Endoplasmic Reticulum Stress-induced Upregulation of STARD1 Promotes Acetaminophen-induced Acute Liver Failure


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Fasting + acetaminophen

VPA + acetaminophen

CYP2E1

NAPQI

protein adducts

N-acetylcysteine

ER stress

STARD1 knockdown

STARD1ΔHep

TUDCA

cholesterol

SH3BP5

p-JNK

STARD1 knockdown

HEPATOTOXICITY

cholesterol

glutathione

glutathione depletion

∆Hep

STARD1 knockdown

HEPATOTOXICITY
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Running title: StARD1 and acetaminophen hepatotoxicity.

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**CONFLICT OF INTEREST:** The authors declare that they have nothing to disclose.

**ABBREVIATIONS:**

ALF, acute liver failure

APAP, acetaminophen

ER stress, endoplasmic reticulum stress

FAH, fumaroylacetoacetate hydrolase

GSH, Glutathione

HH, human adult hepatocytes

mGSH, mitochondrial GSH

NAC, N-acetylcysteine

NAPQI, N-acetyl-p-benzoquinoneimine

NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

OCR, oxygen consumption rates

StARD1, steroidogenic acute regulatory protein 1

TUDCA, tauroursodeoxycholic acid

VPA, valproic acid
ABSTRACT

Background & Aims: Acetaminophen overdose is a major cause of acute liver failure (ALF). Mitochondrial SH3BP5 (also called SAB) and phosphorylation of JNK mediate the hepatotoxic effects of acetaminophen. We investigated the involvement of steroidogenic acute regulatory protein (STARD1), a mitochondrial cholesterol transporter, in this process and sensitization by valproic acid (VPA), which depletes glutathione and stimulates steroidogenesis.

Methods: Non-fasted C57BL/6J mice (control) and mice with liver-specific deletion of STARD1 (Stard1\textsuperscript{ΔHep}), (Sab\textsuperscript{ΔHep}), or JNK1 and JNK2 (Jnk1\textsuperscript{+2ΔHep}) were given VPA with or without acetaminophen. Liver tissues were collected and analyzed by histology and immunohistochemistry and for SH3BP5 acetaminophen metabolism, endoplasmic reticulum (ER) stress, and mitochondrial function. Adult human hepatocytes were transplanted into FRGN mice to create mice with humanized livers.

Results: Administration of VPA before administration of acetaminophen increased the severity of liver damage in control mice. The combination of VPA and acetaminophen increased expression of CYP2E1, formation of NAPQI protein adducts, and depletion of glutathione from liver tissues of control mice, resulting in ER stress and the upregulation of STARD1. Livers from control mice given VPA and acetaminophen accumulated cholesterol in the mitochondria and had sustained mitochondrial depletion of glutathione and mitochondrial dysfunction. Inhibition of ER stress, by administration of tauroursodeoxycholic acid to control mice, prevented upregulation of STARD1 in liver and protected the mice from hepatoxicity following administration of VPA and acetaminophen. Administration of N-acetylcysteine to control mice prevented VPA- and acetaminophen-induced ER stress and liver injury. Stard1\textsuperscript{ΔHep} mice were resistant to induction of ALF by VPA and acetaminophen, despite increased mitochondrial levels of
glutathione and phosphorylated JNK; we made similar observations in fasted
Stard1\textsuperscript{Hep} mice given acetaminophen alone. Sab\textsuperscript{Hep} mice or Jnk1+2\textsuperscript{Hep} mice did not
develop ALF following administration of VPA and acetaminophen. The ability of VPA
to increase the severity of acetaminophen-induced liver damage was observed in FRGN
mice with humanized liver.

**Conclusions:** In studies of mice, we found that upregulation of STARD1 following ER
stress mediates acetaminophen hepatotoxicity via SH3BP5 and phosphorylation of JNK1
and JNK2.

**Key words:** mouse model, signal transduction, lipid, APAP toxicity.
INTRODUCTION

Drug-induced liver injury (DILI) is a major cause of acute liver failure (ALF) and a leading reason for drug withdrawal from the market (1, 2). Unlike intrinsic DILI, which is predictable, reproducible and dose-dependent, idiosyncratic DILI is unpredictable, not strictly dose-dependent, and while rare it accounts for 10-15% of ALF cases in the US (3, 4).

Acetaminophen is one of the most widely used pain relievers worldwide. While relatively safe, acetaminophen is a dose-dependent hepatotoxin that can cause intrinsic DILI. The use of acetaminophen at therapeutic doses for more than a few days has been shown to elevate serum transaminases in one-third of patients (5). Acetaminophen-induced liver damage is characterized by haemorrhagic centrilobular necrosis and high plasma transaminase levels in both humans and animals, which can evolve in some cases to ALF (6). Although acetaminophen is metabolized to its glucuronidated and sulphated non-toxic metabolites in the liver, acetaminophen overdose saturates these pathways and the excess acetaminophen is metabolized by CYP2E1 into the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which is rapidly conjugated with GSH resulting in non-toxic mercapturic acid and cysteine conjugates that are excreted in the urine (7, 8). When glutathione levels are limited, free unconjugated NAPQI reacts with sulfhydryl groups on cysteine and lysine residues, generating NAPQI-protein adducts (acetaminophen-protein adducts) in hepatocytes, particularly in mitochondria, leading to mitochondrial dysfunction and cell death (7-9). SH3BP5 (Sab), a mitochondrial outer membrane protein, has been identified as a key player in acetaminophen-induced hepatotoxicity. Knockdown or liver specific knockout of SH3BP5 inhibited sustained JNK activation and protected against acetaminophen hepatotoxicity (10). Moreover, there is extensive experimental evidence that JNK
activation and its translocation to mitochondria contributes to acetaminophen hepatotoxicity (11, 12).

Valproic acid (VPA) is widely prescribed for epilepsy and used as an anticonvulsant, which is eliminated by biotransformation through multiple pathways, including glucuronidation, cytochrome P-450 metabolism and mitochondrial β-oxidation (13). Besides inducing metabolic syndrome in overweight epileptic patients (14, 15), long-term VPA therapy can cause hepatotoxicity that may progress to idiosyncratic ALF (13, 16). VPA-mediated hepatotoxicity is characterized by necrosis, depletion of endogenous antioxidants and disruption of mitochondrial β-oxidation of fatty acids, which can contribute to hepatic steatosis (16-20). Furthermore, VPA has been shown to inhibit HDAC and GSK3 signaling events (21, 22) and while reported to induce ER stress (23, 24), there are studies showing that VPA protects cells against ER stress-mediated apoptosis and atherosclerosis (25, 26).

As VPA has been shown to deplete glutathione levels and stimulate steroidogenesis by increasing mitochondrial cholesterol trafficking (27, 28), we hypothesized that VPA can sensitize to acetaminophen-mediated ALF independently of fasting by inducing STARD1, a mitochondrial protein that mediates the transport of cholesterol to the mitochondrial inner membrane (29), which results in impaired transport of glutathione into mitochondria (30, 31). Our data reveal that VPA pretreatment in non-fasted mice results in acetaminophen-induced ALF by a mechanism that requires the complimentary action of STARD1 and Sab-JNK1/2.
MATERIALS AND METHODS

Generation of mice with liver-specific STARD1 deletion.

All experimental protocols for animal use and handling met the guidelines of the Animal Care Committee of the Hospital Clinic-Universidad de Barcelona. Male C57BL/6J mice (8 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA). As mice with global STARD1 deletion die soon after birth due to adrenal lipoid hyperplasia (32), we generated mice with liver-specific STARD1 deletion by Cre-lox technology, as described in Supplementary Methods (Supplementary Figure 1A). Homozygous Stard1 floxed animals (STARD1^ff) were crossed with albumin-Cre mice (C57BL/6-TgN(Alb-Cre)^21Mgn, Jackson Laboratories) to obtain Stard1^{ff-AlbCre} (Stard1 Liver specific knockout, STARD1^ΔHep), which exhibited specific deletion of Stard1 in the liver with unchanged expression in steroidogenic tissues and white adipose tissue (Supplementary Fig 1B, C). Mice were backcrossed to C57BL/6J strain for nine generations. 8-10 week old STARD1^{ff} littermates and STARD1^ΔHep mice were treated with VPA, APAP and their combination as described below.

Mice with liver-specific deletion of Sab and JNK1+2.

Sab^ΔHep (C57BL/6N) and JNK1+2^ΔHep (57BL/6J) mice were generated by treatment of Sab^{ff} mice or JNK1+2^{ff} with AAV8.TBG.Cre as described previously (10). JNK1+2^{ff} mice were provided by Dr. Roger J. Davis, UMass Medical School, Worcester, MA.

FRGN mice with humanized liver and expansion of human hepatocytes.

Fah^-/-/Rag2^-/-/Il2rg^-/-/NOD (FRGN) mice were xenotransplanted with human adult hepatocytes (HH), as described before (33), achieving a rate of repopulation of 80-90% as monitored by serum human albumin levels. Experiments to determine susceptibility to VPA and acetaminophen were conducted in 14-weeks old FRGN mice in accordance with the approved Institutional Animal Care Committee at Oregon Health & Science
University of Portland, Oregon and Animal Care Committee of the Hospital Clinic-Universidad de Barcelona. Plateable cryopreserved male HH (#M00995-P) were obtained from BioreclamationIVT Company (Brussels, Belgium). One million viable HH in 100µL of Dulbecco’s modified essential medium were injected intrasplenicly via a 27-gauge needle followed by gradual reduction of NTBC one week after xenotransplantation, as described (33).

**In vivo treatment with VPA and APAP**

8-10 weeks-old C57BL/6J, StARD1\(^{\Delta\text{Hep}}\), Sab\(^{\Delta\text{Hep}}\) and JNK1+2\(^{\Delta\text{Hep}}\) mice were treated with VPA, 400mg/kg s.c., a dose typically used in mice (34, 35), 3 times every 12 hours followed by a single acetaminophen injection (300mg/Kg i.p.). Determination of serum VPA levels (Dimension EXL analyzer, Siemens Healthcare) from mice treated with 400mg/Kg were 38±2.4 µg/ml, similar to the therapeutic range reported in patients under VPA therapy (36). Mice were sacrificed at 2, 4 or 24 hours after giving acetaminophen and serum and liver samples were collected for analyses. In addition, acetaminophen hepatotoxicity was induced in overnight fasted mice. In some cases, tauroursodeoxycholic Acid (TUDCA, Calbiochem; 250mg/Kg i.p.) or N-acetylcysteine (NAC, 2.5 mmol/Kg i.p.) were administered 3 times every 12 hours along with VPA and acetaminophen treatment. Other group of mice were dosed with atorvastatin (10mg/Kg, daily) or vehicle via gavage along with VPA followed by acetaminophen administration.

**Statistical Analyses**

Results are expressed as mean±standard error of the mean (SEM). Statistical significance of mean values were assessed using Student T-test (2 groups) and one or two-way ANOVA followed by Tukey’s Multiple Comparison Post-test (≥ 3 groups). \( P < 0.05 \) was considered statistically significant. Survival curves were constructed using
the method of Kaplan and Meier. The corresponding number of experiments are indicated in the figure legends. Statistics were performed using GraphPad Prism 6 software.
Results

Fasting or VPA pretreatment in non-fasted mice sensitizes to acetaminophen hepatotoxicity.

As fasting sensitzes to acetaminophen-mediated hepatotoxicity (37), we first examined whether fasting potentiated the hepatotoxicity of VPA+acetaminophen coadministration. While repeated VPA dosing to fasted mice did not cause hepatotoxicity, acetaminophen administration induced marked liver injury and centrilobular necrosis (Figure 1A and 1B). However, the combination of VPA+acetaminophen did not further enhance the liver injury caused by acetaminophen alone, indicating that fasting sensitized to acetamionophen hepatotoxicity regardless of VPA pretreatment. In contrast, non-fasted mice were resistant to acetaminophen-induced liver injury when acetaminophen was given in the morning, in agreement with previous results (38), and VPA administration (3 times/12 hours) did not cause liver damage (Figure 1C and 1D). Intestingly, VPA pre-administration in non-fasted mice sensitized to acetaminophen-mediated hepatotoxicity similar to the injury seen in fasted mice following APAP administration (Figure 1C and 1D), with increased ALT release over time, which paralleled the liver injury seen by H&E (Figure 1E, F). We next examined the survival of non-fasted mice to VPA pretreatment followed by the administration of a higher dose of acetaminophen (600mg/Kg). While non-fasted mice were resistant to VPA or acetaminophen alone, mice pretreated with VPA followed by acetaminophen administration died over time with a mortality rate of 40% by 8 days post acetaminophen administration that was similar to the rate observed in fasted mice after being given acetaminophen (Figure 1G). Moreover, treatment with VPA+acetaminophen caused a higher rate of cell death in primary mouse hepatocytes.
Acetaminophen metabolism, glutathione homeostasis and mitochondrial function following VPA+acetaminophen administration in non-fasted mice.

Unlike VPA or acetaminophen alone VPA+acetaminophen coadministration to non-fasted mice induced an early and time-dependent upregulation of CYP2E1 that paralleled the generation of NAPQI-protein adducts, similar to the levels induced by acetaminophen in fasted mice (Figure 2A). Moreover, as glutathione is required for NAPQI detoxification, we next examined glutathione homeostasis in liver homogenate and mitochondrial fraction in non-fasted mice. As seen, total glutathione levels were moderately depleted 2 hours after VPA or acetaminophen alone, which increased by 4 hours post-treatment and recovered to control levels by 24 hours, while total glutathione levels remained significantly depleted at 4 hours after VPA+acetaminophen administration (Figure 2B), consistent with the kinetics of NAPQI-protein adduct formation. However, neither VPA nor acetaminophen alone affected mitochondrial glutathione levels, whereas the combination of VPA+acetaminophen markedly depleted mitochondrial glutathione stores as early as 2 hours post-treatment that remained significantly decreased by 4 hours (Figure 2B). In line with the induction of CYP2E1 or NAPQI-adduct generation, acetaminophen administration to fasted mice depleted total and mitochondrial glutathione levels by 65-75%, similar to the depletion induced by VPA+acetaminophen administration in fed mice.

We next examined mitochondrial function using a metabolic flux analyzer to determine real-time OCR. While VPA or acetaminophen alone did not affect mitochondrial function, VPA+acetaminophen coadministration resulted in a decreased respiratory
control ratio (State III/State IV) with respect to VPA or acetaminophen alone (Figure 2C), indicating impaired mitochondrial respiration.

**VPA+acetaminophen upregulates STARD1 expression in non-fasted mice.**

We next examined the homeostasis of cholesterol and its trafficking to mitochondria, which is known to regulate mitochondrial glutathione (30, 31). Interestingly, increased mitochondrial pool of cholesterol was an early event (4 hours) after giving VPA+acetaminophen (Figure 2D), even though free cholesterol levels increased afterwards as shown by the representative filipin staining at 24 hours (Figure 2E), suggesting the activation of a specific mechanism that triggers the accumulation of free cholesterol in mitochondria. As STARD1 regulates mitochondrial cholesterol trafficking (29), we next examined its expression in response to drug administration. In contrast to VPA or acetaminophen alone, VPA+acetaminophen increased an early expression (4 hours) of STARD1 both at the mRNA and protein level (Figure 2F, G). To examine the pattern of STARD1 expression we performed immunohistochemical analysis in liver sections 24 hours after VPA+acetaminophen administration (Figure 2H), indicating predominant expression in the pericentral area, which is consistent with the characteristic haemorrhagic centrilobular necrosis seen in APAP hepatotoxicity. These findings were accompanied by increased expression of phosphorylated STARD1 (Figure 2G), which is known to increase its activity in the trafficking of cholesterol to mitochondria in steroidogenic cells (39, 40). Moreover, the expression of hepatic MLN64 (also known as STARD3), another member of the STARD family involved in the egress of cholesterol from endosomes to mitochondria (41, 42), did not significantly increased at early time points after VPA+acetaminophen administration (Supplementary Figure 3A), although it increased 24 hours post-treatment.
VPA+acetaminophen induces ER stress and mitochondrial JNK activation in non-fasted mice.

As tunicamycin has been shown to induce the expression of STARD1 by triggering ER stress rather than SREBP2 activation (43) and consistent with the role of ER stress in the regulation of STAR family members (44, 45), we next examined ER stress as a potential mechanism that regulate STARD1 expression. VPA+acetaminophen induced an early (2 hours) increase in the expression of several ER stress markers at the mRNA and protein levels (Figure 3 A, B). The increase of ER stress markers was sustained at 4 hours post-treatment (Figure 3 C, D), suggesting the involvement of different arms of the ER stress pathway (e.g. IRE-1α and PERK). Moreover, immunohistochemical analyses revealed that VPA+acetaminophen increased CHOP expression in liver sections 24 hours post-treatment (Figure 3E), which exhibited predominant pericentral staining, similar to StARD1. Since ERK1/2 phosphorylates and regulates STARD1 activity (46), we next analyzed the expression of hepatic ERK1/2 in mice treated with VPA+acetaminophen. As seen, the phospho-ERK/ERK increased 4 hours after VPA+acetaminophen compared to either drug alone (Figure 3F). In addition, we also examined whether VPA+acetaminophen enhanced the translocation of phosphorylated JNK to mitochondria, which has emerged as a crucial event for acute liver injury (10, 11). As seen, increased translocation of phosphorylated JNK in mitochondria was observed 2 hours after VPA+acetaminophen, similar to the increase seen by acetaminophen treatment in fasted mice (Figure 3G).

Targeting ER stress prevents STARD1 upregulation and protects against VPA+acetaminophen hepatotoxicity in non-fasted mice.

We examined the impact of interfering with ER stress on STARD1 expression and liver injury by VPA+acetaminophen administration. As seen, TUDCA abolished the
induction of ER stress markers CHOP, sXBP1 and p-EIF2α 4 hours post-treatment with VPA+acetaminophen (Figure 4A). In line with these findings, TUDCA prevented the increased expression of CHOP in liver sections (24 hours) after VPA+acetaminophen (Figure 4B). Consistent with the induction of STARD1 by tunicamycin-induced ER stress (44), TUDCA prevented the early (4 hours) STARD1 upregulation by VPA+acetaminophen (Figure 4C, D) and the sustained expression of STARD1 in liver sections as revealed by immunohistochemistry (24 hours) (Figure 4E) and its mitochondrial targeting examined by confocal imaging (Supplementary Figure 4). More importantly and in line with these events, TUDCA prevented the mitochondrial glutathione depletion induced by VPA+APAP (Figure 4F) and protected against liver injury caused by VPA+acetaminophen administration (Figure 4G, H).

Arylating quinones have been shown to induce ER stress (47) and correct protein folding in the ER requires appropriate redox environment. Thus, we addressed whether in addition to the role of NAPQI glutathione depletion can contribute to ER stress. PMH treated with diethylmaleate (DEM) severely depleted total glutathione content and increased the level of CHOP, indicating the onset of ER stress (Supplementary Figure 2B, C). In addition, DEM-induced CHOP upregulation was attenuated by glutathione replenishment with NAC (Supplementary Figure 2C). Moreover, tunicamycin induced CHOP expression, as expected, that was ameliorated by NAC, suggesting a relationship between glutathione and ER stress regulation. Furthermore, in line with these in vitro findings, in vivo NAC administration to mice given VPA+acetaminophen restored total glutathione and mitochondrial glutathione stores (Figure 5A) and, importantly, prevented VPA+acetaminophen-induced ER stress (Figure 5B, C) and subsequent STARD1 induction (Figure 5D, E), resulting in the protection against VPA+acetaminophen-mediated liver injury (Figure 5F, G).
Liver-specific STARD1 deletion protects against acetaminophen hepatotoxicity elicited by VPA or fasting.

STARD1\(^{\Delta\text{Hep}}\) mice were generated by crossing chimeric STARD1\(^{f/f}\) mice with Alb-Cre mice (Supplementary Figure 1A-C). STARD1\(^{\Delta\text{Hep}}\) mice exhibited a marked downregulation of STARD1 expression in liver extracts which did not affect the mitochondrial number and morphology (Supplementary Figure 1D). As seen, VPA+acetaminophen induced the expression of STARD1 in STARD1\(^{f/f}\) mice at the mRNA levels and protein levels in liver homogenates and liver samples as revealed by immunohistochemistry, which were abolished in STARD1\(^{\Delta\text{Hep}}\) mice (Figure 6A-C). This outcome was specific for STARD1 as expression of MLN64 by VPA+acetaminophen was maintained in STARD1\(^{\Delta\text{Hep}}\) mice (Supplementary Figure 3B). As expected, VPA+acetaminophen increased mitochondrial cholesterol levels in STARD1\(^{f/f}\) but not STARD1\(^{\Delta\text{Hep}}\) mice (Figure 6D), which translated in higher levels of mitochondrial glutathione in STARD1\(^{\Delta\text{Hep}}\) mice by VPA+acetaminophen administration (Figure 6E). Consistent with these findings, VPA+acetaminophen-induced liver injury examined by serum ALT levels and H&E was markedly lower in STARD1\(^{\Delta\text{Hep}}\) mice with respect to STARD1\(^{f/f}\) mice (Figure 6F-G, Supplementary Figure 5). Moreover, adenovirus-mediated STARD1 knockdown also prevented VPA+acetaminophen mediated hepatotoxicity in non-fasted mice (Supplementary Figure 6), further validating the relevance of STARD1 activation in the hepatotoxic effects caused by VPA+APAP administration. In addition, similar protection was observed following acetaminophen administration in overnight fasted StARD1\(^{\Delta\text{Hep}}\) mice, without preventing the induction of CYP2E1 and ER stress (Figure 7). Moreover, as a complimentary approach to test the role of cholesterol in VPA+acetaminophen-induced liver injury,
atorvastatin prevented the increase in mitochondrial cholesterol levels and protected against VPA+acetaminophen-mediated liver injury (Supplementary Figure 7).

We examined whether the protection of STARD1\(^{AHep}\) mice was related to a lower translocation of phosphorylated JNK to mitochondria. As seen, the mitochondrial phosphorylation of JNK induced by VPA+acetaminophen was similar in both STARD1\(^{f/f}\) and STARD1\(^{AHep}\) mice (Figure 6H), with similar findings observed in overnight fasted STARD1\(^{f/f}\) and STARD1\(^{AHep}\) mice following acetaminophen administration (Figure 7E). These results indicate that mitochondrial translocation of phosphorylated JNK requires STARD1 to elicit liver injury.

**Mice with liver-specific deletion of Sab and JNK1+2 are protected against VPA+acetaminophen induced liver injury.**

We next examined the role of Sab and JNK in the liver injury in non-fasted mice given VPA+acetaminophen. Non-fasted Sab\(^{AHep}\) mice and JNK1+2\(^{AHep}\) mice were administered with repeated doses of VPA followed by a single acetaminophen administration. While both Sab\(^{f/f}\) mice and JNK1+2\(^{f/f}\) mice were sensitized to acetaminophen-induced liver injury by VPA pretreatment as shown by H&E analyses and serum ALT release, Sab\(^{AHep}\) mice and JNK1+2\(^{AHep}\) mice were markedly protected against VPA+acetaminophen induced liver damage (Supplementary Figure 8A-D). Quite interestingly, while VPA+acetaminophen induced STARD1 and CHOP in Sab\(^{f/f}\) mice and JNK1+2\(^{f/f}\) mice, these effects were prevented in Sab\(^{AHep}\) and JNK1+2\(^{AHep}\) mice, suggesting that Sab/JNK are necessary for ER stress and subsequent STARD1 induction (Supplementary Figure 8E, F). Similar results were observed in fasted JNK1+2\(^{AHep}\) mice after acetaminophen administration (Figure 7F). These findings together with the protection seen in STARD1\(^{AHep}\) mice suggest that STARD1 and Sab-JNK1+2 exert complimentary roles in acetaminophen induced liver injury.
VPA sensitizes to acetaminophen hepatotoxicity in FRGN mice with humanized liver

To address the potential translational relevance of the preceding findings showing the sensitization of VPA to acetaminophen-mediated hepatotoxicity, we next explored the susceptibility of FRGN mice, a genetically engineered model that allows the repopulation of the murine liver with HH, to VPA+acetaminophen induced liver injury. FRGN mice were repopulated with HH by more than 80% as revealed by the human albumin levels in serum and the expression of human fumaroylacetoacetate hydrolase (FAH) (Supplementary Figure 9A, B). Non-fasted FRGN mice were treated with VPA followed by a single dose of acetaminophen and sacrificed 24 after treatment. While non-fasted FGRN mice xenotransplanted with HH were resistant to VPA or acetaminophen their combination caused liver injury, as revealed by increased serum transaminases and H&E analyses (Supplementary Figure 9C, D). In line with these findings, humanized FRGN mice given VPA+acetaminophen exhibited depletion of both total glutathione and mitochondrial glutathione levels (Supplementary Figure 10A), as well as an increase in the expression of ER stress markers (Supplementary Figure 10B), an an early increase in STARD1 mRNA levels (Supplementary Figure 10C) in parallel with the increased STARD1 expression in liver sections (Supplementary Figure 9E) and enhanced MLN64 expression (Supplementary Figure 3D). Moreover, while endogenous staining of F4/80 and myeloperoxidase in humanized FRGN mice was low (48), VPA+acetaminophen induced a mild expression of F4/80 with a minimal staining of myeloperoxidase (Supplementary Figure 10D). To validate whether this outcome reflected the injury of HH rather than the remnant murine hepatocytes by VPA+acetaminophen, we estimated the colocalization of FAH expression with TUNEL as a measure of damaged HH. As seen, most TUNEL positive cells colocalized with...
human hepatocytes expressing FAH (Supplementary Figure 9F, G). Thus, these findings underscore the susceptibility of in vivo repopulation of human adult hepatocytes to VPA+acetaminophen hepatotoxicity.
4. Discussion

Here, we uncover that while non-fasted mice are resistant to VPA or APAP administration, prior VPA dosing, at a concentration that yields therapeutic VPA levels, sensitizes to acetaminophen-mediated hepatotoxicity through the induction of an ER stress-mediated STARD1 upregulation, which promotes mitochondrial cholesterol loading and mitochondrial glutathione deletion.

Our data indicate that VPA+acetaminophen resulted in an early generation of NAPQI-protein adducts formation, a key event involved in acetaminophen-mediated hepatotoxicity, which paralleled the profound depletion of glutathione levels by VPA+acetaminophen in non-fasted mice. While treatment with VPA or acetaminophen alone in non-fasted mice was insufficient to accumulate NAPQI-protein adducts, both drugs caused an early and transient depletion of glutathione, which increased afterwards. These data suggest that in non-fasting conditions the generation of NAPQI from acetaminophen is metabolized at the expense of glutathione stores, implying the existence of a sufficient glutathione reservoir or unrestricted capacity for glutathione neosynthesis to conjugate NAPQI to prevent the NAPQI-protein adduct formation. In line with this scenario, we observed that VPA+acetaminophen administration induced the upregulation of CYP2E1 compared to either drug alone, suggesting that the metabolism of acetaminophen into NAPQI following VPA dosing occurs at a rate that may overwhelm the ability of glutathione to form non-toxic mercapturic conjugates. Thus, the generation of NAPQI by the combination of VPA+acetaminophen but not by VPA or acetaminophen alone may trigger subsequent downstream events leading to hepatotoxicity in non-fasted mice.

While all quinones can generate reactive oxygen species through redox cycling, partially substituted quinones also undergo arylation and Michael adduct formation.
yielding covalent bonds with nucleophiles, such as cysteinyl thiols. The reported cytotoxicity of arylating quinones correlates with their ability to induce ER stress (47). As a partially substituted quinone, NAPQI’s affinity to form Michael adducts with protein thiols can account for the ability of VPA+acetaminophen to induce ER stress. Although we did not examine the identity of the NAPQI-protein adducts not their intracellular site of generation following VPA+acetaminophen, it is conceivable that NAPQI may have formed Michael adducts with ER proteins to underlie the observed ER stress, as well as the covalent binding with intramitochondrial proteins, which are known to be a major target of acetaminophen metabolism (7, 8). In addition to forming Michael protein adducts in the ER, the correct protein folding in the ER requires appropriate redox environment, matched by an appropriate glutathione homeostasis. As ER stress can be regulated by antioxidants and glutathione replenishment (49), the ability of NAPQI to deplete glutathione levels may secondarily contribute to the onset of ER stress caused by VPA+acetaminophen treatment, in parallel with the outcome observed in PMH. In line with this scenario, we observe that NAC prevents the induction of ER stress by VPA+acetaminophen. In addition to the presence of its nucleophilic thiol, which could directly target NAPQI-protein adducts, NAC is a precursor of glutathione stimulating its synthesis by providing the rate-limiting cysteine precursor, and therefore it is conceivable that the protective effect of NAC in VPA+acetaminophen hepatotoxicity may imply a dual mechanism involving the prevention of NAPQI-protein adduct formation and glutathione replenishment, thereby abolishing the onset of ER stress, consistent with recent findings in fasted mice treated with APAP (50). The relevance of the ER stress in the hepatotoxicity caused by VPA+acetaminophen in non-fasted mice is further demonstrated by the time-dependent sequence of events, in which the onset of ER stress by VPA+acetaminophen preceded
liver injury, and by the ability of the chemical chaperone TUDCA to abolish the induction of ER stress markers and protect against VPA+acetaminophen mediated liver injury. Interestingly, both NAC and TUDCA prevented the upregulation of STARD1 by VPA+acetaminophen, consistent with the causal role of ER stress in inducing STARD1 overexpression, underlying a previously unrecognized ER stress-STARD1 axis in acetaminophen hepatotoxicity.

The importance of the induction of STARD1 in the VPA+acetaminophen hepatotoxicity in non-fasted mice is illustrated by the resistance of STARD1\textsuperscript{Hep} mice to VPA+acetaminophen induced liver injury, with similar findings observed in fasted STARD1\textsuperscript{Hep} mice after acetaminophen administration, indicating that STARD1 contributes to acetaminophen hepatotoxicity regardless of the sensitization approach (VPA pretreatment or fasting). Importantly, this outcome is reproduced upon the genetic knockdown of STARD1 in adult wild type mice, discarding the involvement of compensatory effects in the protection of STARD1\textsuperscript{Hep} mice against VPA+acetaminophen induced liver damage. Quite interestingly, non-fasted Sab\textsuperscript{Hep} mice were equally resistant to VPA+acetaminophen hepatotoxicity. These findings complement previous observations in fasted Sab\textsuperscript{Hep} mice given acetaminophen (10, 11) and, consistent with its role as a docking protein for the translocation of phosphorylated JNK to mitochondria, indicate that Sab is a crucial player in acetaminophen-mediated liver injury regardless of the approach of sensitization. Remarkably, our findings in JNK1+2\textsuperscript{Hep} mice indicate that VPA sensitization to acetaminophen hepatotoxicity in non-fasted mice required hepatocyte JNK1+2 activation, which are in line with previous findings of acetaminophen hepatotoxicity in fasted mice (reviewed in 11) but in contrasts with recent findings reporting exacerbated acetaminophen hepatotoxicity in mice with hepatocyte-specific \textit{Jnk1} deletion and global \textit{Jnk2} ablation (51). Although
further work will be required to address this discrepancy, the approach for the genetic deletion of Jnk1 and Jnk2 between Cubero et al. and our current study was different. While we deleted Jnk1 and Jnk2 selectively in hepatocytes in adult mice by AAV8.TBG.Cre injection, Cubero et al used mice with germ line deletion of Jnk1 in hepatocytes and global Jnk2 (51), which could lead to the impairment of JNK’s developmental protective mechanisms. Nevertheless, an intriguing finding from our study is the preservation for increased translocation of p-JNK in mitochondrial fractions from STARD1\textsuperscript{AHeP} mice treated with acetaminophen, which suggests a functional interplay between STARD1 and p-JNK to elicit liver injury. This interplay seems to be exerted at least at two levels: 1) JNK is required for STARD1 expression, possibly through the induction of ER stress; 2) the role of mitochondrial JNK activation in acetaminophen hepatotoxicity is dependent on STARD1. Extensive further research will be required to elucidate the complimentary roles of STARD1 and activated JNK in acetaminophen hepatotoxicity, particularly the elucidation of the mechanisms downstream of JNK involved in the induction of ER stress and subsequent STARD1 activation and how STARD1 sets the adequate physico-chemical scenario in mitochondria for JNK to execute its action in mitochondrial function and liver damage.

To explore the potential relevance of the preceding findings, we investigated the sensitivity of FRGN mice with humanized liver to VPA+acetaminophen and observed that the sensitization by VPA to acetaminophen-mediated hepatotoxicity was recapitulated in vivo in human adult hepatocytes that repopulated the liver of the FRGN mice, as revealed by colocalization of FAH and TUNEL staining. Thus, while VPA+acetaminophen induced a cell autonomous toxic effect of human hepatocytes from FRGN mice, the degree of liver injury in the humanized FRGN mice estimated by the release of serum ALT was lower compared to wild type mice, similar to previous
reports in uPA+/+/SCID mice repopulated with human hepatocytes (52). This outcome may reflect a putative functional immaturity of the repopulating human hepatocytes or the lack of additional factors involved in acetaminophen hepatotoxicity in the liver of humanized FRGN mice, such as inflammatory cells (e.g. macrophages or neutrophils). Whether the reconstitution of human hematopoietic cells in the FRGN mice from transplanted human CD34 stem cells (48) will increase the degree of hepatotoxicity induced by VPA+acetaminophen remains to be investigated.

Finally, we speculate that our findings may have implications in patients with chronic liver disease. There is growing evidence that NAFLD can increase the risk and/or the severity of liver injury induced by different drugs, including acetaminophen (53). Quite interestingly, we have previously reported that patients with NASH exhibit increased expression of STARD1 (54), suggesting that a subset of patients with advanced NAFLD and enhanced free cholesterol content and STARD1 expression may develop liver injury upon acetaminophen consumption.
REFERENCES


19. Cengiz M, Yüksel A, Seven M. The effects of carbamazepine and valproic acid o the erythrocyte glutathione, glutathione peroxidase, superoxide


34. Dowdell KC, Pesniack L, Hoffmann V et al. Valproic acid, a histone deacetylase inhibitor diminishes lymphoproliferation in the Fas deficient


FIGURE LEGENDS

Fig 1. VPA pretreatment sensitizes to acetaminophen hepatotoxicity in non-fasted mice.

A-B, Fasted mice were given VPA (400mg/Kg 3 times/12 hours; N=5 mice), acetaminophen (APAP) (300mg/Kg, N=7 mice) or VPA+APAP (N=6 mice) and sacrificed 24 hours later to examine ALT/AST and H&E analysis. C-D, Non-fasted mice were sacrificed 24 hours after VPA (400mg/Kg 3 times/12 hours; N=5 mice), APAP (300mg/Kg; N=6 mice) or VPA+APAP (N=9 mice) to determine ALT/AST and H&E. E-F, Non-fasted mice were sacrificed at 2, 4 and 24 hours after VPA, APAP or VPA+APAP to examine H&E and ALT levels. G, Time-dependent survival of non-fasted mice given VPA (400mg/Kg 3 times/12 hours), APAP (600mg/Kg) or VPA+APAP. For comparison, survival rate was determined in mice treated with a single injection of APAP (600mg/Kg) after overnight fasting. Scale bars: 100µm. The number of mice in G is shown in the graph. *p<0.05 as indicated.

Fig 2. Mitochondrial dysfunction and STARD1 upregulation by VPA+acetaminophen. A-B, Non-fasted mice were given VPA (400mg/Kg 3 times/12 hours; N≥5 mice), acetaminophen (APAP) (300ng/Kg; N≥5 mice) or VPA+APAP (N≥5 mice) and sacrificed 2 and 4 hours post-treatment for CYP2E1 levels and NAPQI-protein adducts generation or GSH levels in total liver extracts or mitochondrial fraction. As positive control, CYP2E1 and NAPQI-adducts were determined in fasted mice after 2 hours of APAP administration. C, OCR tracings and respiratory control ratio of isolated mitochondria from non-fasted mice treated with VPA (N=4 mice), APAP (N=4 mice) or VPA+APAP (N=4 mice) for 4 hours. D, mitochondrial cholesterol levels of non-fasted mice 4
hours after treatment with VPA (N=9 mice), APAP (N=9 mice) or VPA+APAP (N=9 mice). E, Filipin staining of frozen liver sections from non-fasted mice 24 hours after treatment with VPA, APAP or VPA+APAP. Scale bar: 50µm. F, StARD1 mRNA levels from liver extracts of non-fasted mice 4 hours after treatment with VPA (N=8 mice), APAP (N=8 mice) or VPA+APAP (N=9 mice). G, StARD1 and p-StARD1 expression in liver extracts of non-fasted mice 4 hours after treatment with VPA (N=5 mice), APAP (N=5 mice) or VPA+APAP (N=5 mice). H, Immunohistochemical staining of StARD1 in liver sections from non-fasted mice 24 hours after treatment with VPA, APAP or VPA+APAP. Scale bar: 100µm. *p<0.05 as indicated.

**Fig 3. VPA+acetaminophen induces ER stress in non-fasted mice.**

A, mRNA levels of ER stress markers in liver extracts of non-fasted mice 2 hours after treatment with VPA (400mg/Kg; 3 times/12 hours; N≥5 mice), acetaminophen (APAP) (300mg/Kg; N≥5 mice) or VPA+APAP (N≥5 mice). B, western blots of ER stress markers in liver extracts of non-fasted mice 2 hours after treatment with VPA (400mg/Kg; 3 times/12 hours; N=4 mice), APAP (300mg/Kg; N=4 mice) or VPA+APAP (N=4 mice). C, mRNA levels of ER stress markers 4 hours after treatment with VPA (N≥5 mice); APAP (N≥5 mice) or VPA+APAP (N≥5 mice). D, western blots of ER stress markers in liver extracts of non-fasted mice 4 hours after treatment with VPA (N≥4 mice); APAP (N≥4 mice) or VPA+APAP (N≥4 mice). E, Immunohistochemistry of CHOP expression in livers of non-fasted mice 24 hours after treatment with VPA, APAP or VPA+APAP. Scale bar: 100µm. F, Phospho-ERK activation in liver extracts from non-fasted mice 4 hours after treatment with VPA (N=4 mice), APAP (N=4 mice) or VPA+APAP (N=4 mice). G, Phospho-JNK levels in
mitochondrial fractions from non-fasted mice treated for 2 hours with VPA (N=4 mice), APAP (N=4 mice) or VPA+APAP (N=4 mice). *p<0.05 as indicated.

**Fig 4. TUDCA prevents VPA+acetaminophen induced ER stress and hepatotoxicity in non-fasted mice.** A, western blot of ER stress markers from non-fasted mice given VPA+APAP with or without TUDCA treatment for 4 hours. Control (N=4 mice), TUDCA (N=4 mice), VPA+APAP (N=4 mice), VPA+APAP+TUDCA (N=4 mice). B, CHOP expression in liver sections on non-fasted mice 24 hours post-treatment with VPA+APAP with or without TUDCA administration. Scale bar: 100µm. C, StARD1 mRNA levels in liver extracts 4 hours post VPA+APAP treatment with or without TUDCA administration. Control (N=7 mice), TUDCA (N≥5 mice), VPA+APAP (N=11 mice), VPA+APAP+TUDCA (N=5 mice). D, StARD1 protein levels in liver extracts 4 hours post-treatment as in C. N=4 mice/per group. E, immunohistochemistry analysis of StARD1 examined 24 hours post VPA+APAP treatment with or without TUDCA administration. Scale bar: 100µm. F, total and mitochondrial GSH levels from mice treated with VPA+APAP with or without TUDCA. Control (N=7 mice), TUDCA (N=5 mice), VPA+APAP (N=8 mice), VPA+APAP+TUDCA (N=5 mice). G-H, H&E analyses and transaminases from mice 24 hours after treatment with VPA+APAP with or without TUDCA. Control (N=5 mice), TUDCA (N=5 mice), VPA+APAP (N=16 mice), VPA+APAP+TUDCA (N=7 mice). Scale bar: 100µm. *p<0.05 as indicated.

**Figure 5. NAC restores GSH levels and prevents VPA+acetaminophen-induced ER stress and STARD1 induction in non-fasted mice.** A, GSH levels in liver extracts and mitochondrial fraction from non-fasted given VPA+APAP
for 4 hours with or without NAC treatment. Control (N=5 mice), VPA+APAP (N=6 mice), VPA+APAP+NAC (N=4 mice). B-C, Expression of ER stress markers in liver extracts of mice given VPA+APAP for 4 hours with or without NAC. Control (N=4 mice), VPA+APAP (N=4 mice), VPA+APAP+NAC (N=4 mice). D-E, StARD1 expression following VPA+APAP treatment for 4 hours with or without NAC administration. Control (N=13 mice), VPA+APAP (N=11 mice), VPA+APAP+NAC (N=5 mice). F-G, Serum transaminases and H&E analyses of VPA+APAP-treated mice (24 hours) with or without NAC administration. Control (N=10 mice), VPA+APAP (N=12 mice), VPA+APAP+NAC (N=5 mice). Scale bar: 100µm. *p<0.05 as indicated.

Figure 6. STARD1\(^{\Delta}\text{Hep}\) mice are resistant to VPA+acetaminophen-induced liver injury. A-B, StARD1 expression in STARD1\(^{\text{ff}}\) mice (N≥5) and STARD1\(^{\Delta}\text{Hep}\) mice (N≥5) given VPA+APAP (400mg/Kg 3 times+300mg/Kg) for 6 hours. C, STARD1 expression in liver sections from STARD1\(^{\text{ff}}\) and STARD1\(^{\Delta}\text{Hep}\) mice 24 hours after treatment with VPA+APAP. Scale bar in C. 100µm. D, Mitochondrial cholesterol in STARD1\(^{\text{ff}}\) mice (N=6) and STARD1\(^{\Delta}\text{Hep}\) mice (N=6) 6 hours after treatment with VPA+APAP. E, total GSH and mitochondrial GSH levels in STARD1\(^{\text{ff}}\) mice (N=6) and STARD1\(^{\Delta}\text{Hep}\) mice (N=6) 6 hours after treatment with or without VPA+APAP. F-G, Serum transaminases and H&E analyses in STARD1\(^{\text{ff}}\) mice (N≥8) and STARD1\(^{\Delta}\text{Hep}\) mice (N≥8) 24 hours after treatment with or without VPA+APAP. Scale bar: 100µm. H, Levels of phosphorylated JNK in mitochondrial fractions from STARD1\(^{\text{ff}}\) mice (N≥5) and STARD1\(^{\Delta}\text{Hep}\) mice (N≥5) treated with VPA+APAP for 4 hours. *p<0.05 as indicated.
Figure 7. Fasted STARD1ΔHep mice are protected against acetaminophen hepatotoxicity. A, B, Representative H&E analyses and serum transaminases of overnight fasted STARD1^{f/f} (N=5) and STARD1^{ΔHep} (N=5) mice 24 hours after treatment with acetaminophen (APAP) (300mg/Kg). C, CYP2E1 expression from fasted STARD1^{f/f} (N=4) and STARD1^{ΔHep} (N=4) mice 2 hours after treatment with APAP. D, ER stress markers from fasted STARD1^{f/f} and STARD1^{ΔHep} mice 4 hours after treatment with APAP. E, Mitochondrial p-JNK levels from fasted STARD1^{f/f} (N=4) and STARD1^{ΔHep} (N=4) mice 4 hours after treatment with APAP. F, STARD1 in mitochondrial fraction from fasted JNK1+2^{ΔHep} mice 4 hours after acetaminophen (N=3). Results are the mean±SEM of 3-5 mice. *p<0.05 as indicated
A

2H 4H

CONTROL VPA APAP VPA+APAP FASTED APAP

CYP2e1

β-actin

NAPQI

VADC

55 kDa

42 kDa

35 kDa

B

Liver GSH (nmol/mg prot)

CONTROL VPA APAP VPA+APAP

2h 4h

Mitochondrial GSH (nmol/mg prot)

CONTROL VPA APAP VPA+APAP

2h 4h

D

Mitochondrial free cholesterol (µg/mg prot)

CONTROL VPA APAP VPA+APAP

E

Filipin

CONTROL VPA APAP VPA+APAP

F

StARD1 (gene/housekeeping)

CONTROL VPA APAP VPA+APAP

G

p-StARD1 (30 kDa)

StARD1 (30 kDa)

β-actin (42 kDa)

H

StARD1

CONTROL VPA APAP VPA+APAP
A  

Gene expression (fold to control)

CHOP, ATF-6, sXBP1, PERK

B  

Gene expression (fold to control)

CHOP, sXBP1, tXBP1, p-eIF2α, eIF2α, β-actin

C  

Gene expression (fold to control)

CHOP, BIP, ATF6, ATF4, sXBP1, tXBP1, PDI, PERK

D  

Relative expression

p-IRE1α, IRE1α, sXBP1, tXBP1, p-eIF2α, eIF2α, β-actin

E  

Relative expression

CHOP, sXBP1, tXBP1, p-eIF2α, eIF2α, β-actin

F  

p-ERK/ERK ratio protein levels

p-ERK, ERK, β-actin

G  

p-JNK/TOM20 ratio protein levels

p-JNK, JNK, TOM20

CHOP

CONTROL, VPA, APAP, VPA+APAP
**A**

Liver GSH (nmol/mg prot.)

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Mitochondrial GSH (nmol/mg prot.)

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**B**

Gene expression (fold to control)

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Western blot analysis

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**D**

StARD1 gene/housekeeping

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**E**

StARD1 protein levels (fold change)

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**F**

ALT (U/L)

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**G**

H&E staining

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A

NON TREATED

APAP FASTED

StARD1ff

StARD1ΔHep

B

ALT (U/L)

AST (U/L)

C

CYP2E1

β-actin

D

CHOP

ATF4

p-eIF2α

eIF2α

sXBP1
tXBP1

APAP FASTED

E

p-JNK

JNK

Tom20

F

JNK1+2ff

JNK1+2ΔHep

StARD1

Prohibitin-1