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Pathologic Role of Stressed-Induced Glucocorticoids in Drug-Induced Liver Injury in Mice

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Abstract

We previously reported that acetaminophen (APAP)-induced liver injury (AILI) in mice is associated with a rise in serum levels of the glucocorticoid (GC), corticosterone. In the current study, we provide evidence that endogenous GC play a pathologic role in AILI. Specifically, pretreatment of mice with the GC receptor (GCR) inhibitor, RU486 (mifepristone), protected normal but not adrenalectomized mice from AILI, while pretreatment with dexamethasone, a synthetic GC, exacerbated AILI. RU486 did not affect the depletion of whole liver reduced GSH or the formation of APAP-protein adducts. It also had no effects on the formation of reactive oxygen species or the depletion of mitochondrial GSH or ATP. While RU486 pretreatment also protected against halothane-induced liver injury, it exacerbated concanavalin A (ConA)- and carbon tetrachloride (CCl₄)-induced liver injury, demonstrating the complexity of GC effects in different types of liver injury. Conclusion: These results suggest that under certain conditions, elevated levels of GC might represent a previously unappreciated risk factor for liver injury caused by APAP and other drugs through the diverse biological processes regulated by GCR.

Keywords

Drug-induced liver injury; stress; RU486; acetaminophen; halothane

INTRODUCTION

Drug-induced liver injury (DILI) is a serious health problem that accounts for over 50% of the cases of acute liver failure in the United States [1]. While the occurrence of DILI is quite high due to the estimated >1000 drugs that have been associated with liver injury [2], with the exception of APAP, the incidence for a particular drug is less than 1 percent [2;3]. This low frequency, combined with insufficient knowledge of the underlying mechanisms, makes it impossible to predict who will be susceptible to DILI. Even in the case of APAP, where there is a clear relationship between overdose and liver failure, it is not entirely possible to predict susceptibility to liver injury [4]. As a result, there is considerable interest in

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identifying risk factors of DILI. To date, a number of potential predisposing factors have been associated with DILI in humans (reviewed in [5]), including gender [6], age [6], genetic polymorphisms in drug metabolizing enzymes [7;8;9], major histocompatibility complex [10], cytokines [11], and viral infections [12;13;14] as well as numerous other potential risk factors that have been identified in animal models of AILI [15;16].

As a follow up to our recent observation that the severity of AILI in mice correlated with increased serum levels of corticosterone [17], we have now explored the possibility that corticosterone might also play a role in AILI. We found that pretreatment with the GCR inhibitor, RU486, protected mice from APAP- and halothane-induced liver injury, a surprising finding considering GC are generally thought to be useful for the treatment of immune-mediated DILI due to their immunosuppressive effects [18;19;20].

MATERIALS AND METHODS

Mice and Treatment

Eight week old male C57Bl/6J (stock# 000664) and female Balb/cJ mice (stock# 000651) were obtained from Jackson Laboratories (Bar Harbor, ME) and acclimatized at NIH facilities for one week. Experiments were conducted with the approval of the NHLBI Animal Use and Care Committee and animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86–23 revised 1985). For experiments involving adrenalectomized C57Bl/6J mice, mice were given sterile saline (0.9 %) solution instead of normal drinking water due to surgical removal of the adrenal glands (surgery performed by Jackson Laboratories).

AILI in male C57Bl/6J mice was produced by withholding food supplies overnight (16 hours) to equally deplete hepatic glutathione stores prior to intraperitoneal injection with APAP (300 mg/kg) dissolved in warm saline (25 mg/mL). CCl₄-induced liver injury was similarly produced in fasted male C57Bl/6J mice by intraperitoneal injection with 750 µl/kg CCl₄ in 100 µl sesame oil [21]. Food supplies were restored promptly after APAP and CCl₄. ConA-induced liver injury was produced in male C57Bl/6J mice by intravenous injections of 30 mg/kg ConA dissolved in saline (2.5 mg/mL) [22], while halothane-induced liver injury was produced in female Balb/cJ mice by intraperitoneal injections of 30 mmoles/kg of halothane dissolved in 2 mL of olive oil [23]. Neither ConA- nor halothane-induced liver injury involved fasting.

To block the GCR, 25 mg/kg RU486 [24] was injected intraperitoneally –16 and –2 hours prior to treatment with APAP, ConA, halothane, or CCl₄. Each injection of RU486 contained 100 µl of a stock solution (50 mg RU486 in 1 mL ethanol) diluted 1:10 in sesame oil. Vehicle control mice received intraperitoneal injections of 100 µl of ethanol diluted 1:10 in sesame oil. Due to ethanol use in the vehicle, RU486 was administered at least 2 hours prior to APAP treatment to prevent ethanol from competitively inhibiting CYP2E1 [25], the major P450 isoform responsible for bioactivating APAP to its toxic reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [26].

The specificity of RU486 for the GCR was assessed by administering RU486 (25 mg/kg) to adrenalectomized male C57Bl/6J mice –16 and –2 hours prior to APAP treatment. Furthermore, the effects of exogenous glucocorticoids were tested by injecting the GCR agonist, dexamethasone (31 mg/kg encapsulated dexamethasone-water soluble formulation [Sigma, D2915] corresponding to a total dose of 2 mg/kg dexamethasone in 100 µL warm saline) intraperitoneally –16 and 0 hours before APAP treatment.

Assessment of Liver Injury

Liver injury was evaluated by histopathological examination of hematoxylin- and eosin- (H&E) stained, formalin-fixed, paraffin-embedded liver sections (American Histolabs, Gaithersburg, MD) by light microscopy examination and by serum activity of alanine aminotransferase (ALT) measured with a diagnostic kit (Teco Diagnostics, Anaheim, CA).

Tissue Preparation

Livers were excised and washed in PBS (pH 7.4). Part of the liver was snap frozen in liquid nitrogen and kept at -80°C until further analysis. For immunoblotting, whole-liver homogenates were prepared by homogenizing tissue in 100 mM Tris (pH 7.4) containing 250 mM sucrose, 1 mM EDTA, and a Cocktail Protease Inhibitor tablet (Roche, Indianapolis, IN).

For some experiments, fresh liver tissue was used to isolate hepatic mitochondrial proteins by differential centrifugation [27]. Briefly, liver was homogenized in equal weight/volume of ice-cold isolation buffer consisting of 220 mM mannitol, 70 mM sucrose, 2.5 mM HEPES, 10 mM EDTA, 1 mM EGTA, and 0.1% bovine serum albumin; pH 7.4. Following centrifugation at $600 \times g$ for 8 min at 4°C to remove nuclei and cell debris, the supernatant was transferred to a new tube and centrifuged at $10,000 \times g$ for 10 min at 4°C to pellet the mitochondria. The mitochondrial pellet was washed with 2 mL of ice-cold isolation buffer and analyzed immediately to determine GSH levels.

Protein concentrations were determined using the Bradford assay (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) with bovine serum albumin as the reference protein.

GSH Measurement

GSH was measured in whole liver or mitochondrial extract using a DTNB assay based on Tietze's method [28]. Briefly, 2% (w/v) 5-sulfosalicylic acid containing 1% (w/v) EDTA was added to total liver homogenates (0.1 g tissue in 0.4 mL PBS) and mitochondrial pellets (resuspended in 50 μL PBS) at a 1:1 volume ratio, vigorously vortexed, and centrifuged at $4000 \times g$ for 10 min at room temperature to sediment the precipitated protein [28;29]. The supernatant was then diluted 1:20 (v/v) in a solution of 5, 5'-dithiobis-(2-nitrobenzoic acid) and further incubated for an additional 15 minutes at room temperature. The absorbance reading at 412 nm was used to quantify the concentration of GSH by comparison to a standard curve.

Protein Carbonyl Content Measurement

Carbonylated proteins were quantified in whole liver tissue using a Protein Carbonyl Assay Kit (Cayman Chemicals; Ann Arbor, MI). Briefly, liver samples were homogenized in 4 volumes of phosphate buffer (pH 6.7) containing 1 mmol/L EDTA and nucleic acids were removed by the addition of a 1% streptomycin sulfate solution. Tissue homogenates were then incubated with 2, 4-dinitrophenylhydrazine in 2.5 M hydrochloric acid for 1 hour and read at 370 nm. The assay was performed according to manufacturer's instructions with the exception that the carbonyl content in samples and controls was corrected for protein concentration using the Bradford assay (Coomassie Plus, Bio-Rad).

ATP Measurement

ATP was measured in whole liver using a firefly luciferase assay (ENLITEN ATP Assay, Promega, Madison, WI). Briefly, pieces of liver tissue (10–30 mg) were pulverized in a 1.5 mL eppendorf tube with a small pestle. Following the addition of 80 volumes (v/w) of 0.6 M perchloric acid, the resulting precipitate was spun at $9000 \times g$ for 1 min. At this point, 800

μL of the supernatant was transferred to a new tube and neutralized by adding 200 μL of a solution of 5 M potassium hydroxide and 0.4 M imidazole. After one more centrifugation at $9000 \times g$ for 1 min at room temperature, supernatants were diluted 1:500 in ATP-free water and read on plate reader with luminescence capability and compared to a standard curve of ATP.

Immunoblotting

Liver homogenates (120 μg) were run on 12% SDS-PAGE gels under reducing conditions. Gels were subsequently transferred to nitrocellulose membranes and blocked with 5% (w/v) non-fat dry milk in 0.02 mM Tris (pH 7.4) containing 0.05% Tween 20 (TBS-Tween 20) for 1 hour at room temperature. Primary antibodies [anti-APAP (1:1000; kind gift of Jack Hinson and Neil Pumford, University of Arkansas) and anti-halothane (1:1000) [30]] were diluted in 5% (w/v) BSA in TBS-Tween 20 were applied overnight at 4°C, followed by 3 washes in TBS-Tween 20. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody for 2 hours at room temperature at 1:2000 and washed 3 times in TBS-Tween 20. The protein bands were visualized with Immobilon Chemiluminescent Substrate (Millipore, Billerica, MA) and images were captured using Kodak Image Station 2000RT (Eastman Kodak, Rochester, NY). Anti- β -Actin (1:5000; Millipore) was included as a control to assess protein loading.

Statistics

Statistical analyses comparing means between 3 or more groups were performed using one-way Analysis of Variance (ANOVA) with Bonferroni's Multiple Comparison Test. Statistical analyses comparing means between 2 groups were performed using Student's t-test. All analyses were performed with Prism 4 software (GraphPad Software, San Diego, CA). Differences were considered significant when $P < 0.05$.

RESULTS and DISCUSSION

To investigate the role of corticosterone in AILI, mice were pretreated with RU486 or vehicle control 2 hours prior to APAP treatment. RU486 pretreatment inhibited AILI at all time points up to 24 hours, as determined by reduced serum ALT activity (Fig. 1A), a biomarker of liver injury, and histological evidence of diminished perivenous necrosis (Fig. 1B). Additional experiments were conducted to determine whether the protective effects of RU486 were related to its inhibitory actions on GCRs. RU486 failed to protect adrenalectomized mice from AILI (Fig. 1C), presumably due to the inability of adrenalectomized mice to produce corticosterone. Conversely, pretreatment with a low dose of dexamethasone (2 mg/kg), a synthetic glucocorticoid, appeared to sensitize intact mice to AILI (Fig. 1D). These findings, in conjunction with our earlier observations that the severity of AILI correlated with a rise in serum corticosterone [17], suggest that RU486 protection is mediated, at least in part, through its inhibitory effects on corticosterone/GCR signaling.

In support of this hypothesis, there are multiple clinical reports suggesting that GC-induced stress may also play a role in DILI, as well as other pathologies of the liver, in humans. In one report, there was a higher incidence of liver injury associated with antipsychotic drug use in physically-restrained patients compared to non-restrained patients [31]. Another study concluded that psychosocial stress may exacerbate inflammation and fibrosis in the livers of alcoholics patients [32]. Similarly, a correlation between psychosocial stressors attributable to type 1 personalities and increased severity of chronic hepatitis C was described in another report [33]. Based on these clinical findings, it seems justified to investigate the potential roles of GC in the etiology of liver diseases in greater detail.

Observations that RU486-pretreatment protected mice from AILI as early as 2–4 hours after APAP administration (Fig. 1A) suggested that corticosterone might be involved in an early event in the mechanism of AILI. This hypothesis was tested by examining the effects of RU486 on established early events in the etiology of AILI; however, none of these experiments yielded positive results. Specifically, RU486 had no effects on the bioactivation of APAP into its reactive and toxic metabolite, NAPQI which covalently binds to cellular proteins (Fig. 2A) and depletes cellular glutathione (Fig. 2B), a long established critical event in the initiation of AILI [34]. RU486 pretreatment also appeared to have no effect on AILI-induced mitochondrial injury, another early event in the mechanism of AILI [35]. Specifically, the loss of mitochondrial glutathione following metabolic activation of APAP is thought to severely compromise mitochondrial function, at least in part, by promoting profound oxidative stress, which in turn triggers the opening of MPT pore resulting in reduced ATP generation and depolarization of the membrane causing the mitochondria to swell and rupture [36;37]. However, RU486 had no effect on the depletion of mitochondrial glutathione (Fig. 2C) or the formation of carbonylated proteins, a biomarker of ROS [38] (Fig. 2D), nor did it have an effect on ATP depletion (Fig. 2E).

Although the mechanism(s) by which RU486 protects against AILI remains to be determined, it will likely be complex due to the diverse, and often contradictory, biological pathways mediated by the GCR [39]. In this regard, we found that RU486 pretreatment also attenuated halothane-induced liver injury (Fig. 3A) in addition to AILI. As with APAP, RU486 did not protect mice against halothane-induced liver injury by inhibiting the formation of the toxic reactive metabolite of halothane, trifluoroacetyl chloride [30], as determined by immunoblotting (data not shown). In contrast, RU486 pretreatment exacerbated CCl₄-induced liver injury (Fig. 3B) [40]. In this model, the pathologic effects of RU486 appeared to be related to its ability to inhibit GC-induced release of the anti-inflammatory cytokine, interleukin-10 [40]. RU486 pretreatment also exacerbated ConA-induced liver injury, a T cell-mediated model of liver disease [41], to such an extent that none of the mice survived the experiment (Fig. 3C). The complexity of the effects of GC in DILI is further highlighted by the results of another study in which restraint stress increased the severity of CCl₄-induced liver injury [42]. Similarly, electric foot shock-induced stress exacerbated α -galactosylceramide (α -GalCer)-induced liver injury, a natural killer T (NKT) cell-mediated model of liver injury [43]. Pretreatment with RU486 also decreased the extent of liver injury in this model.

In conclusion, our results suggest that GC could be an important risk factor for DILI, a finding with enormous clinical implications due to the universal nature of stress. Further understanding of the underlying mechanism is needed due to increasing evidence that GC could also be a risk factor for other types of ADRs, including drug-induced skin rash [44] and delayed-type hypersensitivity responses [45]. It is likely that the role of GC and GCR in ADRs is going to be very complex due to the near ubiquitous expression of the GCR [46] and the numerous pathways by which GCR mediate their signaling, including direct effects on transcription following translocation into the nucleus and more rapid, nongenomic effects initiated in the cytoplasm [39].

Acknowledgments

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Abbreviations

APAP acetaminophen

AILI	acetaminophen-induced liver injury
GC	glucocorticoid
GCR	glucocorticoid receptor
ConA	concanavalin A
CCl₄	carbon tetrachloride
DILI	drug-induced liver injury
NAPQI	<i>N</i> -acetyl- <i>p</i> -benzoquinone imine
H&E	hematoxylin and eosin
ALT	alanine aminotransferase
MPT	mitochondrial permeability transition
ROS	reactive oxygen species

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Fig. 1.

Endogenous corticosterone plays a role in AILI. Pretreatment with 25 mg/kg RU486 or vehicle control at -16 h and -2 h prior to treatment with 300 mg/kg APAP protects male C57Bl/6J mice (n=9) against AILI as assessed by (A) measuring serum ALT activity (* $P < 0.05$ versus vehicle control mice at each time point) and confirmed by (B) histological examination of H&E liver sections at 8 h and 24 h post-APAP treatment. Evidence that RU486 protection is mediated through the GCR was obtained by demonstrating that (C) pretreatment with 25 mg/kg RU486 at -16 h and -2 h prior to treatment with 300 mg/kg APAP did not protect adrenalectomized male C57Bl/6J mice (n=9) against AILI and that (D) pretreatment with 2 mg/kg dexamethasone (Dex) at -16 h and 0 h prior to treatment with 300 mg/kg APAP exacerbated AILI in male C57Bl/6J mice (n=8). * $P < 0.05$ versus vehicle control.

Fig. 2.

RU486 does not inhibit APAP metabolism, reactive oxygen species formation, or ATP depletion. For all experiments, male C57Bl/6J mice were pretreated with 25 mg/kg RU486 or vehicle control -16 h and -2 h prior to treatment with 300 mg/kg APAP. The effects of RU486 on APAP metabolism were assessed by comparing (A) immunoblots of APAP-protein adducts 2 h after APAP treatment and (B and C) reduced glutathione (GSH) levels in (B) whole liver homogenate and (C) mitochondria at indicated timepoints. In (A), each lane represents an individual mouse (120 μg/lane) and in (B and C), results represent the mean ± standard error of the mean of 5 mice per group. The effect of RU486 on APAP-induced (D) formation of carbonylated proteins and (E) ATP depletion was assessed by quantifying carbonylated proteins and ATP in whole liver homogenates at indicated timepoints. Results shown represent the mean ± standard at error of the mean of (D) 4 mice or (E) 5 mice per group.

Fig. 3.

RU486 protects mice from liver injury induced by halothane, but not by carbon tetrachloride (CCl_4) or concanavalin A (ConA). Mice were pretreated with 25 mg/kg RU486 or vehicle control -16 h and -2 h prior to treatment with (A) 30 mmoles halothane (B) 750 $\mu\text{l/kg}$ CCl_4 or (C) 30 mg/kg ConA. Serum ALT activity was measured at 8 h and 24 h. Male C57Bl/6J mice were used for all experiments except for those involving halothane (B) in which female Balb/c mice were used. * $P < 0.05$ versus vehicle-pretreated mice at each time point ($n=10$).