Original Article
Mediators of hypoxia in a rat model of sterile-induced acute liver injury

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Abstract: Background: The liver plays a key role in iron homeostasis during injury and hypoxia. Methods: For induction of liver injury, thioacetamide (TAA) was administered intraperitoneally to male Sprague Dawley rats. Animals were sacrificed at 0, 1, 3, 6, 12, 24, 48, 72 and 96 h. Serum, liver, spleen and heart tissues were collected from control and TAA-treated rats. Tissue sections were prepared for immunohistochemical studies. Nuclear and cytoplasmic proteins were isolated for Western blot analysis. Results: Hypoxia inducible factor (HIF)-1α and ED1 positive cells accumulated around the portal field and the interlobular space within 12 hours after TAA administration. Accordingly, Western blot analysis of liver tissue showed an early increase of HIF1α followed by a decrease at 48 h to 96 h. For Erythropoietin (EPO), as well as for HIF1- and -2α, a time-dependent translocation was observed from the cytoplasmic to the nuclear compartment. Conclusion: Our data suggest that the TAA-induced acute liver damage generates HIF-1α dependent rescue mechanisms with translocation of EPO from the cytoplasmic to the nuclear compartment. Enhanced iron transport into the liver could be necessary for increased metabolic activities during repair processes.

Keywords: Thioacetamide (TAA), acute phase injury, hypoxia inducible factor (HIF), erythropoietin (EPO)

Introduction

Severe liver injuries such as acute hepatic failure lead to multi-organ dysfunction with high mortality. Acute liver injury due to adverse drug reactions accounts for more than half of the cases of acute liver failure [1].

Thioacetamide (TAA) has been proposed for induction of liver injury in animal experiments as it causes hepatocellular necrosis after biotransformation to acetamide and TAA-S-oxide, causing hypoxia [2]. One of the hallmarks of acute liver injury is hypoxia. During hypoxia, genes involved in iron uptake and metabolism are induced, leading amongst others - to erythropoiesis. Erythropoietin (EPO) is a glycoprotein hormone of about 30 kDa molecular weight. It is essential for the regulation of the number of red blood cells in response to changes in tissue oxygenation. EPO gene expression is tightly regulated by the hypoxia-inducible factor (HIF)-1, a heterodimeric helix-loop-helix transcription factor consisting of an oxygen-sensitive α subunit and the constitutively expressed β subunit ARNT [3]. The discovery of EPO as a direct target of HIF [4, 5] provided the first evidence that HIF could regulate iron homeostasis and induce rescue mechanisms involved in cell energy metabolism during hypoxic conditions. The activity of HIF members is mainly regulated by oxygen concentrations. When oxygen levels are normal, HIF-1α and HIF-2α undergo post-translational alterations by oxygen-dependent prolyl and asparaginyl hydroxylases that induce their degradation [6]. During low oxygen tension (hypoxia), HIF-α is translocated into the nucleus promoting cell-survival under hypoxic condi-
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Our aim was to investigate the gene and protein expression of EPO, together with the analysis of the two main transcription factors involved in its gene induction, HIF-1α and HIF-2α, in an experimental model of in vivo sterile acute liver damage.

Although they are synthesized primarily by the kidney, EPO and EPO receptors have also been identified in many other organs, such as brain, heart and liver [8, 9]. Several studies have revealed that EPO exerts anti-apoptotic [10, 11], anti-oxidative [12] and anti-inflammatory [13] effects. It has been demonstrated to have a role as a crucial mediator in the resolution of immune response-mediated injury and in the process of resolution of damage and recovery from injury [14].

The concomitant elevation of EPO and pro-inflammatory cytokines in the serum has been recently described in several clinical and experimental situations [15, 16]. Although the circulating levels of EPO have been shown to increase during several pathophysiological conditions, the sites and the regulation of EPO gene expression during acute-phase reactions in the liver have not yet been investigated in detail.

Materials and methods

Materials and methods are briefly summarized below.

Animals

Eight- to twelve-week-old male Sprague-Dawley rats of about 170-200 grams body weight were purchased from Harlan-Winkelmann (Borchen, Germany). The rats were kept under standard conditions with 12 h light/dark cycles and had ad libitum access to fresh water and food pellets. All animals were cared for according to the University's guidelines, German regulations for the protection of animals, and NIH guidelines.

Induction of acute-phase reaction by single-dose TAA administration

Acute liver damage was induced by a single intraperitoneal injection of 300 mg/Kg TAA dissolved in 0.9% sodium chloride (NaCl). Control animals for each time point only received the isotonic saline injection. All animals were killed at time points ranging from 1 to 96 hours after TAA administration under pentobarbital anesthesia [17]. The liver and spleen were taken, frozen in liquid nitrogen, and stored at -80°C for further use.

Visualization of antigens by histochemistry and immunofluorescent staining

In short, we utilized frozen tissue samples to cut cryostat sections of 4 µm (Reichert Jung, Wetzlar, Germany), after cutting sections were air-dried at room temperature and fixed with ice cold acetone for 1 min followed by methanol for 9 min. Tissue sections were hydrated in deionized water.

For peroxidase staining the sections were incubated in a humified chamber with the first antibody against HIF-1α and HIF-2α diluted in PBS for 1 h at room temperature. These procedures were performed as described previously [18].

For immunofluorescence staining, mouse monoclonal anti-EPO (Novus Biologicals, 1:200), mouse monoclonal anti-ED1 (S erotec, 1:100), rabbit polyclonal anti-HIF-1α (Novus Biologicals, 1:200) and rabbit polyclonal anti-HIF2α (Novus Biologicals, 1:200) were used. In short, tissue sections were blocked with 5% bovine serum albumin for 1 hour at room temperature then rinsed in phosphate-buffered saline (PBS) three times for 5 min each and incubated overnight at 4°C with the primary antibody diluted in 0.5% Triton X-100 PBS buffer. The next day, slides were again rinsed in PBS three times for 10 min each. Antigens were visualized using immunofluorescence-conjugated secondary antibodies (with incubation for 1 h at RT), followed by another washing-step in PBS again three times for 10-15 min each. The nuclei were then counterstained with 4,6-diamidino-2-phenylindole (DAPI), washed and mounted with Fluoromount-G. Negative immunostaining was performed by omitting the primary antibody and using an isotype-matched immunoglobulin.

Isolation of nuclear and cytoplasmic protein fractions

Frozen liver tissue was homogenized with an Ultra-turrax TP 18/10, in TRIS-HCl buffer (50 mM, pH 7.4) at 4°C, as detailed in [19]. In the following, homogenates were passed through a 22-G needle and centrifuged for 5 min at 10,000 G. The protein concentration was determined with the BCA (bicinchoninic acid) protein
Western blot analysis

50 μg of whole tissue, cytoplasmic and nuclear proteins were applied per well for polyacrylamide gel electrophoresis using NuPAGE (4%-12% Bis-Tris Gel, Invitrogen, Carlsbad, USA) under reducing conditions [21]. The proteins were then transferred to Hybond-ECL nitrocellulose membranes according to the ECL Western blotting protocol [22]. After transfer, the membranes were blocked in 5% milk and blotted with a mouse anti-EPO (Novus Biologicals) diluted 1:1000, mouse monoclonal anti-HIF-1α (Novus Biologicals) diluted 1:1000, polyclonal rabbit anti-HIF-2α (Novus Biologicals) diluted 1:1000 overnight at 4°C and monoclonal mouse anti-β-actin (Sigma-Aldrich).

Results

Immunofluorescence staining of EPO in liver tissue after TAA-administration

As oxidative stress induces EPO-expression, we analysed EPO in our model of rat liver injury. After 3 h, we found an increase in perinuclear expression of EPO, with mixed nuclear and perinuclear expression at 12 h and 24 h. At 48 h EPO expression returned to the cytoplasm (Figure 1).

Immunohistochemical detection of HIF-1α and HIF-2α in rat liver after TAA-injection

With peroxidase (POD) staining, using antibodies against HIF-1α and HIF-2α, we could detect an accumulation of HIF-1α positive cells around the portal fields of the rat liver 24 h after TAA administration whereas we could not find these changes for HIF-2α+ staining (Supplementary Figure 1). For better visualization of nuclear translocation, immunofluorescent staining was done (Figure 2).

Immunofluorescence staining of HIF-1α and HIF-2α in liver tissue after TAA-administration

For HIF-1α, perinuclear positivity was observed at 12 h, followed by nuclear translocation at 24 h up to 48 h (Figure 2A). Also for HIF-2α, we observed a perinuclear accumulation at 12 hours with nuclear translocation at 24 h, returning to cytoplasmic positivity from 48 h (Figure 2B).

Immunofluorescence staining of ED1 in liver tissue after TAA-administration

An accumulation of ED1 positive cells was visible from 12 h, especially around the portal field (Figure 3), with increased infiltration of ED1 positive macrophages up to 96 h.
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Immunofluorescence staining of EPO in spleen tissue after TAA administration

In spleen immunofluorescence revealed no major change in the expression of EPO after TAA administration (Figure 4).

Immunofluorescence staining of HIF-1α and HIF-2α in spleen tissue after TAA administration

Immunofluorescence staining of TAA-treated rat spleen showed a slight increase in HIF-1α positive immune cells after 3 h with staining of filament-like patterns after 24 h and especially 48 h, suggesting HIF-1α positive small blood vessels (Figure 5A). A slight increase of HIF-2α positive immune cells was seen after 12 h (Figure 5B).

Protein changes of EPO, HIF-1α and HIF-2α in rat liver after TAA administration

In Western Blot analysis, EPO expression was observed from 1-48 h in the cytoplasmic frac-

Figure 2. Immunofluorescence staining of liver sections with antibodies directed against HIF-1α (A) and HIF-2α (B) after single-dose TAA administration. Liver sections at 0 h (Control), 3 h, 12 h, 24 h, 48 h, 96 h. Results show representative pictures of six animals and six slides per time points. Original magnification, × 200. Scale bar equals 50 µm.
Notably, we detected a time-dependent translocation from the cytoplasmic to the nuclear compartment beginning at 48 h until the end of the experiment (96 h). For HIF-1α, an accumulation was found in liver tissue from 1-6 h. In the nuclear fraction, HIF-1α was found from 1-72 h with a maximum from 1-6 h. For HIF-2α, an accumulation of the nuclear protein expression was seen from 1-24 h with a decrease after 3 h and an increase in the cytoplasmic fraction at 6 h (Figure 6).

**Discussion**

In the acute phase of liver tissue damage even if it is indirect, the liver is prone to hypoxic changes. In our model, we could show a time-dependent increase of total hepatic iron content.
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centration, in parallel with an early reduction of the serum iron, a probable mechanism to recover iron from the blood. In contrast, spleen iron levels remained rather stable with a delayed increase at 96 h [23].

Hepatic EPO production has been shown to occur in several clinical and experimental pathological conditions, such as acute liver injury and repair [24], as well as during the processes of chronic hepatic injury with progression to cancer [25]. Furthermore, we previously reported an increase of the hepatic EPO concentration during indirect hepatic injury (turpentine oil) [26].

In this work, our aim was to investigate EPO gene expression together with the analysis of the two main transcription factors involved in EPO gene induction, HIF-1α and HIF-2α. The

Figure 5. Immunohistochemistry of HIF-1α (A) and HIF-2α (B) in rat spleen during aseptic acute phase response induced by TAA. Sections were stained with antibodies directed against HIF-1α and HIF-2α followed by fluorescence immunodetection at 0 h (Control), 3 h, 12 h, 24 h, 48 h and 96 h. Results show representative pictures of six animals and six slides per time points. Original magnification, × 200. Scale bar equals 50 µm.
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Figure 6. Western blot analysis of EPO, HIF-1α and HIF-2α in rat liver of control animals and at different time points after single-dose TAA administration. β-actin was used as a marker for equal loading. Results are representative of three experiments.

TAA rat model is characterized by a marked mononuclear cell infiltration [27, 28]. We previously demonstrated that Kupffer cell-derived acute phase cytokines (IL-6, IL-1β and TNF-α) take part in the induction of proteins involved in iron metabolism [29]. We further confirmed liver damage in TAA-treated rats by showing an elevation of hepatic enzymes in the serum at 24 and 48 h together with a strong increase of the acute-phase cytokines IL-6, IL-1β, TNF-α, and IFN-γ [18]. We now demonstrate an accumulation of ED1 positive cells within the portal area 24 h after TAA-administration together with early nuclear translocation of HIF-1α and HIF-2α. It is known that nuclear translocation of HIF-1α and HIF-2α helps cell survival in hypoxic conditions, promoting EPO-expression. Consequently, we observe an early and high protein expression of HIF-1α in the nuclear fraction of liver tissue, with a slight but constant increase of cytoplasmic EPO expression until 48 hours, and nuclear expression of EPO from 48 hours. This supports the role of EPO for cell survival in stress conditions. At the same time, we observed no major changes of EPO expression in the spleen.

A growing body of clinical and experimental studies supports a possible direct correlation between acute phase cytokines, notably IL-6, and EPO levels in patients with chronic liver disease [30]. Accordingly, Ramadori P. et al. [24] demonstrated an increase in EPO serum levels in two models of acute phase reaction: after intramuscular turpentine oil injection as well as after LPS administration whilst EPO serum concentration and liver gene expression were dramatically reduced in IL-6 KO mice.

We now propose that acute liver damage after TAA-administration induces not only the generation of cytokines (such as IL-6), but also a HIF-1α and -2α dependent EPO production and translocation of EPO from the cytoplasmic to the nuclear compartment.

Nuclear expression of EPO could explain its protective role during inflammatory and not only in hypoxic stress conditions within the liver.

Disclosure of conflict of interest

None.

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Supplementary Figure 1. Immunodetection of hypoxia-inducible factor HIF-1α (A) and HIF-2α (B) in sections of rat livers after single-dose TAA administration. Sections were stained with an antibody directed against HIF-1α followed by peroxidase staining (a-f). Section (a) of (A) shows HIF+ cells in control livers, Section (b-f) show HIF+ cells after TAA injection. The stimulation with TAA induces a time-dependent (24 h, d) accumulation of HIF-1α+ cells in the damaged area around the portal vessels. Results show representative pictures of six animals and six slides per time points. Original magnification, × 100. Scale bar equals 50 µm. P, portal area.