Acetaminophen overdose is the most common cause of acute liver injury (ALI) or acute liver failure in the United States. Its pathogenetic mechanisms are incompletely understood. Additional studies are warranted to identify new genetic risk factors for more mechanistic insights and new therapeutic target discoveries. The objective of this study was to explore the role and mechanisms of nicotinamide phosphoribosyltransferase (NAMPT) in acetaminophen-induced ALI. C57BL/6 Nampt<sup>−/−</sup> (Nampt<sup>−/−</sup>/<sup>−/−</sup>), heterozygous knockout (Nampt<sup>−/−</sup>/<sup>C0</sup>), and overexpression (NamptOE<sup>−/−</sup>) mice were treated with overdose of acetaminophen, followed by histologic, biochemical, and transcriptomic evaluation of liver injury. The mechanism of Nampt in acetaminophen-induced hepatocytic toxicity was also explored in cultured primary hepatocytes. Three lines of evidence have convergently demonstrated that acetaminophen overdose triggers the most severe oxidative stress and necrosis, and the highest expression of key necrosis driving genes in Nampt<sup>−/−</sup>/<sup>−/−</sup> mice, whereas the effects in NamptOE<sup>−/−</sup> mice were least severe relative to Nampt<sup>−/−</sup>/<sup>−/−</sup> mice. Treatment of P7C3-A20, a small chemical molecule up-regulator of Nampt, ameliorated acetaminophen-induced mouse ALI over the reagent control. These findings support the fact that NAMPT protects against acetaminophen-induced ALI. (Am J Pathol 2018, 188: 1640–1652; https://doi.org/10.1016/j.ajpath.2018.04.004)
and toxic consequences of APAP have been found in >10 different genes.9 However, more studies are needed to expand our understanding of the comprehensive relationship between genetic factors and APAP response toward the realization of personalized medicine.

Nicotinamide phosphoribosyltransferase (NAMPT) is a pleiotropic protein that regulates necroptosis, oxidative stress, innate immunity, and cytokine expression.10 Its dysregulation has been implicated in several human diseases. Our previous studies have demonstrated that overexpression of NAMPT was involved in the pathogenesis of acute lung injury,11 whereas down-regulation of Nampt engendered mice more susceptible to ischemic stroke.12 Involve ment of necrosis, innate immunity, and inflammatory cytokines in the pathogenesis of ALF has been well documented.13 Excessive cell death has been identified as a central mechanism of APAP-induced liver injury.13 APAP hepatotoxicity is characterized by extensive oxidative stress.14,15 NAMPT is a key enzyme in mammalian cellular NAD synthesis via an important salvage pathway.16 NAD has emerged as a key regulator of cellular and organismal homeostasis.17 Being a major component of both bioenergetic and signaling pathways, NAD+ mediates several regulatory processes, including attenuating cellular oxidative stress,18 enzyme regulation, control of gene expression and health span, DNA repair, cell cycle regulation, and calcium signaling. Thus, it is conceivable that NAMPT exerts its physiological role at least in part via NAD. NAMPT possesses bioactivities both in the intracellular and in the extracellular environment. There are reports to distinguish the functions of intracellular NAMPT from those of extracellular NAMPT (eNAMPT).19 Although the functions of intracellular NAMPT are well known, much less is known on eNAMPT. eNAMPT can be detected in the human circulation and in many extracellular environments. It is unclear whether eNAMPT is structurally different from intracellular NAMPT and how eNAMPT is secreted from cells. NAMPT can up-regulate antioxidant gene expression.20 Inhibition of NAMPT decreases cell growth and enhances susceptibility to oxidative stress.21 Thus, we hypothesized that overexpression of NAMPT may enhance, whereas down-regulation of NAMPT may attenuate, its antioxidative and antinecroptotic function, resulting in resistance or vulnerability to APAP-induced ALI, respectively. To test this hypothesis, we assessed the role of Nampt in APAP-induced ALI in Nampt wild-type (Nampt+/+), Nampt heterozygous knockout (Nampt+/−), and Nampt overexpressing (NamptOE) C57BL/6 mice. Herein, we report that loss-of-function and gain-of-function studies in mouse models have provided complementary evidence that Nampt has a protective role in APAP-induced liver injury. Mechanistic studies revealed that NAMPT’s protective role is dependent on its enzymatic activity to catalyze hepatocyte synthesis of NAD, which, in turn, secures the cellular defense functions by mediating NAD-dependent genes, possibly in part via the Sirt3-Foxo 3 axis.

Materials and Methods

Animal Studies

To generate the NamptOE mice in C57BL/6 background, full-length mouse liver Nampt cDNA plus a his tag were amplified using mouse Nampt-specific primers with EcoRI adaptors and subcloned into a modified pCAGGS vector to generate the expression vector (pCAGGS-Nampt cDNA). Pronuclear injection and embryo transfer were performed in The Transgenic Animal Core, University of Missouri (Columbia, MO). Positive mice were genotyped by PCR amplification of tail DNA using transgene-specific primers. C57BL/6 Nampt+/− were generated and genotyped, as described previously.22 All mice between 8 and 12 weeks of age were kept in a temperature-controlled facility with a 12-hour light/dark cycle and free access to food and water. For all experiments, food was withdrawn 12 to 15 hours before treatment with APAP, which was obtained from the Sigma-Aldrich Company (St. Louis, MO). The drugs were administered intraperitoneally at the indicated doses in warm saline or vehicle solution. The food was restored 30 minutes after the APAP administration. At the end of the experiments, the animals were sacrificed and blood was collected. Livers were excised, and portions were flash frozen for different assays. All mouse experiments were approved by the University of Missouri Kansas City Institutional Animal Care and User Committee. NamptOE and Nampt+/− mice in C57BL/6 background were generated in our laboratory.

Biochemical Assays

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), liver reduced glutathione (GSH) levels, liver NAD+/NADH levels, liver hydrogen peroxide levels, and catalase activities were measured using a colorimetric ALT assay kit (BioVision Inc., Milpitas, CA), an AST kit (Cat cachem, Oxford, CT), a GSH kit (Promega, Madison, WI), a Fluorimetric NAD/NADH Assay kit (AAT Bioquest, Inc., Sunnyvale, CA), a DetectX Hydrogen Peroxide Colorimetric Detection Kit, and a Catalase Colorimetric Activity Kit (Arbor Assays, Ann Arbor, MI), respectively.

Isolation of Mouse Primary Hepatocytes

Mouse primary hepatocytes were isolated from overnight fasted Nampt+/+ mice with a standard collagenase procedure, as described previously.23,24 Cells were plated in Dulbecco’s modified Eagle’s medium with regular supplements at 37°C in a humidified atmosphere of 5% CO2, 95% air for 24 hours before being subjected to various treatments.

Propidium Iodide Staining for Necrotic Cell Death

Propidium iodide staining of necrotic cell death after the APAP plus or minus various treatments was performed, as
described by Ni et al. After the staining, cells were imaged on an Olympus inverted fluorescent microscope IX71 (Olympus, Tokyo, Japan) using a Texas Red filter.

**Histology and Immunohistochemistry**

Paraformaldehyde-fixed tissue samples were embedded in paraffin, and 5-μm sections were cut. Replicate sections were stained with hematoxylin and eosin for evaluation of necrosis. For the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, liver sections were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN). Mouse liver sections were also stained using an anti-nitrotyrosine antibody (Invitrogen, Carlsbad, CA), according to Ramachandran et al.

**RNA-seq**

RNA sequencing (RNA-seq) of mouse liver RNA was performed in the Core of Genetic Research, Children’s Mercy Hospital (Kansas City, MO), according to previously published protocol, except that sequencing libraries from 1 μg total liver RNA were prepared using the Illumina TruSeq Stranded Total RNA Sample Prep Kit (catalog number RS-122-220; Illumina, San Diego, CA), and a 2 × 101 paired-end high-output sequencing run was performed using the HiSeq 1500 instrument. Each sample was sequenced to an average depth of approximately 63 million reads with >80% of bases >Q30. The resulting base calling (.bcl) files were converted to FASTQ files using Illumina’s CASAVA software version 1.8.1. Mapping of RNA-seq reads and transcript assembly and abundance estimation were conducted using Tuxedo Suite (TopHat v1.3.0/Bowtie v0.12.7/Cufflinks v1.0.3) and reported in fragments per kb of exon per million fragments mapped (FPKM). Our RNA-seq data have been deposited to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE110787).

**RT-PCR**

Quantitative real-time PCR was performed, as described previously.

**Western Blot Analysis**

Selective validations of several protein expressions in liver were performed by Western blot analysis. Briefly, liver tissues were homogenized, separated onto 10% SDS-PAGE, and immunodetected by rabbit anti-mouse polyclonal

---

**Figure 1** Nampt plays an important role in acetaminophen (APAP)—induced hepatocyte toxicity. **A**: Molecular structures of mouse Nampt transgene (pCAGGS-mNampt cDNA) construct. **B**: Nampt gene insertional mutation construct. Nampt<sup>+/−</sup> mice were generated previously from the mouse embryonic stem cell clone (RRR084), which harbors a characterized insertional mutation in intron 7 of mouse Nampt gene via gene-trap vector (pGT0Lxf). **C**: Hematoxylin and eosin staining shows different severity of liver injury after APAP treatment. **D**: Mouse serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Data are expressed as means ± SD (D). n ≥ 6 (D). **P < 0.01 versus their saline controls; ***P < 0.01 versus APAP + Nampt<sup>+/−</sup>. Original magnification, ×100 (C). CMV-IE, cytomegalovirus immediate-early; Ori, origin; pA, polyadenylation signal; SA, splice acceptor.
antibodies to NAMPT (Bethyl Laboratories, Montgomery, TX), glutathione-S-transferase α 3 (Proteintech Group, Chicago, IL), glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Dallas, TX), and mouse monoclonal antibody polycystathionine-β-synthase (CBS; Santa Cruz Biotechnology, Inc.). Glyceraldehyde 3-phosphate dehydrogenase protein assay was included as a housekeeping gene control.

Statistical Analysis

All results were expressed as means ± SD of at least three independent experiments. Comparisons between multiple groups were performed with one-way analysis of variance, followed by a post hoc Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis test, followed by Dunn’s multiple comparisons test. \( P < 0.05 \) was considered significant.

Results

Preparation of \( \text{Nampt}^{OE} \) and \( \text{Nampt}^{+/-} \) Mice

To investigate the role of \( \text{Nampt} \) in APAP-induced ALI, \( \text{Nampt}^{OE} \) and \( \text{Nampt}^{+/-} \) mice were prepared. A molecular structure of the mouse \( \text{Nampt}^{OE} \) transgene (pCAGGS-m\( \text{Nampt} \) cDNA) construct is shown in Figure 1A. \( \text{Nampt} \) gene insertional mutation (\( \text{Nampt}^{+/-} \)) construct is shown in Figure 1B. Genotyping strategies and representative images for \( \text{Nampt}^{OE} \), \( \text{Nampt}^{+/-} \), and \( \text{Nampt}^{+/-} \) mice are presented in Supplemental Figure S1, A and B. The sequences of the genotyping primers are listed in Table 1. Phenotypically, \( \text{Nampt}^{OE} \) mice express higher amounts of serum NAMPT (136.31% ± 1.33%, \( n = 6, \ P < 0.01 \)), whereas \( \text{Nampt}^{+/-} \) mice express lower level of serum NAMPT protein (56.74% ± 3.94%, \( n = 6, \ P < 0.01 \)) than \( \text{Nampt}^{+/-} \) mice, whose levels were arbitrarily set as 100% (Supplemental Figure S1C). Successful preparations of \( \text{Nampt}^{OE} \) and \( \text{Nampt}^{+/-} \) mice have paved the way to investigate the role of \( \text{Nampt} \) in APAP-induced ALI.

Mouse Liver Histology and Serum ALT and AST Levels after APAP Treatment

To determine the role of NAMPT in APAP-induced hepatotoxicity, the mouse liver histologic changes were examined and mouse serum ALT and AST levels were measured 24 hours after APAP overdose. After treatment of mice with 200 mg/kg APAP, the most severe liver injury occurred in \( \text{Nampt}^{+/-} \) mice, as indicated by an extensive blanching of the liver surface. \( \text{Nampt}^{+/-} \) mice had a milder injury, whereas \( \text{Nampt}^{OE} \) mice had the least liver injury compared with the saline control mice (Supplemental Figure S2). Hematoxylin and eosin staining of mouse liver sections (Figure 1C) also showed that the characteristic centrilobular necrosis was most evident in \( \text{Nampt}^{+/-} \) mice, milder in \( \text{Nampt}^{+/-} \) mice, and least apparent in \( \text{Nampt}^{OE} \) mice. There are no apparent differences among the same genotype saline control mice. To examine this difference in earlier time points, \( \text{Nampt}^{+/-} \) and \( \text{Nampt}^{+/-} \) mice were subjected to APAP treatment from 1 to 6 hours. Hematoxylin and eosin staining of liver sections (Supplemental Figure S3) showed more severe necrosis in \( \text{Nampt}^{+/-} \) mice than \( \text{Nampt}^{+/-} \) mice, that could be seen as early as 1 hour, clearly in 3 hours, and obviously in 6 hours. Measurement of serum ALT and AST as indicators of liver cell death demonstrated that serum ALT and AST levels were 10.89 ± 0.86 U/L and 13.04 ± 0.41 U/L in saline control \( \text{Nampt}^{+/-} \) mice, 2124.89 ± 310.00 U/L and 1190.25 ± 311.91 U/L in APAP-treated \( \text{Nampt}^{+/-} \) mice, 25.72 ± 14.85 U/L and 35.72 ± 20.65 U/L in saline control \( \text{Nampt}^{OE} \) mice, 88.07 ± 39.56 U/L and 65.18 ± 21.72 U/L in APAP-treated \( \text{Nampt}^{OE} \) mice, 22.60 ± 3.36 U/L and 26.10 ± 4.46 U/L in saline control \( \text{Nampt}^{+/-} \) mice, and 5181.71 ± 460.37 U/L and 4340.16 ± 674.37 U/L in APAP-treated \( \text{Nampt}^{+/-} \) mice, respectively (Figure 1D). Both the ALT and AST levels are significantly increased in APAP-treated groups versus the saline control group in both \( \text{Nampt}^{+/-} \) and \( \text{Nampt}^{+/-} \) mice (\( P < 0.01 \)). The APAP treatment resulted in significantly higher ALT and AST levels in \( \text{Nampt}^{+/-} \) mice (\( P < 0.01 \)), and significantly lower ALT and AST levels in \( \text{Nampt}^{OE} \) mice (\( P < 0.01 \)), than those seen in \( \text{Nampt}^{+/-} \) mice.

Mouse Liver NAMPT mRNA Levels, Protein Levels, NAD Levels, and Their Serum NAMPT Detection after APAP Treatment

To establish whether NAMPT expression correlated with its role in APAP-induced hepatotoxicity, mouse liver NAMPT mRNA levels were determined by RT-PCR and NAMPT protein levels by Western blot analysis. On the basis of mouse liver NAMPT mRNA level in saline-treated control \( \text{Nampt}^{+/-} \) mice set to 1.0, the mouse liver mRNA level was significantly lower in \( \text{Nampt}^{+/-} \) mice (2.0 ± 1.5-fold decrease, \( n = 3, \ P < 0.05 \)) and the lowest in \( \text{Nampt}^{+/-} \) mice (7.8 ± 0.4-fold decrease, \( n = 3, \ P < 0.01 \)), whereas it was higher in \( \text{Nampt}^{OE} \) mice (2.2 ± 0.4-fold increase, \( n = 3, \ P < 0.05 \)), after APAP treatment (Figure 2A). The similar trend is true of NAMPT liver protein levels (Figure 2B).

To determine whether NAMPT expression correlated with its role in mouse liver NAD synthesis, mouse liver NAD concentration was measured (Figure 2C). Liver NAD concentrations in saline control \( \text{Nampt}^{+/-} \) mice, APAP-treated...
Nampt\(^{+/+}\) mice, APAP-treated Nampt\(^{OE}\) mice, and APAP-treated Nampt\(^{-/-}\) mice were 0.49 ± 0.09, 0.38 ± 0.06, 0.43 ± 0.05, and 0.18 ± 0.09 \(\mu\)mol/L per mg liver protein, respectively. Although liver NAD concentrations in all three Nampt genotypes treated with APAP were lower than those in the saline control mice, Nampt\(^{OE}\) mice had the highest liver NAD level, whereas Nampt\(^{-/-}\) mice had the lowest liver NAD level.

To test the potential utility of NAMPT as a diagnostic and prognostic biomarker in APAP-induced ALI, mouse serum NAMPT levels were detected using Western blot analysis. Mouse serum NAMPT levels after APAP treatment were lowest in Nampt\(^{-/-}\) mice, highest in Nampt\(^{OE}\) mice, and medium in Nampt\(^{+/+}\) mice, which correlated with liver Nampt expression levels (Figure 2D). Furthermore, mouse serum NAMPT levels were negatively correlated with mouse serum ALT levels (Figure 2E).

**Mouse Liver Oxidative Stress after APAP Treatment**

Because GSH is the most abundant cellular thiol antioxidant,\(^{30}\) it was first determined whether abnormal GSH expression may underlie the role of NAMPT in APAP-induced liver toxicity. Thus, the expression levels of those enzymes were surveyed in the GSH synthesis pathway (Figure 3A), liver tissue lysate GSH levels were assayed, and the relationship between liver GSH levels and serum ALT concentrations was analyzed.

RNA-seq analysis revealed that the genes (Mat1, Gnmt, Ahec, Cbs, and Cth) encoding the five enzymes involved in metabolism of methionine to cysteine (Figure 3A) all showed the lowest expression levels in Nampt\(^{-/-}\) mice and the highest expression levels in Nampt\(^{OE}\) mice after APAP treatment (Supplemental Table S1). RT-PCR experiments selectively validated RNA-seq—detected expression of Mat1, Cth, and Cbs genes (Figure 3B). On the basis of the saline control Nampt\(^{+/+}\) group set as 1, APAP caused increased expressions of Mat1, Cth, and Cbs genes in Nampt\(^{OE}\) mice (2.5 ± 1.5, \(P < 0.05\); 2.5 ± 1.1, \(P < 0.01\); and 1.7 ± 0.1, \(P < 0.01\), respectively) and decreased expression in Nampt\(^{+/+}\) (−1.6 ± 0.7, \(P < 0.05\); −3.0 ± 0.5, \(P < 0.01\); and −1.6 ± 0.8, \(P < 0.05\), respectively) and Nampt\(^{-/-}\) (−4.0 ± 0.6, \(P < 0.01\); −10.4 ± 0.5, \(P < 0.01\); and −2.8 ± 0.6, \(P < 0.01\), respectively) mice. Western blot analysis confirmed that two different isoforms of CBS protein displayed the same expression pattern as its mRNA (Figure 3C). Oxidized glutathione and GSH levels were also assayed in liver after APAP treatment. In Nampt\(^{+/+}\) and Nampt\(^{-/-}\) mice, oxidized glutathione levels are significantly increased in APAP-treated versus saline control (136.42 ± 10.86 versus 54.25 ± 3.39 nmol/g liver, \(n = 6\), \(P < 0.01\); 165.45 ± 10.16 versus 96.16 ± 19.85 nmol/g liver, \(n = 6\), \(P < 0.01\), respectively) and decreased expression in Nampt\(^{OE}\) mice, which correlated with liver GSH levels and serum ALT activities. A representative Western blot image of serum NAMPT levels from Nampt\(^{-/-}\) mice with low (L), medium (M), and high (H) ALT level is displayed. Data are expressed as means ± 50 (A and C). \(n ≥ 3\) (A); \(n = 4\) (C). *\(P < 0.05\); **\(P < 0.01\) versus control (Ctrl); \(\dagger\) \(P < 0.05\) versus Nampt + APAP. Ctrl, saline-treated control Nampt\(^{+/+}\) mice.

**Figure 2** Nampt expression levels correlate with NAD production and liver injury after acetaminophen (APAP) treatment. A: Real-time quantitative RT-PCR assays for liver Nampt mRNA after saline or APAP treatments. Fold changes are relative to the saline-treated Nampt\(^{+/+}\) group, which was set to 1.0. Ct values were normalized to β-actin. B: Western blot image of NAMPT and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). C: Mouse liver NAD levels. D: Western blot image of serum NAMPT levels from Nampt\(^{-/-}\), Nampt\(^{OE}\), and Nampt\(^{+/+}\) mice. E: Mouse serum NAMPT levels and their relationship with serum alanine aminotransferase (ALT) activities. A representative Western blot image of serum NAMPT levels from Nampt\(^{-/-}\) mice with low (L), medium (M), and high (H) ALT level is displayed. Data are expressed as means ± 50 (A and C). \(n ≥ 3\) (A); \(n = 4\) (C). *\(P < 0.05\); **\(P < 0.01\) versus control (Ctrl); \(\dagger\) \(P < 0.05\) versus Nampt + APAP. Ctrl, saline-treated control Nampt\(^{+/+}\) mice.

Mouse Liver Oxidative Stress after APAP Treatment

Because GSH is the most abundant cellular thiol antioxidant, it was first determined whether abnormal GSH expression may underlie the role of NAMPT in APAP-induced liver toxicity. Thus, the expression levels of those enzymes were surveyed in the GSH synthesis pathway (Figure 3A), liver tissue lysate GSH levels were assayed, and the relationship between liver GSH levels and serum ALT concentrations was analyzed.

RNA-seq analysis revealed that the genes (Mat1, Gnmt, Ahec, Cbs, and Cth) encoding the five enzymes involved in metabolism of methionine to cysteine (Figure 3A) all showed the lowest expression levels in Nampt\(^{-/-}\) mice and the highest expression levels in Nampt\(^{OE}\) mice after APAP treatment (Supplemental Table S1). RT-PCR experiments selectively validated RNA-seq—detected expression of Mat1, Cth, and Cbs genes (Figure 3B). On the basis of the saline control Nampt\(^{+/+}\) group set as 1, APAP caused increased expressions of Mat1, Cth, and Cbs genes in Nampt\(^{OE}\) mice (2.5 ± 1.5, \(P < 0.05\); 2.5 ± 1.1, \(P < 0.01\); and 1.7 ± 0.1, \(P < 0.01\), respectively) and decreased expression in Nampt\(^{+/+}\) (−1.6 ± 0.7, \(P < 0.05\); −3.0 ± 0.5, \(P < 0.01\); and −1.6 ± 0.8, \(P < 0.05\), respectively) and Nampt\(^{-/-}\) (−4.0 ± 0.6, \(P < 0.01\); −10.4 ± 0.5, \(P < 0.01\); and −2.8 ± 0.6, \(P < 0.01\), respectively) mice. Western blot analysis confirmed that two different isoforms of CBS protein displayed the same expression pattern as its mRNA (Figure 3C). Oxidized glutathione and GSH levels were also assayed in liver after APAP treatment. In Nampt\(^{+/+}\) and Nampt\(^{-/-}\) mice, oxidized glutathione levels are significantly increased in APAP-treated versus saline control (136.42 ± 10.86 versus 54.25 ± 3.39 nmol/g liver, \(n = 6\), \(P < 0.01\); 165.45 ± 10.16 versus 96.16 ± 19.85 nmol/g liver, \(n = 6\), \(P < 0.01\), respectively) and decreased expression in Nampt\(^{OE}\) mice, which correlated with liver GSH levels and serum ALT activities. A representative Western blot image of serum NAMPT levels from Nampt\(^{-/-}\) mice with low (L), medium (M), and high (H) ALT level is displayed. Data are expressed as means ± 50 (A and C). \(n ≥ 3\) (A); \(n = 4\) (C). *\(P < 0.05\); **\(P < 0.01\) versus control (Ctrl); \(\dagger\) \(P < 0.05\) versus Nampt + APAP. Ctrl, saline-treated control Nampt\(^{+/+}\) mice.
respectively). However, the latter increase is significantly higher than that of the former \( (P < 0.01) \), indicating a more severe oxidative stress in \( \text{Nampt}^+/\cdot /C0 \) mice (Figure 3D). In \( \text{Nampt}^{+/+} \) and \( \text{Nampt}^{+/+} \) mice, GSH/oxidized glutathione ratios are decreased significantly in APAP-treated versus saline control \( (38.90 \pm 3.07 \text{ versus } 75.18 \pm 3.05, n = 6, P < 0.01; 26.69 \pm 1.84 \text{ versus } 55.67 \pm 9.17, n = 6, P < 0.01, \text{ respectively}) \). The latter is more significantly decreased than the former \( (P < 0.01) \), reinforcing a more severe oxidative stress status in \( \text{Nampt}^{+/+} \) mice (Figure 3E).

In addition, two representative littermate GSH assays demonstrated that GSH levels in \( \text{Nampt}^{OE} \) mice were significantly higher than littermate \( \text{Nampt}^{+/+} \) controls \( (80\% \text{ versus } 40\%, \text{ respectively}) \), whereas GSH levels in \( \text{Nampt}^{+/+} \) mice were significantly lower than littermate \( \text{Nampt}^{+/+} \) controls \( (45\% \text{ versus } 70\%), \text{ after APAP treatment} \) (Figure 3F). Furthermore, linear regression analysis indicated that GSH levels were negatively correlated with their serum ALT levels in two representative litters after APAP treatment \( (n = 4 \text{ per litter}, r^2 = -0.94 \text{ and } -0.84, \text{ respectively}) \) (Figure 3G).

Figure 3 The lowest reduced glutathione (GSH) synthesis and lower GSH/oxidized glutathione (GSSG) ratio in acetaminophen (APAP)–treated \( \text{Nampt}^{+/+} \) mouse livers. A: Diagram of hepatic methionine metabolism and GSH synthesis. Methionine adenosyltransferase (Mat), S-adenosyl methionine (SAMe), glycine N-methyltransferase (Gnmt), S-adenosyl-L-homocysteine (SAH), adenosylhomocysteine (Ahcy), cystathionine-β-synthase (Cbs), cystathionase (Cth), cysteine (Cys), glutamate-cysteine ligase catalytic subunit (Gclc), glutamate-cysteine ligase, modifier subunit (Gclm), γ-glutamylcysteine (γ-GC), and glutathione synthetase (GSS) are shown. B: Selective validation of mRNA levels by real-time quantitative RT-PCR TaqMan Gene Expression Assays. Fold change values for the expression of each gene relative to those in saline-treated \( \text{Nampt}^{+/+} \) mice are presented. C: Two different CBS protein isoforms were presented in the Western blot analysis. NAMPT was included as a positive control. D: Mouse liver tissue GSSG levels after either saline or APAP treatment were assayed, as described in Materials and Methods. Representative results from \( \text{Nampt}^{OE} \) mice and littermate \( \text{Nampt}^{+/+} \) control mice and \( \text{Nampt}^{+/+} \) mice and littermate \( \text{Nampt}^{+/+} \) control mice are displayed separately. The results are presented on the basis of the value from saline control (Ctrl) mice as 100%. G: Correlation between mouse liver GSH levels and mouse serum alanine aminotransferase (ALT) levels. Mouse liver GSH levels and mouse serum ALT levels were assayed, as described in Materials and Methods. Representative results of correlations between mouse liver GSH levels and mouse serum ALT levels are displayed separately for all mice in litter 1 (left panel) and litter 2 (right panel). Data are expressed as means ± SEM (B, D, and E). n = 3 (B); n = 6 (D). *P < 0.05, **P < 0.01; ***P < 0.001 versus Ctrl; **P < 0.01 versus \( \text{Nampt}^{+/+} \) + APAP. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
To examine further the liver oxidative/antioxidative status in Nampt+/−, NamptOE, and Nampt+/+ mice, the expression levels of liver catalase, an antioxidative gene, were surveyed and the catalase activity and hydrogen peroxide content after exposure to APAP were assayed. RNA-seq detected that catalase expression level was lower in Nampt+/− mice (133.85 ± 41.66 FPKM) and higher in NamptOE mice (516.58 ± 90.58 FPKM) than Nampt+/+ mice (439.63 ± 95.87 FPKM) after APAP treatment (Figure 4A), although all of them were significantly lower than the saline control group (828.83 ± 69.07 FPKM, P < 0.01). The same pattern was true of catalase activities (Figure 4B). After APAP treatment, NamptOE mice had the highest catalase activity (292.67 ± 32.25 U/mL cell lysates), Nampt+/− mice had the lowest catalase activity (145.67 ± 25.78 U/mL cell lysates), and Nampt+/+ mice had the intermediate catalase activity (174.67 ± 4.04 U/mL cell lysates). Nampt+/− liver had a significantly higher hydrogen peroxide level (2.2 ± 0.3; P < 0.01), and NamptOE liver (0.8 ± 0.4; nonsignificant) had a lower hydrogen peroxide level, than that of Nampt+/+ mice after APAP treatment (Figure 4C).

To seek additional evidence to assess liver oxidant stress, it was found that APAP caused the most extensive nitrotyrosine staining, an index of mitochondria oxidative event,31 in Nampt+/− mice, less extensive nitrotyrosine staining in Nampt+/+ mice, and the least staining in NamptOE mice. These results corroborate that APAP caused more severe mitochondrial oxidant stress in Nampt+/− mice and less severe mitochondrial oxidant stress in NamptOE mice than that in Nampt+/+ mice (Figure 4D).

Mouse Liver Necrosis after the APAP Treatment

Necrotic cell death is the hallmark of APAP-induced ALI. Expression levels of mouse liver K18, K8, and RIPK 3, whose overexpression indicates cell necrosis, were determined. RNA-seq analysis indicates that the K18 and K8 mRNA levels are 122.02 ± 8.98 and 109.53 ± 6.05 FPKM in the saline control group, 294.53 ± 59.86 and 257.93 ± 42.46 FPKM in APAP + Nampt+/− group, 93.91 ± 31.15 and 78.00 ± 23.26 FPKM in APAP + NamptOE group, and 665.13 ± 240.45 and 600.23 ± 223.75 FPKM in APAP + Nampt+/+ group, respectively (Figure 5A). It is evident that expression levels of both K18 and K8 were dramatically higher in Nampt+/− mice and significantly lower in NamptOE mice than that in Nampt+/+ mice after APAP treatment. Western blot analysis confirmed that K18 protein had the same expression pattern as its mRNA counterparts (Figure 5B).

RNA-seq analysis also indicated that Ripk3 expression follows the same expression pattern as K18 and K8 (Figure 5C). Ripk3 mRNA levels were 0.47 ± 0.06 FPKM in the saline control group, 0.89 ± 0.37 FPKM in APAP + Nampt+/− group, 0.28 ± 0.11 FPKM in APAP + NamptOE group, and 1.56 ± 0.36 FPKM in APAP + Nampt+/+ group (Figure 5C). From the RNA-seq data, the expression levels of all 84 key genes central to necrotic cell death, as tabulated in the Mouse Necrosis PCR Array by Qiagen (Valencia, CA), were also systematically examined. In the APAP-treated groups, there were 44 genes up-regulated in Nampt+/− mice and 22 genes down-regulated in NamptOE mice relative to Nampt+/+ mice. There are 15 overlapped differentially expressed genes (Figure 5D and Suplemental Table S2), which were up-regulated in Nampt+/− mice and down-regulated in NamptOE mice, suggesting that Nampt may be directly involved in the regulation of these genes.

In addition, formalin-fixed hepatic tissue sections were stained for DNA strand breaks in the three different Nampt genotypes. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays demonstrated that Nampt+/− liver had the most extensive necrosis and DNA...
fragmentation, whereas NampOE liver had the least extensive necrosis and DNA fragmentation, among the three different Nampt genotypes after APAP treatment (Figure 5E).

To explore further the molecular mechanism that may underpin the role of NAMPT in APAP-induced hepatic cell death, the effects of FK866, a known inhibitor of NAMPT enzyme, minus or plus either NAD or nicotinamide mononucleotide (NMN), were determined on APAP-induced Nampt+/+ mouse primary hepatocyte necrosis using propidium iodide staining (Figure 6, A and B). The APAP treatment caused approximately 24% more cell death of Nampt+/+ primary hepatocytes than the control group (30.51% ± 2.23% versus 6.30% ± 0.78%, n = 4, P < 0.01). The APAP plus FK866 treatment engendered approximately 15% more cell death of Nampt+/+ primary hepatocytes than the APAP group (45.29% ± 6.34% versus 30.51% ± 2.23%, n = 4, P < 0.01). The addition of either NAD, the end product of NAMPT reaction, or NMN, a direct product of NAMPT reaction, to the APAP + FK866 treatment significantly attenuated cell death of Nampt+/+ primary hepatocytes compared with the APAP + FK866 group (17.34% ± 0.90% or 15.37% ± 2.06% versus 45.29% ± 6.34%, n = 4, P < 0.016 and P < 0.014, respectively).

Mouse Liver Gene Expression Levels of the Sirtuin and Foxo Families

To investigate possible signal transduction pathways that may transduce the Nampt-mediated effect on APAP-induced liver injury, mouse liver gene expression levels of the sirtuin and foxo families were surveyed by RNA-seq. Sirtuins, which include Sirt1-7, are NAD-dependent deacetylases, which can deacetylate transcription factors, such as the foxo family. Thus, Nampt, which is a known regulator of sirtuins, may exert its effect via a sirtuin-foxo axis. Table 2 presents

![Figure 5](https://example.com/figure5.png)

*Figure 5* Nampt regulates acetaminophen (APAP)–induced hepatocyte necrosis. Mouse liver total RNA from four different groups of mice was isolated and analyzed by RNA sequencing. A: Fragments per kb of exon per million fragments mapped (FPKM) values of Krt18 and Krt8 mRNA levels are presented. B: Western blot analysis of Krt18. C: FPKM values of Ripk3 mRNA levels are presented. D: Venn diagram shows 15 overlapping dysregulated genes in APAP-treated Nampt+/+ mice and Nampt+/- mice relative to APAP-treated Nampt+/+ mice. E: Liver necrosis detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. TUNEL-positive cells are identified by dark purple staining. Data are expressed as means ± SD (A and C), n ≥ 3 (A and C). *P < 0.05 versus saline control; †P < 0.05 versus APAP Nampt+/- group; ‡P < 0.05 versus saline control by one-way analysis of variance. Original magnification, × 100 (E).
that, except for Sirt1 and Sirt6, expression of the remaining Sirtuins is lower in APAP-treated Nampt\textsuperscript{+/+} mice and Nampt\textsuperscript{-/-} mice than that in saline control mice, whereas there is no significant difference in those gene expression levels between APAP-treated Nampt\textsuperscript{OE} mice and saline control mice. Furthermore, in the APAP-treated groups, the expression of Sirt3 stood out as significantly down-regulated in Nampt\textsuperscript{-/-} mice and up-regulated in Nampt\textsuperscript{OE} mice relative to Nampt\textsuperscript{+/+} mice (5.68 ± 1.82 FPKM or 27 ± 6.0 FPKM versus 16.6 ± 3.34 FPKM, n ≥ 3, P < 0.01 and P < 0.05, respectively) (Table 2). In RNA-seq analysis, expression of four members of the foxo gene family (Foxo1, Foxo3, Foxo4, and Foxo6) was detected. Relative to saline controls, the expressions of Foxo1, Foxo3, and Foxo4 genes in Nampt\textsuperscript{-/-} and Nampt\textsuperscript{+/+} mice were all decreased after APAP treatment, whereas in Nampt\textsuperscript{OE} mice, only Foxo3 expression was slightly decreased (Supplemental Table S3). In the APAP-treated groups, except for Foxo6, the trend of expressing Foxo1, Foxo3, and Foxo4 was lower in Nampt\textsuperscript{-/-} mice and higher in Nampt\textsuperscript{OE} mice relative to the Nampt\textsuperscript{+/+} mice (Supplemental Table S4).

**NAMPT May Be a New Therapeutic Target to APAP-Induced ALI**

The therapeutic potential of P7C3-A20 in APAP-induced mouse ALI was evaluated. The data above derived from mice and cell culture all support a protective role of NAMPT in APAP-induced ALI or hepatocyte toxicity. Pool seven (P7) and its third compound (C3), hence named P7C3-A20 chemical structure. Treatment effect of P7C3-A20 on APAP-induced liver injury in C57BL/6J mice. Mice were treated with P7C3-A20 (20 mg/kg per day) 1 and 6 hours after APAP i.p. injection. The serum alanine aminotransferase (ALT) activities were detected 24 hours after APAP administration. Control mice were injected with equal amount of saline without APAP injection. Vehicle (Veh) mice were injected with equal amount of vehicle and treated with i.p.

**Figure 6** NAMPT protects and treats acetaminophen (APAP)—induced hepatocyte necrosis. A: Propidium iodide fluorescence in primary hepatocytes after treatments as indicated: saline (control), 10 mmol/L APAP, 10 mmol/L FK866, 100 μmol/L NAD, and 100 μmol/L nicotinamide mononucleotide (NMN). B: Quantification; for quantification, > 300 cells in four different eye fields were counted in each cultured well. Data are from four different locations of the well. C: P7C3-A20 chemical structure. Treatment effect of P7C3-A20 on APAP-induced liver injury in C57BL/6J mice. Mice were treated with P7C3-A20 (20 mg/kg per day) 1 and 6 hours after APAP i.p. injection. The serum alanine aminotransferase (ALT) activities were detected 24 hours after APAP administration. Control mice were injected with equal amount of saline without APAP injection. Vehicle (Veh) mice were injected with equal amount of vehicle and treated with i.p.

**APAP. Bottom panel:** Representative hematoxylin and eosin staining image of liver tissues. D: Scheme of possible mechanisms of NAMPT’s protective role in APAP-induced mouse liver injury. Data are expressed as means ± SE (B) or means ± SD (C). n = 3 per group (C). **P < 0.01 versus control (Ctrl) group; *P < 0.05 versus the APAP + FK group; †P < 0.01 versus Veh control. Original magnification, ×100 (A and C). GSH, reduced glutathione; NAPQI, N-acetyl-p-benzoquinoneimine.
that can activate or up-regulate function or expression of NAMPT, is an aminopropyl carbazole (MM-447.188; fluorinated analog, C21H14Br2N2O). Its more potent fluorinated analog, P7C3-A20 (Figure 6C), is up to 10 times stronger.32 Thus, we used P7C3-A20 in this study. Eight-week-old male mice were starved overnight, and injected intraperitoneally with APAP at 200 mg/kg. At 1 and 6 hours later, P7C3-A20 was injected intraperitoneally at 20 mg/kg. Vehicle mice as a control were injected with equal amount of vehicle solution. At 24 hours after APAP challenge, mouse serum ALT activity directly, is essential in liver to detoxify the reactive metabolite APAP, acetaminophen; FPKM, fragments per kb of exon per million fragments mapped. NAMPT catalyzes the rate-limiting step in a key salvage pathway of mammalian NAD biosynthesis.33 The reductive power provided by NAD, a universal energy- and signal-carrying molecule,34 drives metabolic reactions, controls enzymatic activity, regulates genetic expression, and allows for the maintenance of cellular oxidation-reduction status. In this study, after APAP treatment, NamptOE liver had the lowest levels of Nampt mRNA, NAMPT protein, and NAD, whereas NamptOE liver had the highest levels of these (Figure 2). These results suggest that decreased NAD synthesis may be a culprit underlying the most severe oxidative stress in NamptOE mouse liver, as indicated by the lowest liver catalase mRNA and catalase activity, the highest liver hydrogen peroxide, and the most extensive nitrotyrosine staining among all groups of mice after APAP treatment (Figure 4). Catalase, a key antioxidant enzyme in the body’s defense against oxidative stress, functions by converting the reactive oxygen species hydrogen peroxide to water and oxygen, thereby mitigating the toxic effects of hydrogen peroxide.35 These experiments revealed the lowest GSH level in NamptOE liver after APAP treatment (Figure 3). GSH, which can scavenge hydroxyl radicals and superoxide directly, is essential in liver to detoxify the reactive metabolite of APAP, NAPQI.36 Excess generation of NAPQI after APAP overdose can lead to profound mitochondrial glutathione depletion, covalent binding, severe impairment of mitochondrial function, and cessation of ATP production, which, in total, results in disruption of ion homeostasis and consequently oncotic necrosis.37 This study found that all five enzyme-encoding genes involved in the metabolism of methionine to cysteine, which is an essential precursor in the GSH synthesis (Figure 3A), had the lowest expression level in NamptOE mice, after APAP treatment (Supplemental Table S1). Decreased synthesis of cysteine may have contributed to the lowest GSH level in NamptOE mice.

### Discussion

The objective of this study was to explore the role and mechanisms of NAMPT in APAP-induced ALI. Herein, we have provided three lines of evidence, including biochemical, histologic, and molecular insights, that consistently showed that NAMPT protects against APAP-induced ALI. This study suggests that NAMPT’s protective role depends on its enzymatic activity to catalyze NAD synthesis. To our knowledge, these results represent the first finding that NAMPT has a protective role in APAP-induced ALI, which adds to the ever-growing list of NAMPT’s pleiotropic functions.38 Most important, this study ushers in NAMPT as a potential new diagnostic and/or therapeutic target in APAP-induced ALI.

NAMPT catalyzes the rate-limiting step in a key salvage pathway of mammalian NAD biosynthesis.33 The reductive power provided by NAD, a universal energy- and signal-
lower in Nampt-deficient mouse livers than Nampt wild-type mouse in baseline (Supplemental Table S5). This observation jibes well with that lower expression of Nampt may dictate lower expression of Gclm, which results in lower production of GSH, leading to lower capacity of antioxidative stress function, thus engendering more susceptible to APAP-induced acute liver injury in Nampt-deficient mice. In addition, there are lower liver expression levels of glutathione S-transferases P1 (Gstp1), which covalently binds APAP metabolites, in Nampt-deficient mice relative to the wild-type mice. Lower Gstp1 expression may be responsible for subsequent higher oxidative stress and necrosis in Nampt-deficient mouse livers. Cytochrome P450 enzymes (CYP2E1) catalyze oxidation of APAP to the reactive toxic metabolite NAPQI in liver.37 The data presented in Supplemental Figure S4 do not show any significant difference in liver protein levels of CYP2E1 after the APAP treatment between Nampt+/− and Nampt+/+ mice. This indicates that more severe liver injury with the APAP treatment in Nampt+/− mice over Nampt+/+ mice is not because of more conversion by CYP2E1 in Nampt+/− mice. Multiple studies have shown that patterns of protein expression may be modulated in mammalian cells in response to oxidative stress.40 Our experiments found that expressions of liver K18, K8, and Ripk3, which are known drivers of cell necrosis,15,26,41 were dramatically higher in Nampt+/− mice and significantly lower in NamptOE mice than Nampt+/− mice after APAP treatment (Figure 5). Necroptosis in APAP toxicity is controversial. In hepatocytes, direct toxicity and intracellular organelle stress (endoplasmic reticulum stress or mitochondrial toxicity) by APAP can activate the intrinsic pathway of apoptosis via mitochondrial outer membrane permeabilization or lead to necrosis by mitochondrial permeability transition.42 Indeed, Ripk3 is implicated in both apoptosis and necrosis.43 Our limited study herein could not exclude the possible mechanism that NAMPT’s protective role is in part because of its attenuation of APAP-induced hepatocyte apoptosis, although the necrotic mechanism has been singled out herein. Systematic examination of the expression levels of 84 key genes central to necrotic cell death44,45 identified 15 genes whose expression may be regulated by NAMPT. The most severe necrosis observed in APAP-treated Nampt+/− mice may be engendered by Nampt-mediated dysregulation of these necrosis-promoting genes. Their highest up-regulations may correlate with the degree of the activation of oxidation-reduction—sensitive transcription factors, perhaps because of the lowest amount of NAD in Nampt+/− mice. Sirt3 is decreased significantly in the APAP-treated Nampt+/− mice and up-regulated significantly in NamptOE mice relative to the Nampt+/− mice (Table 2). Sirt3, residing in mitochondria, is a NAD-dependent deacetylase, which can deacetylate transcription factors, such as the foxo family.46 Foxo3 is a direct target of SIRT3 and functions as a forkhead transcription factor to govern diverse cellular responses to stress.47 Like Sirt3, Foxo3 is also decreased in APAP-treated Nampt+/− mice and up-regulated in NamptOE mice relative to Nampt+/− mice (Supplemental Table S5). Foxo3 can up-regulate a set of genes that are essential for mitochondrial homeostasis.47 It has been shown that SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative stress.47 Thus, lower expression of the Sirt3-Foxo3 axis in APAP-treated Nampt+/− mice may deregulate genes involved in antioxidative stress, leading to the more severe necroptosis in Nampt+/− mice. This reasoning warrants further experimental investigation. Taken together, we put forward possible mechanisms of NAMPT’s protective role in APAP-induced mouse liver injury (Figure 6D). Among Nampt+/−, Nampt+/+, and NamptOE mice, Nampt+/− liver had the lowest production of NAD, which weakens the Sirt3-Foxo3 axis. Disruption of this axis may deregulate a set of key genes in cell homeostasis, resulting in decreased synthesis of GSH. After APAP treatment, the low levels of GSH present in the Nampt+/− liver result in the highest accumulation of the toxic intermediate of APAP, NAPQI, which, in turn, leads to the more severe oxidative stress and eventual hepatocyte necrosis or necroptosis. On the other hand, NamptOE mice produce the highest level of NAD, which affects the similar biochemical and physiological events in the opposite direction, leading to the least hepatocyte necrosis or necroptosis.

In summary, these data suggest that NAMPT protects against APAP overdose—induced ALI via its enzymatic activity to catalyze cellular NAD synthesis, which exerts its effect on hepatocyte homeostasis possibly through the Sirt3-Foxo3 axis. The most severe ALI is correlated with the lowest expression of NAMPT in Nampt+/− mice, which can be readily detectable in mouse serum. Our preliminary results suggest that NAMPT is a genetic biomarker in APAP-induced ALI or ALF. Treatment of P7C3-A20, a small chemical molecule up-regulator of Nampt, ameliorated APAP-induced mouse ALI over the reagent control. Although the detailed molecular mechanisms linking the lowest expression of Nampt or NAD level to the most severe ALI in Nampt+/− mice remain to be elucidated fully, this study has demonstrated that NAMPT may be a potential new diagnostic and therapeutic target to APAP-induced ALI.

Acknowledgments

We thank Suman Chaudhary and Inna Sokoloversusky for excellent technical assistance; Dr. Steven Weinman (The University of Kansas Liver Center and Medical Center, Kansas City, MO) for the helpful intellectual input and critical reading of the manuscript; and Drs. Hartmut Jaeschke and Wen-Xing Ding (University of Kansas Medical Center, Kansas City, MO) for expert and insightful suggestions and comments to usher our mechanistic study in this project during and after our research progress presentation of this project at The University of Kansas Liver Center.

References


Zhang et al