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Early growth response-1 attenuates liver injury and promotes hepatoprotection after carbon tetrachloride exposure in mice

Michele T. Pritchard^{1,*}, Jessica I. Cohen^{1,3}, Sanjoy Roychowdhury¹, Brian T. Pratt¹, and Laura E. Nagy^{1,2,3}

¹Department of Pathobiology, Cleveland Clinic, Cleveland OH, 44195

²Department of Gastroenterology, Cleveland Clinic, Cleveland OH, 44195

³Department of Nutrition, Case Western Reserve University, Cleveland OH 44106

Abstract

Background & Aims—Inflammatory gene expression plays a pathological role in acute and chronic hepatic inflammation, yet, inflammation also promotes liver repair by inducing protective mechanisms to limit collateral tissue damage by priming hepatocytes for proliferation. Early growth response (Egr)-1, a transcription factor that regulates inflammatory gene expression, plays a pathological role in many animal models of acute and chronic inflammatory disease. Here we tested the hypothesis that Egr-1 is beneficial after toxic liver injury.

Methods—Acute liver injury was induced in wild-type and *egr-1*^{-/-} mice by a single injection of carbon tetrachloride (CCl₄). Liver injury, inflammatory, and hepatoprotective gene expression and signaling events were measured 18, 48, and 72h after CCl₄ administration.

Results—Peak liver injury was greater in *egr-1*^{-/-} mice compared to wild-type mice. Enhanced injury in *egr-1*^{-/-} mice was associated with reduced tumor necrosis factor (TNF) α mRNA and protein expression, reduced Akt phosphorylation and nuclear localization of NF κ B-p65 in nuclei of cells in the hepatic sinusoid. Expression of inducible nitric oxide synthase and cyclooxygenase-2, TNF α -regulated genes that have hepatoprotective function, was attenuated in *egr-1*^{-/-} mice compared to wild-type mice. Although plasma interleukin (IL)-6 protein and hepatic accumulation of IL-6, glycoprotein 130, and IL-6 receptor α mRNA in wild-type and *egr-1*^{-/-} mice were equivalent, signal transducer and activator of transcription 3 phosphorylation was attenuated in *egr-1*^{-/-} mice and associated with reduced oncostatin M expression.

Conclusions—In contrast to its role in inflammation-mediated tissue injury in other models, Egr-1 expression promotes protection in the liver after CCl₄ exposure.

Keywords

Early growth response-1; inflammation; hepatoprotection; carbon tetrachloride

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*Corresponding author: Department of Pathobiology – NE40 Cleveland Clinic 9500 Euclid Avenue Cleveland, OH 44195. Tel.: 216-444-8613; Fax: 216-636-1493, pritchm@ccf.org (M.T. Pritchard).

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Introduction

In response to injury, the liver is rapidly repaired to reinstate normal hepatic function and ensure survival of the affected organism; a process for which evolution has afforded a profoundly effective regenerative response. The initial response to acute liver injury, mediated by alcohol abuse, drug overdose, hepatitis virus infection or trauma, includes an increase in pro-inflammatory mediators including lipopolysaccharide (LPS), C3a, C5a, tumor necrosis factor (TNF) α , and interleukin (IL)-6. If these pro-inflammatory signals are inappropriately controlled, injury is enhanced and inflammatory disease can result. However, these signals also promote hepatoprotection. For example, inflammation-mediated induction of the cell cycle in hepatocytes by partial hepatectomy promotes protection against subsequent carbon tetrachloride (CCl₄)-mediated toxic liver injury [13] while inhibition of mitosis by colchicine exacerbates thioacetamide-induced hepatotoxicity in rats [16]. Therefore, signaling pathways activated early in the time course of liver repair serve to induce hepatocyte proliferation, as well as promote hepatoprotection.

Early growth response (Egr)-1 is an immediate early gene that encodes an 80–82kDa transcription factor induced by various growth factors, LPS, hypoxia, and reactive oxygen species (ROS) in many tissues [8,21]. In addition to its requirement for luteinizing hormone β production and female fertility in mice [14], Egr-1 also contributes to the over-expression of TNF α , and many other proinflammatory genes, in animal models of acute and chronic disease where it contributes to disease pathology [21]. By contrast, Egr-1 also contributes to the expression of inflammatory genes involved in tissue repair [29]. Indeed, TNF α is a cytokine of critical importance to liver repair after partial hepatectomy or CCl₄-induced liver injury; deficiency of TNF α or TNF α signaling is detrimental in these model systems [5,28]. Therefore, depending on the context in which Egr-1 is produced and whether or not it promotes appropriate (repair) or exaggerated (acute or chronic disease) expression of inflammatory mediators (i.e. TNF α), its expression and activity could be detrimental or beneficial.

The hepatic response to injury is exquisitely regulated; up and downregulation of inflammatory cytokines and growth factors occurs in a precise and step-wise progression. Misregulation in amplitude or timing has deleterious effects [5,28]. Egr-1 is an important regulator of TNF α . However, Egr-1's expression pattern and its role in CCl₄-induced inflammation and inflammation-mediated hepatoprotective events is not known. Because TNF α is important for hepatoprotection and liver repair after CCl₄ exposure, we hypothesized that Egr-1 expression was required for this process. We tested this hypothesis after induction of acute liver injury with a single exposure of CCl₄ in wild-type and *egr-1*^{-/-} mice.

Materials and Methods

Materials

CCl₄ and olive oil were purchased from Sigma-Aldrich (St. Louis, MO). All primers for real-time reverse transcription PCR (real-time PCR) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primary antibodies were purchased from the following companies: Cytochrome P450 2E1 (CYP2E1) Research Diagnostics, Inc. (Flanders, NJ, USA); Egr-1, NF κ B-p65, phospho (Ser473)-Akt, and phospho (Tyr705)-STAT3, Cell Signaling Technology (Danvers, MA, USA); tErk Upstate (Temecula, CA, USA).

Animals

Female, wild-type (C57BL/6NTac) or *egr-1*^{-/-} (B6.129-*Egr1*^{tm1Jmi} N12) mice (8–12 weeks old) were purchased from Taconic Farms (Germantown, NY) and were originally developed by J. Milbrandt [14]. Animals were housed in standard microisolator cages and fed standard

laboratory chow. All animals received humane care and all procedures were approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

CCl₄ administration and sample collection

CCl₄ was prediluted 1:3 in olive oil before administration. Mice received a single dose at 1 µl/g body weight of CCl₄ administered by intraperitoneal injection using 100 µl Hamilton syringes fitted with 26G 5/8 inch needles. Mice were anesthetized and blood was drawn from the posterior vena cava 18, 48, and 72 hours (h) after CCl₄. Plasma was separated from whole blood by centrifugation. After euthanasia, livers were removed, portions of which were fixed in 10% neutral buffered formalin, preserved in RNAlater (Ambion, Austin, TX) or snap frozen in liquid nitrogen for further analysis.

Liver histology, plasma alanine aminotransferase (ALT), and aspartate aminotransferase activities

For histological analysis, formalin-fixed tissues were paraffin-embedded, sectioned (5 µm) and stained with hematoxylin and eosin. Slides were coded prior to examination and viewed by two separate individuals. Images were captured using an Olympus microscope and digital camera. Plasma samples were assayed for ALT and AST activity using Diagnostic Chemicals Ltd. enzymatic assay kits (Oxford, CT) according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

Apoptotic hepatic DNA fragmentation was detected by TUNEL using the ApopTag Plus fluorescence *in situ* apoptosis detection kit (Millipore, Temecula, CA) and fluorescence quantified as described earlier by Pritchard *et al.* [22].

Cytokine analysis by real-time PCR and enzyme-linked immunosorbent assay (ELISA)

Total RNA was isolated from liver and reverse transcribed as previously described [17]. Real-time PCR amplification was performed using gene-specific primers (Table 1 in Supplemental Data) as previously described [17]. The relative amount mRNA was determined using the comparative threshold (Ct) method by normalizing target cDNA Ct values to that of 18S. Fold induction ratios were calculated relative to olive oil treated controls (basal conditions) for each genotype using the formula: $2^{-\Delta\Delta Ct}$. Statistics were performed on ΔCt values. ELISAs for plasma TNF α and IL-6 were performed as described in [22].

Liver homogenate preparation, electrophoresis, and immunoblotting

Liver homogenates were prepared and immunoblotting performed, as previously described [22].

Statistical analysis

Values reported are means \pm standard error of the mean (SEM). Due to the limited number of age-matched *egr-1*^{-/-} mice, data were collected from several different experiments. The data were analyzed by general linear models procedure (SAS, Carey, NC) followed by least square means analysis of differences between groups, blocking for experiment effects when data from more than one experiment was used in any given data set. Data were log transformed to obtain a normal distribution, if necessary. In all cases, values in graphs with different alphabetical superscripts are significantly different from one another ($p < 0.05$).

Results

Egr-1 deficiency exacerbates liver injury induced by acute CCl₄ exposure

Egr-1 deficiency attenuates disease pathology in many model systems [21]. However, here we report that liver injury after a single injection of CCl₄ was enhanced, not reduced, in *egr-1*^{-/-} mice compared to wild-type mice. Pericentral necrosis was present in livers from both wild-type and *egr-1*^{-/-} mice, but was greater in *egr-1*^{-/-} mice 72h after CCl₄ administration (Fig. 1A). Peak plasma ALT and AST activities, biochemical markers of liver injury, 48h after CCl₄ were greater in plasma from *egr-1*^{-/-} mice compared to wild-type (Fig. 1B and C). ALT and AST activities in *egr-1*^{-/-} mice were also greater than wild-type 72h after CCl₄ administration, but were reduced relative to peak liver injury (Fig. 1B and C). Seventy-two hours after CCl₄, there was an increase in TUNEL-positive cells; this was two-fold greater in livers from *egr-1*^{-/-} mice compared to wild-type mice (Fig. 2). Differences in liver injury between wild-type and *egr-1*^{-/-} mice were not due to altered expression of cytochrome P450 2E1 (CYP2E1), the enzyme responsible for the bioactivation of CCl₄ [33] (Supplemental Data Fig. 1).

Hepatic Egr-1, TNF α , and IL-6 expression after acute CCl₄ administration

TNF α and IL-6 are required for normal hepatoprotection and liver repair after CCl₄ exposure [5,28]. Egr-1 transcriptionally regulates the expression of TNF α in monocytic cells [34]. After a single exposure to CCl₄, hepatic Egr-1 mRNA expression in mice was induced in wild-type mice as early as 1h, peaked at 2h and then decreased below baseline at 18h (Fig. 3A). After CCl₄ administration, Egr-1 protein was expressed in hepatocytes and non-parenchymal cells in the hepatic sinusoid (NPC-HS), presumably Kupffer cells, the resident macrophage in the liver. In NPC-HS, nuclear Egr-1 protein was detected at 1h, peaked at 2h, was reduced 4h after CCl₄ exposure, nearly absent at 8h but increased again at 18h after CCl₄ (Fig. 3B). Egr-1 expression was detected in hepatocyte nuclei at 1h, peaked at 4h, declined at 8h and was absent at 18h after CCl₄ exposure (Fig. 3B). Interestingly, Egr-1 protein was first found in the nuclei of hepatocytes immediately surrounding the portal vein (1h), but by 4h, staining was restricted only to hepatocyte nuclei in the pericentral area (Fig. 3B).

In wild-type mice, CCl₄-induced expression of TNF α mRNA was first detected 8h after CCl₄ administration (Fig. 3C). TNF α was further increased to 6-fold over olive oil controls 18h after exposure (Fig. 3C). Hepatic IL-6 mRNA accumulation was detected prior to hepatic TNF α mRNA expression, 1h after CCl₄ exposure in wild-type mice (Fig. 3D). IL-6 mRNA expression remained elevated over at least 18h in wild-type mice (Fig. 3D).

If Egr-1 is required for TNF α expression after CCl₄, then TNF α expression should be reduced in *egr-1*^{-/-} mice. Eighteen hours after CCl₄, hepatic TNF α mRNA and protein was increased in wild-type mice and *egr-1*^{-/-} mice, but this increase was less in *egr-1*^{-/-} mice (Fig. 4A). In contrast, hepatic IL-6 mRNA and plasma IL-6 protein were not different between mouse strains (Fig. 4C and D). These data support the hypothesis that Egr-1 is an important regulator of TNF α , but not IL-6, expression after acute CCl₄ exposure in mice.

Akt and STAT3 phosphorylation and nuclear NF κ B-p65 localization were reduced in *egr-1*^{-/-} mice after CCl₄ exposure

Signaling events mediated by TNF α , IL-6, and their receptor complexes are among the critical pathways required for hepatocyte priming and hepatoprotection after CCl₄-induced acute injury [28]. Deficiencies in TNF α and tumor necrosis factor (TNFR)1 are associated with reduced nuclear factor κ B (NF κ B) activity and Akt phosphorylation while deficiencies in IL-6 and gp130 are associated with reduced signal transducer and activator of transcription (STAT) 3 and Akt phosphorylation after surgical or toxin-induced liver injury [5,28]. If Egr-1 promotes

hepatoprotection and cell survival during the priming phase of liver repair after CCl₄-induced liver injury through a TNF α -mediated pathway, then markers of NF κ B activation and phosphorylation of Akt should be attenuated in livers of *egr-1*^{-/-} mice. Conversely, STAT3 phosphorylation, largely associated with IL-6 signaling [5,28], should be equivalent in wild-type and *egr-1*^{-/-} mice due to their equivalent expression of IL-6. NF κ B-p65 (p65) translocated to the nuclei of NPC-HS 18h after CCl₄; fewer nuclei were positive in *egr-1*^{-/-} mice (Fig. 5A); p65 was not found in hepatocyte nuclei of either mouse strain at this time point (Fig. 5A). Increased Akt phosphorylation was detected 18h after CCl₄ in wild-type mice compared to olive oil treated controls (Fig. 5C and D). In *egr-1*^{-/-} mice, phosphorylation of Akt was blunted relative to wild-type in both olive oil controls and 18h after CCl₄ exposure and was not significantly increased in response to CCl₄ (Fig. 5C and D). Despite equivalent IL-6 expression between the genotypes, STAT3 phosphorylation was increased only in livers from wild-type mice 18h after CCl₄ administration; STAT3 phosphorylation in *egr-1*^{-/-} mice remained at baseline (Fig. 5E and F). These data suggested that p65 nuclear translocation in NPC-HS as well as phosphorylation of Akt and STAT3 are required to attenuate liver injury in mice after acute CCl₄ administration.

iNOS and COX-2 expression were attenuated in *egr-1*^{-/-} mice after CCl₄ administration

TNF α contributes to the expression of the inducible form of nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 [26,30]. In addition, both iNOS and COX-2 are induced after CCl₄-administration and have well-documented, but divergent, hepatoprotective functions after toxic liver injury or partial hepatectomy [1,4,24,30]. If iNOS and COX-2 expression is dependent on TNF α , then expression of these genes should be attenuated in *egr-1*^{-/-} mice. Hepatic accumulation of iNOS mRNA was induced in both strains of mice 18h after CCl₄ administration, but was lower in *egr-1*^{-/-} mice (Fig. 6A). Similarly, COX-2 mRNA was induced in livers from wild-type and *egr-1*^{-/-} mice, but this induction was attenuated in livers from *egr-1*^{-/-} mice (Fig. 6B).

Reduced expression of IL-6 receptor components or other IL-6 family members that, similar to IL-6, promote STAT3 activation could be responsible for reduced STAT3 phosphorylation in *egr-1*^{-/-} mice, despite normal IL-6 expression. While glycoprotein (gp)130, the receptor chain shared by all IL-6 family members [11], and IL-6R α (gp80), the IL-6-specific receptor component [11], were increased over baseline 18h after CCl₄ exposure, they did not differ between wild-type and *egr-1*^{-/-} mice (Fig. 6C, D). Recently, a hepatoprotective role for the IL-6 family member, oncostatin m (OSM), was described [20]. Hepatic mRNA accumulation of OSM was robustly induced in wild-type mice; however, its induction was attenuated in livers from *egr-1*^{-/-} mice (Fig. 6E). The expression of the specific OSM receptor (OSMR) was induced but not different between the genotypes 18h after CCl₄ exposure (Fig. 6F). Collectively, these data suggested that reduced expression of the hepatoprotective molecules iNOS, COX-2, and OSM may contribute to increased liver injury after CCl₄ exposure in *egr-1*^{-/-} mice.

Discussion

This is the first study to identify a critical role for Egr-1 in hepatoprotection early in the response to CCl₄-induced liver injury in mice. In addition, we have identified differential expression patterns of Egr-1 in hepatocytes vs NPC-HS in response to CCl₄. Reduced expression of TNF α , iNOS, COX-2, and OSM, but not IL-6, was observed in Egr-1-deficient mice. Furthermore, in the absence of Egr-1, phosphorylation of Akt and STAT3, and NF κ B-p65 nuclear localization in NPC-HS were reduced. The end result of this maladaptive response to CCl₄ in livers from *egr-1*^{-/-} mice was enhanced peak liver injury at 48h after CCl₄ compared to wild-type mice (Fig. 1). The more severe hepatic injury phenotype observed in *egr-1*^{-/-}

mice, concomitant with reduced expression of hepatoprotective molecules and their downstream signals, provides evidence that Egr-1 is an important regulator of hepatoprotective pathways after CCl₄-induced acute liver injury.

We discovered a specific spatial and temporal pattern of Egr-1 expression in hepatocytes in response to CCl₄ exposure. Nuclear Egr-1 protein occurred in a transient wave across the liver lobule. It was first observed in periportal (zone 1) hepatocytes, then zone 2 hepatocytes, then pericentral hepatocytes (zone 3); Egr-1 expression disappeared thereafter (Fig. 3). It is likely that priming events, mediated in part by a CCl₄-induced increase in LPS in the portal circulation after CCl₄ exposure, played a role in Egr-1 expression and nuclear localization in periportal hepatocytes. These signaling events may then provide initial Egr-1-mediated hepatoprotective signals after CCl₄ exposure. However, CYP2E1-mediated CCl₄ bioactivation and ROS generation, which occurs in zone 3, likely contribute to robust Egr-1 protein expression and nuclear localization in this area of the liver lobule.

Egr-1 in NPC-HS nuclei, most likely Kupffer cells, was detectable over the entire time course, suggesting that the major changes in Egr-1 mRNA accumulation in total liver reflected changes in Egr-1 expression in hepatocytes (Fig. 3). Early priming events orchestrated by gut-derived products in the portal area may provide the Kupffer cells with critical activation signals to induce synthesis of TNF α . Egr-1 directly contributes to LPS-induced TNF α expression in macrophages [34]. Here, we found that Egr-1 also contributed to TNF α expression in response to CCl₄. In wild-type mice, sufficient TNF α likely induces hepatoprotective mechanisms in periportal (zone 1) hepatocytes and the more distant, zone 2 hepatocytes, limiting CCl₄-induced liver injury. However, in *egr-1*^{-/-} mice, TNF α is reduced and these hepatoprotective mechanisms are attenuated and instead associated with expansion of liver injury (Fig. 1 and 2).

NF κ B DNA binding activity is induced after partial hepatectomy and after CCl₄ exposure. Early NF κ B-DNA binding activity is demonstrable by EMSA using nuclear extracts prepared from hepatocyte-enriched fractions of rat liver 30 min after partial hepatectomy [6]; this is not observed at later time points. More recent literature demonstrates that it is translocation of p65 to the nuclei of NPC-HS which is required for hepatocyte regeneration after partial hepatectomy [3]. We found that p65 nuclear localization in NPC-HS at 18h was reduced in *egr-1*^{-/-} mice relative to wild-type mice; we were unable to detect p65 in hepatocyte nuclei at any time point tested [Fig. 5 and data not shown (1, 2, 4, 8, 36, 48, 72h)]. We propose that reduced TNF α protein in *egr-1*^{-/-} mice 18h after CCl₄ exposure is responsible for reduced p65 nuclear localization in NPC-HS, as TNF α signaling promotes p65 nuclear localization [28].

Despite the well-described roles for TNF α and signaling via TNFR1 for hepatocyte proliferation [28], the mechanisms for TNF α -mediated hepatoprotection are not well understood. One potential downstream target of TNF α is iNOS [25]. Indeed, hepatic expression of iNOS is critical for liver repair in various liver injury models [1,4,24,35]. iNOS produces nitric oxide (NO) from L-arginine; NO is associated with a shift from hepatocyte quiescence to proliferation during the priming phase of liver regeneration [4,24,35]. Induction of proliferative competence in hepatocytes provides a protective function which attenuates persistent liver injury [18]. iNOS/NO can also reduce TNF α expression [19] and protect hepatocytes from TNF α -mediated apoptotic cell death after CCl₄ exposure in mice [24]. Collectively, these reports provide evidence for a critical hepatoprotective and proliferative role for iNOS/NO and suggest mechanisms by which reduced iNOS observed in this study could contribute to enhanced liver injury in *egr-1*^{-/-} mice.

Another target downstream of TNF α expression is COX-2. COX-2 is the inducible COX isoform with known contributions to inflammatory processes [26]. Like iNOS, COX-2 is induced by proinflammatory cytokines including TNF α and IFN γ with roles for NF κ B and C/EBP β in this process [12,32]. Despite the potency of COX-2-mediated inflammation that spurred the wide-spread use of COX-2 inhibitors, the beneficial effects of COX-2 function cannot be overlooked. Indeed, COX-2 is implicated in hepatoprotection in lipopolysaccharide/D-galactosamine, CCl $_4$, acetaminophen, and Concanavalin-A-induced liver injury models and after partial hepatectomy. COX-2 is required to mitigate the proinflammatory production of arachidonic acid by soluble phospholipase (sPL) A2 released from dead and dying hepatocytes after CCl $_4$ -induced toxic liver injury [2]. The hepatoprotective effect of COX-2 is due to its synthesis of prostaglandins from arachidonic acid [1,23,35]. Prostaglandins reduce TNF α expression, improve resistance to apoptotic and necrotic cell death and promote cell cycle progression [1,2,12].

Despite the eventual expression of TNF α in *egr-1*^{-/-} mice, iNOS and COX2 were never induced to levels comparable to wild-type mice (Supplementary Data Fig. 3A and B). Therefore, delayed, but robust, TNF α expression in *egr-1*^{-/-} mice likely contributed to increased tissue injury due to the continued absence of NO and prostaglandins to reduce apoptotic cell death as described above. Increased TUNEL-positive hepatic nuclei in *egr-1*^{-/-} mice 72h after CCl $_4$ support this hypothesis (Fig. 2).

IL-6-mediated signaling is implicated in hepatoprotection in many systems [7,27,31]. Reduced STAT3 phosphorylation was associated only with reduced OSM, an IL-6 family member [9, 20]; CCl $_4$ -induced IL-6, gp130, IL-6R α , and OSMR were not different between wild-type and *egr-1*^{-/-} mice. Indeed, OSM exhibits hepatoprotective function and mediates this function, in part, through STAT3 signaling. For example, *OSMR*^{-/-} mice exhibit impaired regeneration and persistent necrosis after both CCl $_4$ and partial hepatectomy and is associated with reduced STAT3 activation [20]. In addition, OSM gene therapy in rats exposed to dimethylnitrosamine, a potent hepatotoxin, reduces hepatic necrosis, apoptosis, and liver enzyme release and enhances hepatocyte proliferation [10]. Although GC-rich sequences typical of an Egr-1-dependent promoter element exist in the OSM promoter [15], a direct role for Egr-1 in the regulation of OSM is not known. Our data linking reduced OSM expression with reduced STAT3 phosphorylation and hepatoprotection and also with increased necrosis and apoptosis in *egr-1*^{-/-} mice after CCl $_4$ are consistent with these studies but do not preclude potential for other STAT3-activating cytokines such as EGF or amphiregulin in this process [5].

Evolution has afforded the liver several apparently redundant pathways for hepatoprotection and repair after injury. This redundancy is exemplified by studies in which mice deficient in single cytokines, cytokine receptors, or signaling molecules are exposed to hepatotoxins or subjected to partial hepatectomy but recover, albeit with a delay in liver repair processes [28]. Here, we identify CCl $_4$ -induced Egr-1 expression as a very early event in response to liver injury, before TNF α expression but concomitant with IL-6 expression, which places it at a critical point to affect the regulation of several genes and/or pathways involved in hepatoprotection during the liver repair process after CCl $_4$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

LPS	lipopolysaccharide
TNF α	tumor necrosis factor α

IL-6	interleukin 6
CCl ₄	carbon tetrachloride
Egr-1	early growth response 1
CYP2E1	cytochrome P450 2E1
NFκB-p65	nuclear factor κB p65 subunit
ROS	reactive oxygen species
h	hours
ALT	alanine aminotransferase
AST	aspartate aminotransferase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
Ct	threshold cycle
ELISA	enzyme linked immunosorbent assay
SEM	standard error of the mean
STAT3	signal transducer and activator of transcription 3
iNOS	inducible form of nitric oxide synthase
COX-2	cyclooxygenase 2
gp130	glycoprotein 130 kDa
OSM	oncostatin M
OSMR	oncostatin M receptor
TNFR1	tumor necrosis factor receptor 1
NO	nitric oxide

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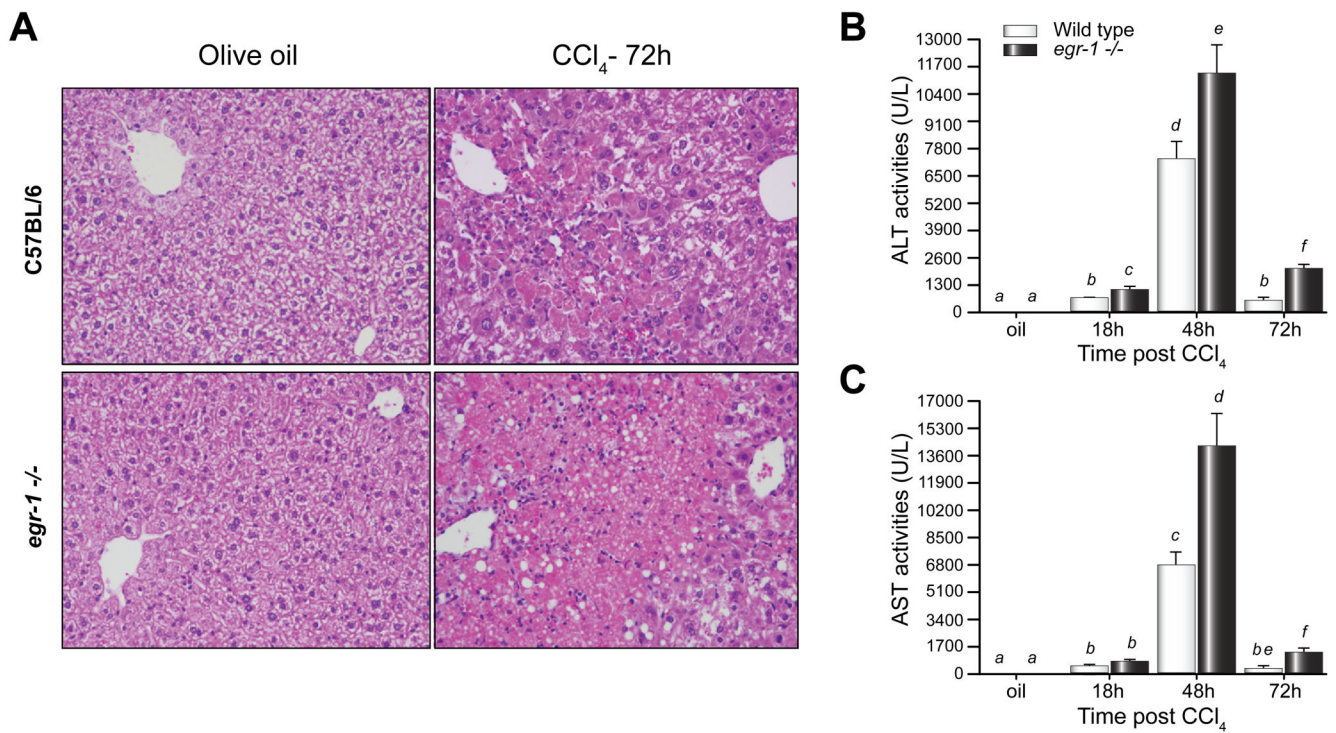


Fig. 1. Absence of Egr-1 exacerbated CCl₄-induced liver injury

(A) Liver histology 72h after CCl₄ administration, Images are representative of n = 4 – 8 mice per group. Hematoxylin and eosin, 100X magnification. Plasma isolated from whole blood was used to determine (B) ALT and (C) AST activities. Bars are means \pm SEM, n = 4–8 mice per group.

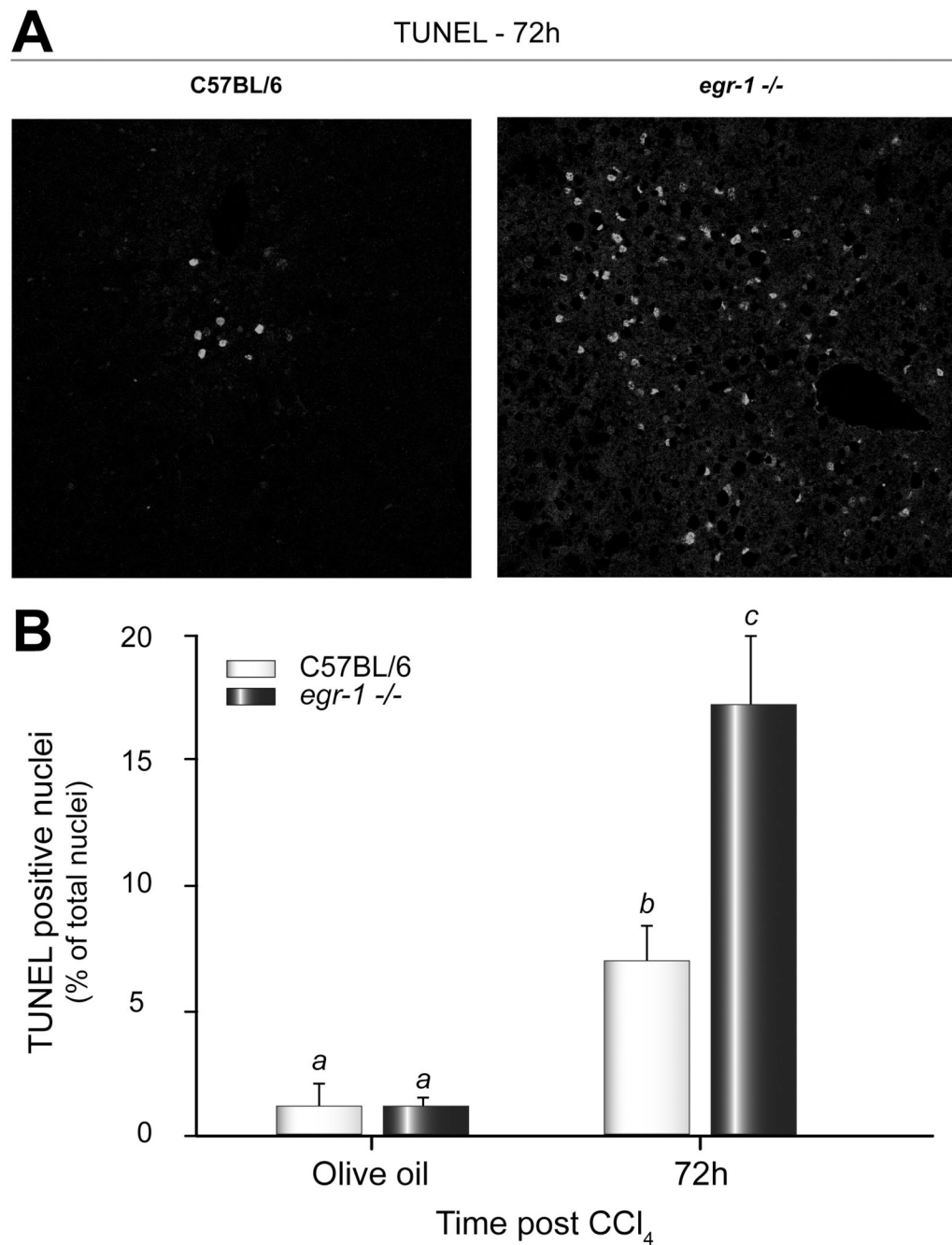


Fig. 2. Hepatocellular apoptosis was enhanced in Egr-1 deficient mice
 (A) Representative 400X images of hepatic TUNEL staining from wild-type and *egr-1*^{-/-} mice 72h after CCl₄. DAPI staining was used to label total hepatic nuclei (not shown). (B) TUNEL quantification. The data were calculated as percent TUNEL positive cells of total DAPI positive hepatic nuclei. Bars are means \pm SEM, n = 3–4 mice per group.

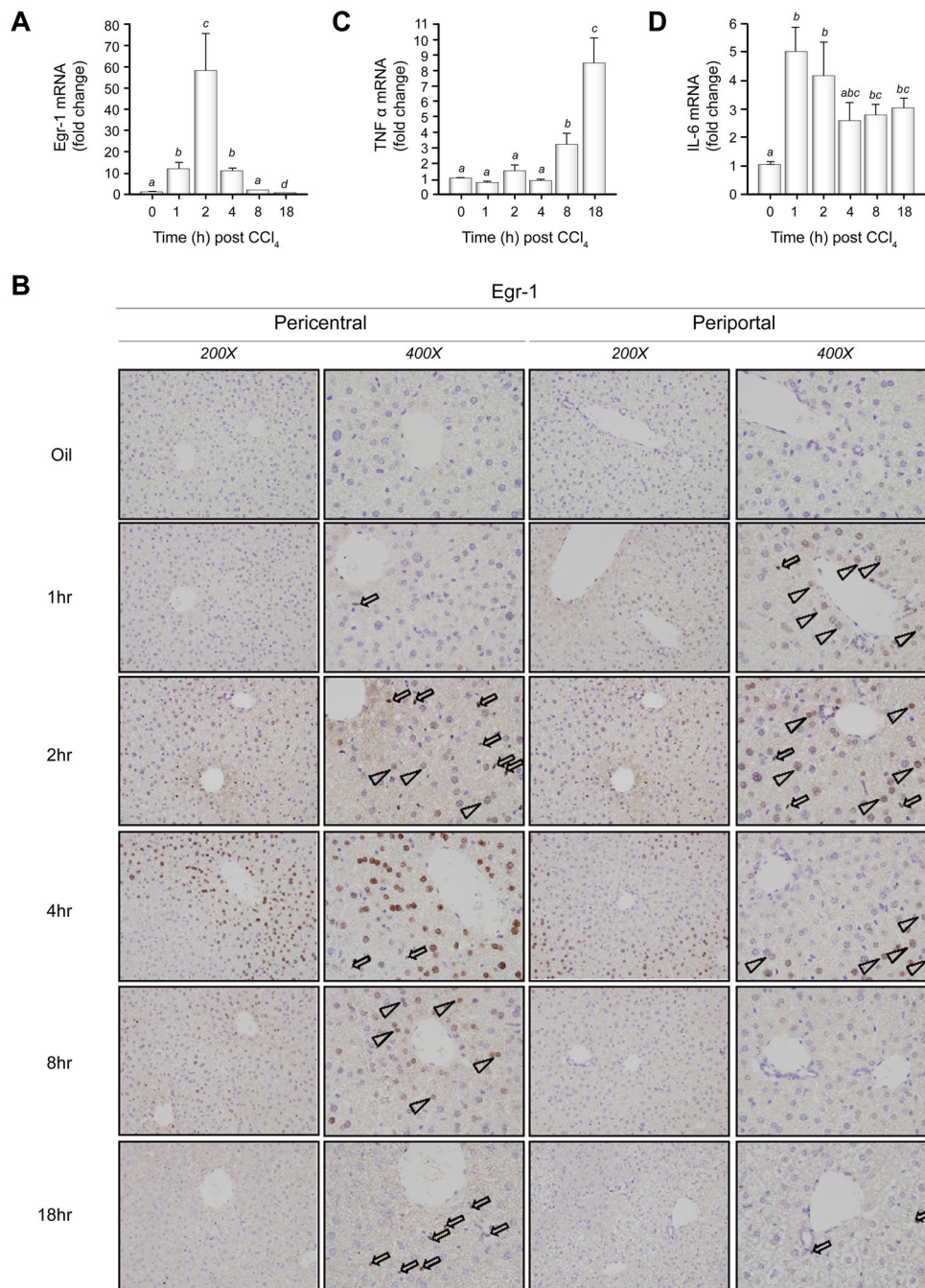


Fig. 3. Egr-1, TNF α , and IL-6 expression after CCl₄ exposure in wild-type mice
 Real-time PCR was utilized to determine mRNA accumulation of (A) Egr-1 (C) TNF α and (D) IL-6. Bars represent means \pm SEM of $n = 4 - 6$ mice per experimental condition. (B) Immunohistochemistry was used to localize Egr-1 protein in liver sections. Open arrowheads indicate Egr-1-positive hepatocyte nuclei, open arrows indicate Egr-1-positive nuclei in NPC-HS. Images are representative of $n = 4$ mice for each experimental condition

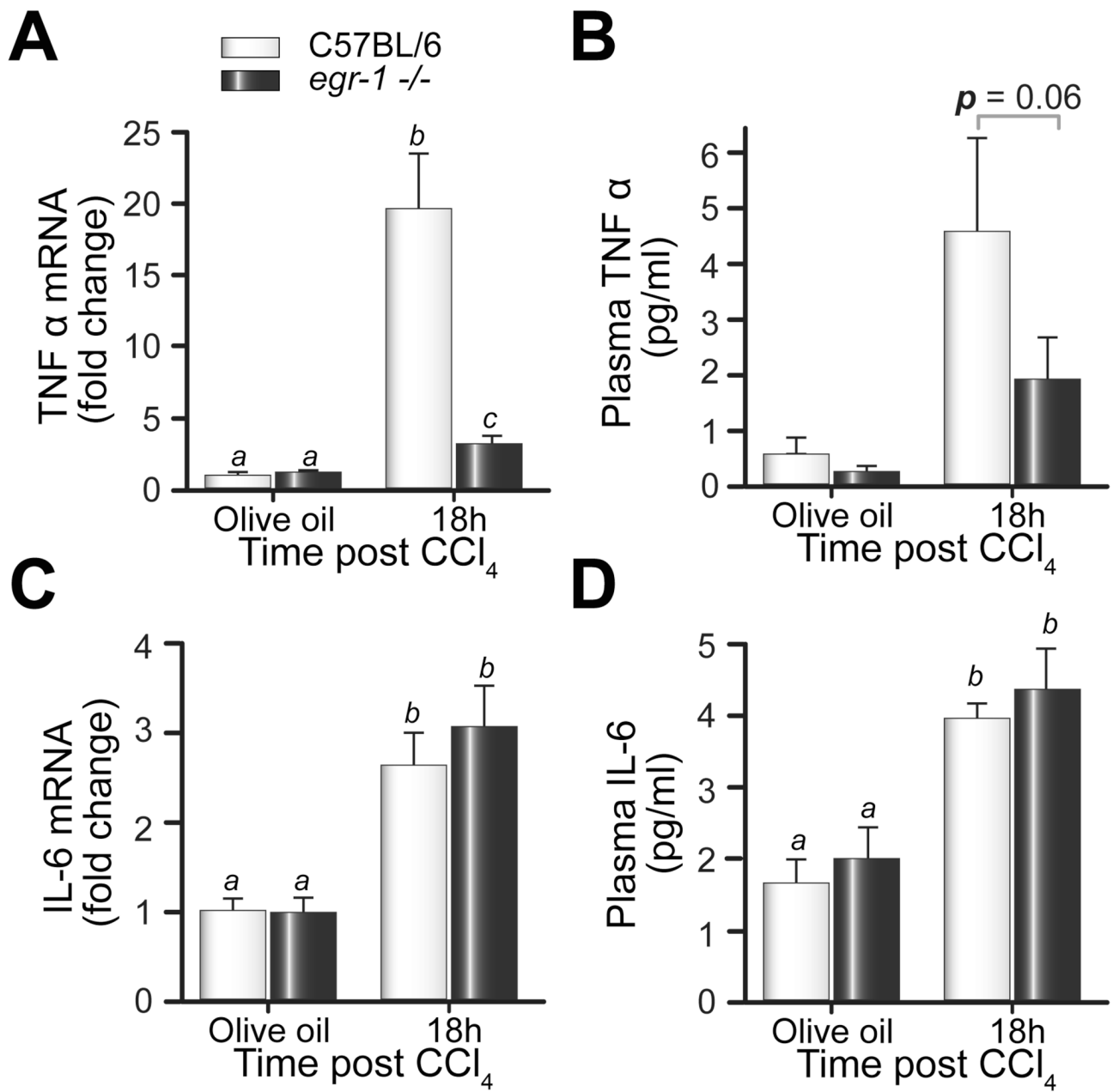


Fig. 4. TNF α and IL-6 expression after CCl₄ exposure
 Eighteen hours after CCl₄ exposure, hepatic mRNA accumulation of (A) TNF α and (C) IL-6, and plasma (B) TNF α and (D) IL-6 protein were determined by real-time PCR and ELISA, respectively. Bars represent means \pm SEM, n = 5–8 mice per group.

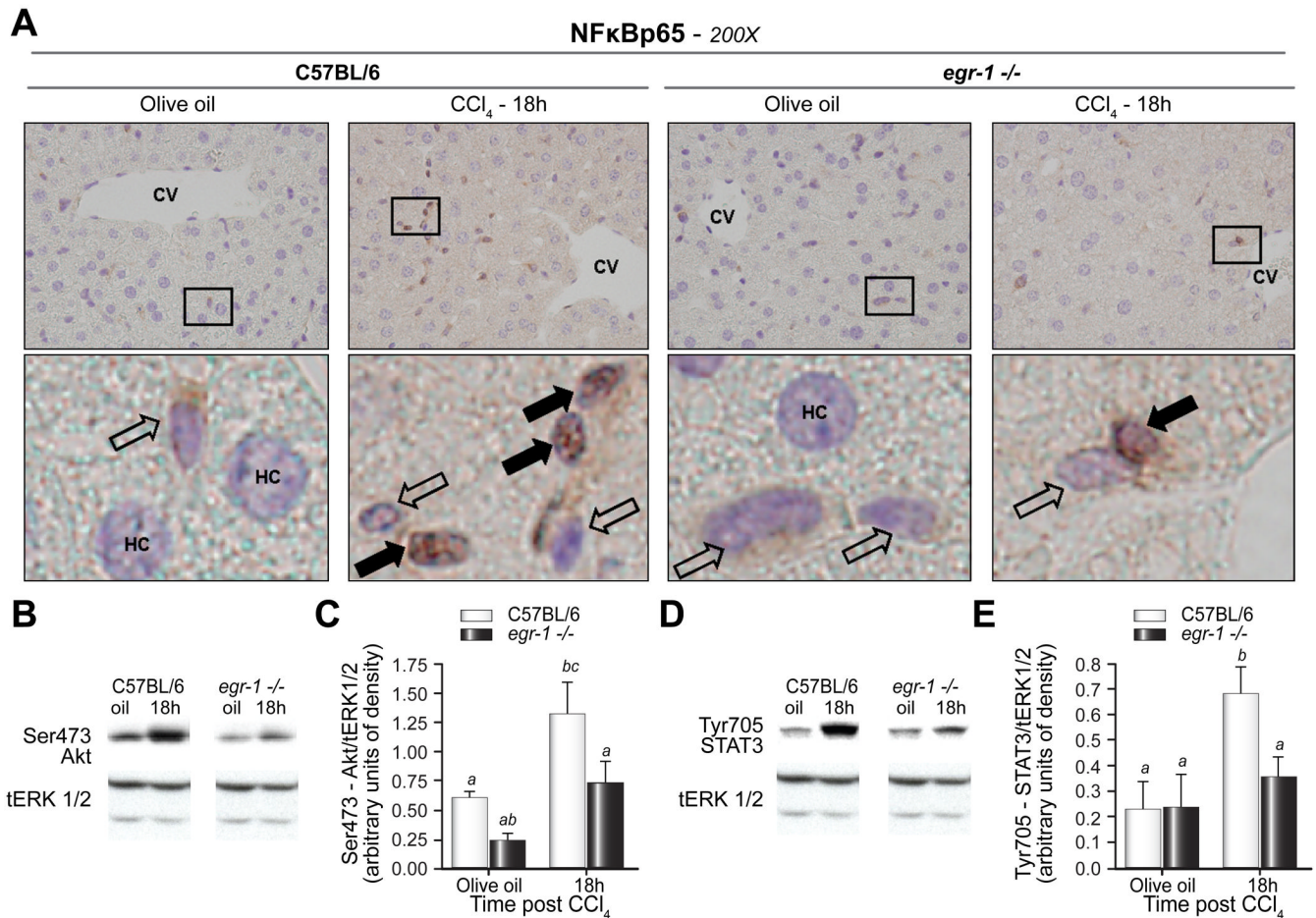


Fig. 5. Nuclear localization of NFκB-p65 in NPC-HS, and phosphorylation of Akt and STAT3 were reduced in *egr-1*^{-/-} mice after CCl₄ exposure

(A) Immunohistochemistry was utilized to localize p65 in liver sections from wild-type and *egr-1*^{-/-} mice. Closed arrows indicate p65-negative nuclei, while open arrows indicate p65-positive nuclei. CV = central vein; HC = hepatocyte. Outlined areas in (A) are shown enlarged below each 200X image. Images are representative of n = 4 – 8 per experimental group. Immunoblots of total hepatic protein were performed to determine expression of (B,C) phospho (Ser473)-Akt, (D,E) phospho (Tyr705)-STAT3 in livers from wild-type and *egr-1*^{-/-} mice. Total (t)-Erk1/2 was used as a loading control. Representative (B) Ser473-Akt and (D) Tyr705-STAT3 immunoblots are shown. Quantification of band intensity measured by densitometry after normalization to tErk1/2 are shown in the bar graphs for (C) Ser-473 Akt and (E) Tyr705-STAT3 from n = 4–5 mice per group. Bars are means ± SEM determined after scanning densitometry of immunoreactive bands.

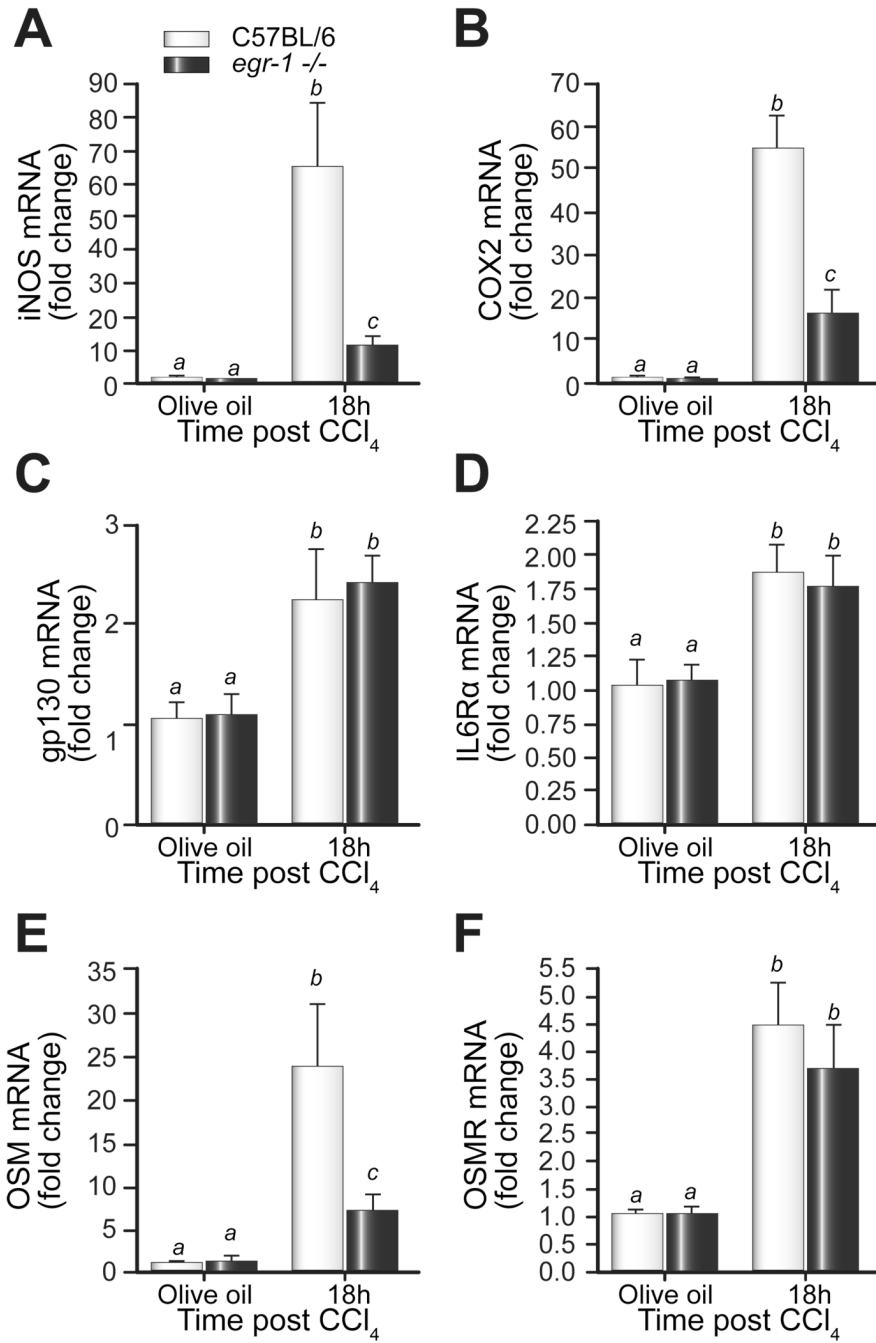


Fig. 6. CCl₄-induced expression of hepatoprotective molecules
 Eighteen hours after CCl₄ exposure, hepatic (A) iNOS, (B) COX-2 (C) gp130 (D) IL-6Rα (E) OSM and (F) OSMR mRNA accumulation was determined using real-time PCR. Bars represent means ± SEM, n = 5–8 mice per group.