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Deficiency of pyruvate dehydrogenase kinase 4 sensitizes mouse liver to diethylnitrosamine and arsenic toxicity through inducing apoptosis☆

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Abstract

Background and Aim: Pyruvate dehydrogenase kinase 4 (PDK4) is a metabolism switch that regulates glucose oxidation and the tricarboxylic acid cycle (TCA cycle) in the mitochondria. Liver detoxifies xenobiotics and is constantly challenged by various injuries. This study aims at understanding how the loss of the metabolism regulator PDK4 contributes to liver injuries.

Methods: Wild-type (WT) and *Pdk4* knockout (*Pdk4*$^{-/-}$) mice of different ages were examined for spontaneous hepatic apoptosis. Juvenile or adult mice of two genotypes were insulted by diethylnitrosamine (DEN), arsenic, galactosamine (GalN)/lipopolysaccharide (LPS), anti-CD95 (Jo2) antibody or carbon tetrachloride (CCl4). Liver injury was monitored by blood biochemistry test. Apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, poly (ADP-ribose) polymerase (PARP) cleavage, and caspase activity assay. Inflammatory response was determined by nuclear factor (NF)-κB activation and the activation of NF-κB target genes. Primary hepatocytes were isolated and cell viability was evaluated by MTS assay.

Results: We showed that systematic *Pdk4*$^{-/-}$ in mice resulted in age-dependent spontaneous hepatic apoptosis. PDK4-deficiency increased the toxicity of DEN in juvenile mice, which correlated with a lethal consequence and massive hepatic apoptosis. Similarly, chronic arsenic administration induced more severe hepatic apoptosis in *Pdk4*$^{-/-}$ mice compared to WT control

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Authors' contributions

J. Choiniere, M.J. Lin and J.Wu performed experiments, analyzed results and prepared the manuscript. L.Wang and J.Wu designed experiments, supervised the work and prepared the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

mice. An aggravated hepatic NF-κB mediated-inflammatory response was observed in *Pdk4*$^{-/-}$ mice livers. *In vitro*, *Pdk4*-deficient primary hepatocytes were more vulnerable to DEN and arsenic challenges and displayed higher caspase activity than wild type cells. Notably, hepatic PDK4 mRNA level was remarkably reduced during acute liver failure induced by GalN/LPS or Jo2 antibody. The diminished PDK4 expression was also observed in CCl4-induced acute liver injury.

Conclusions: PDK4 may contribute to the protection from apoptotic injury in mouse liver.

Keywords

Hepatocytes; Cell death; Liver injury; Pyruvate dehydrogenase kinase 4 (PDK4)

1. Introduction

Pyruvate dehydrogenase kinase 4 (PDK4) inhibits the pyruvate dehydrogenase complex (PDC) through phosphorylating the pyruvate dehydrogenase (PDH, the E1 enzyme of PDC) within the glucose-metabolism pathway. This particular isoform is highly expressed in heart, kidney, and liver tissue in mice and humans.[1] PDK4 protects the development of steatosis induced by a high-saturated fat diet in mice,[2] and its reduced expression associates with insulin sensitivity improvement in postobese patients.[3] Despite important roles in metabolism and bioenergetics, recent studies have uncovered PDK4's unexpected influence in cell cycle,[4] anoikis,[5] drug resistance,[6] vascular calcification,[7] hormone response and tumor progression.[8-11]

Cells with altered metabolic states are prone to defect in response to various stresses and injuries. Apoptosis, or programmed cell death, is activated after significant cellular stress through intrinsic and/or extrinsic pathways, which irradiates irreparable cellular damage.[12] Abnormalities in apoptosis regulation (either impaired or increased) serves as an important component of diseases. Intermingled with other forms of cell death, apoptosis is a prominent feature in hepatic diseases. A putative role of PDK4 in apoptosis has been suggested in HepG2 cells.[13] However, the direct experimental evidence linking PDK4 to hepatic apoptosis is still lacking.

Different causative factors lead to varied severity of hepatic apoptosis. Notably, alcohol, viruses, drugs, fatty acids, toxic bile acids, and immune response can induce hepatic apoptosis.[14] Diethylnitrosamine (DEN) is a putative carcinogenic initiator often used to induce tumor growth in juvenile mouse livers.[15] Typically, wild-type (WT) mice injected intraperitoneally at postnatal day 15 with a single dose (25 mg/kg) of this slow-acting hepato-carcinogen exhibit visible liver tumors after 8–12 months. As an initiator, DEN is metabolically activated by cytochrome p450 (CYP450) proteins, where it acts as an alkylating agent to form deoxyribonucleic acid (DNA)-adducts that are mutagenic.[16] The carcinogenic competency of DEN in livers has been linked to oxidative stress regulators as well as inflammatory signaling.[17-19] The hepatic apoptosis-inducing effect of DEN could also be regulated by death signaling molecules.[20] Arsenic is a metalloid known to be carcinogenic in humans.[21] There are multiple accounts of environmental exposure to arsenic correlated to liver cancer development. Mice liver studies have found that chronic, low-level

exposure to arsenic leads to malignant cell growth.[22] This is likely due to the inhibitory effect that metabolized arsenic exerts upon nearly 200 enzymes important to energy pathways and DNA maintenance and synthesis.[21] It has also been previously described that arsenic suppresses PDK4 expression by activating the histone H3 lysine 9 (H3K9) methyltransferase G9a and abrogates cyclin activity in hepatocellular carcinoma (HCC) cells.[4,23] It is of particular interest to reveal how the deficiency of a metabolic switch molecule such as PDK4 sensitizes/blunts the efficacy of DEN and arsenic, which is important in understanding the interaction between chemical carcinogenesis, especially the development of HCC, and the pleotropic function of PDK4.

In this study, we investigated the effects of DEN and arsenic on systemic PDK4-deficient mice livers and isolated primary hepatocytes. PDK4-deficiency predisposed hepatocytes to apoptotic injury upon DEN and arsenic treatment. This finding may prove useful in understanding the effects DEN and arsenic under a PDK4-deficient condition and further define the role of PDK4 in liver homeostasis and disease progression.

2. Materials and methods

2.1. Mice and treatment

WT and *Pdk4* knockout (*Pdk4*$^{-/-}$) mice were handled in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut.[4,24] All mice used in this study were male and of C57BL/6J (inbred strain) genetic background. Littermates of the same generation were used for all mouse studies performed at the Animal Care Services Facility at the University of Connecticut. WT or *Pdk4*$^{-/-}$ mice were cohoused in clean and ventilated cages bedded with Teklad aspen sani chips (7090A; Envigo Bioproducts, Inc, Madison, WI). Experimental adult mice were fed with irradiated Teklad global 18% protein (2918; Envigo Bioproducts, Inc.). Breeding mice were fed with irradiated sterile Teklad S-2335 mouse breeder diet (7904; Envigo Bioproducts, Inc.). Mouse diet composition is available online (http://www.envigo.com). Mice were housed in a 12-h/12-h light–dark (LD) cycle (light on 6 a.m. to 6 p.m.) with free access to water and food. Diethylnitrosamine (DEN) (N0756; Sigma Aldrich, St. Louis, MO) was diluted in phosphate buffered solution (PBS). Male mice (day 15 postpartum) were intraperitoneally (i.p.) injected with DEN (25 mg/kg body weight (BW)) and the survival status was monitored daily post-injection. Samples were collected under feeding conditions at day 6 after DEN injection due to unexpected death of *Pdk4*$^{-/-}$ mice. Arsenic ($NaAsO_2$) was from Sigma Aldrich (S7400). For arsenic treatment, mice were fed with 50 ppm via distilled drinking water, which was replaced every other day for 4 weeks.[6] D-(+)-galactosamine (GalN) was from Sigma Aldrich (G0500-5G). GalN/LPS injection and anti-Mouse CD95 (Jo2) (554255; BD Pharmingen) injection have been reported previously.[25,26] Carbon tetrachloride (CCl4) was administrated i.p. as 750 uL/kg BW, diluted in corn oil.

2.2. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL staining was performed in an In Situ Cell Death Detection Kit, Fluorescein (11684795910; Sigma-Aldrich) according to the manufacturer's instruction. The nucleus was counterstained with diamidino-phenyl-indole (DAPI). The labeling was detected and

images were taken under a Leica TCS SP8 confocal microscope with 40 or 63 × oil immersion lens in a sequential scanning mode.

2.3. Preparation of nuclear extracts

Tissues were collected, washed, and homogenized in hypotonic buffer (10mM HEPES-KOH (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.2mM Pefabloc® SC, 0.5% NP-40).[27] After incubation on ice for 5 min, lysates were centrifuged for 10 s at 16,000 ×*g* at 4 °C. The supernatants were discarded and the pellets were washed once with hypotonic buffer, and then lysed with high-salt buffer (50mM Tris (pH 7.4), 450mM NaCl, 1% NP-40, 1mM phenyl-methanesulfonyl fluoride (PMSF), 0.2mM Na_3VO_4, 5mM β-glycer-ophosphate, 20% glycerol, 2mM dithiothereiol (DTT)). Following incubation for 10 min on ice, lysates were centrifuged for 15 min at 16,000×*g* at 4 °C, and the supernatants (nuclear extracts) were collected.[28]

2.4. H&E staining

Mouse liver samples were fixed in 10% buffered formalin (#23-245684; Fisher Scientific, Pittsburgh, PA) and sent to the Department of Pathology at University of Connecticut for embedding, sectioning and hematoxylin-eosin staining.[11,29]

2.5. Enzyme-linked immunosorbent assay (ELISA)

Tumor necrosis factor (TNF) was determined with a TNF alpha ELISA Ready-SET-Go kit (#88-7346-22; eBioscience, San Diego, CA). 2mg of tissue lysate per well in 100 μL volume was loaded for the detection. The detection procedures were performed according to the manufacturer's instruction.

2.6. Measurement of nuclear factor (NF)-κB (p65) activity

The transcriptional activity of NF-κB (p65) was measured with a commercial kit (10007889; Cayman Chemical, Ann Arbor, MI) following the manufacturer's instruction using nuclear protein.[30]The activity is determined based on a sensitive colorimetric readout at 450 nm.

2.7. Caspase-3 colorimetric assay

Caspase-3 Colorimetric Assay Kit was from BioVision, Inc. (K106-200; Milpitas, CA). Caspase-8 (KHZ0061) and Caspase-9 (KHZ0101) Colorimetric Protease Assay Kits were from Thermo Fisher Scientific (Waltham, MA). The assays were performed following the manufacturers' protocol.[26] In brief, cells or tissues were lysed in pre-chilled lysis buffer provided in the kit. Cytosolic extracts (200 μg) in 50 μL lysis buffer and 5 μL chromophore substrate were added to 50 μL reaction buffer. The mixtures were set up in a 96-well plate and assayed for caspase activity in terms of the absorbance of 405 nm after incubation in a microplate reader.

2.8. Western blot and reverse transcription-quantitative PCR (RT-qPCR)

The procedures for Western blot and RT-qPCR were well established as described previously.[30,31] Antibodies used for western blot are poly (ADP-ribose) polymerase (PARP) (46D11) (#9532; Cell Signaling Technology, Danvers, MA) and β-actin (sc-47778, C4;

Santa Cruz Biotechnology, Santa Cruz, CA). The primers used in qPCR are *Pdk4* (forward: 5′-TCCCCGCTGTCCATGAAG-3’; reverse: 5′-CGTTCTTTCACAGGCATTTTCTG-3′), *Tnf* (forward: 5′-CAGCCGATGGGTTGTACCTT-3’; reverse: 5′-GGCAGCCTTGTCCCTTGA-3′), baculoviral IAP repeat-containing 3 (*Birc3*) (forward: 5′-TGGGTCAGTCTGCTTCGAGAT-3’; reverse: 5′-AATACGGGCTGCGTGTGTCT-3′), interferon gamma (*Ifnγ*) (forward: 5′-TTGGCTTTGCAGCTCTTCCT-3’; reverse: 5′-TGACTGTGCCGTGGCAGTA-3′), *E-selectin* (forward: 5′-CTTGCATGGCTCAGCTCAAC-3’; reverse: 5′-GGGACTTCCTGGGTCCACTT-3′), growth arrest and DNA-damage-inducible beta (*Gadd45β*) (forward: 5′-CGTTCTGCTGCGACAATGAC-3’; reverse: 5′-GCGCCAGCCTCTGCAT-3′), coiled-coil domain containing 103 (Ccdc103) (forward: 5′-AGCCATGCAGAGCGAGAGA-3’; reverse: 5′-TGCTCATGGCTTGCAACTTC-3′) and *Actb* (forward: 5′-CGATGCCCTGAGGCTCTTT-3’; reverse: 5′-TGGATGCCACAGGATTCCA-3′). For mouse study, both individual and pooled protein and RNA samples were compared to validate the difference between groups.

2.9. Primary hepatocytes isolation, treatment and viability evaluation

Primary hepatocytes isolation and culture has been described previously.[32] In brief, mice were anesthetized and then livers were sequentially perfused with pre-warmed solution I (Hank's balanced salt solution (HBSS) containing 0.1mM EGTA, 5.5mM Glucose, 1% Pen/Strep) for 10 min, and solution II (HBSS containing 1.5mM $CaCl_2$, 5.5mM glucose, 1% Pen/Strep and 0.04% collagenase I) for 15 min using portal vein (PV) cannulation and inferior vena cava (IVC) drainage method. Strained hepatocytes were washed and cultured with William E medium containing 2mM glutamine, 1% Pen/Strep and 5% FBS in type I collagen-coated tissue culture 96-well plates. 6 h later, hepatocytes were treated with DEN or arsenic of different concentrations as indicated for 24 h. Cell viability was evaluated using a MTS assay kit (3580; Promega, Madison, WI).

2.10. Blood biochemistry

The measurement of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) has been described previously.[29,30] Alkaline phosphatase (ALP) was measured in a commercial kit from Pointe Scientific (A7516; Canton, MI) according to the manufacturer’s protocol.

2.11. Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM). Statistical analysis was carried out using the Student’s *t*-test for unpaired data to compare the values between the two groups and one way analysis of variance (ANOVA) among multiple groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pdk4$^{-/-}$ mice exhibited greater rates of spontaneous hepatic apoptosis

Apoptosis is a tightly regulated, and continually occurring process that maintains tissue homeostasis. Uncontrolled apoptosis undermines tissue integrity and underlines the

pathogenesis. The balance between pro-apoptosis and anti-apoptosis mechanisms could be disrupted by either cellular internal environment or external stimulation such as inflammatory signals.[14,33] Although systemic depletion of PDK4 was not lethal in mice,[24] the deficiency of an important metabolism switch molecule might impact the survival of hepatocytes which is constantly challenged with foreign compounds and toxic insults. We evaluated spontaneous apoptosis in WT and *Pdk4*$^{-/-}$ livers by comparing three different ages, *i.e.* 3 weeks, 16 weeks and 12 months. TUNEL analysis showed that apoptotic cells were barely detected in WT mice, however they were increased in older *Pdk4*$^{-/-}$ livers compared to younger mice (Fig. 1A), suggesting an age-dependent progression.

3.2. Pdk4$^{-/-}$ mice were intolerant to chemical toxicant DEN administration

A commonly used model to study the development of liver cancer is to give 15-day-old mice a single dose of DEN (25 mg/kg BW) administration. WT mice injected with DEN are expected to develop liver tumors after 8–12 months.[34] We tested the response of 15-day-old *Pdk4*$^{-/-}$ mice to DEN treatment. Unexpectedly, *Pdk4*$^{-/-}$-DEN mice died within one week, whereas WT-DEN mice showed no visible abnormalities (Figs. 1B and 1C). *Pdk4*$^{-/-}$-DEN mice had significantly reduced BW and liver/BW ratio (Fig. 1D). *Pdk4*$^{-/-}$-DEN livers showed swollen and hemorrhagic appearance (Fig. 1C). The hepatic lobules of *Pdk4*$^{-/-}$-DEN mice lost integrity, appearing visibly irregular and less densely packed, whereas the WT-DEN livers showed normal histological structure (Fig. 1E). Serologically, *Pdk4*$^{-/-}$-DEN mice had higher levels of ALT, AST and ALP than WT-DEN mice, suggesting aggravated liver injury (Fig. 1F).

3.3. Juvenile Pdk4$^{-/-}$ mice exhibited severe hepatic apoptosis upon DEN treatment

TUNEL staining revealed massive hepatic apoptosis in *Pdk4*$^{-/-}$-DEN mice, which was not observed in WT-DEN mice. Apoptotic hepatocytes were barely detected in WT-PBS and were slightly increased in *Pdk4*$^{-/-}$-PBS mice (Fig. 2A). Biochemical detection of apoptosis revealed a marked increase in caspase-3 activity (Fig. 2B, left). Next, western blot analysis was applied in order to detect caspase-mediated cleavage levels of poly PARP protein in *Pdk4*$^{-/-}$-DEN *vs.* WT-DEN livers. It was found that Pdk4$^{-/-}$-DEN mouse livers were the only test group to show detectable levels of cleaved PARP (Fig. 2B, right).

3.4. Pdk4-deficiency sensitized hepatic NF-κB-TNF signaling and enhanced the expression of inflammatory genes after DEN treatment

DEN administration leads to acute hepatic inflammatory response. The NF-κB pathway regulates transcription of inflammatory and anti-apoptotic genes.[35] In the above DEN-insulted mouse livers, we isolated nuclear protein and interrogated NF-κB activity. The NF-κB/p65 subunit activity was significantly induced in *Pdk4*$^{-/-}$-DEN *vs.* WT-DEN livers (Fig. 2C, left), which corresponded to the expression of NF-κB target TNF. In liver tissues, ELISA assay revealed that TNF protein was highly induced by DEN in *Pdk4*$^{-/-}$ livers comparing with WT livers (Fig. 2C, right). In addition, the mRNA expression of NF-κB targets such as *Tnf, Birc3, Ccdc103, iIfnγ, E-selectin,* and *Gadd45β* were more drastically upregulated in *Pdk4*$^{-/-}$-DEN *vs.* WT-DEN livers (Fig. 2D). It was noted that DEN also induced *E-selectin* and *Gadd45β* mRNA in WT-DEN livers. Taken together, the results

suggested that the death of *Pdk4*$^{-/-}$-DEN mice was likely caused by severe hepatic apoptosis due to the activation of NF-κB activation of inflammation signaling.

3.5. Pdk4−/− mice were sensitive to arsenic toxicity

The exposure to arsenic compounds impairs normal liver function through inducing apoptosis.[36] Our previous study revealed that arsenic silenced hepatic PDK4 expression.[23] We insulted 8-week-old WT and *Pdk4*−/− mice with chronic $NaAsO_2$ administration in drinking water for 1 month to serve as an additional model of liver injury to test the hepatic vulnerability of *Pdk4*$^{-/-}$ mice. As expected, TUNEL staining in the liver sections revealed that arsenic intake increased apoptosis in WT livers. We observed spontaneous apoptosis in *Pdk4*$^{-/-}$ livers (− arsenic), which became more severe after arsenic administration (+arsenic) (Fig. 3A). Further, we measured Caspase-3 activity (Fig. 3B) and PARP cleavage (Fig. 3C) in the livers, which was consistent with the TUNEL analysis. Arsenic administration remarkably induced ATL, AST and ALP in *Pdk4*$^{-/-}$ mice serum, but had negligible effect on WT mice (Fig. 3D). We also measured the mRNA expression of NF-κB targets, *Tnf, Birc3, Ccdc103, Ifnγ, E-selectin* and *Gadd45β* (Fig. 3E). After arsenic administration, PDK4-deficiency significantly increased the expression of these NF-κB target genes comparing with WT livers, implying potentiated inflammation signaling.

3.6. Pdk4-deficient hepatocytes were more susceptible to DEN and arsenic insults in vitro

In order to study hepatocyte-specific susceptibility to DEN and arsenic, MTS assays were performed on primary hepatocytes to check cell viability under each treatment condition. Different concentrations of DEN and arsenic were applied to primary hepatocytes isolated from WT and *Pdk4*$^{-/-}$ livers. For hepatocytes treated with DEN, *Pdk4*$^{-/-}$ hepatocytes had an half maximal inhibitory concentration (IC50) (4.4 mM) approximately half of that found in WT hepatocytes (8.6 mM) (Fig. 4A, top). Similarly, *Pdk4*$^{-/-}$ hepatocytes were also intolerant to arsenic with an IC50 of 41.4 μ.M, which was significantly lower than 31.2 μM in WT hepatocytes (Fig. 4A, bottom). These results suggest that PDK4 may actively be involved in the detoxification of DEN and arsenic. Notably, caspase-3, −8, and −9 activities were significantly higher in the *Pdk4*$^{-/-}$ hepatocytes than in WT hepatocytes after DEN and arsenic treatment (Fig. 4B). Collectively, the results indicated that loss of PDK4 function sensitized hepatocytes to apoptotic death.

3.7. PDK4 mRNA was downregulated in acute liver injuries

GalN/LPS and Jo2 induce acute liver failure through triggering hepatic apoptosis.[37,38] Apoptosis intermingles with other forms of cell death in CCl4-induced acute liver injury. In terms of the results that PDK4 protected hepatocytes from apoptosis in mice, we interrogated PDK4 mRNA level in the above three acute liver injury mouse models. GaIN LPS, Jo2 and CCl4 were i.p. administered and liver tissues were collected for qPCR analysis. All three treatments resulted in marked reduction of PDK4 mRNA levels in liver tissue in a time dependent manner (Fig. 5).

4. Discussion

PDK4 is well known for its role in regulating metabolism through inhibiting the pyruvate dehydrogenase complex.[1] As a metabolic checkpoint of glucose oxidation and TCA cycle, the role of PDK4 beyond metabolism remains elusive and understudied. In this study, we reveal that the deficiency of PDK4 sensitizes the liver to DEN and arsenic toxicity, inducing hepatic apoptosis.

When comparing untreated WT and *Pdk4*$^{-/-}$ livers, TUNEL staining revealed that *Pdk4*$^{-/-}$ mouse livers exhibited significant markers for apoptosis, while WT mice showed little to no sign of apoptosis as expected in untreated mice (Fig. 1A). The spontaneous apoptosis observed in *Pdk4*$^{-/-}$ mice indicates that PDK4 plays a role in hepatic apoptosis. Whether or not PDK4 interacts directly with the apoptosis pathway or indirectly via metabolic instability is currently under investigation. It is know that PDK4 functions in the mitochondria to regulate energy production. A novel concept of mitochondria damage checkpoint (mitocheckpoint) arises to link genomic instability and apoptosis.[39] Here, our study suggests that loss of a mitochondrial molecule such as PDK4 might activate this checkpoint to induce apoptosis. Since loss of PDK4 leads to spontaneous apoptosis in the absence of stimuli, PDK4 inhibitor might be particularly useful in triggering apoptosis in cancer cells.

Pdk4$^{-/-}$ mice also demonstrated surprising intolerance to DEN administration, showing extensive histological hepatic damage (Fig. 1E). DEN treatment is a commonly used model to study liver cancer progression in mice. Normally DEN administration leads to tumor growth over a period of multiple months, but in this study *Pdk4*$^{-/-}$ mice died approximately one week after DEN treatment (Fig. 1B). DEN treated *Pdk4*$^{-/-}$ had significantly decreased liver to BW ratio, confirming loss of normal liver functions, thus the increased sensitivity to DEN's hepatic toxicity (Fig. 1D). TUNEL staining revealed apoptosis as a major cause of the loss of liver integrity in the *Pdk4*$^{-/-}$ mice (Fig. 2A), which is confirmed by the biochemistry characteristics of apoptosis, increased caspase-3 activity and the cleavage of PARP. Along with increased apoptosis, DEN and arsenic treated *Pdk4*$^{-/-}$ mouse livers showed significant signs of inflammatory response. Normally, apoptotic cell death avoids triggering inflammation. However, the inefficiency to clean massive apoptosis by phagocytes also induces inflammation which conversely aggravates apoptosis.[40] Therefore, the more drastic inflammatory response in *Pdk4*$^{-/-}$ livers may fuels the apoptosis process (Figs. 2C, 2D and 3E). The relationship between PDK4 and inflammation maybe another direction for future studies; especially, it will be important to examine the role of PDK4 in inflammation cells, such as Kupffer cells.

The kidney and liver are the major targets for arsenic-induced toxicity. Chronic arsenic exposure is associated with liver diseases.[41,42] Arsenic silences the expression of PDK4.[23] *Pdk4*$^{-/-}$ livers as well as primary hepatocytes are susceptible to arsenic insult (Figs. 3 and 4), which is consistent with the results from DEN treatment. How PDK4 coordinates the detoxification of these xenobiotic remains to be determined. Further, whether PDK4 overexpression can protect xenobiotic toxicity needs further investigation. It has been

appreciated that PDK4 has a protective role in apoptotic liver injury induced by different apoptosis-inducers, such as Jo2 and GalN/LPS.[29]

Although this current study reveals that loss of *Pdk4* results in increased apoptosis, our lab previously showed that loss of *Pdk4* in mice resulted increased cellular proliferation within the liver.[4] Why does PDK4 curb apoptosis, seemingly *de novo*, whereas inhibit proliferation? A potential explanation for this paradox is that the increased hepatocyte proliferation may likely represent a compensatory response to the accelerated cell death in *Pdk4*$^{-/-}$ livers as a means to maintain liver mass and metabolic homeostasis. Together, our findings point to this possible compensatory mechanism in which cellular proliferation increases in response to apoptotic events in *Pdk4*$^{-/-}$ livers, especially when the liver is challenged with xenobiotics. There is increasing recognition that cell death and tumor repopulation, the opposite process of tumor cell loss, are closely intertwined. The phenomenon of compensatory proliferation is evolutionarily conserved in death-induced tissue regeneration.[43] Thus, it is useful to examine the role of PDK4 in liver regeneration in the future.

As a mitochondrial protein, the non-mitochondrial functions of PDK4 are still poorly understood. As a kinase, additional PDK4's substrates as well as new PDK4 interacting proteins remain to be identified and characterized. Additional questions include how PDK4 shuttles between cytosol and mitochondria. Considering PDK4 is downregulated during acute liver hepatic injuries (Fig. 5), elucidating the regulatory role of PDK4 under pathological conditions such as liver fibrosis and steatosis may further unravel its importance in liver diseases.

In summary, loss of PDK4 resulted in spontaneous hepatic apoptosis, while DEN treatment further increased incidence of apoptosis and significantly reduced mouse survival rate. Chronic arsenic treatment also induced hepatic apoptosis in *Pdk4*-deficient mice. These results reveal PDK4's influence on apoptosis in the liver and provoke further investigation in order to understand PDK4's functions.

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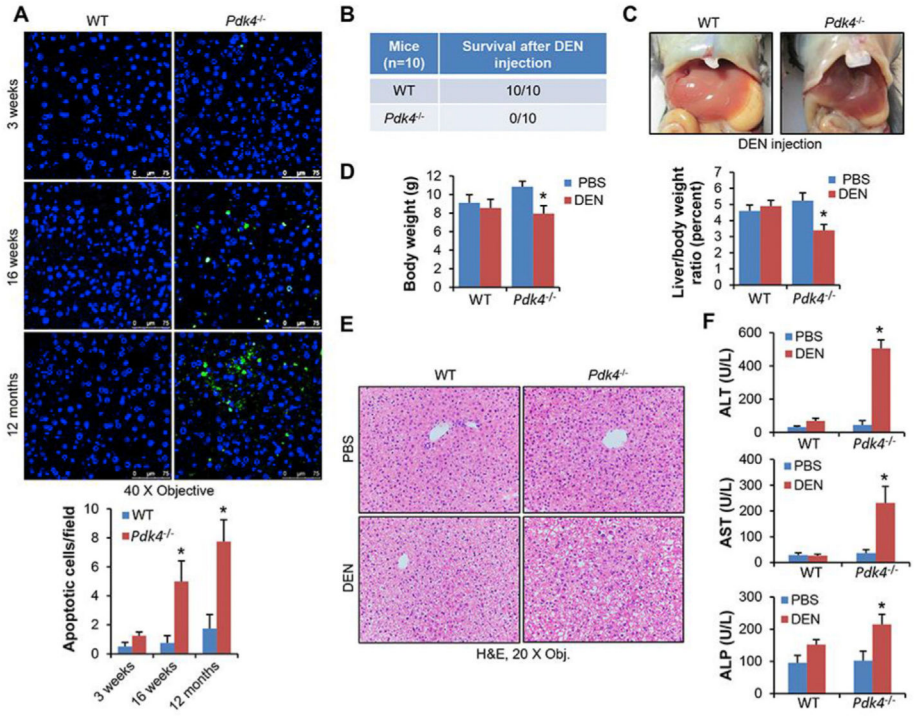

Fig. 1. Systematic *Pdk4*-deficiency in mice led to spontaneous hepatic apoptosis and the *Pdk4*-deficient juvenile were intolerant to DEN administration.

(A) Top, TUNEL staining of liver sections from WT and *Pdk4*$^{-/-}$ mice at different ages as indicated. Bottom, the histogram represents statistical analysis of apoptotic cells in 20 fields ($N = 10$ mice/group). *$P < 0.01$ *vs.* WT. **(B)** The survival statistics of 15-day-old WT and *Pdk4*$^{-/-}$ mice intraperitoneally injected with a single dose of DEN (25 mg/kg BW). **(C)** Gross liver morphology of WT and *Pdk4*$^{-/-}$ mice at day 6 post-injection with DEN. **(D)** BW and liver/BW ratio. *$P < 0.01$ *vs.* *Pdk4*$^{-/-}$-PBS. **(E)** H&E staining of liver sections in WT and *Pdk4*$^{-/-}$ mice injected with PBS or DEN. **(F)** Serum ALT, AST and ALP levels were determined in WT and *Pdk4*$^{-/-}$ mice injected with PBS or DEN. *$P < 0.01$, *Pdk4*$^{-/-}$ *vs.* WT. Abbreviations: WT, wild-type; Pdk4, pyruvate dehydrogenase kinase 4; DEN, diethylnitrosamine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PBS, phosphate buffered solution; Obj., Objective; BW, body weight.

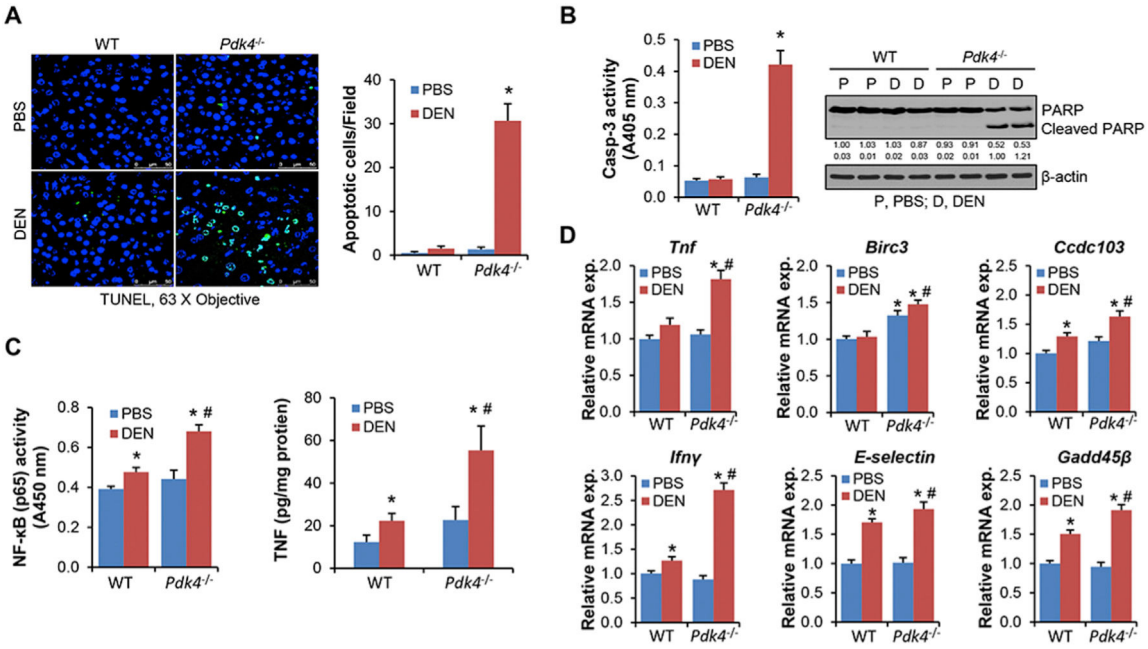

Fig. 2. *Pdk4*$^{-/-}$ juvenile mice exhibited severe hepatic apoptosis accompanied with elevated NF-κB signaling and enhanced expression of inflammatory genes upon DEN treatment.

(A) TUNEL staining of liver sections in 15-day-old WT and *Pdk4*$^{-/-}$ mice injected with PBS or DEN to determine hepatic apoptosis. The histogram represented statistical analysis of 20 fields ($N = 10$ mice/group). *$P < 0.01$ *vs.* WT-DEN. **(B)** Caspase-3 activity (left) and Western blot of cleaved PARP protein (right) in liver tissue lysates from WT and *Pdk4*$^{-/-}$ mice injected with PBS (P) or DEN (D). Each group represents a pooled sample (equal amounts of protein) from 5 individual mice with triplicate assays. Data are shown as mean ± SEM. *$P < 0.01$ *vs.* WT-DEN. **(C)** Measurement of liver NF-κB (p65) activity with nuclear extracts (left) and ELISA of liver TNF protein expression (right) from WT and *Pdk4*$^{-/-}$ juvenile mice injected with PBS or DEN. **(D)** qPCR of mRNA expression of NF-κB (p65) target genes in the mice livers described in (C). Each group represents a pooled sample (equal amounts of protein or RNA) from 5 individual mice with triplicate assays. Data are shown as mean ± SEM. *$P < 0.01$ *vs.* WT-PBS; #$P < 0.01$, *Pdk4*$^{-/-}$-DEN *vs.* WT-DEN. Abbreviations: WT, wild-type; Pdk4, pyruvate dehydrogenase kinase 4; DEN, diethylnitrosamine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PBS, phosphate buffered solution; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; exp., expression; PARP, poly (ADP-ribose) polymerase; Birc3, baculoviral IAP repeat-containing 3; Ccdc103, coiled-coil domain containing 103; Ifnγ, interferon gamma; Gadd45β, growth arrest and DNA-damage-inducible beta.

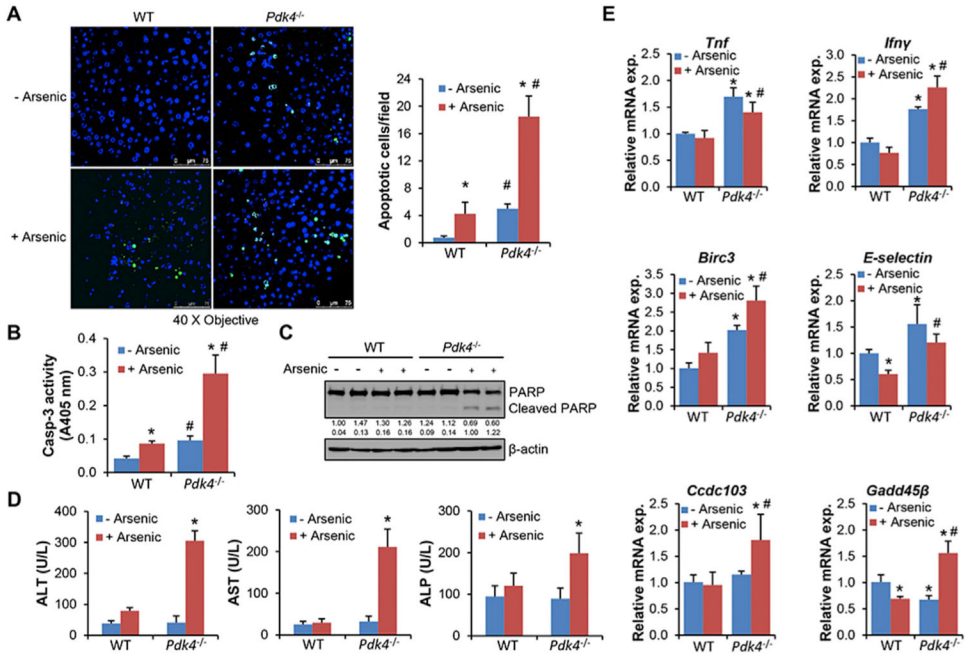

Fig. 3. Chronic arsenic administration aggravates hepatic apoptosis in *Pdk4*$^{-/-}$ mice compared to WT mice.

(A) 8-week-old WT and *Pdk4*$^{-/-}$ mice were fed with 50 ppm $NaAsO_2$ via distilled drinking water for 4 weeks. Left, TUNEL staining of liver sections to examine apoptosis. Right, the histogram represents statistical analysis of apoptotic cells in 20 fields (N = 10 mice/group). **(B)** Measurement of Caspase-3 activity in the livers from arsenic treated mice described in (A). **(C)** Western blot of PARP cleavage. **(D)** Serum ALT, AST and ALP levels were determined in WT and *Pdk4*$^{-/-}$ mice with or without arsenic administration. **(E)** qPCR of mRNA expression of NF-κB (p65) target genes in the mice livers described in (A). Each group represents a pooled sample (equal amounts of protein or RNA) from 5 individual mice with triplicate assays. A, B: *P < 0.01, Arsenic (+) *vs.* (−); #P < 0.01, *Pdk4*$^{-/-}$ *vs.* WT. D: *P < 0.01, *Pdk4*$^{-/-}$ *vs.* WT. E: *P< 0.01 *vs.* WT-Arsenic (−); #P<0.01, *Pdk4*$^{-/-}$-Arsenic (+) *vs.* WT-Arsenic(+). Abbreviations: WT, wild-type; Pdk4, pyruvate dehydrogenase kinase 4; Casp, Caspase; DEN, diethylnitrosamine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PARP, poly (ADP-ribose) polymerase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; exp., expression; Tnf, tumor necrosis factor; Birc3, baculoviral IAP repeat-containing 3; Ccdc103, coiled-coil domain containing 103; Ifnγ, interferon gamma; Gadd45β, growth arrest and DNA-damage-inducible beta.

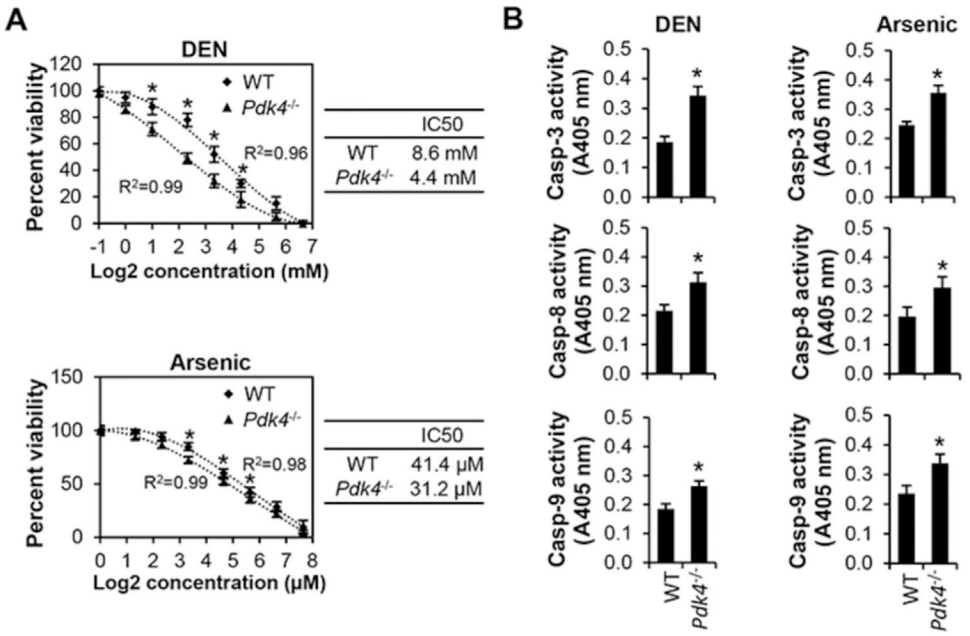

Fig. 4. *Pdk4*-deficient cells were more susceptible to DEN and arsenic treatment *in vitro*.

(A) MTS assay to determine cell viability. Primary hepatocytes from WT and *Pdk4*$^{-/-}$ mice were treated with DEN or arsenic for 24 h. Data were plotted as percent viability versus log 2 transformed concentrations. **P* < 0.01 WT *vs*. *Pdk4*$^{-/-}$. **(B)** Caspase-3, −8 and −9 activities in WT and *Pdk4*$^{-/-}$ primary hepatocytes treated with DEN (10 mM) or arsenic (40 µM) for 24 h. Data are shown as mean ± SEM. **P*< 0.01 *vs*. WT. Abbreviations: DEN, diethylnitrosamine; Casp, Caspase; WT, wild-type; Pdk4, pyruvate dehydrogenase kinase 4; IC50, half maximal inhibitory concentration.

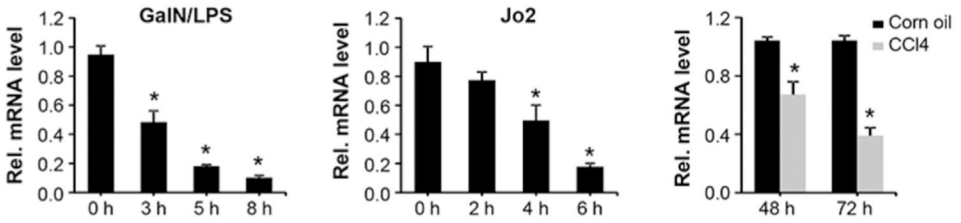

Fig. 5. PDK4 mRNA was downregulated in acute liver injuries.

qPCR of PDK4 mRNA in GalN/LPS (left), Jo2 (middle) and CCl4 (right) intraperitoneally-injected mice livers. GalN: 700 mg/kg BW; LPS: 50 μg/kg BW; Jo2: 10 μg/mouse; CCl4: 750 uL/kg BW, diluted in corn oil. Livers were collected at the indicated time points. Each group represents a pooled sample (equal amounts RNA) from 5 individual mice with triplicate assays. *$P < 0.05$ *vs.* 0 h or Corn oil. Abbreviations: GalN, galactosamine; LPS, lipopolysaccharides; Jo2, anti-mouse CD95 antibody; CCl4, carbon tetrachloride; Rel., Relative; BW, body weight.