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Protection of Nicotinic Acid against Oxidative Stress-Induced Cell Death in Hepatocytes Contributes to Its Beneficial Effect on Alcohol-induced Liver Injury in Mice

Xiaobing Dou^{a,b}, Chen Shen^b, Zhigang Wang^{b,d}, Songtao Li^b, Ximei Zhang^b, and Zhenyuan Song^{b,c}

^aCollege of Life Science, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, P. R. China. 310053

^bDepartment of Kinesiology and Nutrition, University of Illinois at Chicago, Chicago, IL 60612

^cDepartment of Pathology, University of Illinois Medical Center, Chicago, IL 60612

^dCollege of Pharmacy, Harbin Medical University, Harbin, Heilongjiang, P. R. China. 150081

Abstract

Oxidative stress plays a pathological role in the development of alcoholic liver disease. In this study, we investigated the effects of nicotinic acid (NA) supplementation on H₂O₂-induced cell death in hepatocytes and alcohol-induced liver injury in mice. Hepatocytes were exposed to H₂O₂ (0–0.4 mM) for 16 hours after a 2-hour pretreatment with NA (0–100 μM). Cell viability, intracellular glutathione and total NAD contents were determined. In animal experiments, male C57 BL/6 mice were exposed to Lieber-De Carli liquid diet (+/– ethanol with/without NA supplementation (0.5%, w/v) for 4 weeks. Nicotinic acid phosphoribosyltransferase (NaPRT) is the first enzyme participated in the NA metabolism, converting NA to nicotinic acid mononucleotide (NaMN). In NaPRT-expressing Hep3B cells, H₂O₂-induced cell death was attenuated by NA, whereas in NaPRT-lost HepG2 cells, only NaMN conferred protective effect, suggesting that NA metabolism is required for its protective action against H₂O₂. In Hep3B cells, NA supplementation prevented H₂O₂-induced declines in intracellular total NAD and GSH/GSSG ratios. Further mechanistic investigations revealed that conservation of Akt activity contributed to NA's protective effect against H₂O₂-induced cell death. In alcohol-fed mice, NA supplementation attenuated liver injury induced by chronic alcohol exposure, which was associated with alleviated hepatic lipid peroxidation and increased liver GSH concentrations. In conclusion, our findings indicate that exogenous NA supplementation may be an ideal choice for the treatment of liver diseases involved oxidative stress.

Keywords

Nicotinic acid; Hydrogen peroxide; Oxidative stress; Glutathione; Alcohol; Liver

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Corresponding author: Zhenyuan Song, Ph. D., Department of Kinesiology and Nutrition, University of Illinois at Chicago, 1919 W Taylor Street, RM 627, Chicago, IL 60612, USA, Phone: (312)-996-7892, Fax: (312)-413-0319, song2008@uic.edu.

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1. Introduction

Oxidative stress refers to various deleterious processes resulting from an imbalance between the excessive formation of reactive oxygen species and limited antioxidant defenses. Reactive oxygen species (ROS) play a dual role in a variety of normal physiological and pathological conditions [1]. At physiological low levels, ROS have been indicated as mediators and redox messengers in various vital cellular processes and intracellular signaling networks. In contrast, at certain pathological conditions, excessive ROS accumulation promotes cell death by inducing oxidative damage to cellular macromolecules, including lipids, proteins, and DNA [2–4]. Increased damage caused by ROS has been shown to determine the cell fate by inducing cell cycle arrest and apoptosis [5]. Normal tissues maintain intracellular redox homeostasis by balancing the equilibrium of ROS generation with the elimination of generated ROS [6].

All aerobic organisms are subject to a certain level of physiological oxidative stress from mitochondrial respiration. The intermediates that are formed, such as superoxide and hydrogen peroxide (H_2O_2), can lead to the production of toxic oxygen radicals that can cause lipid peroxidation and cell injury. To prevent this, a variety of intracellular antioxidant processes evolves to prevent intracellular ROS accumulation, thereby protecting against ROS induced cellular damage. Glutathione is a tri-peptide synthesized from glycine, cysteine, and glutamate in two ATP-dependent steps. Intracellular glutathione exists in reduced (GSH) and oxidized (GSSG) states. In normal cells, more than 90% of the total glutathione pool is GSH and less than 10% exists in the form of GSSG. During oxidative stress condition, the endogenously produced hydrogen peroxide is reduced by GSH in the presence of selenium-dependent GSH peroxidase (GPx). In the process, GSH is oxidized to GSSG, which in turn is reduced back to GSH by GSSG reductase (GR) at the expense of NADPH, forming a redox cycle. Therefore, an increased GSSG-to-GSH ratio has been considered to be a strong indicative of oxidative stress [7, 8].

Oxidative stress has been implicated in the pathogenesis of a variety of liver diseases, including alcoholic liver disease, non-alcoholic fatty liver disease, hepatocellular carcinoma, to name a few. Due to its critical participation in cellular oxidative stress defense system, the approach aimed at increasing intracellular GSH levels represents a promising therapeutic choice in the prevention and treatment of these diseases. Many compounds have been reported to confer hepatoprotective effect via enhancing intracellular production of GSH either directly or indirectly [9–11].

Nicotinic acid (niacin) has been used for decades to prevent and treat atherosclerosis due to its well-established anti-dyslipidemic effects [12,13]. Nicotinic acid has the remarkable ability to increase HDL cholesterol levels while decreasing triglyceride, LDL cholesterol and lipoprotein (a) levels, thereby improving the total plasma lipid profile [12]. Although the underlying mechanisms remained incompletely understood, both inhibition of lipolysis in adipose tissue via the activation of the nicotinic acid receptor HCA₂ (GPR109A) on adipocytes and suppression of VLDL secretion from the liver has been documented [14]. In addition to its anti-dyslipidemic functions, in recent years, accumulated evidence from in vitro and in vivo studies supported that nicotinic acid possesses potent antioxidant properties [15–17]. Nicotinic acid deficiency was associated with increased oxidative stress. In human aortic endothelial cells (HAEC), nicotinic acid inhibited vascular inflammation by decreasing endothelial ROS production and subsequent LDL oxidation and inflammatory cytokine production, key events involved in atherogenesis [15]. Using both animal and cell culture model, Wu et al recently showed that nicotinic acid supplementation inhibited vascular inflammation via Nrf2 regulated heme oxygenase-1 (HO-1) induction [18].

Given the pathological role of oxidative stress in the development of various liver diseases and reported antioxidant function of nicotinic acid, this study was aimed to investigate whether nicotinic acid supplementation could protect against oxidative stress-induced cell death in hepatocytes. We found that nicotinic acid prevented H₂O₂-induced oxidative stress and protected hepatocytes from H₂O₂-induced cell death. Our results also demonstrated that long-term supplementation of nicotinic acid attenuated chronic alcohol induced oxidative stress and liver injury.

2. Materials and Methods

2.1. Chemicals

Except indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animal Model

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) weighing 25 ± 0.5 g (means $\pm$ SD) were fed *ad libitum* with ethanol-containing (ethanol-derived calories were increased from 30% to 36% during the first 4 weeks, 2% increase each week) or isocaloric control liquid diet (Bioserv, Frenchtown, NJ) for 4 weeks. For NA supplementation, NA was added into ethanol-containing liquid diet at 0.5% (w/v). Food intake and body weight were recorded daily and weekly, respectively. Mice were euthanized and plasma and liver tissue samples harvested at the end of the experiment.

2.3. Cell Culture

HepG2 cells and Hep3B cells, two human hepatoma cell lines, and AML-12 cells, a nontumorigenic mouse hepatocyte cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) or DMEM/F-12 medium containing 10% (v/v) fetal bovine serum, 2mM glutamine, 5 U/ml penicillin, and 50 ug/ml streptomycin at 37°C in a humidified O₂/CO₂ (19:1) atmosphere.

2.4. MTT Assay

For MTT assay, cells were seeded at a density of 2×10^4 cells/well on 96-well culture plates and incubated overnight. After the corresponding treatment, the medium was removed, and cell viability was evaluated by assaying the ability of functional mitochondria to catalyze the reduction of thiazolyl blue tetrazolium bromide (MTT) to a formazan salt by mitochondrial dehydrogenases.

2.5. LDH Release Assay

For LDH release assay, LDH activity in the medium was determined spectrophotometrically at 340 nm by following the rate of NAD⁺ reduction in the presence of L-lactate.

2.6. Flow Cytometry Analysis of Cell Death

The effects of NA and H₂O₂ exposure on cell viability were determined by staining with propidium iodide (PI), using the commercially available kit (Annexin V-FITC Apoptosis Detection Kit I; BD Biosciences Pharmingen). PI were added to the cellular suspension as in the manufacturer's instructions, and sample fluorescence of 10,000 cells was analyzed by flow cytometry (C6 Flow Cytometer, Accuri Cytometers Inc., MI).

2.7. Hoechst Staining

Hoechst 33342 is used for specifically staining the nuclei of living or fixed cells and tissues. Thus, it allows for the measurement of apoptosis within cells. Half an hour before the end of the incubation with the indicated stimulus, Hoechst was added to each well of 24-well plates at a final concentration of 1 μ M. At the completion of the incubation, the cells were washed three times with ice-cold PBS, and then the fluorescence was measured by fluorescent microscope. All data are representative of at least three independent experiments.

2.8. Intracellular Total NAD (NAD⁺ +NADH) Measurement

Intracellular total NAD level in cell lysate were measured using NAD/NADH quantification kit in accordance with the manufacturer's instructions (Catalogue number: K337-100 Biovision Inc, CA). All data are representative of at least three independent experiments.

2.9. Intracellular GSH/GSSG Measurement

GSH and GSSG in the whole liver tissues or cultured cells were measured using Oxiselect Total Glutathione (GSSG/GSH) Assay Kits in accordance with the manufacturer's instructions (Cell Biolabs, CA). The data were expressed as nmol/mg protein. All data are representative of at least three independent experiments.

2.10. Western Blot

Hepatocytes were lysed in RIPA buffer and the isolated proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to 0.45 μ M polyvinylidene difluoride (PVDF) membrane. After transferring, membranes were blocked in 1% BSA in PBS with 0.1% Tween-20 and probed with anti-PARP (Novus Biologicals, Littleton, CO), anti-phospho-Akt, anti-Akt (Cell Signaling Technology, Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence substrate kit were used in detection of specific proteins.

2.11. GPx and Catalase Activity Assay

GPx and catalase activity in cell lysate were measured using GPx assay kit (Cayman Chemical Company, MI) and Catalase Activity Assay Kit (MBL International, MA) in accordance with the manufacturer's instructions. All data are representative of at least three independent experiments.

2.12. Statistical Analysis

All data were expressed as mean $\pm$ SD. Statistical analysis was performed using a one-way ANOVA and was analyzed further by Newman-Keuls test for statistical difference. Differences between treatments were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Nicotinic acid (NA) protects hepatocytes from H₂O₂-induced cytotoxicity

H₂O₂ is an important form of active oxygen. Owing to its high permeability across cell membrane, H₂O₂ has been widely used as a classical reagent to trigger oxidative stress in cultured cells, including hepatocyte. In the present study, H₂O₂ exposure of cultured hepatocytes was employed as our *in vitro* experimental model. Hep3B and HepG2, two human hepatoma cell lines, AML-12, a non-transformed mouse hepatocyte cell line were used in our cell culture studies. Hepatocytes were exposed to exogenous H₂O₂ at various concentrations for 16 hours and cell viability was determined by LDH release measurement and MTT assay, respectively. As shown in Fig. 1, exogenous H₂O₂ exposure reduced cell

viability in both Hep3B and AML-12 cells in a dose-dependent fashion (1A). H₂O₂-induced hepatocyte cell death was further confirmed by Western blot detection for cleaved form of PARP-1 in Hep3B cells (1B). To examine NA's protective effect, we pretreated Hep3B cells with NA at 25, 50, 100 μ M for 2 hours before 0.2 mM H₂O₂ exposure. Cell death was measured 16 hours later. As shown in Fig. 2A, NA pretreatments indeed protected hepatocytes against H₂O₂-induced cytotoxicity. At 50 μ M, NA almost completely prevented cell death induced by various concentrations of H₂O₂ exposure (Fig. 2B). These observations were further confirmed by Hoechst staining (Fig. 2C), Western blot detection for cleaved form of PARP-1 (Fig. 2D), as well as flow cytometry assay (Fig. 2E).

3.2. The protective effects of NA depend on its intracellular metabolism

NA serves as one of substrates (another one is nicotinamide) in the salvage pathway of NAD synthesis. The first enzyme in this pathway is NA phosphoribosyltransferase (NAPRT), converting NA to NA mononucleotide (NaMN), which is then converted into NA adenine dinucleotide, and lastly into NAD (Fig. 3A). To determine whether the protective effect of NA in H₂O₂-induced cytotoxicity requires its intracellular metabolism, HepG2, a human hepatoma cell line, was also utilized based on the fact that in comparison to Hep3B cells, HepG2 cells only express minimal NAPRT mRNA (Fig. 3B). Both Hep3B and HepG2 cells was pretreated with NA for 2 hours, followed by a 16-hour 0.2mM H₂O₂ exposure. As shown in Fig. 3C, unlike that seen in Hep3B cells, NA could not confer protective effects in HepG2 cells. In contrast, when HepG2 cells were pretreated with NaMN, the product of NAPRT-catalyzed reaction, H₂O₂-induced cytotoxicity was significantly reduced (Fig. 3D), indicating that intracellular metabolism of NA is required for its protective effect. To further confirm this notion, we measured intracellular NAD concentrations after NA treatment in both Hep3B and HepG2 cells. As shown in Fig. 3E & F, the protective effect of NA in Hep3B cells was associated with significantly elevated intracellular NAD levels, whereas NAD levels in HepG2 cells were not affected by NA supplementation (Fig. 3F). Conversely, when HepG2 cells were treated with NaMN, intracellular NAD levels were significantly increased (Fig. 3F), confirming that NA's protection against H₂O₂ cytotoxicity involves its intracellular metabolism.

3.3. NA prevents H₂O₂-induced alteration of intracellular redox status in hepatocytes

Altered intracellular redox status (GSH/GSSG) plays a fundamental role in H₂O₂-induced cell death in a variety of types of cells. To determine whether alleviation of H₂O₂-induced intracellular redox status change contributes to the protective effect of NA on H₂O₂-induced cytotoxicity, we measured intracellular GSH and GSSG levels in Hep3B cells. As shown in Fig. 4A & B, H₂O₂ exposure at 0.2 mM resulted in increased oxidation of GSH to GSSG, and the GSH/GSSG ratio fell significantly within 30 minutes after H₂O₂ addition. Although the intracellular redox status recovered to certain degrees in the later time points, due to activated compensatory mechanisms, it was still lower than those of control and hepatocytes treated with NA alone (Fig. 4C). Pretreatment with 50 μ M NA for 2 hours prevented H₂O₂-induced GSH reduction and GSSG elevation (Fig. 4A & B), leading to almost complete recovery of GSH/GSSG ratio (Fig. 4C). When buthionine sulfoximine (BSO), a potent inhibitor for glutathione synthesis, was added into the media before NA pretreatment, the protective effect of NA was dramatically compromised (Fig. 4D). As expected, BSO aggravated H₂O₂-induced cell death. Interestingly, our results in this experiment also showed that NA protected not only H₂O₂-induced, but also BSO-induced hepatocyte cell death, suggesting that maintenance of intracellular redox status only partially contributed to NA's beneficial effects, other unknown mechanisms are also involved.

3.4. NA increased activities of the enzymes involved in H₂O₂ metabolism

Glutathione peroxidase (GPx) and catalase are two major enzymes metabolizing H₂O₂ to H₂O. To determine whether NA is able to regulate activities of these two enzymes, the enzyme activities of GPx and catalase were measured using commercially available assay kits. As shown in Fig. 5, H₂O₂ exposure increased activities of both enzymes when compared to the untreated cells, which may represent a protective mechanism. Intriguingly, NA pretreatment further increased the enzyme activities.

3.5. Prevention of H₂O₂-induced Akt suppression contributes to NA's beneficial effect

Signaling pathways involved in H₂O₂-induced cell death are cell-type specific and dose-dependent. Among these, suppression of Akt kinase plays a critical role in cell death caused by a variety of oxidative insults, including H₂O₂. To determine the potential involvement of Akt kinase activity, we measured phosphorylated Akt levels in Hep3B cells exposed to H₂O₂ with/without NA pretreatment by Western blot. As shown in Fig. 6A & B, NA pretreatment prevented H₂O₂-induced decrease in phosphorylated Akt protein levels. To further determine the critical role of conservation of Akt activity in this process, LY294002, a specific Akt inhibitor, was added to the media before NA supplementation. As shown in Fig. 6C, inhibition of Akt kinase activity abolished the protective effects of NA, confirming that conservation of Akt kinase activity indeed plays a key role in NA's protective effect in H₂O₂-induced cell death in hepatocytes.

3.6. NA supplementation prevents chronic alcohol induced oxidative stress and liver injury

Oxidative stress plays a pathologic role in the development of alcoholic liver disease. Our *in vitro* studies provide strong evidence that NA can function as an antioxidant protecting hepatocytes against H₂O₂-induced cytotoxicity. These observations prompt us to examine the potential preventive role of NA supplementation in alcoholic liver disease. To do that, the well-established Lieber-De Carli liquid diet feeding mice model of ALD was used and NA was supplemented into the liquid diet (0.5%, w/v) from the beginning of the feeding. Animals were killed 4 weeks later. Our results demonstrated that NA supplementation alleviated liver injury induced by chronic alcohol exposure, evidenced by significantly decreased plasma ALT levels when compared with AF group (Fig. 7A). Attenuation of liver injury by NA supplementation was associated with alleviated oxidative stress, illustrated by decreased hepatic TBA contents compared to AF group (Fig. 7B). Similar to our observations in cell culture studies, NA supplementation prevented alcohol-induced hepatic GSH reduction and GSSG elevation, thereby improved liver GSH/GSSG ratio (Fig. 7C).

4. Discussion

In the present study, we examined the effect of NA on oxidative stress-induced cell death in hepatocytes and investigated the underlying mechanisms involved. Using H₂O₂ exposure of cultured hepatocytes as our *in vitro* experimental model, our data demonstrated that NA protects hepatocytes against H₂O₂-induced cytotoxicity. Taking advantage of the fact that HepG2 cells only express minimal level of NA phosphoribosyltransferase (NAPRT), the first enzyme catalyzing salvage pathway of NAD synthesis using NA as a substrate, we showed that the beneficial effect of NA depends on its intracellular conversion to NAD. The protective effect of NA on H₂O₂-induced cytotoxicity in Hep3B cells was concomitant with significantly increased intracellular NAD levels, whereas in HepG2 cells, the lack of protection by NA was associated with unchanged intracellular NAD contents. Further mechanistic investigations revealed that NA prevents H₂O₂-induced alteration in intracellular redox status (GSH/GSSG ratio) and downregulation of Akt activation. Finally, using well-established alcoholic liver disease mouse model, we demonstrated that long-term

NA supplementation endowed beneficial effect on ALD, which was associated with alleviated oxidative stress.

In addition to its well-established regulatory effects on lipid metabolism, several recent studies demonstrated that NA status was also associated with cell viability and oxidative stress. For instance, a three-week feeding of NA-deficient diet altered p53 expression and impaired etoposide-induced cell cycle arrest and apoptosis in rat bone marrow cells [16]. HaCaT keratinocytes cultured with NA-deficient medium develop a decreased growth rate due to an increase in apoptotic cells and an arrest in the G(2)/M phase of the cell cycle. Additionally, long-term culture with NA-deficient medium of these cells resulted in accumulation of reactive oxygen species and increased DNA damage [19]. On the other hand, exogenous supplementation of NA protects against UVB radiation-induced apoptosis in cultured human skin keratinocytes [20]. Ex vivo supplementation with nicotinic acid protects from DNA-damage-induced cell death in human peripheral blood mononuclear cells [21]. A very recent animal study showed that NA improved ischemia-induced neovascularization in diabetic mice by enhancement of endothelial progenitor cell functions independent of changes in plasma lipids [22]. In the present study, we directly tested beneficial effect of NA in hepatocytes via exposing the cells with H₂O₂, a representative membrane-permeable oxidant and the most abundant ROS in cells. In consistent with many previous studies, our data clearly showed that NA protected hepatocytes against oxidative stress-induced cell death.

Metabolically, NA serves as one of substrates in the salvage pathway of NAD synthesis. The first enzyme in this pathway is NA phosphoribosyltransferase (NAPRT), converting NA to NA mononucleotide (NaMN), which is then converted into NA adenine dinucleotide, and lastly into NAD [23]. Our initial observation that, unlike Hep3B cells, NA lost protective effect on H₂O₂-induced cell death in HepG2 cells promoted us to examine whether these two types of hepatocytes expressed different levels of NAPRT. As expected, in comparison to Hep3B cells, HepG2 cells only express minimal levels of NAPRT, indicating that intracellular conversion to NAD is required for protection. This notion was indeed supported by the observations that NaMN, the product of NAPRT, at the same concentration as NA, protected HepG2 cell against H₂O₂-induced cell death. Similar to NA, NaMN significantly increased intracellular NAD levels.

Glutathione (GSH) plays central roles in the defense against H₂O₂-induced oxidative damage. Replenishment of GSH with NAC, a glutathione precursor, effectively prevented H₂O₂-induced oxidative stress and cell death in a variety of types of cells, including hepatocytes [24]. In consistence with many previous reports [25, 26], in the present study, H₂O₂ exposure of hepatocytes caused remarkable intracellular GSH decrease and GSSG increase, leading to significantly fall in GSH/GSSG ratio. Pretreatment with NA efficiently prevented H₂O₂-induced intracellular redox alteration. Intriguingly, unlike NAC, NA treatment alone did not induce markedly increase in intracellular GSH levels. Although BSO, a potent inhibitor of glutathione synthesis, offset NA's protective effect, it also aggravates H₂O₂-induced cell death. Unexpectedly, we also observed that NA partially protects against cell death induced by BSO exposure, suggesting that maintenance of intracellular redox status only partially contributes to NA's beneficial effect. Other unknown mechanisms are obviously involved in this process. Although the exact mechanisms involved in the effect of NA on the glutathione homeostasis are still elusive and under investigation, a couple of hypothesis can be proposed based on previous reports and data obtained in this study. 1. NA increases intracellular NADPH levels. Glutathione reductase is an enzyme that reduces GSSG to GSH, thereby alleviating oxidative stress-induced GSH depletion. For every mole of GSSG, one mole of NADPH is required to reduce GSSG to GSH. Previous study by Yan et al. demonstrated that NA increased intracellular NADPH

levels via upregulating the expression of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the pentose phosphate pathway and the principal source of cellular NADPH [27]. Therefore, it is likely that in hepatocytes, NA can increase NADPH levels either through the same mechanism or through NADPH production using NAD as a substrate. 2. NA enhances glutathione synthesis via Nrf2 activation. A very recent study using human coronary artery endothelial cells (HCAECs) showed that NA at mM levels induced Nrf2 activation, a major transcription factor in regulating glutathione synthesis [18]. Although much lower concentrations of NA were used in our study, it is still possible that in hepatocytes NA at these levels may activate Nrf2. Nevertheless, detailed mechanistic studies are warranted.

Signaling pathways involved in H₂O₂-induced cell death are cell-type specific and dose-dependent [28–30]. Many studies report that H₂O₂ is implicated in most of the growth factor-inducing signaling cascades, including PI3K/Akt and JNK pathway, which is dependent on cell types and dose. Among these, suppression of Akt kinase plays a critical role in cell death caused by H₂O₂. Consistent with a recent study by Iwakami et al [29], we found that H₂O₂ exposure significantly decreased phosphorylated Akt protein levels in Hep3B cells. Pretreatment with NA prevented H₂O₂-induced Akt suppression. Furthermore, LY294002, an Akt inhibitor, offset NA's protective effect, confirming that conservation of Akt kinase activity indeed plays a key role in NA's prevention of cell death induced by H₂O₂ in hepatocytes.

Both oxidative stress and decreased intracellular NAD⁺/NADH ratio, due to hepatic ethanol metabolism, contributes to the onset and progression of alcoholic liver disease (ALD) [31, 32]. During ethanol metabolism, the reactions catalyzed by both alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) use NAD⁺ as coenzyme, leading to increased intracellular NADH/NAD⁺ ratio. Our cell culture experiments provided evidence that NA supplementation not only prevented oxidative stress, but also increased intracellular NAD levels in hepatocytes. To test the clinical relevance of our in vitro observations, the well-established Lieber-De Carli diet feeding mouse model of ALD was employed. Our results clearly showed that long-term supplementation alleviated liver injury induced by chronic alcohol exposure. Comparable to our cell culture observations, NA supplementation prevented alcohol-induced hepatic GSH depletion and alleviated oxidative stress in the liver. Therefore, data from both in vitro and in vivo experiments in this study support that NA can confer protection against oxidative stress-induced cytotoxicity in hepatocytes. Further investigations are required to test the potential therapeutic function of NA in liver diseases with oxidative stress being a pathological factor, such as ALD.

In summary, our data demonstrate that NA is capable of protecting hepatocytes against H₂O₂-induced cell death. The protection involves its intracellular conservation to NAD and prevention of GSH depletion. Mechanistic investigations suggest that conservation of Akt signaling pathway plays a critical role in NA's beneficial effect. Our in vivo studies using mouse model of ALD suggest that NA may serve as a safe and efficacious therapeutic choice for oxidative stress-related liver diseases. Continued attempts to clarify the underlying mechanisms of NA in protection against oxidative stress induced cell death will pave the way in exploiting preventive and/or therapeutic strategies for a variety of types of liver diseases.

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Abbreviations

NA	nicotinic acid
H₂O₂	Hydrogen peroxide
ROS	reactive oxygen species
GSH	glutathione
GPx	glutathione peroxidase
NAD	Nicotinamide adenine dinucleotide
NaPRT	nicotinic acid phosphoribosyltransferase
NaMN	nicotinic acid mononucleotide
NaAD	nicotinic acid adenine dinucleotide
BSO	buthionine sulfoximine

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Fig. 1. H_2O_2 exposure induces hepatocyte cell death. (A) Both Hep3B and AML-12 cells were exposed to exogenous H_2O_2 at indicated concentrations for 16 hours. Cell viability was determined by LDH release measurement and confirmed by MTT assay. Exogenous H_2O_2 exposure reduced cell viability in both Hep3B and AML-12 cells in a dose-dependent fashion. All values are denoted as means $\pm$ SD from three or more independent studies. * $p < 0.05$ vs. UT. (B) H_2O_2 exposure induced PARP-1 cleavage in a dose-dependent manner. Hep3B cells were treated with exogenous H_2O_2 at indicated concentrations for 16 hours. Cell lysates were collected and subjected to Western blot detection for cleaved form of PARP-1.

Fig. 2. Nicotinic acid (NA) protects hepatocytes from H₂O₂-induced cytotoxicity. Hep3B cells were pretreated with NA for 2 hours before H₂O₂ exposure. Cell death was measured 16 hours later. (A) NA (25, 50, 100 μM) pretreatments protected hepatocytes against 0.2mM H₂O₂-induced cytotoxicity in a dose-dependent manner. Bars with different letters differ significantly ($p < 0.05$). (B) NA at 50μM almost completely prevented hepatocyte cell death induced by various concentrations of H₂O₂ (0.2, 0.4, 0.6 mM) exposure. * $p < 0.05$ vs. control cells. (C-E) The protective effects of NA against H₂O₂-induced cytotoxicity were further confirmed by Hoechst staining (C), Western blot detection for cleaved form of PARP-1 (D), and flow cytometry assay (E).

Fig. 3. The protective effects of NA depend on its intracellular metabolism. (A) Intracellular conversion of NA to NAD⁺. NaPRT: nicotinic acid phosphoribosyltransferase; NaMN: nicotinic acid mononucleotide; NaAD: nicotinic acid adenine dinucleotide. (B) NaPRT gene expression in HepG2 and Hep3B cells. All values are denoted as means ± SD from three or more independent studies. * $p < 0.05$ vs. Hep3B cells. (C) NA pretreatment had no effect on H₂O₂-induced cytotoxicity in HepG2 cells. All values are denoted as means ± SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$). (D) NaMN pretreatment protected HepG2 cells against H₂O₂-induced cytotoxicity. All values are denoted as means ± SD from three or more independent studies. Bars with different

letters differ significantly ($p < 0.05$). (E) NA treatment elevated intracellular NAD⁺ concentrations in a dose-dependent fashion in Hep3B cells. All values are denoted as means $\pm$ SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$). (F) NaMN, but not NA, significantly increased intracellular NAD⁺ levels in HepG2 cells. All values are denoted as means $\pm$ SD from three or more independent studies.

Fig. 4. Maintenance of intracellular redox status contributes to NA's protective effect against H₂O₂-induced cell death. Hep3B cells were pretreated with NA for 2 hours before H₂O₂ exposure. Intracellular glutathione (both GSH and GSSG) levels were measured. (A-C) Time-course changes of intracellular GSH and GSSG concentrations after exposure to H₂O₂ (0.2 mM). Pretreatment with 50 μ M NA for 2 hours alleviated H₂O₂-induced GSH depletion, GSSG elevation, and fall in GSH/GSSG ratio. All values are denoted as means $\pm$ SD from three or more independent studies. * Significantly different from other treatments at same time point. ($p < 0.05$). (D) Inhibition of glutathione synthesis with BSO compromised NA's protective action. BSO was added into the media for 1 hour before NA pretreatment in Hep3B cells.

Cell death was measured 16 hours later after H₂O₂ exposure. All values are denoted as means $\pm$ SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$) .

Fig. 5. NA increased activities of the enzymes involved in H₂O₂ metabolism. Hep3B cells were pretreated with NA for 2 hours before H₂O₂ stimulation for 16 hours. The enzyme activities of glutathione peroxidase (GPx) and catalase were measured. (A) H₂O₂ exposure elevated GPx activity. NA pretreatment further increased the enzyme activity. All values are denoted as means $\pm$ SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$). (B) H₂O₂ exposure increased catalase activity. NA pretreatment further elevated the enzyme activity. All values are denoted as means $\pm$ SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$).

Fig. 6.

Conservation of Akt activation contributes to NA's beneficial effect. (A & B) NA pretreatment prevented H₂O₂-induced Akt suppression in Hep3B cells. Hep3B cells were pretreated with NA (50 μ M) for 2 hours, followed by exogenous H₂O₂ exposure for indicated time. Whole cell lysates were collected and subjected to Western blot for p-Akt, total-Akt, and actin. All values are denoted as means $\pm$ SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$). (C) LY294002, an Akt kinase inhibitor, abolished NA's protective effect in H₂O₂-induced cytotoxicity in Hep3B cells. Hep3B cells were pretreated with LY294002 (10 μ M) for 1 hour before NA and H₂O₂ exposure. Cell viability was determined 16 hours later. All values are denoted as means $\pm$

SD from three or more independent studies. Bars with different letters differ significantly ($p < 0.05$).

Fig. 7. NA supplementation prevents chronic alcohol induced oxidative stress and liver injury. Male C57BL/6 mice were pair-fed liquid diets with or without ethanol for 4 weeks. For NA supplementation, NA was supplemented in liquid diet in a dose of 0.5% (w/v). (A) NA supplementation significantly decreased plasma ALT levels induced by chronic alcohol consumption. Data are expressed as the mean $\pm$ SD (n = 6 mice per group). Bars with different letters differ significantly ($p < 0.05$). (B) NA supplementation alleviated hepatic TBA contents compared to AF group. Data are expressed as the mean $\pm$ SD (n = 6 mice per group). Bars with different letters differ significantly ($p < 0.05$). (C) NA supplementation prevented alcohol-induced hepatic GSH reduction and GSSG elevation, thereby improved

liver GSH/GSSG ratio. Data are expressed as the mean $\pm$ SD (n = 6 mice per group). Bars with different letters differ significantly ($p < 0.05$). PF: pair feeding; AF: alcohol feeding.