraacetic acid (EGTA)/collagenase perfusion procedure and plated on collagen coated dishes. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research, Regensburg, Germany), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. All experiments involving human tissues and cells have been carried out in accordance to "WMA International Code of Medical Ethics" (30).

Cell Culture

Human hepatoma cell lines HepG2, HepG2.2.15 (10), Huh7.5 (11) and PLC (PLC/PRF/5 ATCC CRL-8024) were cultivated in DMEM/1% MEM (Invitrogen/Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), penicillin (100 units/mL), and streptomycin (10 μg/mL) and maintained at 37°C, 5% CO_2. HepG2 cells (2×10^5) were seeded and, after 24 h, cells were stimulated with 200 μmol/L tBHQ (tert-butylhydrochinone) for 24 h.

Promoter Constructs, Transfections and Luciferase Reporter Gene Assays

The 5′-flanking region of human ALR gene (gene ID 2671) residing from –733 to +240 bp (32) was amplified by PCR and inserted into pGL2-basic plasmid. HepG2 and Huh-7 cells (2×10^5) were transiently cotransfected with ALR promoter construct and caNrf2 (constitutively active Nrf2) or dnNrf2 (transdominant negative Nrf2) expression plasmids (0.5 μg each) (10), using the siPORT XP-1 method (Ambion/Life Technologies). Cells were lysed 24 h after transfection and the luciferase activity was determined. To determine the transfection efficiency, pRL-TKRenilla-vector was cotransfected and the promoterless vector pGL2-Basic served as negative control.

Nrf2$^{-/-}$ Mouse Model

Male Nrf2$^{-/-}$ mice in the C57BL/6 background were provided by M Yamamoto (University of Tsukuba, Japan) (33). As controls, age-matched male C57BL/6 mice were obtained from Charles River

Laboratories (Sulzfeld, Germany). Mice (8 to 10 wks old) were anesthetized by intraperitoneal (IP) injection of ketamine (100 mg/g bodyweight [bw])/xylazine (5 mg/g bw) and subjected to two-thirds partial hepatectomy as described previously (34). At different stages after injury, they were euthanized by CO_2 inhalation, and the remaining livers were harvested. For each time point studied, at least four animals were harvested. All experiments were approved by the local authorities.

Transgenic HBV Mouse Model

The HBV transgenic mouse model used in this study is based on a 1.3-fold HBV genome and has been initially described elsewhere (35). Liver samples were derived from 10–12-wk-old transgenic mice or the corresponding sex and age matched wild-type (WT) littermates.

HCV Replicating Cells and HBV-Infected Primary Human Hepatocytes

The plasmids pJFH1/J6 and pJFH1/J6_GND were described recently (36). *In vitro* transcription, electroporation of HCV RNAs and infection of Huh7.5 cells with HCV were performed as described (37). The transfection efficiency was up to 65%. RNA was isolated 72 h after electroporation. Primary human hepatocytes were infected with HepAD38-derived supernatant as described (38). Cells were harvested 8 d after infection.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from cultivated cells, mouse and human liver tissues using RNeasy kit (Qiagen, Hilden, Germany). Subsequently, first strand cDNA was synthesized using 1 μg of total RNA and virus-reverse transcription reaction (Promega, Madison, WI, USA). For cDNA synthesis of samples from HCV and HBV experiments, RNA isolation was performed using TRIzol (Invitrogen/Life Technologies) and 5 μg of total RNA were treated with DNase I followed by first-strand synthesis using SuperscriptII reverse transcriptase (Invitrogen/Life Technologies).

Quantification of mRNA Expression by Real-Time PCR

Transcript levels of NQO1, ALR, GSTα1 and 18S ribosomal RNA were quantified using the RT-PCR (LightCycler, Roche, Penzberg, Germany). Primers used to amplify human ALR were: forward 5′-CAC AAT GAA GTG AAC CGC AAG-3′, reverse 5′-CAC CCA ACT GAG ACA CAA CAG-3′, mouse ALR: forward 5′-CAC AGG ATC GGG AAG AAT TG-3′, reverse 5′-ATT CCT CGC AGG GGT AAA AC-3′, human NQO1: forward 5′-GCA CTG ATC GTA CTG GCT CA-3′, reverse 5′-GAA CAC TCG CTC AAA CCA G-3′, mouse NQO1: forward 5′-GGC TGC TTG GAG CAA AAT AG-3′, reverse 5′-TTC TCT GGC CGA TTC AGA G-3′, mouse GSTα1: forward 5′-CCG GAA GAT TTG GAA AAG C-3′, reverse 5′-TTT GGT GGC GAT GTA GTT GA-3′, 18S rRNA: forward 5′-GTA ACC CGT TGA ACC CCA TT-3′, reverse 5′-CCA TCC AAT CGG TAG TAG CG-3′. The PCR reaction was evaluated by melting curve analysis.

Western Blot Analysis

Total protein fractions (20 μg/ lane) were separated by 14% SDS-PAGE under reducing conditions using 100 mmol/L DTT. Proteins were transferred onto polyvinylidene fluoride membranes and incubated with anti-ALR (1:440), anti-β-actin (1:1000) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and anti-GAPDH (1:1000) (New England Biolabs GmbH, Frankfurt, Germany) antibodies, and developed with ECL reactions (Pierce, Rockford, IL, USA).

Immunohistochemistry

Immunostaining was performed as described recently (39). Briefly, sections of paraffin-embedded liver samples were deparaffinized and incubated with anti-ALR antibody (0.26 μg/mL) overnight at 4°C. Immunostaining was performed by incubation with secondary mouse anti-rabbit antibody (DAKO, Hamburg, Germany) for 1 h and development using APAAP complex (DAKO) and Fast Red Chromogen (Roche). Signal intensity of staining was defined as follows: 0, no staining; 1 weak staining; 2 when moderate staining; and 3, strong staining.

Statistical Analysis

Significant differences in paired and nonpaired samples were evaluated using Wilcoxon signed-rank *t* test and Mann-Whitney *U* test, respectively.

RESULTS

Human ALR Promoter Harbors an Antioxidant Response Element (ARE)

Previous studies exploring gene expression profiles induced by chemopreventive compounds such as phenethyl isothiocyanate (29) and sulforaphane (28), found enhanced ALR mRNA expression in WT ($Nrf2^{+/+}$) mice compared with KO ($Nrf2^{-/-}$) mice. This gene induction primarily occurs via transcription factor Nrf2 acting on ARE located at the 5′-flanking region of these genes. The ARE [5′-G(/A)TGAC(/G)NNNGCA(/C)-3′] motif is a *cis*-acting element governing the regulation of multiple antioxidant genes (3). To identify these potential ARE motifs in the ALR promoter, we examined the human ALR (gene ID 2671) genomic locus and revealed one putative ARE sequence in the human ALR promoter. This ARE sequence [5′-TGACCCGGCA-3′] is located in the proximal promoter region, from approximately –27 to –19 bp of the initial ATG codon. Furthermore, *in silico* analysis of the ALR promoter indicated that potential AREs [5′-TGGCCCCGCT-3′] also were found from –27 to –19 bp within the mouse and rat ALR promoter region.

Nrf2 Induces Promoter Activity and Expression of ALR

Chemopreventive agents and antioxidants such as tBHQ were shown to stimulate Nrf2, by enabling its release from Keap 1, followed by its translocation into the nucleus and heterodimerization with other transcription factors, such as Jun and small Maf (6). Therefore, we incubated HepG2 cells and PHH with tBHQ to activate Nrf2 and analyze if activated Nrf2 has an impact on ARE-

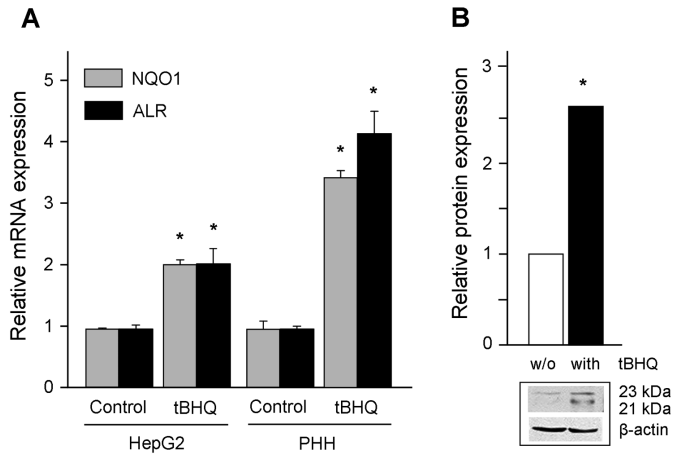


Figure 1. Specific Nrf2 inducer activates ALR expression. (A) mRNA expression of NQO1 and ALR was analyzed after stimulation with tBHQ (200 μmol/L, 24 h) in HepG2 and PHH by qRT-PCR. Untreated cells served as control and 18S ribosomal RNA expression was determined for normalization (two independent experiments in triplicates, mean ± SD). (B) ALR protein expression in cellular lysates of HepG2 cells stimulated without or with tBHQ (200 μmol/L, 24 h) was analyzed by Western blotting followed by densitometry. **P* < 0.05 differs from corresponding control.

regulated gene expression. In HepG2 cells and PHH we observed enhanced mRNA expression of ALR and NQO1 after tBHQ treatment, the latter gene known to be regulated by Nrf2 (Figure 1A). Induced ALR expression after tBHQ treatment was confirmed for ALR protein expression by Western blotting (Figure 1B). Furthermore, to investigate the involvement of Nrf2 activation in ARE-regulated ALR expression, we cotransfected HepG2 and Huh-7 cells with an ARE harboring luciferase reporter construct (–733 to +240) of ALR and constitutively active (caNrf2) or transdominant negative mutant of Nrf2 (dnNrf2) expression plasmid. The reporter gene assay demonstrates that expression of caNrf2 induced and expression of dnNrf2 slightly reduced ALR promoter activity (Figure 2A). In addition, quantitative mRNA analysis after transfection of HepG2 and Huh-7 cells with caNrf2 or dnNrf2 revealed increased or unchanged expression, respectively, of ALR and NQO1 (Figure 2B). These results could be confirmed for ALR protein expression by Western blotting (Figure 2C). Taken together, these data demonstrate that Nrf2 induces expression of ARE-regulated genes including ALR *in vitro*, by activation with antioxidants leading to enhanced promoter activity, mRNA and protein expression.

ALR Expression Is Diminished in Livers of Hepatectomized Nrf2$^{-/-}$ Mice

Recently, Nrf2 was demonstrated to play an essential role in liver regeneration, since Nrf2-deficient mice show significantly delayed liver regeneration after partial hepatectomy (8). To investigate whether ALR expression is dependent on Nrf2 activation *in vivo* under circumstance of liver regeneration, we used a two-thirds-partial-hepatectomy mouse model applied to Nrf2 WT and Nrf2$^{-/-}$ mice. Expression of NQO1 and GSTα mRNA, both genes being regulated by Nrf2, is reduced significantly in Nrf2$^{-/-}$ compared with Nrf2 WT mice at 0, 6, 10, 24 h and 0, 6, 10 h after hepatectomy, respectively (Figures 3A, B). ALR mRNA expression is significantly abolished at 0 and 10 h after hepatectomy in Nrf2$^{-/-}$ compared with Nrf2 WT mice (Figures 3C, D). This finding could be confirmed for ALR protein expression by Western blotting (see Figure 3D) with reduced ALR levels in Nrf2$^{-/-}$ mice compared with Nrf2 WT at 0 and 10 h after hepatectomy. These data show that activation of Nrf2 is

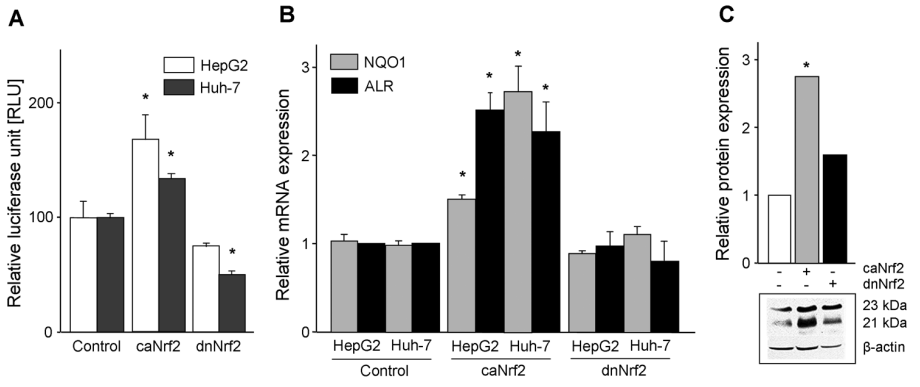


Figure 2. Nrf2 expression induces ALR expression. (A) For reporter-gene assay HepG2 and Huh-7 cells were cotransfected with luciferase-reporter construct harboring ALR promoter (–733 to +240) with putative ARE sequence and caNrf2 or dnNrf2 expression plasmids (all 0.5 μg each). Cotransfection with pcDNA3 vector served as control and was set 1 (three independent experiments in triplicates, mean ± SEM). (B) For mRNA expression analysis, HepG2 and Huh-7 cells were transfected with caNrf2 or dnNrf2 expression plasmid (1 μg, 24 h) and mRNA expression of ALR as well as NQO1 were analyzed by quantitative RT-PCR. Transfection with pcDNA3 vector served as control and was set 1. 18S ribosomal RNA expression was determined for normalization (two independent experiments in triplicates, mean ± SEM). (C) Western blot analysis of cellular lysates of HepG2 cells transfected with caNrf2 or dnNrf2 expression plasmids was performed followed by densitometric analysis of 21- and 23-kDa ALR band. **P* < 0.05 differs from corresponding control.

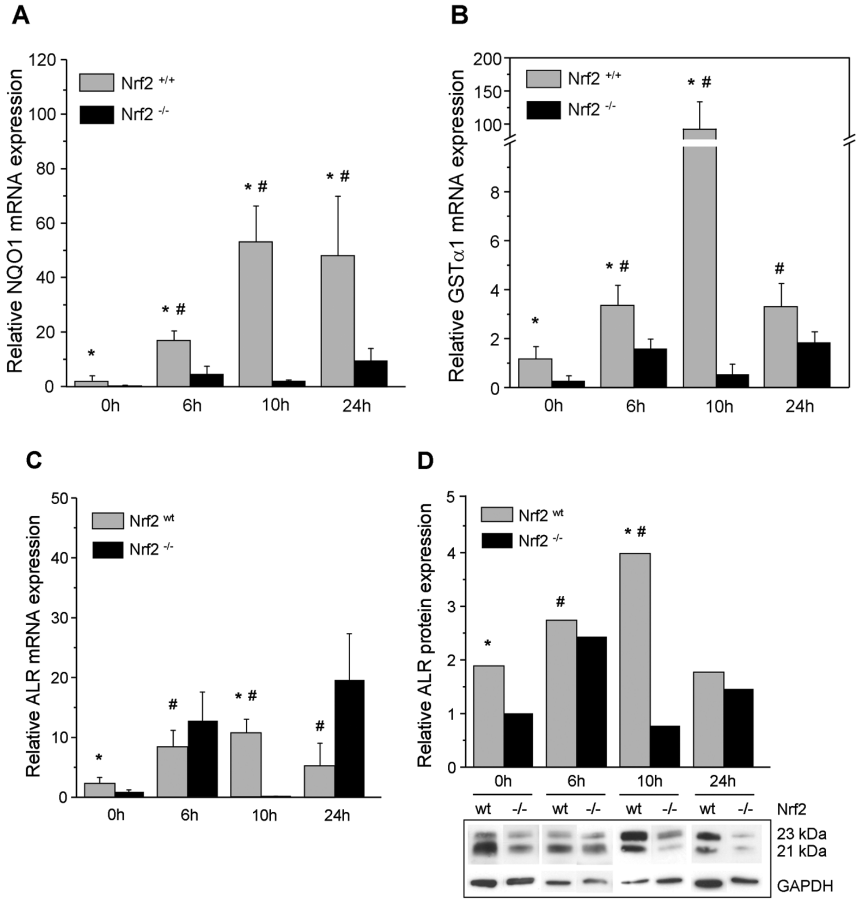


Figure 3. Reduced expression of ARE-regulated genes in Nrf2$^{-/-}$ mice after partial hepatectomy. A two-thirds partial hepatectomy using Nrf2 WT or Nrf2$^{-/-}$ mice was performed inducing liver regeneration. Liver tissue samples were taken at indicated time points after surgery. (A–C) mRNA expression of NQO1 (A) and GST (B), and ALR (C) was determined by quantitative RT-PCR analysis. 18S ribosomal RNA expression was measured for normalization (four independent experiments, mean ± SEM). (D) Western blot analysis of liver tissue homogenates from Nrf2 WT and Nrf2$^{-/-}$ mice after two-thirds partial hepatectomy was performed followed by densitometric analysis of 21- and 23-kDa ALR band at the indicated time points (one of four mice are presented). *P < 0,05 Nrf2 WT differs from Nrf2$^{-/-}$; #P < 0.05 differs from 0 h.

mandatory for expression of ARE-regulated genes including, at least in part, ALR, under normal conditions and circumstances of liver regeneration.

ALR Expression Is Enhanced in HBV-Positive Cells

Activation of Nrf2 followed by expression of ARE-regulated genes was not only shown for chemopreventive and antioxidant agents, but also reported to be mediated by hepatitis B virus (HBV) (10), while hepatitis C virus (HCV) inhibits Nrf2 activation (11). Therefore, we analyzed the potential impact of HBV and HCV infection on ALR expression *in vitro* and *in vivo*. Expression of ALR mRNA is increased in cells expressing HBV specific proteins (after infection) as demonstrated by qRT-PCR analysis of HBV-positive stable cell lines HepG2.2.15 and PLC compared with HBV-negative HepG2 cells and PHH (Figure 4A). Specificity of HBV-mediated activation of NQO1 expression in HBV-positive stable cell lines was shown previously (10). Furthermore, ALR mRNA expression is significantly enhanced 8 d post infection in HBV-infected versus noninfected PHH (Figure 4B), while no difference in ALR expression was observed in HCV-positive (replicating Huh7.5 cells) compared with HCV-negative cells (replication-deficient Huh7.5 cells). Additionally, ALR protein expression is increased in HBV-positive liver tissue homogenates from HBV transgenic mice compared with WT mice demonstrated by Western blot analysis (Figure 4C).

Moreover, analysis of liver tissue samples from patients with HCV and HBV infection for ALR mRNA expression by RT-qPCR reveals enhanced ALR expression in HBV-positive versus HCV-positive liver tissues (Figure 5A). Liver samples were nontumorous tissues with signs of cirrhosis, fibrosis and inflammation. Furthermore, we performed immunohistochemical analysis of hepatocellular carcinoma (HCC) samples and nontumorous liver samples adjacent to HCC and found ALR expression solely in hepatocytes and cholangiocytes as described earlier (Figure 5C) (48). ALR immunoreactivity was analyzed using an intensity score and revealed enhanced ALR protein expression in nontumorous liver tissue from patients with HBV infection compared with liver tissue from patients without HBV infection or alcohol intoxication (Figures 5B, C). Interestingly, we could not find a correlation of ALR expression in the corresponding adjacent HCC samples.

DISCUSSION

In this study, for the first time, we show that expression of protective and antiapoptotic protein ALR is regulated by activation of the antioxidant Nrf2/ARE system. We demonstrate that overexpression of constitutive active Nrf2 resulted in enhanced expression of an ARE specific gene, NQO1, as well as ALR (Figure 2). Furthermore, a typical inducer of ARE driven genes, tBHQ, confirmed these observations by increasing mRNA and protein expression of NQO1 and ALR (Figure 1). It is widely accepted

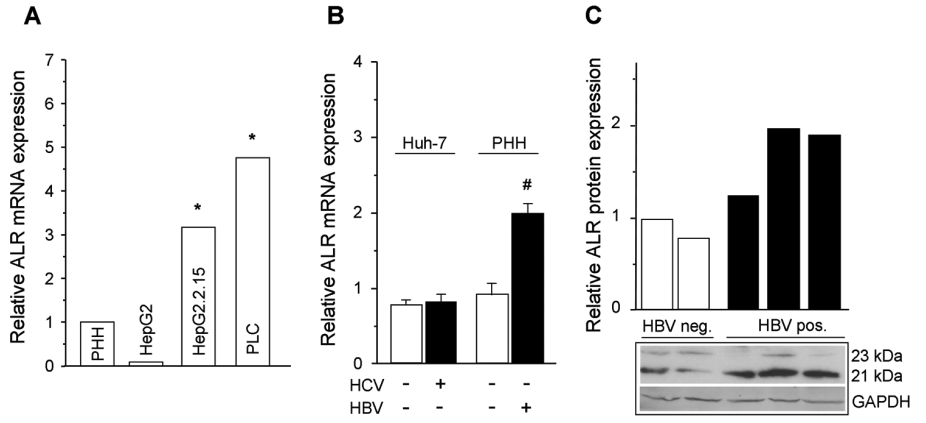


Figure 4. HBV infection, known to induce Nrf2 activity, enhances ALR expression. (A) ALR mRNA expression in HBV-positive hepatoma cell lines (HepG2.2.15, PLC) compared with noninfected PHH and HepG2 cells was analyzed by qRT-PCR. 18S ribosomal RNA expression was used for normalization. *$P < 0.05$ differs from PHH and HepG2. (B) Huh7.5 cells were transfected with HCV replicon pJFH/J6 and replication-deficient construct pJFH/J6_GND (served as control) as well as PHHs were infected with HBV or not. HCV replicating cells were analyzed 72 h after electroporation, the HBV-infected PHH 8 d after infection for ALR mRNA expression. 18S ribosomal RNA expression was used for normalization (mean ± SEM). #$P < 0.05$ differs from noninfected PHH. C) Western blot analysis of liver tissue homogenates derived from HBV-transgenic mice or the corresponding WT animals were performed followed by densitometric analysis of 21- and 23-kDa ALR band.

that enhanced oxidative stress is associated with increased Nrf2 activity and, as a consequence, upregulated antioxidant gene expression. Within this study we present some evidence that ALR, in addition to known antioxidant genes, is a protein regulated in response to Nrf2 activation to address cellular oxidative stress. This is underlined by observations that ALR reduces cell damage and apoptosis upon oxidative stress (13,24) and also improves renal function by reducing the extent of tubular injury in a renal ischemia/reperfusion model in which injury is known to be mediated by oxidative stress (40).

Performing an ALR promoter analysis, we found the presence of a *cis*-acting regulatory element ARE in the 5′-flanking region of ALR gene. Interestingly, it was reported that the ALR 5′-flanking region contains a core promoter (–66/ –1 bp) proximal of the ALR start codon, which showed an initiatorlike element with three tandem repeats (41). This promoter sequence, able to activate the ALR promoter, harbors the so far unrecognized ARE element (–27/–19 bp) which is located between the initiatorlike element and the initial start codon of the ALR gene. It would be of interest if these two elements act in a combinatorial manner to regulate ALR expression and if this might explain different expression profiles. Furthermore, the ALR promoter region revealed putative binding sites for AP1/AP4 (42), SP1 and HNF-4α (43), all of them located upstream of –49 bp from the transcriptional start site of the ALR gene. Recently, we found additional putative binding sites in an intronic ALR promoter and could confirm binding of Foxa2 (HNF-3β) to this ALR promoter region (+276/+282 bp) (32). Subsequently, ALR promoter was activated and ALR expression increased, which could be amplified by IL-6 treatment (32). Therefore, Foxa2 or IL-6, in addition to Nrf2, might have an impact on expression of ALR after partial hepatectomy in mice (Figure 3C), since it has been reported that Foxa2 is upregulated consistently during liver regeneration (44) and IL-6 was shown to be slightly induced after injury in Nrf2 WT and KO mice (8). Furthermore, this might explain the not fully blunted ALR expression in the Nrf2 KO model at 6 and 10 h post hepatectomy (Figure 3C). In addition, the observed increase in ALR expression in HCC samples despite of different underlying etiology (Figure 5B), might only in part be dependent on Nrf2, but also on Foxa2 activity. Foxa2 was reported to be upregulated in livers from patients with HCC, suggesting a potential role in tumorigenesis (45).

The roles of Nrf2 in toxin detoxification and cancer prevention have been well established, and a novel function of Nrf2 in liver regeneration was demonstrated recently. Deficiency in this transcription factor resulted in enhanced oxidative stress in the normal and particularly in the injured liver due to reduced expression of ROS-detoxifying enzymes (8). This was confirmed in our study and, in addition, we found diminished ALR expression levels in normal and hepatectomized livers (Figure 3). Furthermore, under pathological conditions, besides hepatocyte proliferation, enhanced hepatocyte apoptosis can occur after hepatectomy (46). Using the Nrf2 KO model, the number of apoptotic cells after hepatectomy increased severalfold compared with WT controls (8). This indicates enhanced ROS-mediated apoptosis and that Nrf2 regulated gene expression can abate this process. ALR is one of these Nrf2 regulated genes able to reduce apoptosis, which is supported by a recent report demonstrating that ALR administration induces antiapoptotic gene expression, inhibits hepatocyte apoptosis and reduces ROS-induced cell damage in a rat model of partial hepatectomy (13). Enhanced oxidative stress in the Nrf2 KO model is responsible for an impairment of insulin/IGF signaling and subsequent inhibition of PI3K-Akt pathway, explaining increased apoptosis and reduced liver regeneration (8). Therefore, ALR supports liver regeneration by reducing intracellular-generated ROS, enabling activation of survival and proliferation signaling through PI3K-Akt pathway by growth factors or even by ALR itself (22).

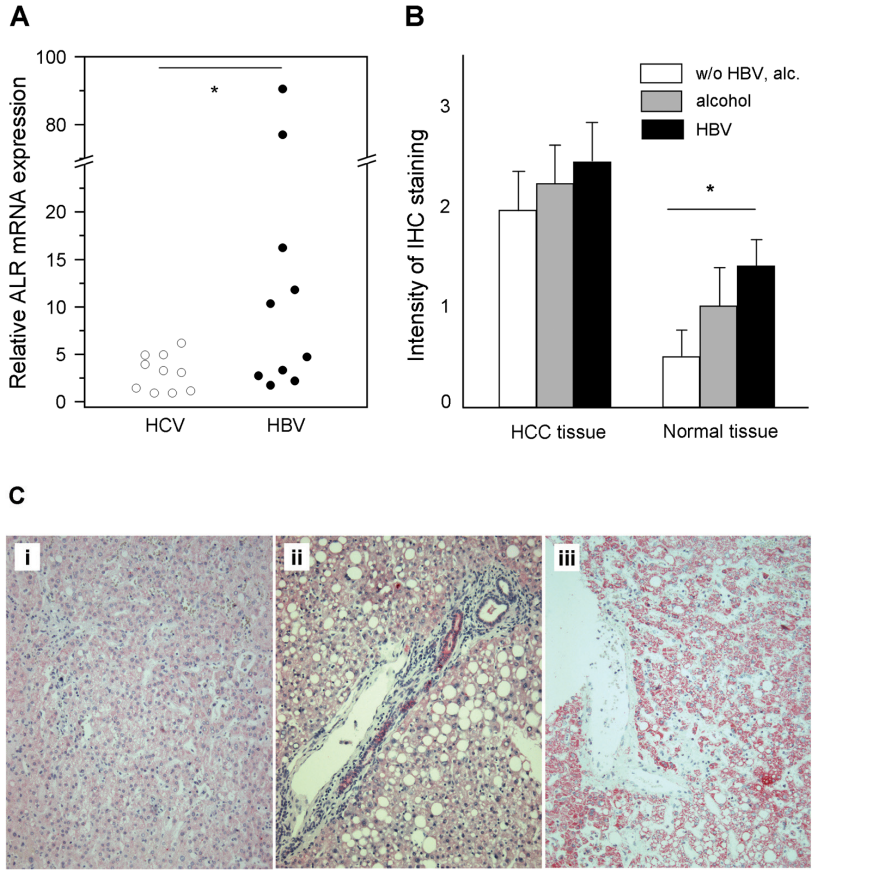


Figure 5. Increased ALR expression in HBV-positive liver tissue. (A) ALR mRNA expression in liver tissue samples from patients with positive HCV (n = 10) or HBV (n = 10) infection was analyzed by quantitative RT-PCR analysis. 18S ribosomal RNA expression was measured for normalization, $*P < 0.05$. (B) Sections of liver tissue samples from patients with HCC and nontumorous adjacent liver tissue were analyzed for ALR immunoreactivity. Etiology of hepatocellular carcinoma (HCC) samples: without (w/o) HBV infection and no alcohol n = 12, alcohol n = 14, HBV infection n = 7; nontumorous liver tissue adjacent to HCC: without (w/o) HBV infection and no alcohol n = 5, alcohol n = 4, HBV infection n = 8. Intensity of anti-ALR staining was categorized from 0 to 3 (values are declared as mean ± SEM). $*P < 0.05$ HBV differs from w/o HBV, alc. (C) ALR protein immunoreactivity in nontumorous liver tissue sections adjacent to HCC as described in (B), (i) without (w/o) HBV infection and no alcohol, (ii) alcohol intoxication and iii) HBV infection. ALR expression was detected in the majority of hepatocytes (i–iii) and in cholangiocytes (ii), but was negative in nonparenchymal liver cells (48). (i–iii, magnification 250×).

In a previous report, we have shown that HBV induces a strong activation of a variety of cytoprotective genes that are Nrf2/ARE-regulated (10). In line with this observation, we found ALR expression induced in HBV-infected, but not in HCV-infected, cells *in vitro* and *in vivo* (Figures 4, 5), which is in agreement with some recent findings reporting a HCV-dependent inhibition of Nrf2/ARE-regulated genes (11). In addition, it was reported that Nrf2 protects from toxin-induced liver injury and fibrosis (9), which seems to be contradictory to chronic HBV infection as a causative factor for liver fibrosis, cirrhosis or HCC. Permanent activation of the inflammatory process after chronic infection is characterized by increased ROS levels. Induction of Nrf2/ARE-regulated genes, such as ALR, by HBV protects HBV-positive cells and thereby ensures viral replication. It would be intriguing to know how a loss in Nrf2 activation may affect HBV-associated pathogenesis in liver diseases. Furthermore, in HCCs, overexpression of ARE-regulated genes was found to be advantageous to these tumors (47). Enhanced ALR expression in hepatic tumors was reported earlier (39,48), but a correlation of increased expression of ALR with HBV-associated HCCs could not be confirmed. The overexpression of many proteins, which function as antioxidants and detoxifying enzymes, is likely to protect the tumor from elimination by mechanisms that are based on increased electrophiles or radicals and therefore count for chemoresistance and tumor promotion (47).

CONCLUSION

In summary, we present evidence that ALR is one of the ARE-regulated genes, which can be induced by Nrf2 activation. Therefore, ALR expression as a consequence of a functional ARE/Nrf2 system accounts for efficient liver regeneration and protection of cells from oxidative stress, for example, after HBV infection. Furthermore, based on the crucial role of Nrf2 in the protection of hepatocytes from acetaminophen (7), ethanol (49) and bile acid (50) toxicity, we hypothesize a beneficial effect of ALR on liver damage caused by these noxious effects and postulate a functional role of ALR in the pathogenesis of steatosis, NASH and fibrosis.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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