Acetaminophen Overdose-~~induced~~ Induced Liver Injury in Mice ~~Is~~ is Mediated by Peroxynitrite Independently of ~~the~~ Cyclophilin D-~~regulated~~ Regulated Permeability Transition

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Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. ~~that~~ While ~~is~~ safe ~~at~~ in therapeutic doses~~.~~ , ~~However, when administered overdose,~~overdoses of APAP can cause liver damage in humans and mice. Despite extensive research ~~for~~ over several decades, the underlying molecular mechanisms of hepatocyte injury are still not fully understood, limiting the development and therapeutic application of novel cytoprotective agents ~~in~~ against APAP-induced liver injury (Jaeschke & Bajt 2006; ~~&~~ Saito et al. 2010)~~.~~ ~~What has become~~It ~~is clear is that~~ ~~m~~Mitochondria play a key role in both the early stages of cell injury (interactions of the thiol-intermediate reagent, *N*-acetyl-*p*-benzoquinone imine~~,~~ (NAPQI), with glutathione and proteins, accompanied by antioxidants and nitrative stress) ~~And~~ and the subsequent ~~phase~~ propagation phase (signaling followed by hepatocellular death)~~, mitochondria appear to play a key role~~ (Cover et al. 2005; ~~&~~ Hanawa et al. 2008). Evidence ~~has been shown,shows~~suggests that ~~after~~ exposure of hepatocytes to APAP in vitro or in vivo~~,~~ facilitates ~~mitochondria easily undergo~~ permeabilization of the mitochondrial outer membrane ~~occurs easily~~, ~~thus~~ which ~~inducing~~ induces necrotic cell death, ~~largely~~ primarily through caspase-independent mechanisms. How ~~exactly~~ NAPQI and its subsequent signaling events lead to mitochondrial permeabilization ~~at present~~ is ~~not~~ currently unknown ~~at present~~. ~~It has been suggested that the~~The process may involve ~~the transition of~~ mitochondrial permeability transition (mPT)~~. The mPT is a functional term that~~, which ~~involves~~ causes the sustained opening of a megapore ~~that encompasses~~across both the ~~internal~~ inner and ~~external~~ outer mitochondrial ~~membrane~~membranes.~~,~~ This ~~allowing~~ allows the exchange of solutes of <1.5 kDa, leading to mitochondrial swelling, external membrane ~~rupture~~ rupture, and release of proapoptotic ~~proteins .~~ proteins. Although the

Commented [CP1]: Dear Author,

It is a pleasure to edit your paper. I will do my best to ensure that all editorial issues are picked up and the document is thoroughly proofread.

Please go through all my amendments carefully to ensure that I have not changed your intended meaning. Please read all my comments carefully as I will make suggestions to improve the text.

I wish you good luck with the publication process.

Commented [CP2]: I corrected this to align with the reference list. Please confirm that you were referring to these papers.

Commented [CP3]: I corrected this to align with IUPAC naming conventions (with the positional symbols italicized).

Commented [CP4]: It is unclear how this information fits in with cell injury. Perhaps you mean "… (caused by the toxic APAP metabolite, *N*-acetyl-*p*-benzoquinone imine [NAPQI], which depletes glutathione, leading to oxidative stress and protein nitration)"?

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physiological properties of the mPT pore have been ~~well~~ extensively studied, ~~the~~ its molecular nature ~~of this pore~~ remains poorly defined. ~~Originally,~~ ~~the~~The ~~ADP / ATP~~ ADP/ATP translocator (ANT) and ~~the voltage~~ voltage-dependent anionic transporter (VDAC) ~~have been~~were initially attributed crucial roles, but these have recently been drawn into question ~~a crucial role, but this concept had to be~~was ~~recently reviewed~~ disputed recently, ~~it was found that~~because due to the occurrence of permeability transition ~~could still occur~~ in the mitochondria of ANT or VDAC knockout mice ~~were still Capable of~~ , ~~being subjected to~~ ~~On the other hand,~~Genetic ~~the genetic~~ studies support a major role for ~~cytophilin~~ cyclophilin D (CypD) matrix protein ~~appears to be a critical actor involved~~ in the regulation of the mPT pore. Studies ~~of~~using ~~Mitochondrial~~ mitochondria ~~studies~~ isolated from mice with a genetic deletion of CypD ~~have~~ clearly demonstrated ~~that these mitochondria were much more resistant~~increased resistance to mPT inducers ~~than the~~compared to ~~to~~ wild-type mitochondria (~~though~~ although they were not fully protected). As an alternative to ~~the~~ genetic deletion of CypD, pharmacological inhibition ~~, e.g.,~~for instance, ~~with~~using cyclosporin A (CsA) or other specific cyclophilin ligands ~~,~~ can also disrupt the interaction ~~of~~ between CypD ~~with~~ and the mPT pore ~~can also be disrupted by pharmacological inhibition, eg with cyclosporin A (CsA) or other specific cyclophilin ligands~~. Therefore, the ~~demonstration of~~ protective effects ~~provided~~ demonstrated by CsA against ~~the effects of toxic~~ drug~~s~~g toxicity ~~has~~ have ~~been widely~~ been widely used to ~~make an the argument for~~support ~~mPT~~ the involvement of mPT.

Based on ~~this~~ the ~~concept of~~ cytostatic effects of CsA, a number of independent studies have provided experimental evidence that mPT ~~could~~ is indeed ~~be~~ implicated in APAP-induced liver toxicity. However, ~~one caveat is that~~ CsA~~,~~ given at high doses, as ~~used~~ in some of the mouse studies, may inhibit drug transporters in the ~~domain of the~~ canalicular membrane domain and ~~also~~ induce cholestasis. This could alter the kinetics of APAP ~~and / or~~ and/or its metabolites. ~~In addition, and importantly~~Importantly, CsA ~~not only~~ binds not only to mitochondrial CypD but ~~also~~ to other forms of cyclophilin, including cytosolic CypA. The ~~CypA / CsA~~ CypA/CsA complex is subsequently linked to calcineurin, a ~~Ca2 + / calmodulin~~ Ca^{2+}/calmodulin-activated ~~serine / threonine~~ serine/threonine phosphatase that ~~has been~~is mechanically involved in the immunosuppressive effects of CsA. ~~Finally,~~ CsA has also been shown to exert other calcineurin-independent effects on ~~NH 2~~ NH2-terminal ~~terminal~~ kinase (JNK) signaling. Therefore, the role

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Commented [CP8]: Please remember to include a citation here. This sentence refers to published work.

Commented [CP9]: Latin abbreviations are reserved for parentheses and tables; in running text, we prefer the full English forms.

Commented [CP10]: The relevant studies should be cited here.

Commented [CP11]: Should this be cJun NH2-terminal kinase (JNK) signaling?

of CypD-dependent mPT in ~~the~~ APAP hepatotoxicity, based solely on the protective effects ~~provided by~~of CsA, should be ~~reviewed~~investigated. ~~In fact~~Notably, studies ~~in~~ on isolated hepatocytes ~~have provided evidence~~suggest that ~~with increasing time and cell stress,~~ CsA eventually loses its protective effects ~~towards~~ against APAP-induced cell injury with increasing time and cell stress. However, ~~it is not known~~ whether this occurs in vivo is unknown~~,~~. Additionally, ~~and, more importantly,~~ the mechanism of "~~"insensitive to~~ CsA insensitivity"~~"~~ ~~of~~ to APAP toxicity ~~has remained~~is remains obscure ~~enigmatic~~difficult to understand.

~~The~~ This ~~aim of this~~ study ~~was to~~ investigated whether APAP ~~exerts~~ caused mitochondrial permeabilization, either through mPT ~~and / or~~ or ~~through~~ other mechanisms, ~~independent~~ independently of CypD.~~,~~ ~~using~~ We used both ~~the~~ in vivo pharmacological inhibitors of CypD and a genetic approach with ~~deficient~~ CypD-deficient (*Ppif*$^{-/-}$) ~~Mice~~mice. The data suggest that high doses of APAP induce mitochondrial peroxynitrite stress that directly triggers mitochondrial permeabilization without the involvement of CypD.

Results

Pharmacological inhibition or genetic depletion of mitochondrial CypD ~~does~~ did not protect against ~~the~~ APAP hepatotoxicity ~~of APAP~~. A previously characterized mouse model was used ~~T~~to investigate the mechanistic role of CypD-controlled mPT versus other modes of cell death in APAP-induced liver injury~~, a previously characterized mouse model was used~~. ~~20~~ Twenty ~~Acetaminophen~~ ~~acetaminophen~~APAP (600 mg~~-~~/~~-~~day) ~~was~~ were given to male wild-type ~~males~~ ~~male~~ mice (*Ppif*$^{+/+}$ Kg, ip). As expected, APAP caused typical centrilobular necrosis, which was evident at 8 h ~~post dose~~after dosing and became more severe at 24 h, ~~parallel~~ paralleling ~~to~~ the highly increased activity of plasma ALT (~~Fig~~ Fig. 1A, B, D)~~.~~ ~~Because~~ The~~the~~ choice of solvent ~~may have~~can significantly ~~effects~~ affect ~~on~~ APAP bioactivation ~~and / or~~ and/or the subsequent recruitment of immune cells and thus ~~on~~ the extent of liver injury~~,~~. Therefore, we first ~~determined~~ compared the effects of Solutol HS-15, used in ~~Para Parenteral~~ parenteral administration of lipophilic compounds, ~~and compared it with~~to those of the hot saline solution used to dissolve APAP. ~~It was found that Solutol HS-15, in~~ In contrast to

Commented [CP12]: This is slightly confusing. I think you're saying that a total of 600 mg APAP was given to the mouse each day in 20 doses. Is this correct? If so, I would suggest rewriting this as "**APAP (20 x 30 mg) was**". If my understanding is incorrect, please feel free to contact me. I am more than happy to help rephrase this so that your intention is clearly expressed.

Commented [CP13]: This seems to refer to the administration of acetaminophen and should be moved to the relevant place in the sentence.

Is "**Kg**" referring to the weight of the mouse? If so, the SI unit for "**kilogram**" is "**kg**."

Commented [CP14]: Is this "**alanine transaminase**?"

It is generally best practice to introduce acronyms into the text with their expanded forms, e.g., "**alanine transaminase (ALT)**." Only widely accepted acronyms (**DNA**, **RNA**, **DMSO**, etc.) should be used without first defining them.

However, journals often have a list of abbreviations/acronyms that can be used without first defining them in the text. Please check whether "**ALT**" is on their list. If it is, this comment can be ignored.

~~dimethylsulfoxide~~dimethyl sulfoxide, Solutol HS-15 had no apparent effects on plasma ALT activity (Table 1). Therefore, Solutol HS-15 was used as ~~a~~ the ~~vehicle for~~ excipient in all subsequent experiments.

Previous reports ~~from various laboratories~~ have shown that CsA ~~can~~ effectively protects mouse hepatocytes from APAP-induced injury both in vitro and in vivo. However, CsA may have a number of off-target effects, including those ~~not related~~ unrelated to CypD. ~~To avoid these confounding factors, the CsA analog,~~ Debio 025, a CsA analog ~~which~~ that ~~is a more~~ selectively inhibits mitochondrial CypD ~~mitochondrial inhibitor~~ and inhibits the immune system (via calcineurin-mediated pathways) at least ~~whose is >~~3,000 times less ~~potent potency to at inhibitinginhibit the immune system (via the calcineurin-mediated pathways) is> 3,000 times less~~ than ~~The~~ CsA, was used to avoid these confounding factors. Debio 025 (10 ~~mg / kg~~ mg/kg, ip) was injected 1.5 h after APAP administration (when APAP bioactivation was largely complete~~d~~ and NAPQI had consumed ~~most~~ the majority of ~~the~~ hepatic GSH ~~was already consumed by NAPQI~~) ~~was injected, thus minimizing~~to minimize drug- -drug ~~interactions .~~ interactions. Surprisingly, ~~it was found that~~ Debio 025 did not afford ~~protect~~ protection from ~~APAP~~ APAP-induced hepatotoxicity (Fig. 1C, D). A pilot study revealed ~~that there was~~ a similar lack of protective effects when ~~administering~~ Debio 025 was administered simultaneously with APAP (data not shown), indicating that the lack of protection was not simply due to the late administration of the CypD inhibitor. These findings suggest ~~that, in~~an additional ~~-~~mode of mitochondrial permeabilization induced by high doses of APAP ~~to the~~other than ~~CypD~~ CypD-dependent ~~mode of~~ mPT~~, there may be another mode of mitochondrial permeabilization induced by high doses of APAP~~.

To corroborate these findings and ~~to totally~~ exclude ~~any~~ possible drug interactions due to the presence of ~~the~~ pharmacological inhibitors, we ~~then~~ determined the extent of APAP-induced liver injury in a CypD depletion mouse ~~genetic~~ model ~~of CypD depletion~~ (*Ppif* mice) (~~Figure~~ Fig. 2A-). We first ~~had to check~~ ~~checked~~confirmed that ~~the APAP bioactivation rates of these~~ CypD-deficient mice ~~exhibited similar rates of APAP bioactivationwere~~had similar APAP bioactivation rates to ~~those of as their the~~ wild-type controls. ~~Therefore~~Specifically, hepatic GSH consumption (a marker established for the extension of NAPQI formation) was measured for 90

Commented [CP15]: The accepted abbreviation for "**dimethyl sulfoxide**" is "**DMSO**." Please confirm whether this can be used by checking the journal guidelines.

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Commented [CP19]: Should this be *Ppif*$^{-/-}$?

min ~~after~~ following ~~administering~~administration of a hepatotoxic dose of APAP ~~in~~ to *Ppif*$^{-/-}$ mice and their wild-type littermates ~~for the first 90 min (a marker established for the extension of NAPQI formation)~~. Although *Ppif*$^{-/-}$ mice ~~had~~ initially had higher GSH levels (+ 30%), no significant differences were found in the ~~extent~~ level of GSH depletion between the two genotypes (~~Figure~~ Fig. 2B). We then evaluated the degree of liver injury after 4, ~~8~~ 8, and 24 h in both *Ppif*$^{+/+}$ and *Ppif*$^{-/-}$ mice injected with APAP (600 mg~~/~~/kg, ip). ~~In line~~Consistent with the ~~results of the~~ Debio 025 experiments, the *Ppif*$^{-/-}$ mice were not protected from APAP toxicity at this high dose~~,~~ but developed typical centrilobular necrosis ~~after 24 h,~~ after 24 h, ~~whose~~ similar ~~in expression was not different from~~ to ~~that of in the Wild~~ wild-type animals~~,~~ ~~after 24 h~~ (Fig. 2D). ~~Taken together~~Overall, these data indicate that, at the high dose used here, ~~the~~ mitochondrial signaling involved in APAP hepatotoxicity includes ~~an~~ a ~~mode~~ CypD-independent mode~~mode of CypD, at least at this high dose~~. In contrast, a recent report suggests that the ~~at much lower doses,~~ inhibition of the CypD pathway ~~may still~~ allows cytoprotection when lower doses are administered~~, as shown in a recent report~~.

Commented [CP20]: Although it is not essential here, including the *p*-value would strengthen this statement.

Commented [CP21]: Please remember to include a citation here. This sentence refers to published work.

References

Commented [CP22]: The in-text citations are not numbered, so these references do not need to be numbered. Instead, they should be organized alphabetically.

1. Jaeschke H, Bajt M, (2006). Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol. Sci.*, (89), 31–41 2006.

2. Saito C, Lemasters J, Jaeschke H (2010). c-Jun *N*-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. *Toxicol. Appl. Pharmacol*., (246), 8–17.

3. Cover C, Mansouri A, Knight T, Bajt M, Lemasters J, Pessayre D, Jaeschke H (2005). Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. *J. Pharmacol. Exp. Ther*., (315), 879–887.

4. Hanawa N, Shinohara M, Saberi B, Gaarde W, Han D, Kaplowitz N (2008). Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J. Biol. Chem*., (283), 13565–13577.