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Induction of the nuclear factor HIF-1α in acetaminophen toxicity: Evidence for oxidative stress

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Abstract

Hypoxia inducible factor (HIF) controls the transcription of genes involved in angiogenesis, erythropoiesis, glycolysis, and cell survival. HIF-1α levels are a critical determinant of HIF activity. The induction of HIF-1α was examined in the livers of mice treated with a toxic dose of APAP (300 mg/kg IP) and sacrificed at 1, 2, 4, 8, and 12 h. HIF-1α was induced at 1–12 h and induction occurred prior to the onset of toxicity. Pre-treatment of mice with *N*-acetylcysteine (1200 mg/kg IP) prevented toxicity and HIF-1α induction. In further studies, hepatocyte suspensions were incubated with APAP (1 mM) in the presence of an oxygen atmosphere. HIF-1α was induced at 1 h, prior to the onset of toxicity. Inclusion of cyclosporine A (10 μM), an inhibitor of mitochondrial permeability transition, oxidative stress, and toxicity, prevented the induction of HIF-1α. Thus, HIF-1α is induced before APAP toxicity and can occur under non-hypoxic conditions. The data suggest a role for oxidative stress in the induction of HIF-1α in APAP toxicity.

Keywords: Acetaminophen; Oxidative stress; Mitochondrial permeability transition; Hypoxia inducible factor; Hypoxia; Glutathione; Nitric oxide; Hepatotoxicity

Hypoxia inducible factor (HIF) is a critical transcription factor that is regarded as a master regulator of oxygen homeostasis. It regulates many of the genes involved in angiogenesis, erythropoiesis, glycolysis, iron metabolism, and cell survival. Because of the number of parameters that affect HIF induction and the many downstream targets of this signal transduction factor, HIF is considered to be a generalized stress response gene regulator [1].

HIF is composed of two subunits, HIF-1α and HIF-1β, both of which are constitutively expressed. HIF-1α protein is undetectable in most cell systems due to proline hydroxylation and rapid degradation by the ubiquitin–proteasome system [2]. With hypoxia, HIF-1α protein levels increase due to decreased proline hydroxylation (an oxygen-dependent enzyme), decreased ubiquitination, and degradation [3]. As a result, HIF-1α binds to HIF-1β, forming a heterodimeric complex that activates hypoxia-responsive elements of target genes, such as vascular endothelial growth factor (VEGF). In addition to hypoxia, oxidative stress may promote HIF-1α induction [4,5].

In the following study, we examined the induction of HIF-1α in the livers of acetaminophen (APAP)-treated mice and in freshly isolated mouse hepatocytes incubated with APAP. Acetaminophen toxicity is mediated by metabolism through cytochrome P-450 enzymes to form the reactive metabolite *N*-acetyl-benzoquinone imine (NAPQI). This metabolite reacts with glutathione (GSH) leading to its depletion and covalently binds to proteins [6]. These events lead to increased production of reactive oxygen and nitrogen species, mitochondrial permeability transition

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(MPT), and toxicity [7,8]. The data presented herein demonstrate that HIF-1α is induced very early in APAP toxicity and suggest that HIF-1α induction may be a result of oxidative stress.

Materials and methods

Reagents. Acetaminophen (APAP, paracetamol) was obtained from Sigma Chemical Co. (St. Louis, MO). Immunopure Peroxidase Suppressor and Coomassie Plus Protein Assay Reagent were purchased from Pierce Chemical Co. (Rockford, IL). *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes), Heparin sodium salt Grade 1-A: from porcine intestinal mucosa, penicillin G sodium salt, RPMI-1640 medium with glutamine, without sodium bicarbonate, and without phenol red, propidium iodide solution of 1 mg/mL in water, *N*-acetyl-L-cysteine (NAC), Percoll, and Trypan blue 0.4% solution were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase A from "*Clostridium histolyticum*" was acquired by Roche Laboratories. Cyclosporine A was obtained from Bedford Laboratories. DTT (dithiothreitol—Cleland's reagent of electrophoresis purity) was acquired from Bio-Rad Laboratories (Hercules, CA). ALT Colorimetrics was obtained from TECO Diagnostics (Anaheim, CA). Other chemicals were of analytical grade obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Fisher Scientific (Toronto, Ontario, Canada).

Animals. Six-week-old male B6C3F1 mice (mean weight, 26.5 g) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). All animal experimentation was in accordance with the criteria of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by University of Arkansas for Medical Sciences Animal Care and Use Committee. Methods for administration of APAP and termination of experiments were performed as previously described [9,10]. In initial experiments, mice were dosed with APAP (300 mg/kg i.p. in saline) and sacrificed at 1, 2, 4, 8, and 12 h after APAP ($n = 5$ per time point). Control mice ($n = 5$) received saline only. In subsequent experiments, mice were dosed with *N*-acetylcysteine (NAC; 1200 mg/kg IP) immediately prior to APAP (300 mg/kg i.p. in saline) and sacrificed at 12 h ($n = 5$). Other mice received APAP with saline ($n = 5$) or saline only ($n = 5$). Following anesthesia with CO_2, blood was removed from the retro-orbital plexus and mice were subsequently euthanized under a CO_2 atmosphere. Livers were removed and samples were snap-frozen in liquid nitrogen.

Hepatocyte isolation and incubation. Freshly isolated hepatocytes were prepared from male B6C3F1 mice by collagenase perfusion following the modified method of Grewal and Racz [7,11]. The hepatocytes were incubated at a concentration of 1,000,000 cells/mL in RPMI-1640 (supplemented with 25 mM Hepes, 10 IU heparin/mL, and 500 IU penicillin G/mL) in 125 mL Erlenmeyer flasks at 37 °C under an atmosphere of 95% O_2–5% CO_2. APAP (1 mM) was added to experimental hepatocytes, but no APAP was added to control flask. Some incubations contained cyclosporine A (10 μM).

Nuclear extraction for HIF-1α and immunoblot assay. Nuclear extraction for HIF-1α was performed on frozen liver sections and freshly isolated hepatocytes using the Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's recommended protocol. For liver sections, 0.3 g of frozen tissue was ground to a powder and then resuspended in 1.95 mL of hypotonic buffer, containing 3 μL of 1 M DTT and 3 μL of detergent. Suspensions were spun at 850*g* for 5 min at 4 °C to pellet the nuclei. The supernatant was poured off and 0.30 mL of lysis buffer was added to the pellet. The resulting material was homogenized and the resulting homogenate was incubated on ice for 30 min to extract the nuclear proteins. A final spin at 14,000*g* for 30 min at 4 °C was performed to remove the nuclear debris. For freshly isolated hepatocytes, cells were washed and suspended with 3 mL ice-cold PBS/phosphatase inhibitors and incubated for 10 min. Suspensions were centrifuged at 500 rpm for 5 min at 4 °C. The resulting pellet was resuspended in 0.5 mL of 1× Hypotonic Buffer and incubated on ice for 15 min. Suspensions were centrifuged at 14,000*g* for 30 s at 4 °C. The supernatant was removed and the pellet was resuspended in 50 μL lysis buffer and incubated for 15 min on ice to extract the nuclear fraction. The suspension was centrifuged at 14,000*g* for 10 min at 4 °C to remove nuclear debris. For immunoblots, an anti-HIF-1α monoclonal antibody (1:1000) from Novus Biologicals (Littleton, CO) was used according to the manufacturer's protocol. Fifty microgram protein of nuclear extract was loaded per lane on a 4–20% Precise Protein Gel (Pierce, Rockford, IL). After running under non-reducing conditions, the proteins were transferred onto nitrocellulose membranes. Membranes were then subsequently blocked for 1 h in TBS/Tween 20 buffer with 5% non-fat dried milk and incubated with the primary antibody, diluted in TBS/Tween 20 with 5% non-fat dried milk at 4 °C overnight. After washing, membranes were incubated with a 1:2000 dilution of HRP-conjugated anti-mouse IgG for 1 h at room temperature in TBS/Tween 20 with 5% non-fat dried milk. After another wash series, membranes were exposed to Supersignal Chemiluminescent Substrate (Pierce) for 5 min, then exposed to radiographic film and developed.

Biochemical analysis. Serum alanine aminotransferase (ALT) was determined with a spectrophotometric diagnostic kit (Teco Diagnostics, Anaheim, CA). Glutathione (GSH) was measured by a colorimetric method using Ellman's reagent as previously modified by Mitchell et al. [12].

Statistical analysis. Results are expressed as means ± SE. Comparisons between multiple groups were by one-way analysis of variance followed by the Tukey HSD post hoc test; $p \leqslant 0.05$ was considered statistically significant. SPSS Version 10.0 (SPSS Inc, Chicago, IL) was used for statistical analyses.

Results

HIF-1α induction in livers of APAP-treated mice

In initial experiments, the toxicity of APAP was examined in the B6C3F1 male mouse. Toxicity, as demonstrated by elevation of serum ALT, was apparent at 4 h and persisted at 12 h (Table 1) [6]. Following an initial depletion, GSH recovered by 8 and 12 h to levels higher than control levels (Table 1). Western blot analysis were performed to examine the induction of HIF-1α in the APAP-treated mice. Significant elevation of HIF-1α was apparent by 1 h and remained elevated in all time points examined (Fig. 1), despite the recovery in GSH levels at 8 and 12 h (Table 1). These data demonstrate that HIF-1α induction occurs very early following APAP and occurs *before* biochemical evidence of toxicity.

Effect of NAC on HIF-1α induction in livers of APAP-treated mice

The effect of NAC on HIF-1α induction in APAP toxicity was examined. NAC, the antidote used in the treatment

Table 1
Serum ALT and hepatic GSH in mice treated with acetaminophen (300 mg/kg)

Time (h)	Serum ALT (IU/L)	SEM	Normalized hepatic GSH (%)	SEM (%)
0	20.7	0.8	100.0	8.0
1	31.8	3.0	25.6*	11.4
2	72.4	13.7	28.2*	11.3
4	1364.3*	138.0	35.4*	28.6
8	3737.8*	288.4	117.4	27.7
12	2039.6*	292.4	293.1*	9.5

* Significant difference from control mice ($p < 0.05$), designated as time 0.

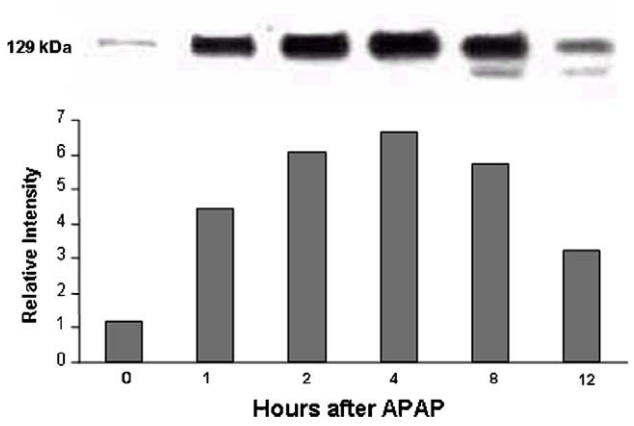

Fig. 1. Time course for induction of hepatic HIF-1α following APAP treatment of mice. Mice were treated with APAP (300 mg/kg IP) and sacrificed at the indicated times. Hepatic nuclear fractions from each treatment group ($n = 5$ per group) were pooled and the relative amount of HIF-1α was determined by immunoblot analysis as described in Materials and methods. Toxicity data are presented in Table 1.

of APAP overdose patients [13], increases hepatic GSH levels [6,14,15]. Mice were pre-treated with either NAC (1200 mg/kg) or saline immediately before APAP (300 mg/kg IP). Control mice received saline only. All mice were sacrificed at 12 h. NAC significantly decreased toxicity as determined by measurement of serum ALT (Fig. 2A). Western blot analysis for HIF-1α showed minimal HIF-1α protein in the controls and substantial induction in the APAP-treated mice at 12 h. Moreover, HIF-1α induction was dramatically reduced in mice treated with APAP plus NAC. Mice pre-treated with NAC had HIF-1α levels similar to those of controls (Fig. 2B; lane 3). The data suggest an important role for the metabolic activation of APAP and GSH depletion in the initiation of HIF-1α induction.

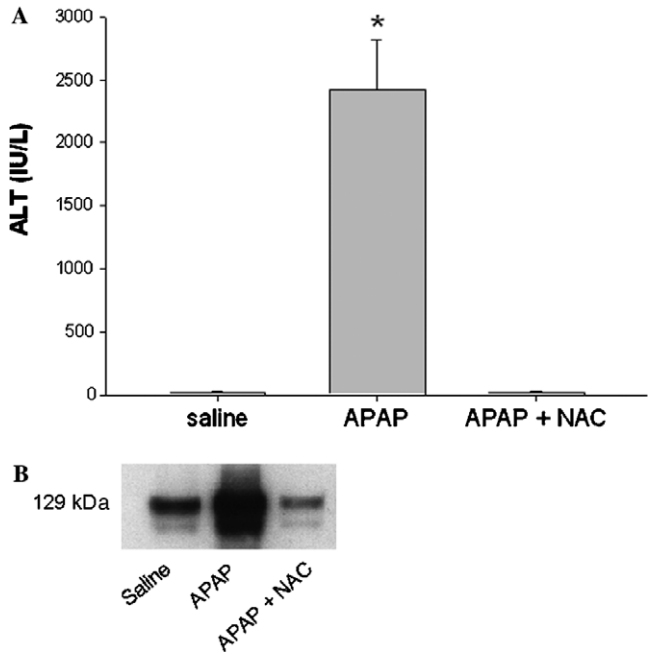

Fig. 2. Effect of NAC on hepatic HIF-1α induction following APAP treatment of mice. Mice ($n = 5$ per group) were treated with saline (lane 1), APAP (300 mg/kg; lane 2) or APAP plus NAC (1200 mg/kg; lane 3) and sacrificed at 12 h. (A) Serum ALT (means ± SE) was determined as a measure of hepatic toxicity, $^{*}p < 0.05$. (B) Hepatic nuclear fractions from each treatment group were combined and the relative amount of HIF-1α was determined using immunoblot analysis.

Induction of HIF-1α in freshly isolated mouse hepatocytes treated with APAP

The effect of APAP on HIF-1α induction was determined in vitro using freshly isolated mouse hepatocytes. The hepatocytes were incubated for 2 h with APAP (1 mM). Control hepatocytes were incubated in media alone. Samples were removed at 0.5, 1.0, 1.5, and 2.0 h from each experimental group and Western blot analysis for HIF-1α were performed. These data showed minimal HIF-1α induction in controls (Fig. 3A; lanes 1 and 2) while

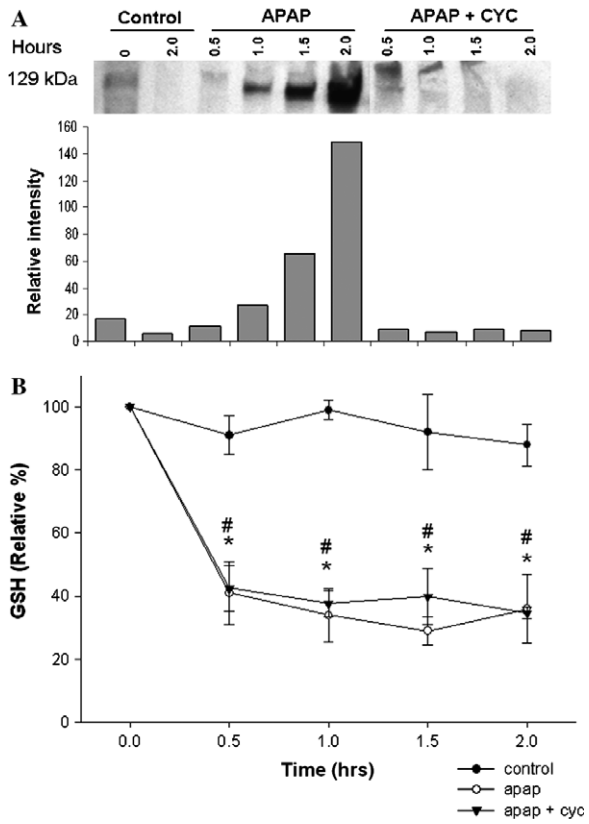

Fig. 3. Time course for HIF-1α induction in freshly isolated mouse hepatocytes incubated with APAP and the effect of CYC on HIF-1α induction. Hepatocytes were incubated with media alone (control), APAP (1 mM), or APAP (1 mM) plus CYC (10 μM). The experiment was performed in triplicate using three different mice. (A) At the designated times, the experiments were stopped and the nuclear fractions from the mice of each group were pooled and analyzed for HIF-1α induction using immunoblot assays. (B) In a separate experiment, the effect of APAP and APAP plus CYC on GSH depletion was determined. APAP significantly reduced GSH levels ($^{\#}p < 0.05$), as did APAP plus CYC ($^{*}p < 0.05$). GSH depletion was not significantly different between APAP alone and APAP plus CYC at any time. Each data point represents the means ± SE of three separate experiments.

APAP-treated cells had increased HIF-1α levels at 1.0 and 1.5 h, with maximum levels at 2.0 h (Fig. 3A; lanes 3–6). Importantly, the incubations were performed in a 95% oxygen, 5% CO_2 atmosphere. Thus, APAP-mediated induction of HIF-1α in the hepatocytes was not mediated by hypoxia.

Effect of cyclosporine A on HIF-1α induction in hepatocytes treated with APAP

Cyclosporine A (CYC) is an inhibitor of MPT and MPT has been shown to be mechanistically important in APAP toxicity [7]. MPT occurs as a result of increased oxidative stress and leads to increased oxidative stress [16,17]. Hepatocytes were incubated with APAP plus CYC (10 μM) and samples were removed at the indicated times for analysis of HIF-1α. Western blot analysis showed that induction of HIF-1α protein was completely abolished in the APAP/CYC-treated hepatocytes (Fig. 3; lanes 7–10). In a subsequent study, GSH levels were examined in the hepatocytes incubated with APAP or with APAP plus CYC. GSH depletion is known to be a requirement for the development of APAP toxicity. GSH was depleted by approximately 40% by 0.5 h and remained depleted for the duration of the experiment. GSH levels were comparable in the APAP/CYC and the APAP-treated hepatocytes (Fig. 3B). Thus, CYC did not alter GSH levels, indicating that the effect of CYC did not involve inhibition of APAP metabolism and suggesting a role for MPT and oxidative stress in HIF-1α induction in APAP toxicity.

Discussion

In this manuscript, we report that a toxic dose of the commonly used analgesic APAP causes induction of hepatic HIF-1α (Fig. 1). HIF-1 is a transcriptional activator that functions as a master regulator of O_2 homeostasis. HIF-1 target genes include those that encode proteins that increase O_2 delivery and mediate adaptive responses in cells. Major mechanisms for HIF-1α induction are growth factor-stimulated signal transduction pathways and hypoxia [18,19]. Oxidative stress is another mechanism which has been reported to cause induction of HIF-1α [20–22]. Nitrosylation by reactive nitrogen leading to HIF-1α stabilization has been implicated as a critical mechanism of induction [23–25].

The mechanism of HIF-1α induction in the livers of APAP-treated mice is unclear. The role of hypoxia in HIF-1α induction is well documented and a role for hypoxia in HIF-1α induction in APAP-treated mice cannot be ruled out. APAP causes pooling of blood in the livers of treated mice [26] and this could lead to tissue hypoxia. However, comparison of the previously reported time course for the development of hepatic congestion [26] with our data on HIF-1α induction suggests that hypoxia may not have played a significant role. Walker and co-workers [26] did not observe centrilobular hepatic congestion before 3 h in APAP-treated mice. The finding that HIF-1α induction occurred by 1 h (Fig. 1) suggests that induction may have occurred by another mechanism.

The finding that NAC blocked HIF-1α induction (Fig. 2B) clearly shows that HIF-1α induction occurs as a result of the toxic metabolite NAPQI. This metabolite is detoxified by GSH leading to its depletion [14,15]. NAC increases GSH synthesis, thus preventing the metabolite from depleting GSH and covalently binding to protein [14,15]. Depletion of GSH results in alteration of the redox balance within the hepatocyte and in the absence of GSH, the reactive metabolite of APAP covalently binds to cysteine groups on proteins. We recently reported that these events initiate oxidative stress and MPT leading to toxicity in freshly isolated hepatocytes [7].

To further understand the mechanism of HIF-1α induction, assays were performed using freshly isolated mouse hepatocytes. These hepatocytes have high levels of cytochrome P-450 enzymes necessary for the metabolism of APAP to the toxic metabolite and are very sensitive to the toxic effects of APAP [7]. As shown in Fig. 3A, APAP caused a substantial induction of HIF-1α by 1 h in the hepatocytes, the time that we previously found maximal GSH depletion in the hepatocytes. Thus, GSH depletion and HIF-1α induction are both early events in vivo and in vitro. Since the hepatocyte assays were performed under a 95% oxygen atmosphere, the mechanism was not hypoxia.

Our laboratory and others previously described the importance of MPT in APAP toxicity in freshly isolated mouse hepatocytes [7,8]. MPT occurs as a result of oxidative stress and leads to oxidative stress [16]. Since the MPT inhibitor CYC was shown to block oxidative stress and toxicity [7], we determined the effect of CYC on the induction of HIF-1α. As shown in Fig. 3A, CYC prevented induction of HIF-1α *and* did not alter GSH levels (Fig. 3B). These data suggest a potential role for oxidative stress in APAP-mediated induction of HIF-1α. Reactive nitrogen has been reported to be a mechanism of induction of HIF-1α [23–25,27] and it may be the major mediator of oxidative stress in APAP toxicity. In previous work, we showed that APAP toxicity correlates with APAP covalent binding to proteins and with the formation of nitrated tyrosine residues [6,28]. Nitrotyrosine is a biomarker of peroxynitrite which is formed by nitric oxide (NO) and superoxide, and is an important mechanism in oxidative stress. Also, we have previously reported that increased NO synthesis is an early event in APAP toxicity [29]. Further research is necessary to determine mechanisms of hepatic HIF-1α induction following APAP and its physiological importance.

Hepatic HIF-1α levels were still maximally induced at 8 h in APAP-treated mice, a time after complete development of toxicity with lysis of hepatocytes (4–6 h). Thus, the data provided no evidence that HIF-1α induction in hepatocytes leads to toxicity. Since HIF-1α induction occurs before toxicity, we postulate that induction occurs in some cells that subsequently die and some cells that do

not die. We postulate that the cells that recover following APAP toxicity are important in the subsequent process of hepatocyte regeneration that occurs after the toxicity. We previously reported that hepatic VEGF levels were markedly increased 8 h after the administration of a toxic dose of APAP to mice and that inhibition of VEGF decreased hepatocyte regeneration [10,30]. Since HIF-1α induction has been reported to be an important regulator of VEGF synthesis, we postulate that HIF-1α induction is a key critical event that initiates hepatocyte repair. Indeed, a recent report described the temporal upregulation of HIF-1α prior to the onset of VEGF synthesis in a rat model of partial hepatectomy and postulated that HIF-1α may have a role in the regeneration of the sinusoidal endothelium after liver injury [31]. Further study will be required to confirm that VEGF is a gene target of HIF-1α induction in APAP toxicity and to elucidate the mechanisms of cellular communication that allow for recovery of the organ in sublethal APAP toxicity.

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