D-Galactosamine/Lipopolysaccharide-Induced Hepatotoxicity Downregulates Sirtuin 1 in Rat Liver: Role of Sirtuin 1 Modulation in Hepatoprotection

M. K. KEMELO[1], L. WOJNAROVÁ[1], N. KUTINOVÁ CANOVÁ[1], H. FARGHALI[1]

[1]Institute of Pharmacology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Received February 27, 2014
Accepted April 23, 2014
On-line June 5, 2014

Summary

D-Galactosamine/Lipopolysaccharide (D-GalN/LPS) is a well known model of hepatotoxicity that closely resembles acute liver failure (ALF) seen clinically. The role of sirtuin 1 in this model has not yet been documented. However, there have been a number of studies about the cytoprotective effects of resveratrol, a SIRT1 activator, in the liver. This study was aimed at elucidating the roles of SIRT1 protein expression or catalytic activity in D-GalN/LPS model of hepatotoxicity. ALF was induced in male Wistar rats by intraperitoneal injection of D-GalN and LPS. Some groups of animals were pretreated with resveratrol and/or EX-527 (SIRT1 inhibitor). The effects of these treatments were evaluated by biochemical and Western blot studies. D-GalN/LPS treatment was able to induce hepatotoxicity and significantly increase all markers of liver damage and lipid peroxidation. A dramatic decrease of SIRT1 levels in response to D-GalN/LPS treatment was also documented. Resveratrol pretreatment attenuated D-GalN/LPS-induced hepatotoxicity. EX-527 blocked the cytoprotective effects of resveratrol. However, both resveratrol and EX-527 pretreatments did not exhibit any significant effect on SIRT1 protein expression. Collectively, these results suggest that downregulation of SIRT1 expression is involved in the cytotoxic effects of D-GalN/LPS model and SIRT1 activity contributes to the cytoprotective effects of resveratrol in the liver.

Key words

SIRT1 • Resveratrol • EX-527 • D-galactosamine/Lipopolysaccharide • Hepatotoxicity

Corresponding author

M. K. Kemelo, Institute of Pharmacology, First Faculty of Medicine, Charles University in Prague, Albertov 4, 128 00 Prague 2, Czech Republic. E-mail: mighty.kemelo@lf1.cuni.cz

Introduction

Liver is a metabolically active organ responsible for biotransformation and clearance of xenobiotics from the body. It is an important target of drugs and pathogens that may initiate liver cell damage and compromise its overall function (Hong *et al*. 2009). Currently, there is no way to compensate for the absence of liver function in the long term and massive hepatic destruction often necessitates the need for liver transplantation (Chan *et al*. 2009). There is therefore an intensive search of safe, affordable and readily available agents that can protect the liver from fulminant damage (Cengiz *et al*. 2013).

The general strategy for prevention of liver damage includes reduction of reactive metabolites by using antioxidants (Bansal *et al*. 2005). Natural polyphenolic compounds such as resveratrol, quercetin, curcumin and silymarin possess antioxidant properties and anti-inflammatory effects and have been the subject of considerable research as liver protectants (Rivera *et al*. 2008, Haddad *et al*. 2011, Cerny *et al*. 2011, Lekic *et al*. 2013). Interest in resveratrol has skyrocketed over recent years due to its cytoprotective effects in many organs. For instance, it has been proven to be effective in attenuating vascular endothelial inflammation (Chen *et al*. 2013), diabetic nephropathy (Wen *et al*. 2013) and cholestatic liver injury (Ara *et al*. 2005). Moreover, our experimental

Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

studies, both *in vivo* and *in vitro*, demonstrated that resveratrol is effective in protecting hepatocytes against D-GalN/LPS-induced hepatotoxicity (Farghali *et al*. 2009). However, the exact mechanism by which resveratrol exerts its cytoprotective effects is still elusive.

One of the hypotheses is that resveratrol allosterically activates an NAD^+-dependent histone deacetylase SIRT1 which has multifaceted functions and plays a critical role in cellular stress responses (Howitz *et al*. 2003). On activation, SIRT1 can deacetylate and turn on anti-inflammatory and antioxidant factors such as FOXO (Brunet *et al*. 2004, Hasegawa *et al*. 2008, Tanno *et al*. 2010). The many positive health benefits of SIRT1 can also be explained in part by inhibition of pro-inflammatory factors such as NF-κB (Yeung *et al*. 2004, Farghali *et al*. 2013). This notion is also supported by the finding that SIRT1 deficiency in experimental animals exacerbates conditions such as nephrosclerosis and hyperglycemia which are normally ameliorated by resveratrol treatment (Wang *et al*. 2011, Vasko *et al*. 2014). Nonetheless, the validity of direct SIRT1 activation by resveratrol has been challenged by many researchers. Some studies suggest that activation of SIRT1 by resveratrol is an experimental artifact and resveratrol's health benefits and sirtuins are not related (Beher *et al*. 2009). Besides SIRT1, there are other potential target molecules such as AMPK that may be involved in the aforementioned cytoprotective effects of resveratrol (Biasutto *et al*. 2012).

This ambiguity prevents development of more potent resveratrol-like compounds which are promising liver protectants. The goal of the present study was to elucidate the roles of SIRT1 protein expression and catalytic activity in D-GalN/LPS model of hepatotoxicity.

Materials and Methods

Chemicals

Lipopolysaccharide from Escherichia coli K-235 (LPS), D-galactosamine hydrochloride (D-GalN), resveratrol (3,4′,5-trihydroxy-trans-stilbene, 5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol, ≥99 % GC), EX-527 (6-chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide, ≥98 % HPLC), Tris-HCl, Nonidet P40 Substitute, dimethyl sulfoxide (DMSO), isopropyl alcohol, Tween 20, 2-thiobarbituric acid, tetraethoxypropane, trichloroacetic acid (TCA), sodium dodecyl sulphate, ammonium persulfate, methanol, glycine, N,N,N′,N″-tetramethylethylenediamine, 2-mercaptoethanol, bromophenol blue, glycerol, N,N′-methylenebis (acrylamide), NaCl, KCl, Na_2HPO_4, KH_2PO_4, ammonium molybdate tetrahydrate, hydrogen peroxide, filter paper, nitrocellulose membrane, anti-mouse IgG (whole molecule)-peroxidase antibody and mouse monoclonal anti-B-actin antibody were purchased from Sigma-Aldrich (Prague, Czech Republic). SirT1 (1F3) mouse mAb antibody was from Cell Signaling Technology through Biotech A.S. (Prague, Czech Republic). Non-fat dry milk was from Biotech A.S. (Prague, Czech Republic). Water for injection 100 % w/v was from Baxter (Czech Republic, Prague). Bio-Rad protein assay dye reagent was from Bio-Rad (Prague, Czech Republic).

Animals

Male Wistar rats, 250-400 g body weight, were purchased from Velaz-Lysolaje, Czech Republic. They were given water and a standard granulated diet *ad libitum*. They were maintained under standard conditions (12-h light-dark cycle, 22±2 °C temperature and 50±10 % relative humidity). The animals received humane care in accordance with the ethical guidelines of the First Faculty of Medicine, Charles University in Prague.

Experimental design

The animals were allowed to acclimatize to the vivarium for seven days before being used in the experiments.

Then they were randomly divided into five groups of six animals each and treated as follows:

- Group 1 – Control: DMSO (500 µl/kg) + saline (1 ml/kg)
- Group 2 – resveratrol (2.3 mg/kg)
- Group 3 – D-GalN (400 mg/kg) + LPS (10 µg/kg)
- Group 4 – resveratrol (2.3 mg/kg) + D-GalN (400 mg/kg) + LPS (10 µg/kg)
- Group 5 – EX-527 (1 mg/kg) + resveratrol (2.3 mg/kg) + D-GalN (400 mg/kg) + LPS (10 µg/kg)

The above doses were selected based on our previous experimental studies (Farghali *et al*. 2009, Cerny *et al*. 2011, Lekic *et al*. 2013). All treatments were administered intraperitoneally. Group 1 received only DMSO and physiologic solution. Group 2 was given resveratrol dissolved in DMSO. Group 3 got D-GalN and LPS dissolved in physiologic solution. Group 4 was pretreated with resveratrol 60 min before induction of hepatic failure. Group 5 was pretreated with EX-527 30 min before resveratrol treatment that was followed

60 min later by D-GalN/LPS treatment. At the end of treatment period (6 h), the animals were anesthetized with diethylether and then euthanized by exsanguination. Their blood samples were immediately collected into heparinized tubes for biochemical investigations. Their liver samples were excised and either homogenized for further biochemical analysis or snap-frozen in liquid nitrogen for Western blot studies.

Biochemical investigations

The extent of liver damage was assessed by detecting the levels of transaminases (ALT, AST) and bilirubin in plasma using commercially available diagnostic kits from Synlab (Prague, Czech Republic). Conjugated dienes (CD) and thiobarbituric acid reacting substances (TBARS) were measured in liver homogenate as previously described by Farghali *et al*. (2009).

Immunoblotting

Liver samples were homogenized and lysed in NP40 lysis buffer supplemented with protease and phosphatase inhibitors. Equivalent amounts of lysate protein, 20 μg of protein measured by the Bradford method, were then subjected to 10 % SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. After blocking the nitrocellulose membranes by incubation with Tris-buffered saline containing 5 % non-fat milk (for 1 h at room temperature), the membranes were incubated with primary antibodies overnight at 4 °C. The primary antibodies used were SIRT1 (1:1000 dilution, Cell Signaling Technology) and beta actin (1:5000, Sigma Aldrich). The following day, the membranes were washed in TBST and incubated with anti-mouse IgG (whole molecule)-peroxidase antibody (1:80000, Sigma Aldrich) at room temperature for 1 h. Proteins were visualized by enhanced chemiluminescence (GeneTiCA s.r.o., Prague, Czech Republic). Densitometric analysis was performed using the Quantity One software (Bio-Rad, Prague, Czech Republic).

Statistical analyses

All data are expressed as mean ± SEM of six animals used in each group. Statistical evaluation of the data was performed using one way ANOVA followed by Tukey-Kramer comparison test. $P<0.05$ was considered to have statistical significance.

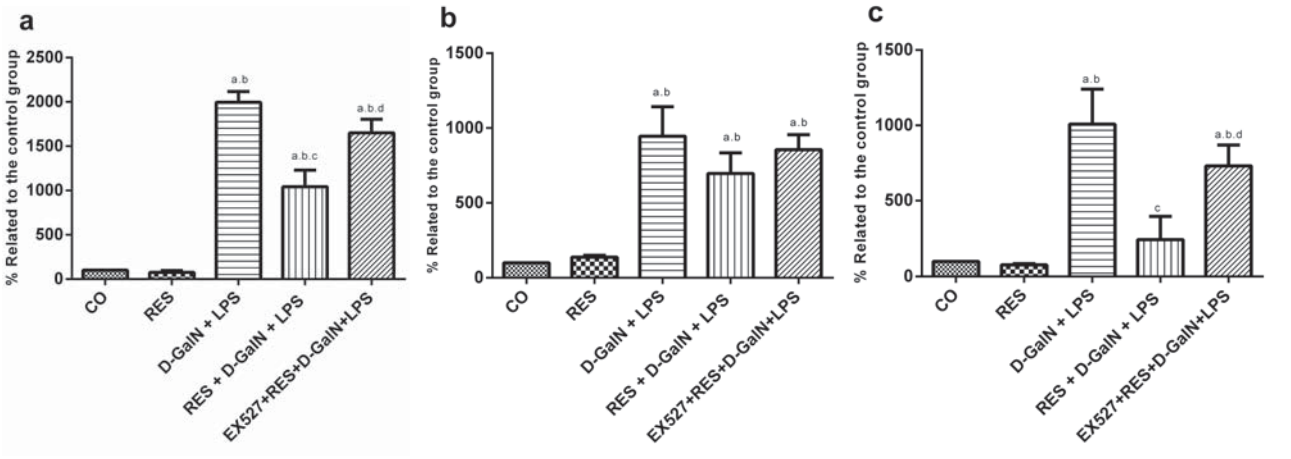

Fig. 1. Effects of resveratrol and EX-527 pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma levels of alanine aminotransferase ALT (**a**), aspartate aminotransferase AST (**b**) and bilirubin (**c**). CO, control group; RES, 2.3 mg/kg resveratrol; D-GalN + LPS, 400 mg/kg D-galactosamine with 10 μg/kg lipopolysaccharide; RES + D-GalN + LPS, 2.3 mg/kg resveratrol + D-GalN + LPS; EX-527 + RES + D-GalN + LPS, 1 mg/kg EX-527 plus combination of previous substances. Data are expressed as means ± SEM (n=6). [a] $P<0.05$ versus CO, [b] $P<0.05$ versus the RES, [c] $P<0.05$ versus D-GalN + LPS, [d] $P<0.05$ versus RES + D-GalN + LPS

Results

Effect of resveratrol and EX-527 pretreatment in D-GalN/LPS-induced liver injury

We first sought to define the role of resveratrol and EX-527 pretreatment in D-GalN/LPS-induced liver injury. For this, we measured the levels of ALT, AST and bilirubin in plasma (Fig. 1). Treatment of animals with D-GalN/LPS was able to induce hepatotoxicity as evidenced by a significant increase in transaminases and bilirubin levels relative to the negative control groups (CO and RES). There was over 20-fold increase in ALT

levels and slightly less with AST and bilirubin. Resveratrol alone had no significant effects on these markers. However, resveratrol pretreatment in D-GalN/LPS rats significantly lowered the ALT and bilirubin levels. There was also the same trend with AST, despite the statistical non-significance. These findings demonstrate that resveratrol was effective in attenuating D-GalN/LPS induced hepatotoxicity. EX-527, on the other hand, blocked the effects of resveratrol and significantly increased the ALT and bilirubin levels. EX-527 is one of the few available SIRT1 inhibitors which combine high potency with specificity. Hence this finding provides a clear indication that the catalytic activity of SIRT1 is required for the cytoprotective effects of resveratrol.

Effect of resveratrol and EX-527 pretreatment on lipid peroxidation in D-GalN/LPS treated rats

To firmly establish the role of resveratrol and EX-527 pretreatment in D-GalN/LPS-induced liver injury, we measured the levels of lipid peroxidation using TBARS and CD in homogenate (Fig. 2). Both CD and TBARS were significantly enhanced after D-GalN/LPS treatment reflecting increased peroxidation. Resveratrol pretreatment reduced the levels of both markers by more than a fold. The anti-peroxidative effects of resveratrol were blocked by EX-527 as evidenced by a significant increase in both the TBARS and CD levels. The extent of lipid peroxidation corresponds to the liver function tests above (Fig. 1) because lipid peroxidation is an index of oxidative stress (Niki 2008).

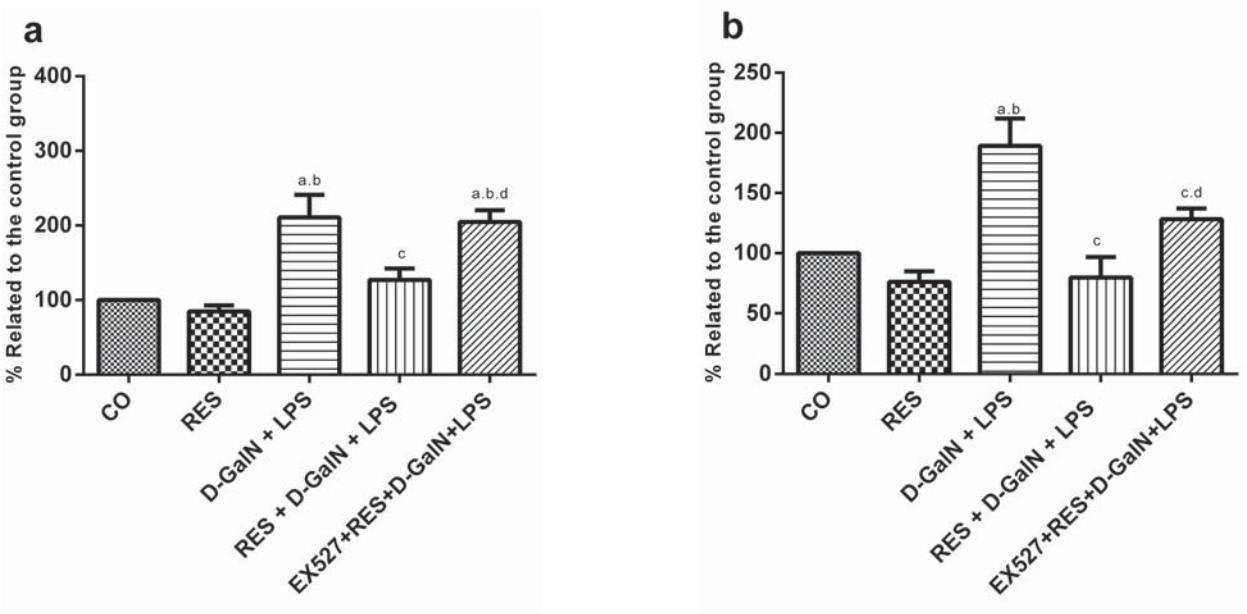

Fig. 2. Effects of resveratrol pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (LPS/D-GalN) on the formation of (**a**) Conjugated dienes (CD) and (**b**) Thiobarbituric acid reactive substances (TBARS) in liver homogenate. CO, control group; RES, 2.3 mg/kg resveratrol; D-GalN + LPS, 400 mg/kg D-galactosamine with 10 µg/kg lipopolysaccharide; RES + D-GalN + LPS, 2.3 mg/kg resveratrol + D-GalN + LPS; EX-527 + RES + D-GalN + LPS, 1 mg/kg EX-527 plus combination of previous substances. Data are expressed as mean ± SEM (n=6). [a] $P<0.05$ versus CO, [b] $P<0.05$ versus the RES, [c] $P<0.05$ versus D-GalN + LPS, [d] $P<0.05$ versus RES + D-GalN + LPS

Effect of resveratrol and EX-527 pretreatment on SIRT1 expression levels in D-GalN/LPS treated rats

A Western blot analysis was performed to confirm if SIRT1 is detected in the liver and how its expression is affected by resveratrol or EX-527 pretreatment. As shown in Figure 3, we found that SIRT1 was ubiquitously expressed in liver samples from all the animal groups. Resveratrol alone, did not have any statistically significant effect on the total endogenous amount of SIRT1. However, treatment with D-GalN/LPS dramatically decreased SIRT1 expression levels. In spite of an increasing trend on the blot, resveratrol pretreatment of D-GalN/LPS rats did not have any statistical significance on SIRT1 expression. Likewise, there was no significant change in SIRT1 expression levels in response to EX-527 pretreatment. This suggests that there may be other ways of modulating the aforementioned biochemical effects of resveratrol (Fig. 1 and Fig. 2) in the liver rather than alterations in SIRT1 expression.

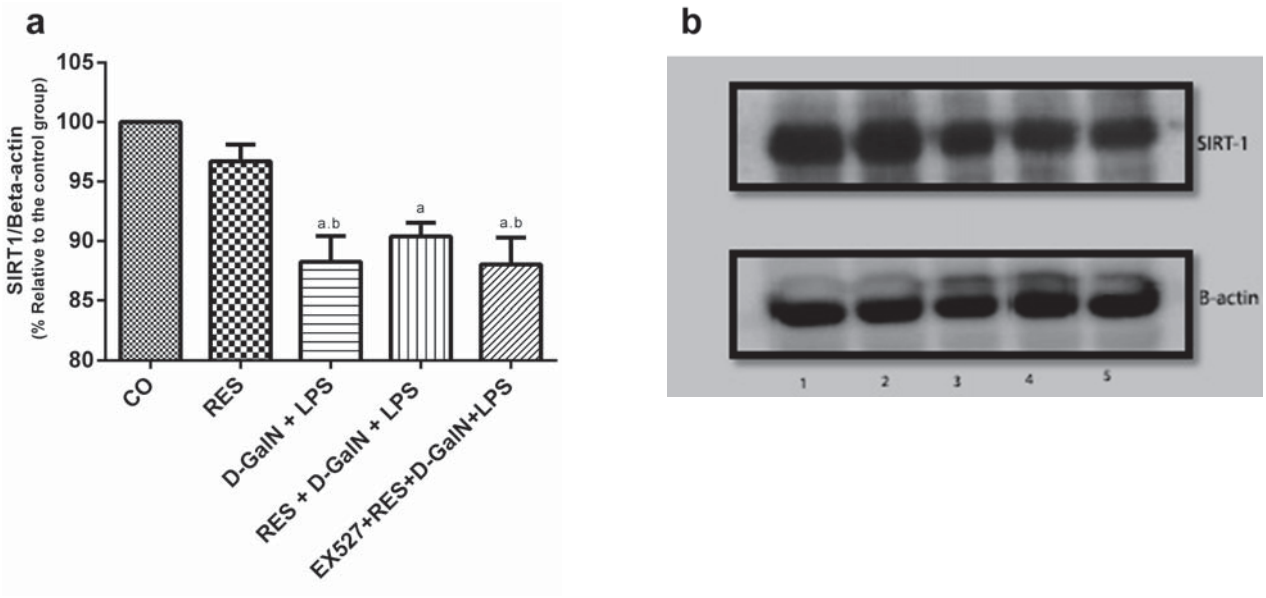

Fig. 3. Effects of resveratrol and EX-527 pretreatment on SIRT1 expression. (**a**) Quantification of SIRT1 expression levels by densitometry. Band intensity measurements were done using Bradford software. In each panel, the intensity of a given band was normalized to the intensity of the corresponding β-actin band. CO, control group; RES, 2.3 mg/kg resveratrol; D-GalN + LPS, 400 mg/kg D-galactosamine with 10 μg/kg lipopolysaccharide; RES + D-GalN + LPS, 2.3 mg/kg resveratrol + D-GalN + LPS; EX-527 + RES + D-GalN + LPS, 1 mg/kg EX-527 plus combination of previous substances. Data are expressed as mean ± SEM (n=6). [a] $P<0.05$ versus CO, [b] $P<0.05$ versus the RES. (**b**) Representative Western blot images lanes: 1) CO, 2) RES, 3) D-GalN + LPS, 4) RES + D-GalN + LPS, 5) EX-527 + RES + D-GalN + LPS

Discussion

Acute liver failure is one of the most challenging conditions in internal medicine. It occurs when the previously healthy liver cells are seriously injured and die giving rise to complications such as jaundice, coagulopathy and encephalopathy within few days (McDowell *et al*. 2010). Most common causes of ALF are viral hepatitis and drug toxins (Gotthardt *et al*. 2007). Its prognosis is dismissal and in most cases orthotopic liver transplantation is the only definitive curative treatment (Russo and Parola 2011). However, the scarcity of donors often precludes transplantation (Smith and Murphy 2008). There is therefore an intensive search of therapeutic strategies to prevent the onset of ALF by preventing apoptotic cell death of hepatocytes in experimental models (Hirono *et al*. 2001).

D-GalN/LPS-induced acute liver injury in experimental animals is a well-known *in vivo* model that closely resemble ALF seen clinically (Kosai *et al*. 1999). In this model, LPS, an endotoxin, activates macrophages and Kupffer cells to produce TNF-α. Through complex signaling cascades, TNF-α activates caspases and transcription factors such as NF-κB leading to cell demise (Silverstein 2004, Bradham *et al*. 2008). D-GalN on the other hand selectively depletes uridine nucleotides in the liver, inhibits RNA synthesis in hepatocytes and potentiates the acute toxicity of LPS (Alcorn *et al*. 1992, Lekic *et al*. 2011). The combined effects of these two agents produce a more severe form of liver injury consistent with ALF (Leist *et al*. 1995). In this study, 10 μg/kg of LPS and 400 mg/kg of D-GalN markedly increased the plasma levels of transaminases confirming that fatal liver injury occurred within six hours of treatment. ALT is the most reliable, sensitive and specific marker of liver injury (Dufour *et al*. 2000). It is abundant in hepatocytes and is released into serum as a result of hepatocellular damage, so its level in plasma approximates the extent of liver damage (Hsueh *et al*. 2011). Likewise, D-GalN/LPS treatment augmented lipid peroxidation as shown by increase in the TBARS and conjugated dienes. Lipid peroxidation alters the physical and chemical properties of cell membranes and their fluidity resulting in cytolysis and cell death (Pradeep *et al*. 2009). The levels of bilirubin were also increased in response to D-GalN/LPS treatment. Bilirubin plays an important role as an antioxidant by scavenging peroxyl radicals and preventing oxidation of fatty acids and proteins (Mayer 2000). Its activity is augmented in oxidative stress as an adaptive mechanism. Of interest, our Western blots revealed a significant and dramatic decrease in SIRT1 expression levels after D-GalN/LPS treatment (Fig. 3). The precise mechanism by which D-GalN/LPS treatment represses SIRT1 expression was

not investigated in this study. However, several studies suggest that generation of ROS plays a key role in the cytotoxic effects of this model (Uchikura *et al*. 2004). For instance, LPS may execute induction of iNOS and subsequent peroxynitrite anion which can oxidize a wide array of molecules within cells including lipids and DNA (Szabó and Ohshima 1997, Morikawa *et al*. 2004, Pacher *et al*. 2007, Lekic *et al*. 2013). Moreover, some recent studies have shown that microRNAs such as miR-34a can downregulate SIRT1 expression in response to oxidative stress and therefore augment liver damage (Yamakuchi 2012, Choi and Kemper 2013). In brief, our studies add to the mounting evidence that SIRT1 expression is decreased to some extent by the degree of oxidative stress.

Pretreatment with resveratrol ameliorated D-GalN/LPS-induced liver damage as evidenced by a decrease in transaminases and other markers of oxidative stress. Interestingly, resveratrol pretreatment did not have any significant effect on SIRT1 expression level when compared to D-GalN/LPS treatment. This demonstrates that there are other ways in which resveratrol exerts its cytoprotective effects in the liver, beside upregulation of SIRT1 expression reported in some studies (Wang *et al*. 2013). SIRT1 expression and activity can be modulated at different levels. One school of thought is that resveratrol allosterically activates SIRT1. It binds to the non-catalytic N-terminus of SIRT1 to cause a conformational change that lowers its Michaelis constant (Howitz *et al*. 2003). SIRT1 in turn deacetylates and suppresses transcription factors such as NF-κB responsible for induction of pro-inflammatory cytokines and pro-apoptotic factors (Yeung *et al*. 2004). SIRT1 also upregulates FOXO-dependent antioxidants such as catalase and MnSOD which protect against oxidative stress-induced cellular apoptosis (Hasegawa *et al*. 2008, Tanno *et al*. 2010). However, the hypothesis that resveratrol is a bona fide SIRT1 agonist has been challenged by many authors (Beher *et al*. 2009, Baur *et al*. 2012). SIRT1 is not the only resveratrol-sensitive molecule that may have protective downstream effects. Another potential resveratrol target is the main metabolic regulator, AMPK (Centeno-Baez *et al*. 2011). SIRT1 and AMPK mutually coexist, share many common targets and have many overlapping cytoprotective effects (Ruderman *et al*. 2010, Farghali *et al*. 2013). It is also possible that SIRT1 and AMPK are interdependent and resveratrol activates SIRT1 through AMPK (Park *et al*. 2012). While the exact mechanism of resveratrol is yet unknown, within the experimental conditions of the present study, it seems that SIRT1 expression does not contribute to the cytoprotective effects of resveratrol in the liver.

To further demonstrate the role of SIRT1 catalysis in the cytoprotective effects of resveratrol, we pretreated another group of animals with a SIRT1 inhibitor, EX-527. EX-527 was chosen because it is more potent than other available SIRT1 inhibitors such as nicotinamide, splitomicin and sirtinol (Solomon *et al*. 2006). Furthermore, EX-527 is more selective for SIRT1 than other closely related histone deacetylases (Napper *et al*. 2005). However, its inhibition mechanisms are not fully understood. SIRT1 couples lysine deacetylation to NAD hydrolysis to yield nicotinamide and O-acetyl-ADP-ribose (Jackson and Denu 2002, Blander and Guarente 2004). Kinetic analyses suggest that EX-527 binds to the SIRT1 C-pocket after release of nicotinamide and prevent the release of O-acetyl-ADP-ribose (Napper *et al*. 2005, Gertz *et al*. 2013). Despite non-significant/ negligible effects on SIRT1 expression levels, EX-527 significantly blocked the protective effects of resveratrol and augmented liver damage (Fig. 1 and Fig. 2). Taken together, these findings confirm that the catalytic activity of SIRT1 plays a key role in the cytoprotective effects of resveratrol in the liver. If the enzymatic activity of SIRT1 is inhibited, then the protective effects of resveratrol are also concomitantly blocked.

In conclusion, we affirm our previous findings that resveratrol is protective against D-GalN/LPS induced hepatotoxicity in rodents. Resveratrol has antioxidant properties and protects cells against lipid peroxidation. Inhibition of SIRT1 by EX-527 renders resveratrol ineffective and exacerbates D-GalN/LPS-induced liver injury. According to our study, SIRT1 downregulation is an involved step in the hepatotoxic effects of D-GalN/LPS treatment but the roles of resveratrol and EX-527 on SIRT1 expression were not documented in this study.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by the research program PRVOUK-P25/LF1/2 and grants GAUK-916314 and IGA MZ NT/14017-3/2013. The authors would like to thank Libuse Slehobrova for her skillful technical assistance.

Abbreviations

AMPK, adenosine monophosphate-activated protein kinase; D-GalN, D-galactosamine; FLF, fulminant liver failure; FOXO, forkhead box-O; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; NAD, nicotinamide adenine dinucleotide; NF-kB, nuclear factor-kappaB; ROS, reactive oxygen species; SIRT1, sirtuin 1, silent information regulator T1; TNF-α, tumor necrosis factor alpha

References

ALCORN JM, FIERER J, CHOJKIER M: The acute phase response protects mice from D-galactosamine sensitization to endotoxin and tumor necrosis factor-alpha. *Hepatology* **15**: 122-129, 1992.

ARA C, KIRIMLIOGLU H, KARABULUT AB, COBAN S, AY S, HARPUTLUOGLU M, KIRIMLIOGLU V, YILMAZ S: Protective effect of resveratrol against oxidative stress in cholestasis. *J Surg Res* **127**: 112-117, 2005.

BANSAL AK, BANSAL M, SONI G, BHATNAGAR D: Protective role of Vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chem Biol Interact* **156**: 101-111, 2005.

BAUR JA, MAI A, GUARENTE L: Revelations into resveratrol's mechanism. *Nat Med* **18**: 500-501, 2012.

BEHER D, WU J, CUMINE S, KIM KW, LU SC, ATANGAN L, WANG M: Resveratrol is not a direct activator of SIRT1 enzyme activity. *Chem Biol Drug Des* **74**: 619-624, 2009.

BIASUTTO L, MATTAREI A, ZORATTI M: Resveratrol and health: the starting point. *Chembiochem* **13**: 1256-1259, 2012.

BLANDER G, GUARENTE L: The Sir2 family of protein deacetylases. *Annu Rev Biochem* **73**: 417-435, 2004.

BRADHAM CA, PLÜMPE J, MANNS MP, BRENNER DA, TRAUTWEIN C: Mechanisms of hepatic toxicity. I. TNF-induced liver injury. *Am J Physiol* **275**: G387-G392, 2008.

BRUNET A, SWEENEY LB, STURGILL JF, CHUA KF, GREER PL, LIN Y, TRAN H, ROSS SE, MOSTOSLAVSKY R, COHEN HY, HU LS, CHENG HL, JEDRYCHOWSKI MP, GYGI SP, SINCLAIR DA, ALT FW, GREENBERG ME: Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**: 2011-2015, 2004.

CENGIZ N, KAVAK S, GÜZEL A, OZBEK H, BEKTAŞ H, HIM A, ERDOĞAN E, BALAHOROĞLU R: Investigation of the hepatoprotective effects of Sesame (Sesamumindicum L.) in carbon tetrachloride-induced liver toxicity. *J Membr Biol* **246**: 1-6, 2013.

CENTENO-BAEZ C, DALLAIRE P, MARETTE A: Resveratrol inhibition of inducible nitric oxide synthase in skeletal muscle involves AMPK but not SIRT1. *Am J Physiol* **301**: E922-E930, 2011.

CERNÝ D, LEKIĆ N, VÁŇOVÁ K, MUCHOVÁ L, HOŘÍNEK A, KMONÍČKOVÁ E, ZÍDEK Z, KAMENÍKOVÁ L, FARGHALI H: Hepatoprotective effect of curcumin in lipopolysaccharide/-galactosamine model of liver injury in rats: relationship to HO-1/CO antioxidant system. *Fitoterapia* **82**: 786-791, 2011.

CHAN AC, FAN ST, LO CM, LIU CL, CHAN SC, NG KK, YONG BH, CHIU A, LAM BK: Liver transplantation for acute-on-chronic liver failure. *Hepatol Int* **3**: 571-581, 2009.

CHEN ML, YI L, JIN X, LIANG XY, ZHOU Y, ZHANG T, XIE Q, ZHOU X, CHANG H, FU YJ, ZHU JD, ZHANG QY, MI MT: Resveratrol attenuates vascular endothelial inflammation by inducing autophagy through the cAMP signaling pathway. *Autophagy* **9**: 2033-45. 2013.

CHOI SE, KEMPER JK: Regulation of SIRT1 by MicroRNAs. *Mol Cells* **36**: 385-392, 2013.

DUFOUR DR, LOTT JA, NOLTE FS, GRETCH DR, KOFF RS, SEEFF LB: Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. *Clin Chem* **46**: 2027-2049, 2000.

FARGHALI H, CERNÝ D, KAMENÍKOVÁ L, MARTÍNEK J, HORÍNEK A, KMONÍCKOVÁ E, ZÍDEK Z: Resveratrol attenuates lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats: role of nitric oxide synthase 2 and heme oxygenase-1. *Nitric Oxide* **21**: 216-225, 2009.

FARGHALI H, KUTINOVÁ CANOVÁ N, LEKIĆ N: Resveratrol and related compounds as antioxidants with an allosteric mechanism of action in epigenetic drug targets. *Physiol Res* **62**: 1-13, 2013.

GERTZ M, FISCHER F, NGUYEN GT, LAKSHMINARASIMHAN M, SCHUTKOWSKI M, WEYAND M, STEEGBORN C: Ex-527 inhibits Sirtuins by exploiting their unique NAD^+-dependent deacetylation mechanism. *Proc Natl Acad Sci USA* **110**: E2772-E2781, 2013.

GOTTHARDT D, RIEDIGER C, WEISS KH, ENCKE J, SCHEMMER P, SCHMIDT J, SAUER P: Fulminant hepatic failure: etiology and indications for liver transplantation. *Nephrol Dial Transplant* **22**: 5-8, 2007.

HADDAD Y, VALLERAND D, BRAULT A, HADDAD PS: Antioxidant and hepatoprotective effects of silibinin in a rat model of nonalcoholic steatohepatitis. *Evid Based Complement Alternat Med* **2011**: article ID 647903, 2011.

HASEGAWA K, WAKINO S, YOSHIOKA K, TATEMATSU S, HARA Y, MINAKUCHI H, WASHIDA N, TOKUYAMA H, HAYASHI K, ITOH H: Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression. *Biochem Biophys Res Commun* **372**: 51-56, 2008.

HIRONO S, NAKAMA T, TSUBOUCHI H: Molecular mechanisms of D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure in mice and the effects of therapeutic agents. In: *Trends in Gastroenterology and Hepatology: Millennium 2000*. Niigata, 2001, pp 59-62.

HONG JY, LEBOFSKY M, FARHOOD A, JAESCHKE H: Oxidant stress-induced liver injury in vivo: role of apoptosis, oncotic necrosis, and c-Jun NH2-terminal kinase activation. *Am J Physiol* **296**: G572-G581, 2009.

HOWITZ KT, BITTERMAN KJ, COHEN HY, LAMMING DW, LAVU S, WOOD JG, ZIPKIN RE, CHUNG P, KISIELEWSKI A, ZHANG LL, SCHERER B, SINCLAIR DA: Small molecule activators of Sirtuins extend Saccharomycetescerevisiae lifespan. *Nature* **425**: 191-196, 2003.

HSUEH CJ, WANG JH, DAI L, LIU CC: Determination of alanine aminotransferase with an electrochemical nano Ir-C biosensor for the screening of liver diseases. *Biosensors* **1**: 107-117, 2011.

JACKSON MD, DENU JM: Structural identification of 2'- and 3'-O-acetyl-ADP-ribose as novel metabolites derived from the Sir2 family of beta -NAD^+-dependent histone/protein deacetylases. *J Biol Chem* **277**: 18535-18544, 2002.

KOSAI K, MATSUMOTO K, FUNAKOSHI H, NAKAMURA T: Hepatocyte growth factor prevents endotoxin-induced lethal hepatic failure in mice. *Hepatology* **30**: 151-159, 1999.

LEIST M, GANTNER F, BOHLINGER I, TIEGS G, GERMANN PG, WENDEL A: Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am J Pathol* **146**: 1220-1234, 1995.

LEKIĆ N, CERNÝ D, HOŘÍNEK A, PROVAZNÍK Z, MARTÍNEK J, FARGHALI H: Differential oxidative stress responses to D-galactosamine-lipopolysaccharide hepatotoxicity based on real time PCR analysis of selected oxidant/antioxidant and apoptotic gene expressions in rat. *Physiol Res* **60**: 549-558, 2011.

LEKIĆ N, CANOVÁ NK, HOŘÍNEK A, FARGHALI H: The involvement of hemeoxygenase 1 but not nitric oxide synthase 2 in a hepatoprotective action of quercetin in lipopolysaccharide-induced hepatotoxicity of D-galactosamine sensitized rats. *Fitoterapi* **87**: 20-26, 2013.

MAYER M: Association of serum bilirubin concentration with risk of coronary artery disease. *Clin Chem* **46**: 1723-1727, 2000.

McDOWELL TORRES D, STEVENS RD, GURAKAR A: Acute liver failure: a management challenge for the practicing gastroenterologist. *Gastroenterol Hepatol (NY)* **6**: 444-450, 2010.

MORIKAWA A, KOIDE N, SUGIYAMA T, MU MM, HASSAN F, ISLAM S, ITO H, MORI I, YOSHIDA T, YOKOCHI T: The enhancing action of D-galactosamine on lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells. *FEMS Immunol Med Microbiol* **41**: 211-218, 2004.

NAPPER AD, HIXON J, McDONAGH T, KEAVEY K, PONS JF, BARKER J, YAU WT, AMOUZEGH P, FLEGG A, HAMELIN E, THOMAS RJ, KATES M, JONES S, NAVIA MA, SAUNDERS JO, DiSTEFANO PS, CURTIS R: Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. *J Med Chem* **48**: 8045-8054, 2005.

NIKI E: Lipid peroxidation products as oxidative stress biomarkers. *Biofactors* **34**: 171-180, 2008.

PACHER P, BECKMAN JS, LIAUDET L: Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* **87**: 315-424, 2007.

PARK SJ, AHMAD F, PHILP A, BAAR K, WILLIAMS T, LUO H, KE H, REHMANN H, TAUSSIG R, BROWN AL, KIM MK, BEAVEN MA, BURGIN AB, MANGANIELLO V, CHUNG JH: Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell* **148**: 421-433, 2012.

PRADEEP HA, KHAN S, RAVIKUMAR K, AHMED MF, RAO MS, KIRANMAI M, REDDY DS, AHAMED SR, IBRAHIM M: Hepatoprotective evaluation of Anogeissus latifolia: in vitro and in vivo studies. *World J Gastroenterol* **15**: 4816-4822, 2009.

RIVERA H, SHIBAYAMA M, TSUTSUMI V, PEREZ-ALVAREZ V, MURIEL P: Resveratrol and trimethylated resveratrol protect from acute liver damage induced by CCl_4 in the rat. *J Appl Toxicol* **28**: 147-155, 2008.

RUDERMAN NB, XU XJ, NELSON L, CACICEDO JM, SAHA AK: AMPK and SIRT1: a long-standing partnership? *Am J Physiol* **298**: E751-E760, 2010.

RUSSO FP, PAROLA M: Stem and progenitor cells in liver regeneration and repair. *Cytotherapy* **13**: 135-144, 2011.

SILVERSTEIN R: D-galactosamine lethality model: scope and limitations. *J Endotoxin Res* **10**: 147-162, 2004.

SMITH M, MURPHY P: A historic opportunity to improve organ donation rates in the UK. *Br J Anaesth* **100**: 735-737, 2008.

SOLOMON JM, PASUPULETI R, XU L, MCDONAGH T, CURTIS R, DISTEFANO PS, HUBER LJ: Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol* **26**: 28-38, 2006.

SZABÓ C, OHSHIMA H: DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide* **1**: 373-385, 1997.

TANNO M, KUNO A, YANO T, MIURA T, HISAHARA S, ISHIKAWA S, SHIMAMOTO K, HORIO Y: Induction of manganese superoxide dismutase by nuclear translocation and activation of SIRT1 promotes cell survival in chronic heart failure. *J Biol Chem* **285**: 8375-8382, 2010.

UCHIKURA K, WADA T, HOSHINO S, NAGAKAWA Y, AIKO T, BULKLEY GB, KLEIN AS, SUN Z: Lipopolysaccharides induced increases in Fas ligand expression by Kupffer cells via mechanisms dependent on reactive oxygen species. *Am J Physiol* **287**: G620-G626, 2004.

VASKO R, XAVIER S, CHEN J, LIN CH, RATLIFF B, RABADI M, MAIZEL J, TANOKUCHI R, ZHANG F, CAO J, GOLIGORSKY MS: Endothelial sirtuin 1 deficiency perpetrates nephrosclerosis through downregulation of matrix metalloproteinase-14: relevance to fibrosis of vascular senescence. *J Am Soc Nephrol* **25**: 276-291, 2014.

WANG P, DU B, YIN W, WANG X, ZHU W: Resveratrol attenuates $CoCl_2$-induced cochlear hair cell damage through upregulation of Sirtuin1 and NF-κB deacetylation. *PLoS One* **8**: e80854, 2013.

WANG RH, KIM HS, XIAO C, XU X, GAVRILOVA O, DENG CX: Hepatic Sirt1 deficiency in mice impairs mTorc2/Akt signaling and results in hyperglycemia, oxidative damage, and insulin resistance. *J Clin Invest* **121**: 4477-4490, 2011.

WEN D, HUANG X, ZHANG M, ZHANG L, CHEN J, GU Y, HAO CM: Resveratrol attenuates diabetic nephropathy via modulating angiogenesis. PLoS One **8**: e82336, 2013.

YAMAKUCHI M: MicroRNA regulation of SIRT1. *Front Physiol* **3**: Article 68, 2012.

YEUNG F, HOBERG JE, RAMSEY CS, KELLER MD, JONES DR, FRYE RA, MAYO MW: Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* **23**: 2369-2380, 2004.

Sirtuin 1 Modulation in Rat Model of Acetaminophen-Induced Hepatotoxicity

L. WOJNAROVÁ[1], N. KUTINOVÁ CANOVÁ[1], H. FARGHALI[1], T. KUČERA[2]

[1]Institute of Pharmacology, First Faculty of Medicine, Charles University, Prague, Czech Republic, [2]Institute of Histology and Embryology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Received September 15, 2015
Accepted September 25, 2015

Summary

Sirtuin 1 (SIRT1) is involved in important biological processes such as energy metabolism and regulatory functions of the cell cycle, apoptosis, and inflammation. Our previous studies have shown hepatoprotective effect of polyphenolic compound resveratrol, which is also an activator of SIRT1. Therefore, the aim of our present study was to clarify the role of SIRT1 in process of hepatoprotection in animal model of drug-induced liver damage. Male Wistar rats were used for both *in vivo* and *in vitro* studies. Hepatotoxicity was induced by single dose of acetaminophen (APAP). Some rats and hepatocytes were treated by resveratrol or synthetic selective activator of sirtuin 1 (CAY10591). The degree of hepatotoxicity, the activity and expression of the SIRT1 were determined by biochemical, histological and molecular-biological assessments of gained samples (plasma, liver tissue, culture media and hepatocytes). Resveratrol and CAY attenuated APAP-induced hepatotoxicity *in vivo* and *in vitro*. Moreover, both drugs enhanced APAP-reduced SIRT1 activity. Our results show that modulation of the SIRT1 activity plays a role in hepatoprotection. Synthetic activators of SIRT1 would help in understanding the role of SIRT1 and are therefore a major boost towards the search for specific treatment of liver disease.

Key words

SIRT1 • Resveratrol • Acetaminophen • CAY10591 • Liver

Corresponding author

L. Wojnarová, Institute of Pharmacology, First Faculty of Medicine, Charles University, Albertov 4, 128 00 Prague 2, Czech Republic. E-mail: lea.wojnarova@lf1.cuni.cz

Introduction

Drug-induced liver injury (DILI) is the most frequent cause of hepatic dysfunction and it is the most common cause of acute liver failure in the United States. Over 1000 medications and herbal products have been associated in the development of DILI. In many studies, acetaminophen-induced liver injury is commonly used as a standard hepatotoxicity model in rodents to test the hepatoprotective effects of herbal and other compounds. Acetaminophen (APAP, paracetamol) belongs to the most widely used analgetic and antipyretic drugs. Overdose can cause hepatotoxicity in experimental animals and humans (Brune *et al.* 2015, Ingawale *et al.* 2014, Jaeschke *et al.* 2013, McGill *et al.* 2012). But what is a maximum safe dose of APAP? The safety of this drug and its toxic reactions are influenced by a large number of factors, both known and unknown (Kaplowitz *et al.* 2004). It is well established that APAP is detoxified by three major pathways. The majority of APAP dose is conjugated with glucuronate and sulfate and remaining part of APAP especially in its excess is metabolized by an alternative pathway known as cytochrome P450 oxidase enzyme system (mainly CYP2E1). The latter metabolic pathway leads to the formation of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is detoxified especially by glutathione (GSH) to form APAP-SG conjugate (Kučera *et al.* 2011, Roušar *et al.* 2009). In the case of GSH depletion, this metabolite covalently binds to cysteine groups on protein, forming APAP-protein adducts in the cell and in mitochondria (Jaeschke *et al.* 2013, Wang *et al.* 2015). It results in

conformational and functional changes in cellular proteins, increased reactive oxygen and reactive nitrogen species production (ROS, RNS), lipoperoxidation, mitochondrial permeability and transition pore opening. These are key events leading to cell death (Bajt *et al.* 2006, Du *et al*. 2015, Jaeschke *et al*. 2013, Karthivashan *et al.* 2015, Roušar *et al.* 2009). Despite the processes mentioned above, the detailed understanding of the mechanism of APAP toxic effect still remains unknown (Kaplowitz *et al.* 2004, Roušar *et al*. 2009).

Our previous studies have shown hepatoprotective effect of resveratrol in lipopoly-saccharide/D-Galactosamine (LPS/D-GAlN) model of hepatotoxicity (Černý *et al*. 2009, Farghali *et al*. 2014). Resveratrol pretreatment led to the overall improvement in hepatotoxic markers and morphology after the hepatic insult. Several studies have also highlighted the hepatoprotective properties of resveratrol (Bishayee *et al.* 2010). Resveratrol (trans-3,4′,5-trihydroxystilbene) is a polyphenolic compound found in a plant sources such as peanuts, grapes and red wine and it´s one of the extensively studied natural product, with wide ranging biological activity and clinical potentials. Many studies have suggested that resveratrol possess many pharmacological actions including anti-aging, anticarcinogenic, anti-inflammatory, and antioxidant properties (Wang *et al.* 2015). Moreover, resveratrol is also the most widely investigated activator of sirtuin 1 (SIRT1, silent information regulator T1) (Baur 2010, Kutinová Canová *et al.* 2012, Ruderman *et al.* 2010, Silva and Wahlestedt 2010).

SIRT1 is the evolutionarily conserved NAD^+-dependent histone/protein deacetylase whose function is linked to cellular metabolism. It plays a role in metabolism of numerous tissues, including liver, muscle, adipose tissue, heart, and endothelium (Chang and Guarente 2014). Moreover, an important target for SIRT1 is nuclear factor-kappa B (NF-κB), a regulator of many processes, including the cell cycle, proliferation, apoptosis, and inflammation (Yang *et al.* 2007). Several small-molecule SIRT1 activators, among others e.g. CAY1059 and SRT1720, were synthesized with higher pharmacologic potency compared with the traditionally studied SIRT1 activator resveratrol (Feige *et al*. 2008, Nayagam *et al*. 2006).

The main goal of the present study was to clarify the role of SIRT1 in the process of hepatotoxicity/ hepatoprotection in animal model of APAP-induced acute liver injury and to investigate SIRT1 activation and protein expression under treatment with natural SIRT1 activator resveratrol and selective synthetic SIRT1 activator CAY10591 (CAY) both *in vivo* and *in vitro*.

Materials and Methods

Materials

CAY10591 was obtained from Cayman Pharma (Neratovice, Czech Republic) and collagenase type IV from Sevapharma (Prague, Czech Republic). Bio-Rad protein DC assay dye reagent was from Bio-Rad (Prague, Czech Republic). Acetaminophen, resveratrol (3,4′,5-trihydroxy-trans-stilbene, 5-[(1E)-2-(4-hydroxyphenyl) ethenyl]-1,3-benzenediol ≥99 % GC), SIRT1 assay kit, PEG400, saline, tris-HCl, Nonidet P40, dimethyl sulfoxide (DMSO), isopropyl alcohol, Tween 20, 2-thiobarbituric acid, tetraethoxypropane, trichloroacetic acid, sodium dodecyl sulphate, ammonium persulfate, methanol, glycine, N,N,N′,N″-tetramethylethylene-diamine, NaCl, KCl, Na_2HPO_4, KH_2PO_4, filter paper, nitrocellulose membrane, anti-mouse IgG (whole molecule) peroxidase antibody and mouse monoclonal anti-beta-actin antibody, protease and phosphatase inhibitor cocktails, and other standard chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic). SirT1 (1F3) mouse mAb antibody was from Cell Signaling Technology through BioTech (Prague, Czech Republic). Non-fat dry milk was also from BioTech. Water for injection 100 % w/v was obtained from Baxter (Czech Republic, Prague).

Animals

Outbred male Wistar rats (Velaz-Lysolaje, Czech Republic) of 200-350 g body weight were used throughout the present studies. Rats were allowed to tap water and standard granulated diet *ad libitum* and were maintained under standard light (12/12h light/dark), temperature (22±2 °C) and relative humidity (50±10 %) conditions. All rats received humane care in compliance with the general guidelines of the First Faculty of Medicine, Charles University in Prague. The study protocol was approved by the Faculty Ethical Committee.

Drug treatments

The rats were divided randomly into five groups of six animals each and treated as follows: 1. Control – polyethyleneglycol 400 (40 %) + DMSO, 2. CAY – CAY 0.5 mg/kg in DMSO, 3. APAP – acetaminophen 1 g/kg in PEG 400 (40 %), 4. APAP+CAY – acetaminophen 1 g/kg

in PEG 400 (40 %) + CAY 0.5 mg/kg in DMSO, 5. APAP+RES – acetaminophen 1 g/kg in PEG 400 (40 %) + RES 30 mg/kg in DMSO.

To induce acute liver injury in rats, 1 g/kg of APAP was injected intraperitoneally from a 0.2 g/ml solution in 40 % PEG 400 (PEG 400 in saline). It was followed after one hour by intraperitoneal injection of CAY10591 or resveratrol, both dissolved in DMSO of total volume of 1.5 ml/kg. Twenty-four hours after APAP application, the animals were anesthetized with diethylether and then euthanized by exsanguination. Blood samples were collected into heparinized tubes. Plasma was immediately isolated by centrifugation at 4,000 rpm for 10 min and used for assessment of alanine aminotransferase (ALT), total bilirubin and nitric oxide (NO) as nitrite (NO_2^-) levels. Their liver samples were immediately homogenized in cooled Tris-HCl buffer or snap frozen in liquid nitrogen for Western blot analysis. The liver homogenates were used for determination of total lipid peroxidation assessed as thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD).

Isolation and culture of primary rat hepatocytes

Hepatocytes were isolated from untreated rats using the standard two phase collagenase perfusion method (Berry *et al*. 1991). Separated hepatocytes were counted and cell viability was assessed by trypan blue exclusion method. The hepatocyte cell viability was greater than 85 %. Cells were seeded on collagen-coated polystyrene Nunclon™ dishes at density of 104 000 viable cells/cm^2. They were incubated in complete medium (William´s medium E, penicillin/streptomycin 1 %, glutamine 1 %, insulin 0.06 %, FBS-fetal bovine serum 5 %) at 37 °C in a humid atmosphere with 5 % CO_2 throughout the study. Unattached hepatocytes were removed 3 h after seeding and remaining hepatocytes further cultured in fresh complete medium overnight. Hepatocytes were then treated with fresh medium alone or with acetaminophen (APAP, 5 mM). Thirty minutes later, vehicle with DMSO (0.1 % of final concentration), resveratrol (RES, 20 μM) or CAY10591 (CAY, 30 μM) were added to respective hepatocyte cultures. After 24 h, medium samples were collected for biochemical analysis and hepatocyte viability was assessed by MTT test. At the end of experiments, some cultured hepatocytes were washed by cooled phosphate buffered saline and lysed in RIPA buffer containing protease inhibitor cocktail. The homogenates were centrifuged at 14,000 g for 10 min at 4 °C and thereafter used for SIRT1 activity measurement.

MTT/cell viability test

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used both to assess the optimal non-toxic concentration of drugs suitable for our study and to measure hepatocyte viability at the end of *in vitro* experiments following test description by Kutinova Canova *et al*. (2008).

Biochemical analysis

ALT and bilirubin concentrations in the medium or plasma samples were measured using customized diagnostic kits according manufacturer´s instruction (Vian Diagnostics, Prague, Czech Republic).

Medium NO_2^-, the stable end-product of NO oxidation, was determined spectrofotometrically by using Griess reagent (1 % sulfanilamide, 0.1 % naphtylethylendiamine, 2.5 % trihydrogenphosphoric acid). The absorbance at 540 nm was recorded and the NO_2^- levels were calculated from $NaNO_2$ standard curve.

Evaluation of oxidative stress parameters

The measurement of homogenate levels of TBARS and conjugated dienes were carried out according to Farghali *et al.* (2009).

SIRT1 deacetylase activity assay

SIRT1 deacetylase activity was evaluated in 5 μl of the whole liver lysate as well as cultured hepatocyte lysate according to manual instruction of fluorometric SIRT1 Assay Kit (Sigma-Aldrich). The measured fluorescence was directly proportional to deacetylation activity of the SIRT1 enzyme in the sample. All measurements were performed in duplicate and the results were reported as arbitrary units of relative fluorescence per 1 mg of lysate protein (assessed by Bio-Rad protein DC assay).

Histological evaluation

After the excised liver (1 cm^3) fixation in 4 % paraformaldehyde in PBS, thin tissue paraffin sections (5 μm) were cut by microtome, stained with hematoxylin and eosin and examined by light microscope.

Immunoblotting

Isolated liver samples were lysed with RIPA buffer (2 M TRIS, 5 M NaCl, 0.5 M EDTA, NP-40, NAF) and homogenized with an electric homogenizer. The samples were then centrifuged for 20 min at 12,000 rpm at 4 °C and supernatant was collected. The

cell lysates were mixed (1:1) with sample buffer (2 x Laemmli buffer 950 µl+50 µl of β-mercaptoethanol) and then heated for 10 min at 90 °C. Proteins (assessed by Bio-Rad protein DC assay) from the tissue samples were separated on 10 % SDS-acrylamide gel and transferred to a nitrocellulose membrane by electrophoresis overnight at 4 °C. Membranes were blocked for 2 h with 5 % non-fat milk in Tris-buffered solution, at 4 °C. Membranes were then washed in a washing buffer (NaCl, KCl, Na_2HPO_4, KH_2PO_4, Tween, H_2O). They were then incubated with mouse primary antibody against SIRT1 (1:1,000) or beta-actin primary antibody (1:5,000) and followed with corresponding secondary rabbit antibody anti-mouse IgG HRP conjugate (1:80,000). For visualization was used chemiluminescence labeling with Super Signal West Pico Chemiluminescent Substrate (GeneTiCA, Prague, Czech Republic). Bands were detected with the use Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic). Optical densities of SIRT1 and beta-actin bands were normalized by the corresponding loading control and then to the mean of the corresponding control group (Lekić *et al.* 2013).

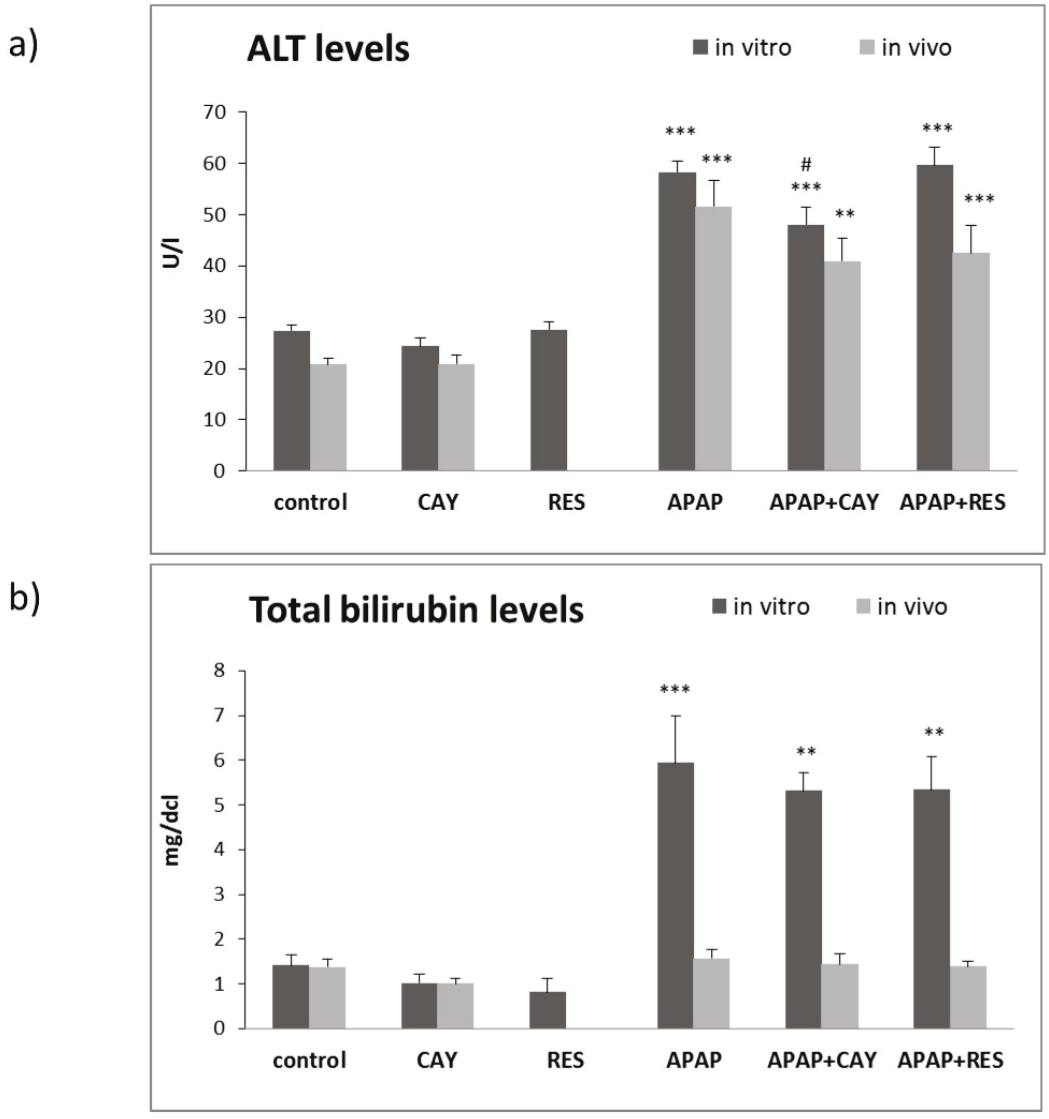

Fig. 1. Effect of specific SIRT1 activator, CAY10591, and resveratrol treatments on hepatocyte function in acute APAP-induced hepatocyte/liver injury *in vitro* and *in vivo* expressed as medium or plasma levels of **a**) Alanine aminotransferase ALT and **b**) Bilirubin. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30 µM *in vitro*, 0.5 mg/kg *in vivo*); RES (Resveratrol: 20 µM *in vitro*, 30 mg/kg *in vivo*); APAP (Acetaminophen: 5 mM *in vitro*, 1 g/kg *in vivo*); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen and Resveratrol in the stated doses). Data are expressed as means ± SEM (n=6): ** P<0.01, *** P<0.001 vs. respective control; # P<0.05 vs. APAP *in vitro*.

Statistical analysis

The statistical significance of differences of mean scores was determined using one-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparisons test (Graph-Pad Prism 4.03, Graph Pad Software, San Diego, CA, USA). P-value less than 0.05 was considered to be statistically significant. Data were expressed as means ± SEM (standard error of mean). All experiments were performed in means of 6 animals per group for *in vivo* experiments and 6 hepatocyte cultures per group of 3 independent *in vitro* experiments.

Results

Effects of CAY in comparison with RES treatment on liver function in APAP treated rats in vitro and in vivo

We measured the levels of ALT and bilirubin in plasma to detect the extent of hepatotoxicity (Fig. 1a). The APAP treatment in rats produced significant two-fold increase of ALT release ($P<0.001$) compared to the control group both *in vitro* and *in vivo*. Moreover, treatment with RES and CAY after induction of hepatotoxicity significantly lowered the ALT parameters *in vivo*. Interesting difference was in *in vitro* experiments, where only CAY treatment slightly reduced ALT, whereas RES did not influence the resulting values. Significant increase of bilirubin, fivefold higher, was observed in hepatocyte cultures compared to the control. There was a tendency of resveratrol and CAY to reduce APAP-increased total bilirubin levels, both *in vitro* and *in vivo* (Fig. 1b).

Effects of CAY in comparison with RES treatment on NO_2^- in vitro and in vivo and oxidative stress parameters in APAP treated rats in vivo

Figures 2a and 2b demonstrate that APAP treatment produced moderate increase of lipid peroxidation as evidenced by the formation of TBARS and conjugated dienes (CD), respectively, as a result of oxidative stress in liver. Single APAP treatment significantly increased both TBARS and CD ($P<0.05$) in homogenate. CAY following APAP treatment slightly reduced only CD levels in contrast with RES, which reduced the levels of both markers. Moreover, APAP significantly increased medium NO_2^- levels ($P<0.001$) *in vitro*. On the other hand, NO_2^- plasma levels were not significantly affected by any treatment even though CAY and resveratrol slightly reduced it (Fig. 2c).

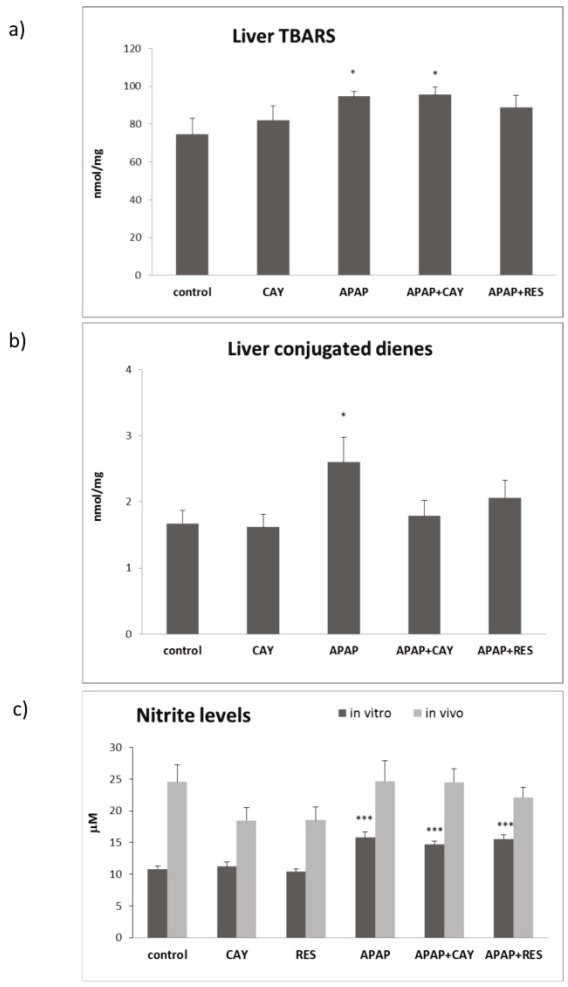

Fig. 2. Effect of specific SIRT1 activator, CAY10591 in comparison with resveratrol treatment in APAP-induced hepatocyte/liver injury on the formation of **a**) Thiobarbituric acid reactive substances (TBARS) and **b**) Conjugated dienes (CD) *in vivo*, and **c**) NO_2^- production *in vitro* and *in vivo*. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30 μM *in vitro*, 0.5 mg/kg *in vivo*); RES (Resveratrol: 20 μM *in vitro*, 30 mg/kg *in vivo*); APAP (Acetaminophen: 5 mM *in vitro*, 1 g/kg *in vivo*); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen and Resveratrol in the stated doses). Data are expressed as means ± SEM (n=6): * $P<0.05$, *** $P<0.001$ vs. respective control.

Effects of CAY in comparison with RES treatment on hepatocyte viability in vitro and relative SIRT1 activity in vitro and in vivo

Measurement of hepatocyte viability (using MTT test) in *in vitro* experiments and relative SIRT1 activity both *in vitro* and *in vivo* are shown in Figure 3. APAP treatment significantly reduced viability ($P<0.001$) of cultured hepatocytes in comparison to the untreated control group and the group treated only by RES or CAY. MTT test showed that RES and CAY did not have toxic effect on hepatocytes in cell culture and that CAY more

potently increased APAP-reduced hepatocyte viability (Fig. 3a). APAP also markedly reduced SIRT1 enzyme activity (by 34 % *in vitro* and 20 % *in vivo*) as shown in Figure 3b. Furthermore, RES and especially CAY increased SIRT1 activity compared to control and APAP treatments. The same trend was observed as *in vitro* as *in vivo*. In summary, the relationship between activity and viability was indirectly proportional.

Effect of RES, CAY and APAP on SIRT1 expression levels in rats

In comparison with SIRT1 activity mentioned above (Fig. 3b), there were no significant changes on the SIRT1 expression in Western blot analysis (Fig. 4). According to our analysis, single dose treatment with APAP, RES and CAY had no effect on the total endogenous amount of SIRT1 expression.

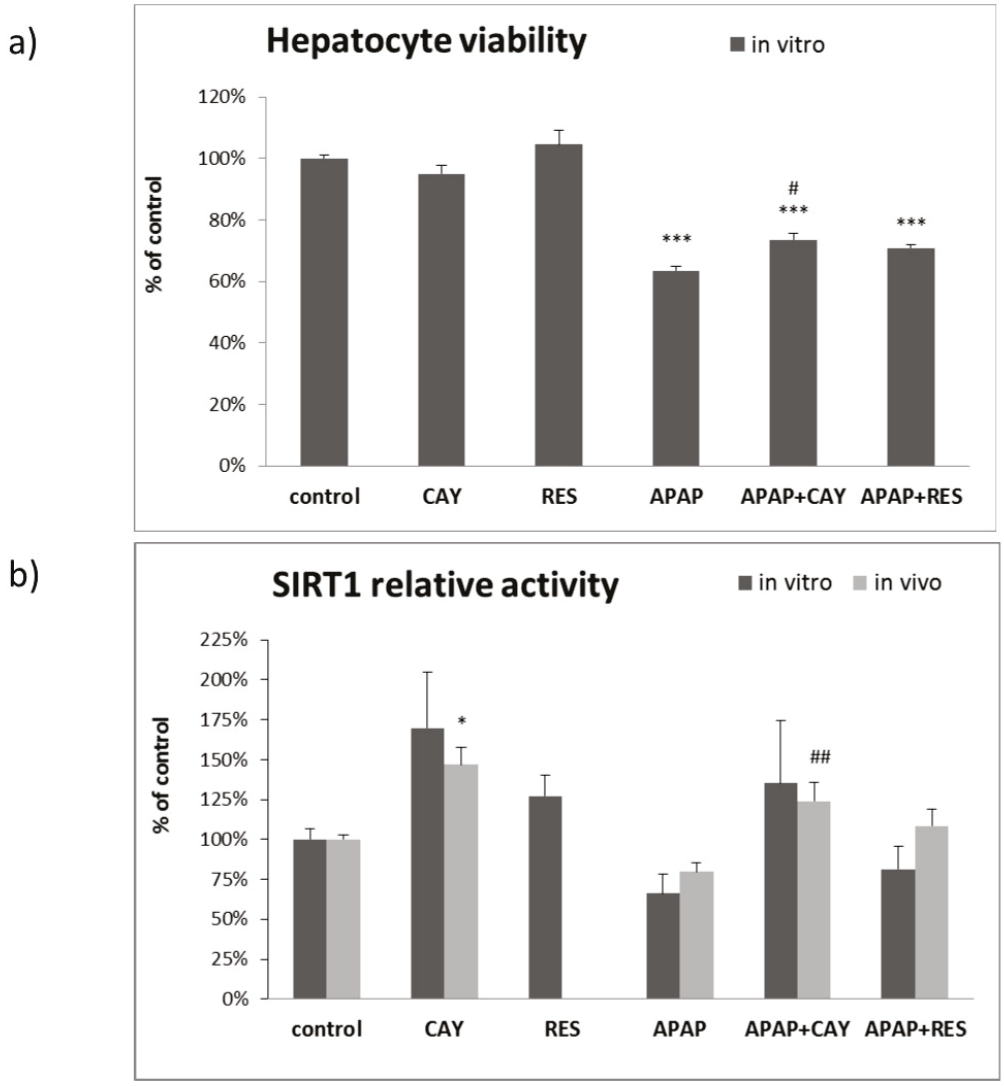

Fig. 3. Effect of specific SIRT1 activator, CAY10591, in comparison with resveratrol treatment in APAP-induced hepatocyte/liver injury on **a**) Hepatocyte viability in *in vitro* experiments and **b**) SIRT1 relative activity in *in vitro* and *in vivo* experiments. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30 μM *in vitro*, 0.5 mg/kg *in vivo*); RES (Resveratrol: 20 μM *in vitro*, 30 mg/kg *in vivo*); APAP (Acetaminophen: 5 mM *in vitro*, 1 g/kg *in vivo*); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen and Resveratrol in the stated doses). Data are expressed as means ± SEM (n=6 for MTT test and *in vivo* SIRT1 activity, n=3 for *in vitro* SIRT1 activity): * P<0.05, *** P<0.001 vs. respective control; # P<0.05, ## P<0.01 vs. respective APAP group.

Histological observations

The general structure of the liver parenchyma of all rats was well-preserved. Liver trabecules were arranged radially around the central vein and portal spaces were well visible (Fig. 5). No signs of steatosis, inflammation (except of rare small mononuclear infiltrates), hepatocellular necrosis or fibrosis were observed in the rat liver of control (Fig. 5a) and CAY

(Fig. 5b). However, there were visible several mitotic hepatocytes in otherwise normal liver parenchyma of these rats, especially in CAY treated ones (Fig. 5c). Histological changes in the liver induced by APAP were not significant (Fig. 5d). APAP caused slight increase in the appearance and number of apoptotic hepatocytes (Fig. 5e) and apoptotic bodies surrounded by a mononuclear infiltrate (Fig. 5f). No mitotic hepatocytes were found in the liver of APAP treated rats. Liver parenchyma had normal morphology after application of APAP followed by CAY (Fig. 5g) or resveratrol (Fig. 5h) to rats.

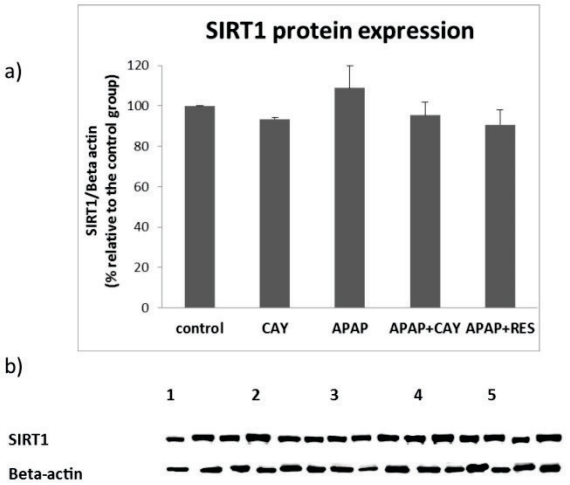

Fig. 4. Effect of specific SIRT1 activator, CAY10591, in comparison with resveratrol treatment in APAP-induced liver injury *in vivo* on **a**) quantification of SIRT1 expression levels by densitometry. The intensity of each panel was normalized to the intensity of corresponding beta-actin band. Control (24 hour-vehicle treated rats); CAY (CAY10591: 0.5 mg/kg); APAP (Acetaminophen: 1 g/kg); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen 1 g/kg and Resveratrol 30 mg/kg). Data are expressed as means ± SEM (n=3). **b**) Western blot images are shown as three samples of each treated group: 1. Control, 2. CAY, 3. APAP, 4. APAP+CAY, 5. APAP+RES.

Discussion

Our and other previous studies demonstrated that small polyphenolic molecules with antioxidant properties such as resveratrol reduced parameters of liver injury in fulminant hepatic failure induced by LPS/D-GAlN combination (Farghali *et al.* 2014, Kemelo *et al*. 2014, Lekić *et al.* 2013). We chose acute rat APAP intoxication followed by potentially hepatoprotective compounds RES and CAY for our present study. The liver impairment was much lower after APAP treatment than after LPS/D-GAlN and did not lead to fulminant hepatic failure. We used this model of mild hepatic impairment because it more resembles the human APAP-induced liver injury with potentially following pharmacological intervention. Despite it was shown that rats are resistant to APAP hepatotoxicity in compare to mice (hepatotoxic dose is 400-600 mg/kg for mice with contrast of 1-2 g/kg for rats) (McGill *et al*. 2012), some recent publications found that human hepatocytes are much more resistant to APAP toxicity than hepatocytes originated from other species (Jemnitz *et al*. 2008). Many factors (e.g. interspecies variability in expression and/or activity of metabolizing enzymes and membrane transporters, formation of NAPQI adducts, oxidative stress, disruption of mitochondrial function, protein nitrosylation, DNA damage among others) contribute to processes leading to APAP-induced liver damage. The electrophilic metabolite NAPQI, which is mainly produced by CYP2E1, is conventionally thought to initiate the toxic processes in liver. Surprisingly, some of CYP2E1 activity data do not support that only formation of reactive metabolite alone can explain interspecies variability after APAP intoxication (Jemnitz *et al.* 2015). Moreover, liver regeneration, which is final outcome of APAP-induced sublethal liver injury, begins at about 12 h and continue until about 72 h post-dosing and can thus attenuate the final biochemical and histological parameters of hepatotoxicity evaluated from samples collected on a single late time point (e.g. 24 h) (Jaeschke *et al*. 2013).

In the present study, APAP significantly increased ALT and total bilirubin (specific markers of liver injury) levels with the same trend *in vivo* as *in vitro*. RES and CAY were administrated after pretreatment of APAP, and both RES and CAY significantly reduced ALT and also total bilirubin levels in *in vivo* experiments and enhanced APAP-reduced hepatocyte viability *in vitro*. As expected and in accordance with other studies (Kučera *et al*. 2012, McGill *et al.* 2012), we did not observe marked hepatocyte necrosis in liver of APAP treated rats as evidenced from histological findings. Interestingly, there were visible several mitotic hepatocytes in otherwise normal liver parenchyma of CAY treated rats. It can be explained by induction of hepatocyte proliferation due to increased SIRT1 activation leading to regulation SIRT1/p53 signaling pathway (Braidy *et al*. 2011, Hwang *et al.* 2013, Jaeschke *et al.* 2013, Wang *et al.* 2015).

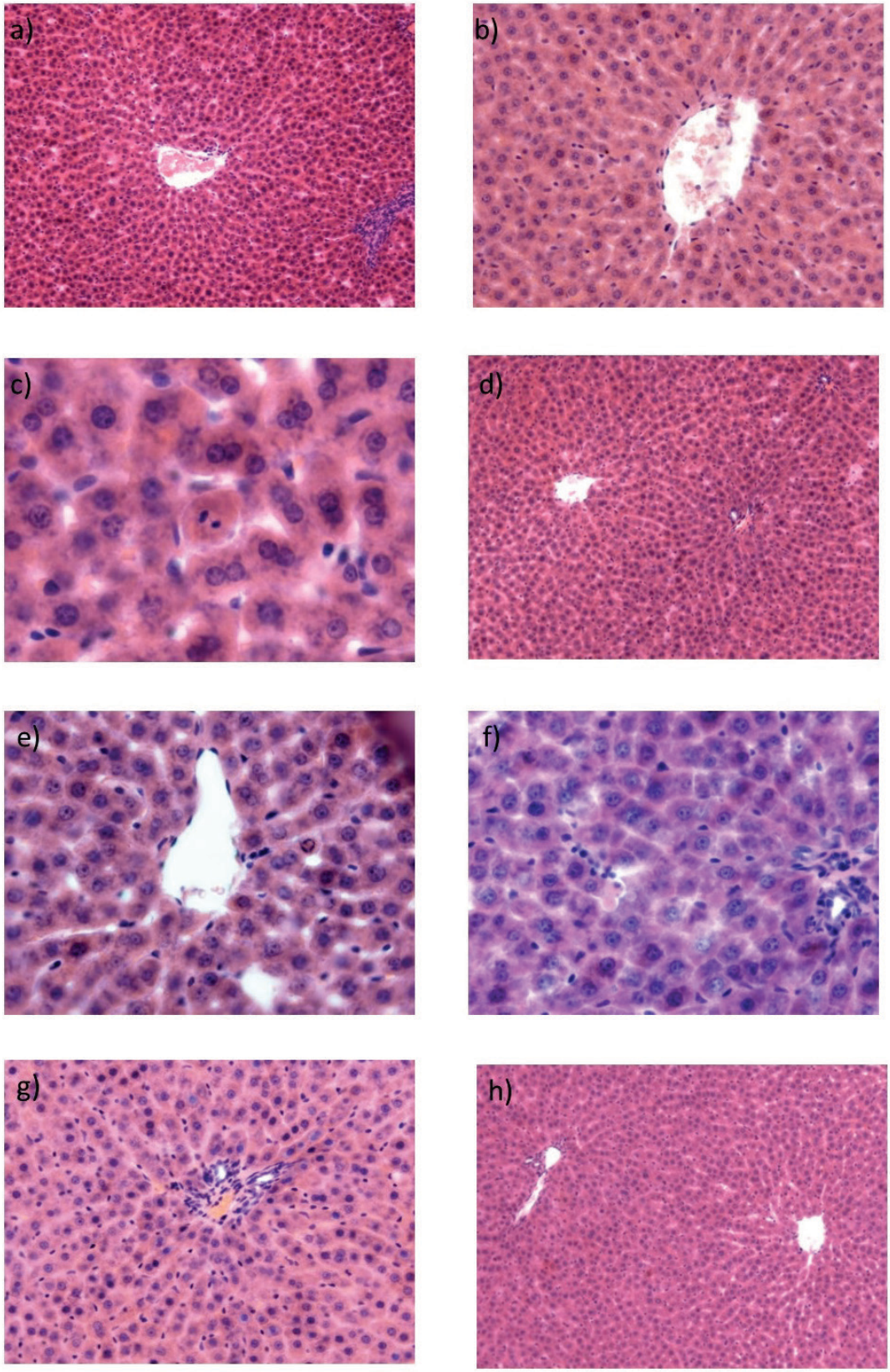

Fig. 5. Representative histopathological samples of livers taken from (**a**) control rats and animals treated with (**b,c**) CAY (CAY10591); (**d,e,f**) APAP (Acetaminophen); (**g**) APAP + CAY; and (**h**) APAP + RES (combination of Acetaminophen and Resveratrol) for 24 h. Hematoxylin and eosin (magnification x 200 with a detailed view in c,e,f).

In the case of RES, its hepatoprotetive effect may be due to its well-known antioxidant effects. Moreover, Wang *et al.* (2015) have found that repeated RES pretreatment of mice significantly increased SIRT1 protein expression and prevented APAP-induced hepatotoxicity revealed as decreased overall necrosis, possibly by inhibition of CYP-mediated APAP bioactivation, and regulation of SIRT1/p53 signaling pathway. RES can by this way promote hepatocyte proliferation and thus facilitate liver regeneration after APAP-induced liver injury. In the case of CAY, which is a selective activator of SIRT1, it can be hypothesized that its hepatoprotective effect may be due to the same SIRT1/p53 signaling pathway as RES. In contrast with study of Wang *et al.* (2015), there were no significant changes on the SIRT1 expression in Western blot analysis. This difference can be explained by only one application of lower dose (30 mg/kg) of RES in our experimental settings instead of several day lasting repeated high dose RES pretreatments in the study of Wang *et al*. (2015) that could induce SIRT expression. However, the SIRT1 activity was highly increased by RES and CAY addition to APAP both *in vivo* and *in vitro* that was accompanied by reduction of oxidative stress markers and hepatocyte/liver injury in our study. Furthermore, APAP reduced SIRT1 activity was accompanied simultaneous enhanced oxidative damage as evidences from increased TBARS, CD, and NO_2^- levels and pronounced liver and hepatocyte injury both *in vivo* and *in vitro*. Indeed, some recent studies have shown that SIRT1 is a redox sensitive molecule that can be inactivated by oxidative stress (Braidy *et al.* 2011, Hwang *et al.* 2013).

SIRT1 itself has multiple biological activities including gene silencing, stress resistance, apoptosis, regulation of cell proliferation, aging, inflammation, and acts as molecular sensor of nutritional status and protects against oxidative stress (Hwang *et al*. 2013, Kutinová Canová *et al.* 2011). However, it should be kept in mind that SIRT1 expression and activity can be modulated at different levels. It was shown that the age-related increasing SIRT1 expression in rat liver and other organs is accompanied by a simultaneous reduction of SIRT1 activity (Braidy *et al*. 2011). Moreover, RES is thought to activate SIRT1 allosterically (Farghali *et al.* 2013). It binds to the non-catalytic N-terminus of SIRT1 to cause a conformational change that lowers its Michaelis constant (Howitz *et al.* 2003). SIRT1 in turn deacetylates and suppresses transcription factors such as NF-κB responsible for induction of proinflammatory cytokines and pro-apoptotic factors (Yeung *et al.* 2004, Kemelo *et al.* 2014). Moreover, SIRT1 is also involved in regulation of SIRT1/p53 and other antiapoptotic (e.g. SIRT1/p66shc) signaling pathways (Shan *et al*. 2015, Wang *et al*. 2015). It can explain the almost complete absence of apoptotic hepatocytes in liver tissue sections of RES+APAP and CAY+APAP treated rats connected with enhanced SIRT activity unlike APAP alone.

In conclusion, resveratrol and CAY attenuated APAP-induced hepatotoxicity *in vivo* and *in vitro* in our study. Moreover, both drugs enhanced APAP-reduced SIRT1 activity but not SIRT1 expression. Therefore, our results show that modulation of the SIRT1 activity plays a role in hepatoprotection. To better understand the role of SIRT1 in hepatoprotection helped us novel specific SIRT1 activator CAY10591. Several other small molecules are being synthesized with higher pharmacologic potency compared with the polyphenolic SIRT1 activator resveratrol. In addition, class III histone deacetylases (sirtuins) are becoming increasingly recognized as important epigenetic drug targets in various diseases (Huber *et al*. 2011) thus suggesting modulation of sirtuin activity could provide an interesting and novel therapeutic option.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The authors would like to thank Libuse Slehobrova and Marcela Blazkeova for their skilful technical assistance. This work was supported by the institutional program PRVOUK-P25/LF1/2 and research grant GAUK 916314.

References

BAJT ML, COVER C, LEMASTERS JJ, JAESCHKE H: Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. *Toxicol Sci* **94**: 217-225, 2006.

BAUR JA: Biochemical effects of SIRT1 activators. *Biochim Biophys Acta* **1804**: 1626-1634, 2010.

BERRY MN, EDWARDS AM, BARRIT JG: *Isolated Hepatocytes Preparation, Properties and Applications*. Elsevier, Amsterdam, 1991.

BISHAYEE A, DARVESH AS, POLITIS T, MCGORY R: Resveratrol and liver disease: from bench to bedside and community. *Liver Int* **30**: 1103-1114, 2010.

BRAIDY N, GUILLEMIN GJ, MANSOUR H, CHAN-LING T, POLJAK A, GRANT R: Age-related changes in NAD^+ metabolism oxidative stress and Sirt1 activity in Wistar rats. *PLoS One* **6**: e19194, 2011.

BRUNE K, RENNER B, TIEGS G: Acetaminophen/paracetamol: a history of errors, failors and faile decisions. *Eur J Pain* **19**: 953-963, 2015.

CHANG H-C, GUARENTE L: SIRT1 and other sirtuins in metabolism. *Trends Endocrinol Metab* **25**: 138-145, 2014.

ČERNÝ D, KUTINOVÁ CANOVÁ N, MARTINEK J, HOŘÍNEK A, KMONÍČKOVÁ E, ZIDEK Z, FARGHALI H: Effects of resveratrol pretreatment on tert-butylhydroperoxide induced hepatocyte toxicity in immobilized perifused hepatocytes: Involvement of inducible nitric oxide synthase and hemoxygenase-1. *Nitric Oxide* **20**: 1-8, 2009.

DU K, MCGILL MR, XIE Y, BAJT ML, JAESCHKE H: Resveratrol prevents protein nitration and release of endonucleases from mitochondria during acetaminophen hepatotoxicity. *Food Chem Toxicol* **81**: 62-70, 2015.

FARGHALI H, ČERNÝ D, KAMENÍKOVÁ L, MARTÍNEK J, HOŘÍNEK A, KMONÍČKOVÁ E, ZÍDEK Z: Resveratrol attenuates lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats: role of nitric oxide synthase-2 and heme oxygenase-1. *Nitric Oxide* **21**: 216-225, 2009.

FARGHALI H, KUTINOVÁ CANOVÁ N, LEKIC N: Resveratrol and related compounds as antioxidants with an allosteric mechanism of action in epigenetic drug targets. *Physiol Res* **62**: 1-13, 2013.

FARGHALI H, KUTINOVÁ CANOVÁ N, ZAKHARI S: Hepatoprotective properties of extensively studied medicinal plant active constituents: possible common mechanisms. *Pharmaceut Biol* **53**: 1-11, 2014.

FEIGE JN, LAGOUGE M, CANTO C, STREHLE A, HOUTEN SM, MILNE JC, LAMBERT PD, MATAKI C, ELLIOTT PJ, AUWERX J: Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation, *Cell Metab* **8**: 347-358, 2008.

HOWITZ KT, BITTERMAN KJ, COHEN HY, LAMMING DW, LAVU S, WOOD JG, ZIPKIN RE, CHUNG P, KISIELEWSKI A, ZHANG LL, SCHERER B, SINCLAIR DA: Small molecule activators of sirtuins extend *Saccharomycetes cerevisiae* lifespan. *Nature* **425**: 191-196, 2003.

HUBER K, SUPERTI-FURGA G: After the grape rush: Sirtuins as epigenetic drug targets in neurodegenerative disorders. *Bioorg Med Chem* **19**: 3616-3624, 2011.

HWANG JW, HONGWEI Y, CAITO S, SUNDAR IK, RAHMAN I: Redox regulation of SIRT1 in inflammation and cellular senescence. *Free Radic Biol Med* **61**: 95-110, 2013.

HY, NI B, ENTZEROTH M, STUNKEL W: 12SIRT1 modulating compounds from high-throughput screening as anti-inflammatory and insulin-sensitizing agents to study biological effects of stimulated SIRT1 enzyme. *J Biomol Screen* **11**: 959-967, 2006.

INGAWALE DK, MANDLIK KM, NAIK SR: Models of hepatotoxicity and the underlying cellular, biochemical and immunological mechanism(s): a critical discussion. *Enviroment Toxicol Pharmacol* **37**: 118-133, 2014.

JAESCHKE H, WILIAMS CD, MCGILL MR, XIE Y, RAMACHANDRAN A: Models of drug-induced liver injury for evaluation of phytotherapeutics and other natural products. *Food Chem Toxicol* **55**: 279-289, 2013.

JEMNITZ K, VERES Z, MONOSTORY K, KOBORI L, VERECZKEY L: Interspecies differences in acetaminophen sensitivity of human, rat, and mouse primary hepatocytes. *Toxicol In Vitro* **22**: 961-967, 2008.

KAPLOWITZ N: Acetaminophen hepatoxicity: what do we know, what don't we know, and what do we do next? *Hepatology* **40**: 23-26, 2004.

KARTHIVASHAN G, ARUSELVAN P, FAKURAZI S: Pathways involved in acetaminophen hepatotoxicity with specific targets for inhibition/downregulation. *RSC Adv* **5**: 62040-62051, 2015.

KEMELO MK, WOJNAROVÁ L, KUTINOVÁ CANOVÁ N, FARGHALI H: D-galactosamine/lipopolysaccharide-induced hepatotoxicity downregulates sirtuin 1 in rat liver: role of sirtuin 1 modulation in hepatoprotection, *Physiol Res* **63**: 615-623, 2014.

KUČERA O, ROUŠAR T, STAŇKOVÁ P, HAŇÁČKOVÁ L, LOTKOVÁ H, PODHOLA M, ČERVINKOVÁ Z: Susceptibility of rat non-alcoholic fatty liver to the acute toxic effect of acetaminophen. *J Gastroenterol Hepatol* **27**: 323-330, 2012.

KUTINOVÁ CANOVÁ N, MARTINEK J, KMONIČKOVÁ E: Modulation of spontaneous and lipopolysaccharide-induced nitric oxide production and apoptosis by D-galactosamine in rat hepatocyte culture: the significance of combinations of different methods. *Toxicol Mech Methods* **18**: 63-74, 2008.

KUTINOVÁ CANOVÁ N, GAIER N, FARGHALI H: Perspectives on pharmacological and clinical benefits from sirtuin 1 activators in oxidative damage (in Czech). *Cas Lek Cesk* **151**: 187-189, 2012.

LEKIC N, KUTINOVÁ CANOVÁ N, HOŘÍNEK A, FARGHALI H: The involvement of heme oxygenase 1 but not nitric oxide synthase 2 in a hepatoprotective action of quercetin in lipopolysaccharide-induced hepatotoxicity of D-galactosamine sensitized rats. *Filoterapia* **87**: 20-26, 2013.

McGILL MR, WILLIAMS CD, XIE Y, RAMACHADRAN A, JAESCHKE H: Acetaminophen-induced liver injury in rats and mice: Comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. *Toxicol App Pharmacol* **264**: 387-394, 2012.

NAYAGAM VM, WANG X, TAN YC, POULSEN A, GOH KC, NG T, WANG H, SONG YEUNG F, HOBERG JE, RAMSEY CS, KELLER MD, JONES DR, FRYE RA, MAYO MW: Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* **23**: 2369-2380, 2004.

ROUŠAR T, PAŘÍK P, KUČERA O, BARTOŠ M, ČERVINKOVÁ Z: Glutathione reductase is inhibited by acetaminophen-glutathione conjugate in vitro. *Physiol Res* **59**: 225-232, 2010.

RUDERMAN NB, XU XJ, NELSON L, CACICEDO JM, SAHA AK, LAN F, IDO Y: AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* **298**: E751-E760, 2010.

SHAN W, GAO L, ZENG W, HU Y, WANG G, LI M, ZHOU J, MA X, TIAN X, YAO J: Activation of the SIRT1/p66shc antiapoptosis pathway via carnosic acid-induced inhibition of miR-34a protects rats against nonalcoholic fatty liver disease. *Cell Death Dis* **6**: e1833, 2015.

SILVA JP, WAHLESTEDT C: Role of Sirtuin 1 in metabolic regulation. *Drug Discov Today* **15**: 781-791, 2010.

WANG Z, JIANG Y, FAN X, TAN H, ZENG H, WANG Y, CHEN P, HUANG M, BI H: Hepatoprotective effect of resveratrol against acetaminophen-induced liver injury is associated with inhibition of CYP-mediated bioactivation and regulation of SIRT-p53 signaling pathways. *Toxicol Lett* **236**: 82-89, 2015.

YANG SR, WRIGHT J, BAUTER M, SEWERYNIAK, KODE A, RAHMAN I: Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. *Am J Physiol Lung Cell Mol Physiol* **292**: L567-L576, 2007.

Differentiated modulation of signaling molecules AMPK and SIRT1 in experimentally drug-induced hepatocyte injury

Lea Njeka Wojnarová, Nikolina Kutinová Canová*, Mahak Arora, Hassan Farghali

Institute of Pharmacology, First Faculty of Medicine, Charles University and General University Hospital in Prague

Brief summary

The study originality consists in the use of mutual combinations of AMPK and SIRT1 activators and inhibitors in well-established experimental model of drug/acetaminophen-induced primary rat hepatocyte injury.

Unlike others, our results suggest that SIRT1-mediated hepatoprotective effects could be partially AMPK independent providing the differentiated modulation of AMPK and SIRT1 activity for hepatocyte injury treatment.

* Name and address of corresponding author:

Nikolina Kutinová Canová, M.D., Ph.D.;

Institute of Pharmacology, 1st Faculty of Medicine, Charles University

Albertov 4, 128 00 Prague 2, Czech Republic

Tel/Fax: +420 224968104

E–mail: ncano@lf1.cuni.cz

ABSTRACT

Aim: Currently available medicines have little to offer in terms of supporting the regeneration of injured hepatic cells. Previous experimental studies have shown that resveratrol, a less specific activator of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1), can effectively attenuate acute liver injury. Therefore, the aim of herein presented experimental study was to clarify whether modulation of AMPK and SIRT1 activity can modify drug/paracetamol (APAP)-induced hepatocyte damage *in vitro*.

Methods: Primary rat hepatocytes were pretreated with mutual combinations of specific synthetic activators and inhibitors of SIRT1 and AMPK and followed by toxic dose of APAP. At the end of cultivation, medium samples were collected for biochemical analysis of alanine-aminotransferase activity and nitrite levels. Hepatocyte viability, thiobarbituric reactive substances, SIRT1 and AMPK activity and protein expression were also assessed.

Results: The harmful effect of APAP was associated with decreased AMPK and SIRT1 activity and protein expression alongside enhanced oxidative stress in hepatocytes. The addition of AMPK activator (AICAR) or SIRT1 activator (CAY10591) significantly attenuated the deleterious effects of AMPK inhibitor (Compound C) on the hepatotoxicity of APAP. Furthermore, CAY10591 but not AICAR markedly decreased the deleterious effect of APAP in combination with SIRT1 inhibitor (EX-527).

Conclusion: Our findings demonstrate that decreased AMPK activity is associated with the hepatotoxic effect of APAP which can be significantly attenuated by the administration of the SIRT1 activator. These findings suggest that differentiated modulation of AMPK and SIRT1 activity could therefore provide an interesting and novel therapeutic opportunity in the future to combat hepatocyte injury.

Keywords: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR); adenosine monophosphate protein kinase (AMPK); CAY10591; enzyme activation; hepatocyte protection; sirtuin 1 (SIRT1)

INTRODUCTION

Liver diseases represent a significant cause of morbidity and mortality and account for approximately 2 million deaths per year worldwide[1]. In addition, liver injury belongs to the reason for black box warnings, drug non-approval, or removing an approved drug from the market[2,3]. Drug-induced liver injury (DILI) is a rare complication but continues to increase as a major cause of acute hepatitis. Population-based studies suggest that the overall incidence of DILI may be as high as 10 to 15 cases per 100,000 patient-years[1,3,4]. DILI can present clinically with multiple manifestations such as acute hepatitis, cholestasis and jaundice, nodular regenerative hyperplasia, or sinusoidal obstruction syndrome[5]. Many drugs inducing severe DILI have been reported to cause an enhanced reactive oxygen species/adenosine triphosphate (ROS/ATP) ratio in primary human hepatocytes, indicating that oxidative stress is followed by hepatic cellular damage, one of the most important mechanisms of DILI[6]. Among them, acetaminophen (APAP, paracetamol) which is one of the safest analgesic and antipyretic drugs at recommended therapeutical doses, however, can cause severe hepatic damage and acute liver failure commonly known as APAP-induced liver injury (AILI) at high, toxic, doses[4]. Therefore, APAP overdose in rodents is frequently used as a hepatotoxic model to test the hepatoprotective potential of herbal and other compounds[5,7,8].

Many plants have emerged as a great source of pharmaceutical products. It has been reported in the publication of Bhaargavi *et al.* (ref.[9]) that about 160 phytoconstituents from 101 medicinal herbs have hepatoprotective action. Many plants have been used to mitigate diverse liver diseases, of which the favorite ones include for example silymarin from *Silybum marianum* and curcumin from *Curcuma longa*[9,10]. Today, the main problem with herbal medicines is that many plants are consumed as polyherbal formulations where multiple constituents work synergistically. The active component responsible for the pharmacological and therapeutical effects in most cases remains unknown. So today, the worldwide research of potent hepatoprotective drugs have led towards the screening of numerous plant products, their purification and characterization of various bioactive compounds, and searching for their probable mode of action[11].

Previous experimental studies on resveratrol[12,13], silymarin[14], curcumin[15], and quercetin[16] at our institute have shown definite hepatoprotective properties with alteration in some intracellular signaling molecules which contributed to these effects. In addition, many other studies have suggested that polyphenol resveratrol (2,3,40-trihydroxystilbene) has anti-

inflammatory, antioxidant, anti-aging, and anti-carcinogenic properties that might be pertinent to chronic diseases and/or longevity in humans. Resveratrol, among others, has been described[17] as an activator of silent information regulator T1 (SIRT1) that can also increase adenosine-5´-monophosphate-activated protein kinase (AMPK) phosphorylation and reduce the oxidative stress biomarkers in laboratory settings[10,18-20]. However, there is still an open question of whether resveratrol can activate SIRT1 directly or indirectly through AMPK or act independently[21].

The sirtuins are a family of evolutionarily conserved NAD^+-dependent histone/protein deacetylases that are expressed in mammalian cells and have been studied in many tissues, including liver, skeletal muscle, adipose tissue, pancreas (β-cells), brain, and endothelium. A common feature about the activity of sirtuins as fuel-sensing molecules is their dependence on intracellular levels of nicotinamide adenine dinucleotide (NAD) in its oxidized (NAD^+) or reduced form (NADH). Seven human sirtuin isoforms (SIRT1–7) were identified. SIRT1 has been found to enhance insulin sensitivity and secretion, decrease oxidative stress and inflammatory activity, and help in glucose and lipid metabolism[22]. AMPK is a fuel-sensing enzyme that is activated by a decrease in a cell's energy state as reflected by an increased AMP/ATP ratio and/or ADP/ATP ratio. AMPK plays a key role in many physiological processes as homeostasis of glucose/lipid, insulin signaling, body weight, food intake, and mitochondrial biogenesis and it is a big therapeutical player in many metabolic diseases such as diabetes or obesity, or tumorigenesis[23,24]. There are some similarities between AMPK and SIRT1 because since AMPK and SIRT1 have a regulatory impact on each other and share many common target molecules[18,19,25,26]. Therefore, we focused our research work on these two molecules and their signaling pathways.

The objectives of this experimental study were to link up to the previous publication Wojnarová *et* al. 2015 (ref.[13]) and explain in more detail the interconnection between AMPK and SIRT1 by the assistance of their synthetic activators and inhibitors in primary rat hepatocytes and mainly to reveal the exact role of AMPK in APAP-induced hepatotoxicity. The effect of the selective agonists and/or antagonists in their original mutual combinations haven´t been evaluated in the previous experimental studies.

MATERIALS AND METHODS

Materials

5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine (Compound C), 6-Chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide (EX-527) and 6-Chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide (CAY10591) were obtained from Cayman Pharma (Cayman chemical company, USA). Collagenase type IV was coming from Sevapharma (Prague, Czech Republic). Bio-Rad protein DC assay dye reagent, Laemmli buffer, and β-mercaptoethanol were from Bio-Rad (Prague, Czech Republic). Water for injection 100% w/v was obtained from Baxter (Prague, Czech Republic). Acetaminophen, trypan blue, William´s medium E, penicillin/streptomycin, glutamine, bovine insulin, foetal bovine serum (FBS), phosphate-buffered saline (PBS), tris-HCl, NaF (sodium fluoride), Nonidet P40, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), N,N,N′,N″-tetramethylethylenediamine, sulfanilamide, naphtylethylendiamine, trihydrogenphosphoric acid, bovine serum albumin (BSA), anti-mouse and anti-rabbit IgG (whole molecule) peroxidase antibodies and mouse monoclonal anti-beta-actin antibody, protease and phosphatase inhibitor cocktails, and other standard chemicals were purchased from Sigma-Aldrich (Merck KGaA, Czech Republic). SirT1 (1F3) mouse mAb antibody and AMPKα and phospho-AMPKα (Thr172) rabbit mAb antibodies were from Cell Signaling Technology through BioTech (Prague, Czech Republic). Non-fat dry milk was also from BioTech.

Animals

Experiments were carried out by hepatocytes isolated from 12-14 week old outbred male Wistar rats provided by Velaz (Lysolaje, Czech Republic). Rats were maintained under standard conditions (12-h light/dark cycle, 22±2 °C ambient temperature and 50±10% relative humidity) and provided with tap water and fed by standard granulated diet *ad libitum*.

Ethics Statement

All rats used received humane care in compliance with the general Guiding Principles in the Use of Animals in Charles University, the First Faculty of Medicine. The animal experimental protocol was approved by the Ministry of Agriculture and Ministry of Education, Youth and Sports of the Czech Republic and by the Faculty Ethical Committee under No. MSMT-9445/2018-8 and 70030/2013-MZE-17214, respectively.

Isolation, culture, and treatment of primary rat hepatocytes

Hepatocytes were isolated from untreated rats using the standard, two-phase collagenase perfusion method[27]. The hepatocytes viability was greater than 85% as assessed by the trypan blue exclusion method. Cells were then seeded on collagen-coated polystyrene Nunclon™ dishes (SchoellerParma, Prague, Czech Republic) at a density of 104,000 viable cells.cm^{-2}. They were incubated in complete medium (William´s medium E, 1% penicillin/streptomycin, 1%glutamine, 0.06% insulin, 5% FBS) at 37°C in a humid atmosphere with 5% CO_2 throughout the study[28]. Unattached cells were removed 3 hours after seeding and the remaining hepatocytes were further cultured in a fresh complete medium overnight. Hepatocytes were then pretreated with fresh medium containing either DMSO (0.1 % of final concentration) or SIRT1 and AMPK modulators at concentrations listed in Table 1. 30 min later, acetaminophen (APAP, 12.5 mM) was added to respective hepatocyte cultures. After 4 or 24 hours, medium samples were collected for biochemical analysis, and hepatocyte viability was assessed by MTT test. At the end of experiments, some cultured hepatocytes were washed by cooled PBS and lysed in RIPA buffer (2 M TRIS, 5 M NaCl, 0.5 M EDTA, NP-40, NAF) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Merck KGaA) and homogenized with an electric homogenizer. The chilled (4 °C) cell homogenates were centrifuged at 14,000 g for 10 min and resulting supernatants were sampled and stored at -80 °C for further measurements.

MTT/cell viability test

Tetrazolium salt (MTT) was used both to assess the optimal non-toxic concentration of drugs suitable for our study (Table 1) and to measure hepatocyte viability at the end of *in vitro* experiments following test description by Kutinová Canová *et al.* (ref.[29]).

Biochemical analysis

ALT (alanine aminotransferase) catalytic concentrations in the samples of treated cultivation media were measured using a customized diagnostic kit according to manufacturer´s instruction (Vian Diagnostics, Prague, Czech Republic).

Medium nitrite (NO_2^-), the stable end-product of nitric oxide (NO) oxidation, was detected spectrophotometrically by using Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% trihydrogenphosphoric acid). The absorbance at 540 nm was recorded and the NO_2^- values were subtracted from $NaNO_2$ standard curve[28].

Evaluation of oxidative stress parameters

The analysis of thiobarbituric acid reactive substances (TBARS) in hepatocyte lysates was carried out according to Farghali *et al.* (ref.[12]). This method uses the reaction of lipid peroxidation products, especially malondialdehyde (MDA) and thiobarbituric acid (TBA), which lead to the formation of MDA-TBA2 adducts named TBARS. TBARS (red-pink color) is determined spectrophotometrically[30]. The results were calculated as the molar amount per 1 mg of lysate protein (assessed by Bio-Rad protein DC assay).

Caspase-3 ELISA assay

The instructions by the manufacturer of Rat Caspase 3 ELISA Kit (LifeSpan BioSciences, Inc, Seattle, USA) were followed to detect caspase-3 proenzyme in hepatocyte lysates.

SIRT1 activity assay

Deacetylase activity of SIRT1 enzyme was determined in lysed hepatocytes using commercial fluorometric SIRT1 Assay Kit (Sigma-Aldrich) as described previously[13].

Immunoblotting

The cell lysates were mixed (1:1) with sample buffer (2 x Laemmli buffer 950 µL + 50 µL of β-mercaptoethanol) and then heated for 10 min at 90 °C. Proteins (assessed by Bio-Rad protein DC assay) from the cell samples were separated on 10% SDS-acrylamide gel (TGX™ FastCast™ Acrylamide Solutions by Bio-Rad) and transferred to a nitrocellulose membrane (Hybond ECL, Cytiva, Prague, Czech Republic) by electrophoresis. Membranes were blocked for 2 hours with 5% non-fat milk or 5% BSA in 10x Tris Buffered Saline with Tween 20 (TBST). Membranes were then washed in TBST washing buffer and incubated with either mouse primary antibody against SIRT1 (1:1,000), beta actin primary antibody (3:15,000), rabbit primary antibody against AMPK (1:1,000) or pAMPK (1:1,000) and followed with corresponding secondary rabbit antibody anti-mouse or anti-rabbit IgG HRP conjugate (3:20,000 or 1:80,000). For visualization, Super Signal West Pico Chemiluminescent Substrate (GeneTiCA, Prague, Czech Republic) was used. Bands were exposed by the Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic). Optical densities of SIRT1, pAMPK, AMPK, and beta-actin bands were normalized by the appropriate loading control and then to the mean of the corresponding control group[16].

Statistical analysis

Data are stated as means ± SEM (standard error of the mean) from at least 3 independent *in vitro* experiments. All the data passed the normality test and the significance of differences between the groups was assessed by one-way analysis of variance (ANOVA) followed by Turkey-Kramer comparison test (Graph-Pad Prism 4.03, Graph Pad Software, San Diego, CA, USA). *P*-value less than 0.05 was reckoned to indicate a statistically significant difference.

RESULTS

Effects of specific AMPK modulators (activator – AICAR and inhibitor - Compound C) and SIRT1 specific activator (CAY10591) on APAP-induced primary rat hepatocyte injury and oxidative stress markers

The rate of hepatocyte damage was assessed as cell viability (by using MTT test, Fig. 1A) and ALT release from hepatocytes to cultivation medium (Fig. 1B). Moreover, oxidative stress markers like the end products of inducible nitric oxide synthesis represented by medium NO_2^- levels (Fig. 1C), and TBARS formed in hepatocytes (Fig. 1D) were evaluated.

Single APAP treatment significantly reduced the viability of cultured hepatocytes and increased ALT, nitrite, and TBARS levels (Fig. 1). Our experiments have shown that the AMPK inhibitor, Compound C (CC), significantly amplified APAP-induced hepatotoxic effect in all observed parameters. Interestingly, AICAR and CAY10591 pretreatments lowered the hepatotoxic and pro-oxidative effects of the APAP+CC combination. The cell death induced by APAP was related to necrosis rather than apoptosis as evidenced by unaffected caspase-3 levels (Fig. 1E). Interestingly, CC in combination with APAP significantly decreased caspase-3 proenzyme suggesting cleavage of inactive pro-caspase-3 to active caspase-3 and induction of apoptosis.

Effects of specific SIRT1 modulators (activator – CAY10591 and inhibitor – EX-527) and specific AMPK activator (AICAR) on APAP-induced primary rat hepatocyte injury and oxidative stress markers

Pretreatment with EX-527, an inhibitor of SIRT1, slightly enhanced APAP toxicity (Fig. 2). Above that, the addition of CAY10591 significantly decreased the toxic effect of combination EX-527+APAP. On the other hand, AICAR mildly lowered the ALT release (Fig. 2C) from cultured hepatocytes induced by the combination of EX-527+APAP but did not increase hepatocyte viability (Fig. 2A).

Similarly, TBARS levels (Fig. 2D) imitated previously mentioned results on cell viability (Fig. 2A) and ALT levels (Fig. 2C). Mainly, CAY10591 but not AICAR pretreatment significantly suppressed the formation of TBARS markedly induced by the combination of EX-527+APAP. Neither CAY10591 nor AICAR had a significant effect on the highly increased nitrite production by hepatocytes after EX-527+APAP application (Fig. 2B).

Effects of acetaminophen (APAP), specific modulators of AMPK (activator – AICAR and inhibitor - Compound C) and SIRT1 (activator - CAY10591 and inhibitor EX-527) on AMPK activity *in vitro*

Our results from *in vitro* experiments demonstrate that the hepatotoxic effect of APAP was coupled with a significant decrease in AMPK activity (Fig. 3). This was apparent already after 4 hours of hepatocyte incubation when AICAR and CAY10591 in combination with APAP significantly increased AMPK activity and CC alone decreased it as suggested (Fig. 3A). However, the suppression of AMPK activity by APAP was not further influenced by neither AMPK nor SIRT1 modulators after 24 hours (Fig. 3B, C).

Effects of acetaminophen (APAP), specific modulators of SIRT1 (activator – CAY10591 and inhibitor – EX-527) and specific AMPK activator (AICAR) on SIRT1 activity and protein expression

APAP significantly inhibited the deacetylase activity and expression of SIRT1 in cultured hepatocytes incubated for 4 and 24 hours, respectively (Fig. 4). As expected, CAY10591 but not AICAR significantly increased SIRT1 activity, whereas EX-527 and even AMPK inhibitor (Compound C) markedly decreased it (Fig. 4A). Interestingly, the addition of AICAR to EX+APAP further decreased SIRT1 protein levels (Fig. 4B).

DISCUSSION

In most cases, liver diseases are associated with inflammation and oxidative stress that can lead to the destruction of liver parenchyma, fibrosis, and cirrhosis with loss of liver function[31,32]. APAP-raised oxidative stress and mitochondrial dysfunction play the key role also in the pathogenesis of acute APAP-induced liver injury[33]. Concurrently, hepatotoxicity caused by APAP overdose is a major clinical problem in a number of Western countries[34]. Despite the clinical significance of human APAP toxicity, the only one existing approved antidote is N-acetylcystein (NAC). Unfortunately, NAC therapy has some limitations. It was observed that NAC protects hepatocytes by scavenging the reactive adduct of APAP (N-acetyl-p-benzoquinone imine, NAPQI) in the cytosol and ROS/peroxynitrite inside the mitochondria. Moreover, NAC increases mitochondrial ATP production[35]. Newer possible pharmacotherapeutic interventions have alternate its mechanisms of action, mainly targeting mitochondrial dysfunction[34]. In addition, hepatoprotective effect has been also demonstrated on metformin, first-line treatment in type 2 diabetes mellitus. Several animal studies really

demonstrated that metformin attenuates APAP-induced liver injury by not otherwise specified antioxidant properties or probably through inhibition of c-Jun N-terminal kinase (JNK) signaling along with stimulation of hemeoxygenase (HO)-1 expression, resulting in hepatoprotection against oxidative stress[34,36,37]. Although the fundamental mechanism of metformin has not been fully clarified its dual activating effect on AMPK/SIRT1 should be taken into the consideration. It was demonstrated that metformin decreases liver gluconeogenesis and ketosis-conveyed inflammatory response through activation of AMPK protein expression leading to SIRT1 induction in mice and porcine hepatocytes, respectively[38,39]. Other works rather, however, showed that metformin reduces lipid accumulation by SIRT1 stimulation independently of AMPK (ref.[40]) or by acting primarily through AMPK independently of SIRT1 when increasing SIRT1 activity simultaneously[41]. Similarly, earlier studies of our research group have shown that another less specific SIRT1 activator, resveratrol, can effectively attenuate acute liver injury, as demonstrated by the quantification of serum ALT/AST levels, oxidative stress parameters, and via histological examination[12,13]. In addition, it was previously reported that resveratrol has also the ability to activate AMPK and this activation is much faster than generally required for SIRT1 activation even in hepatic cell models. Moreover, many experimental studies have shown that the effect of resveratrol is crucially dependent on AMPK activation[32,42,43]. Evidence for this was obtained in the AMPKγ3 knock-out mouse model where defective AMPK prevents the activation of SIRT1 upon resveratrol treatment[43]. Moreover, SIRT1 and AMPK have been shown to play many similar roles and sharing many target molecules[26].

Therefore, the current study aimed to investigate the regulation of the SIRT1/AMPK pathway *in vitro* and to reveal their possible protective effect in drug-induced acute hepatotoxicity. For this purpose, we used small synthetic molecules - CAY10591 (CAY, activator of SIRT1), EX-527 (EX, SIRT1 inhibitor), AICAR (AMPK activator), and Compound C (CC, AMPK inhibitor) and evaluated effects of their original mutual combinations in primary rat hepatocytes cultured with APAP. Because APAP-induced liver injury has become a standard model frequently used in pharmacology and toxicology to test the hepato-protective potential of different compounds and moreover, it can be rapidly triggered by a single dose[8], we used this model for the study presented here. Our current experiments confirmed the results of early studies that the cell death induced by APAP is related to necrosis rather than apoptosis because caspase-3 levels were not affected. In this regard, Ramachandran and Jaeschke reported that it

is not apoptosis but oncotic necrosis which is a respective form of cell death within APAP-induced liver injury both *in vitro* and *in vivo*[44,45].

Here presented results demonstrate that the hepatotoxic effect of APAP is associated with a significant decrease in AMPK activity throughout *in vitro* experiments. The same results were observed by Kang *et* al. 2016 (ref.[46]). They used rat and human hepatocytes cultured in a collagen sandwich and treated by APAP alone or combination of APAP+AICAR. They revealed that APAP down-regulates and deactivates AMPK and thus inhibits AMPK downstream effects (e.g. autophagy, fusion and mitochondrial biogenesis) leading to cellular stress and damage which could be prevented and reversed by AICAR[46]. AMPK is a serine-threonine kinase heterotrimer that is composed of a catalytic α-subunit (α1 and α2) and two β and γ regulating subunits, which are allosterically activated by low levels of adenosine triphosphate (ATP) and high levels of adenosine monophosphate (AMP)[47]. The alpha subunit is responsible for switching the phosphate of ATP to target proteins[48]. Furthermore, AMPK sets in motion alterations in mitochondrial biogenesis and function that could more chronically intensify the efficiency of a cell to give rise to ATP and weaken oxidative stress and other potentially harmful cellular events[43]. It was found that APAP-induced liver injury causes hepatocyte depletion of ATP by inhibiting mitochondrial function. Hwang *et al.* (ref.[47]) observed that APAP administration declines active phosphorylation of the Thr172 active site of AMPK, following a model resembling to that observed with ATP loss. They hypothesized that ATP depletion caused by APAP might correlate with inhibition of AMPK activity. Further explanation could be the duration of time after administration of APAP[47].

Compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) also known as dorsomorphin, is the only small ATP-competitive AMPK inhibitor that has been broadly utilized to study the AMPK signaling pathway. Our experiments have demonstrated that the AMPK inhibitor, CC, significantly amplified APAP-induced hepatotoxic effect in all observed parameters. These results are consistent with article of Jiang *et* al. 2012 (ref.[49]) who revealed that treatment with CC aggravated APAP-induced hepatotoxicity in mice by inactivating AMPK and abolished the hepatoprotective effect of medicinal fungus (*Sanghuangporus sanghuang, SS*) in this *in vivo* model. Further studies confirmed that AMPK plays a key role in the protection against APAP-induced liver injury[49,50]. Furthermore, it was shown that inhibition of AMPK activity by CC or by transfection with a dominant negative form of AMPK near entirely suppressed autophagy in hepatocytes[51]. It can be explained by the direct effect of AMPK on the mammalian target of rapamycin (mTOR). mTOR among others

inhibits autophagy, which is a crucial catabolic process in the cell including hepatocytes[52]. The activation of AMPK inhibits mTOR and thus increasing autophagy[53]. When autophagy was enhanced by treatment with rapamycin, APAP-induced necrosis was significantly inhibited in cultured primary hepatocytes and mouse liver[54]. As autophagy and apoptosis are interrelated and play important role in liver injury[55], we can hypothesize that inhibition of autophagy by CC due to AMPK inhibition could lead to caspase-3 activation with consequent apoptosis and the intensification of APAP-induced hepatotoxicity in our study. The role of AMPK in this process can be supported by the fact that the AMPK activator, AICAR, reversed the detrimental effect of CC on APAP-induced hepatotoxicity.

Interestingly, we have observed that pretreatment both with AICAR (aminoimidazole-4-carboxamide riboside), adenosine analogue that selectively activates AMPK, and SIRT1 synthetic activator, CAY10591, significantly increased the AMPK activity already after 4 hours of hepatocyte incubation with APAP. However, AMPK activity was not influenced or only slightly increased with AICAR and CAY10591, respectively, after 24 hours. One of the explanations of this time-dependent trend could be the short half-life of AICAR in cells[56] and maybe also similar for CAY10591. The explanation for CAY10591-enhanced AMPK activation could be that SIRT1 deacetylates the AMPK kinase LKB1 (liver kinase B1), leading to elevated phosphorylation and activation of AMPK[20,43]. Other important and shared targets are peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1α (PGC-1α) and nuclear factor kappa B (NF-κB), a central mediator of pro-inflammatory signaling pathways precipitated by cytokines[31]. It has been demonstrated that the activated AMPK could stifle NF-κB signaling through its downstream target molecules such as SIRT1, Forkhead box O (FOXO), and PGC-1α, and hereafter diminish the expression of inflammatory factors[47,57]. The relationship between NF-κB and SIRT1 is antagonistic, decreased nuclear SIRT1 level/activity increases NF-κB RelA/p65 activity and amplifies pro-inflammatory gene expression[32,58]. Interestingly, this relationship was confirmed in the publication of Rada *et al.* (ref.[2]) which revealed that *in vivo* administration of the NF-κB inhibitor defended against APAP-mediated acute hepatotoxicity.

According to our results, the hepatotoxic effect of APAP was associated with decreased SIRT1 activity and protein expression and enhanced oxidative stress. Previously, we demonstrated that APAP 24-hour treatment reduced rat hepatocyte SIRT1 enzyme activity (by 34 % *in vitro* and 20 % *in vivo*) even at lower doses that did not affect SIRT1 protein expression[13]. The same trend was also reported in the publication of Rada *et al.* (ref.[2]) where

SIRT1 protein levels were decreased in the liver of humans and mice in APAP-induced liver injury. Hypoxia, nutrient deprivation, DNA damage, and oxidative stress have been reported to control SIRT1 expression at both transcriptional and post-translational levels. In addition, earlier studies reported that liver specific SIRT1 deficiency caused an increase in ROS production[59,60]. Moreover, SIRT1 regulates the levels of inflammation and protects against oxidative stress which plays a key role in the pathogenesis of DILI where the overproduction of ROS, including free radicals, and reactive nitrogen species (RNS) can lead to damage of cellular components[61]. In accordance with that are our results demonstrating that pretreatment with the activator of SIRT1 significantly suppressed oxidative stress (e.g. the formation of TBARS) induced by APAP alone and in combination with SIRT1 inhibitor.

To investigate what and how important role does SIRT1 plays in the process of hepato-protection we performed also experiments with EX-527. EX-527 is widely used as a SIRT1 inhibitor both *in vitro* and *in vivo* with high potency and significant isoform selectivity[62]. Ex-527 inhibits Sirt1 ~100-fold more potently than Sirt2 and Sirt3. Density analysis suggests that EX-527 blocks catalysis by occupying SIRT1's C-pocket preventing the release of O-acetyl-ADP-ribose[63]. Our current data implied that pretreatment with EX-527 only slightly enhanced APAP toxicity. Our Western blot analysis surprisingly showed that EX-527 down-regulated SIRT1 expression. Besides that, the combination of SIRT1 inhibitor and APAP treatment slightly aggravated SIRT1 protein levels regardless addition of SIRT1 activator – CAY and especially AMPK activator – AICAR. Above that, the addition of CAY10591 significantly decreased the toxic effect of combination EX+APAP suggesting that primarily a change in catalytic activity rather than SIRT1 protein expression plays a role in the hepatoprotective action of SIRT1 modulators against APAP-induced hepatotoxicity[21]. Likewise, we hypothesized that the addition of AICAR in the combination with EX-527+APAP could increase the expression of SIRT1 protein which was not confirmed. Despite downregulation of SIRT1 expression, administration of this genuine combination (e.g. AICAR+EX+APAP) was not accompanied by worsening of hepatotoxicity, nonetheless, it would be interesting to determine whether decreased SIRT1 expression was preceded by its increased activity induced by AICAR as a negative feedback loop. One of the possible mechanisms how the AMPK could activate the SIRT1 is through an indirect increase in cellular NAD^+ levels[43]. Reciprocally, this is consistent with our results demonstrating that CC alone significantly reduced SIRT1 activity. Therefore, the above discussion illustrates that AMPK and SIRT1 pathways are closely related.

Hence, pharmacologic modulation of AMPK and SIRT1 activity could be a future major step in the understanding of DILI.

CONCLUSIONS

By using *in vitro* experimental model of drug-induced acute hepatotoxicity, we have demonstrated that the toxic effect of APAP on primary rat hepatocytes is associated with significantly reduced AMPK activity, SIRT1 activity and protein expression, and increased oxidative stress which is in accordance with discussed literature. Our experiments have surprisingly shown that the AMPK activator (AICAR) does not alleviate the potent hepatotoxic effect of acetaminophen (APAP) whereas administration of AMPK inhibitor (Compound C, CC) significantly aggravated APAP toxicity. On the contrary, the genuine addition of AICAR or SIRT1 activator (CAY10591) significantly suppressed the negative hepatotoxic effects of the combination of APAP+CC. In addition, AICAR in contrast to CAY10591 did not attenuate the toxic action of APAP in yet untested combination with SIRT1 inhibitor (EX-527). Taken together, our results from *in vitro* experiments originally suggest that hepatoprotective effects of SIRT1 against APAP toxicity could be at least partially independent of AMPK activity. Therefore, the differentiated modulation of AMPK and SIRT1 activity, especially by their specific synthetic activators, could provide an interesting and novel therapeutic option for hepatocyte injury in the future.

Acknowledgments and funding

We would like to thank Mrs. Jana Plačková and Mrs. Libuše Šlehobrová for their technical assistance in the laboratory and in the menagerie. This work was supported by Charles University under the project Progress Q25/LF1, the grant GA UK 190/54/251753, and the specific academic research program SVV 260 523.

Author contributions

1st Author: Lea Njeka Wojnarová (LNW) as a postgraduate student led and realized here presented in vitro experiments in all their phases. She prepared and wrote the entire manuscript.

2nd Author, the corresponding author: Nikolina Kutinová Canová, the supervisor and leader of the research group who organized and carried out together with her postgraduate students (LNW and MA) all the experiments mentioned here, participated in the collection, evaluation and interpretation of results, and in preparation of the manuscript, including figures and tables. As a holder of a certificate for work with laboratory animals, she manipulated and led work with rats.

3rd Author: Mahak Arora (MA) as a postgraduate student helped with hepatocyte isolation and cultivation, and finalization of the manuscript including analysis for American English.

4th Author, the senior author: Hassan Farghali, who encouraged the idea of experimental testing of natural and synthetic modulators of AMPK and SIRT1 in liver diseases and hepatocyte injury. He also worked in improving the article concept and the overall English language.

Conflict of interest disclosure

The authors declare no conflict of interest concerning the publication of this manuscript.

REFERENCES

1. Asrani SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. J Hepatol 2019;70(1):151-71.
2. Rada P, Pardo V, Mobasher MA, García-Martínez I, Ruiz L, Gonzalez-Rodriguez A, Sanchez-Ramos C, Muntané J, Alemany S, James LP, Simpson KJ. SIRT1 controls acetaminophen hepatotoxicity by modulating inflammation and oxidative stress. Antioxid Redox Signal 2018;28(13):1187-208.
3. McGill MR, Jaeschke H. Biomarkers of drug-induced liver injury. Adv Pharmacol 2019;85:221-39.

4. Rangnekar AS, Fontana RJ. An update on drug induced liver injury. Minerva Gastroenterol Dietol 2011;57:213-29.
5. Iorga A, Dara L, Kaplowitz N. Drug-induced liver injury: cascade of events leading to cell death, apoptosis or necrosis. Int J Mol Sci [serial on internet] 2017;18(5): Article No. 1018. Available from: https://www.mdpi.com/1422-0067/18/5/1018
6. Kuna L, Bozic I, Kizivat T, Bojanic K, Mrso M, Kralj E, Smolic R, Wu GY, Smolic M. Models of drug induced liver injury (DILI)–current issues and future perspectives. Curr Drug Metab 2018;19(10):830-8.
7. Jaeschke H, Williams CD, McGill MR, Xie Y, Ramachandran A. Models of drug-induced liver injury for evaluation of phytotherapeutics and other natural products. Food Chem Toxicol 2013;55:279-89.
8. McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H. Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. Toxicol Appl Pharmacol 2012;264(3):387-94.
9. Bhaargavi V, Jyotsna GS, Tripurana R. A Review on hepatoprotective activity. Int J Pharm Life Sci 2014;5(3):690-702.
10. Farghali H, Canová NK, Zakhari S. Hepatoprotective properties of extensively studied medicinal plant active constituents: possible common mechanisms. Pharm Biol 2015;53(6):781-91.
11. Dey P, Saha MR, Sen A. Hepatotoxicity and the present herbal hepatoprotective scenario. Int J Green Pharm 2013;7(4):265-73.
12. Farghali H, Černý D, Kameníková L, Martínek J, Hořínek A, Kmoníčková E, Zídek Z. Resveratrol attenuates lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats: role of nitric oxide synthase 2 and heme oxygenase-1. Nitric Oxide-Biol Chem 2009; 21:216-25.
13. Wojnarová L, Kutinová Canová N, Farghali H, Kučera T. Sirtuin 1 modulation in rat model of acetaminophen-induced hepatotoxicity. Physiol Res 2015;64:S477-S487.
14. Farghali H, Kmoníčková E, Lotková H, Martínek J. Evaluation of calcium channel blockers as potential hepatoprotective agents in oxidative stress injury of perfused hepatocytes, Physiol Res 2000;49:261-8.
15. Černý D, Lekić N, Váňová K, Muchová L, Hořínek A, Kmoníčková E., Zídek Z, Kameníková L, Farghali H. Hepatoprotective effect of curcumin in

Lipopolysaccharide/D-Galactosamine model of liver injury in rats: relationship to HO-1/CO antioxidant system. Filoterapia 2011;82:786-91.

16. Lekić N, Kutinová Canová N, Hořínek A, Farghali H. The involvement of hemeoxygenase 1 but not nitric oxide synthase 2 in a hepatoprotective action of quercetin in lipopolysaccharide-induced hepatotoxicity of D-galactosamine sensitized rats. Filoterapia 2013;87:20-6.
17. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 2003;425:191-6.
18. Ruderman NB, Xu J, Nelson L, Cacicedo JM, Saha AK, Lan F, Ido Y. AMPK and SIRT1: a long-standing partnership? Am J Physiol Endocrinol Metab 2010;298:E751-60.
19. Farghali H, Kutinová Canová N, Lekic N. Resveratrol and related compounds as antioxidants with an allosteric mechanism of action in epigenetic drug targets. Physiol Res 2013;62:1-13.
20. Lan F, Weikel KA, Cacicedo JM, Ido Y. Resveratrol-Induced AMP-Activated Protein Kinase Activation Is Cell-Type Dependent: Lessons from Basic Research for Clinical Application. Nutrients [serial on internet] 2017;9: Article No. 751. Available from: https://www.mdpi.com/2072-6643/9/7/751
21. Farghali H, Kemelo MK, Kutinová Canová N. SIRT1 Modulators in Experimentally Induced Liver Injury. Oxid Med Cell Longev [serial on internet] 2019: Article No. 8765954. Available from: https://www.hindawi.com/journals/omcl/2019/8765954/
22. Silva JP, Wahlested C. Role of Sirtuin 1 in metabolic regulation. Drug Discov Today 2010;15:781-91.
23. Kim J, Yang G, Kim Y, Kim J, Ha J. AMPK activators: mechanisms of action and physiological activities. Exp Mol Med [serial on internet] 2016;48: Article No. 224. Available from: https://www.nature.com/articles/emm201616
24. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 2007;9:218-24.
25. Hubbard BP, Sinclair DA. Small molecule SIRT1 activators for the treatment of aging and age-related diseases. Trends Pharmacol Sci 2014;35:146-54.

26. Kutinová Canová N, Gaier N, Farghali H. Perspektivy farmakologického a klinického přínosu aktivátorů sirtuinu 1 při oxidativním poškození [Perspectives on pharmacological and clinical benefits from sirtuin 1 activators in oxidative damage]. Cas Lek Cesk 2012;151:187-9.
27. Berry MN, Edwards AM, Barrit JG. Isolated Hepatocytes Preparation, Properties and Applications. 1st ed. Amsterdam: Elsevier Science; 1991.
28. Černý D, Canová NK, Martínek J, Horínek A, Kmonícková E, Zídek Z, Farghali H. Effects of resveratrol pretreatment on tert-butylhydroperoxide induced hepatocyte toxicity in immobilized perifused hepatocytes: involvement of inducible nitric oxide synthase and hemoxygenase-1. Nitric Oxide-Biol Chem 2009;20(1):1-8.
29. Kutinová Canová N, Martínek J, Kmoníčková E. Modulation of spontaneous and lipopolysaccharide-induced nitric oxide production and apoptosis by d-galactosamine in rat hepatocyte culture: the significance of combinations of different methods. Toxicol Mech Methods 2008;18:63-74.
30. De Leon ADJ, Borges CR. Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay. J Vis Exp [serial on internet] 2020: Article No. 159. Available from: https://www.jove.com/t/61122/evaluation-oxidative-stress-biological-samples-using-thiobarbituric
31. Del Campo JA, Gallego P, Grande L. Role of inflammatory response in liver diseases: Therapeutic strategies. World J Hepatol 2018;10:1-7.
32. Wang SW, Wang W, Sheng H, Bai YF, Weng YY, Fan XY, Zheng F, Zhu XT, Xu ZC, Zhang F. Hesperetin, a SIRT1 activator, inhibits hepatic inflammation via AMPK/CREB pathway. Int Immunopharmacol [serial on internet] 2020;89(partB): Article No. 107036. Available from: https://www.sciencedirect.com/science/article/pii/S1567576920327831¨
33. Yan M, Huo Y, Yin S, Hu H. Mechanisms of acetaminophen-induced liver injury and its implications for therapeutic interventions. Redox Biol 2018;17:274-83.
34. Jaeschke H, Akakpo JY, Umbaugh DS, Ramachandran A. Novel Therapeutic Approaches Against Acetaminophen-induced Liver Injury and Acute Liver Failure. Toxicol Sci 2020;174:159-67.
35. Akakpo JY, Jaeschke MW, Ramachandran A, Curry SC, Rumack BH, Jaeschke H. Delayed administration of N-acetylcysteine blunts recovery after an acetaminophen overdose unlike 4-methylpyrazole. Arch Toxicol 2021;95(10):3377-3391.

36. Saeedi Saravi SS, Hasanvand A, Shahkarami K, Dehpour AR. The protective potential of metformin against acetaminophen-induced hepatotoxicity in BALB/C mice. Pharm Biol 2016;54(12):2830-2837.
37. Tripathi SS, Singh S, Garg G, Kumar R, Verma AK, Singh AK, Bissoyi A, Rizvi SI. Metformin ameliorates acetaminophen-induced sub-acute toxicity via antioxidant property. Drug Chem Toxicol 2019:1-9.
38. Caton PW, Nayuni NK, Kieswich J, Khan NQ, Yaqoob MM, Corder R. Metformin suppresses hepatic gluconeogenesis through induction of SIRT1 and GCN5. J Endocrinol 2010;205(1):97-106.
39. Xu T, Lu X, Arbab AAI, Wu X, Mao Y, Loor JJ, Yang Z. Metformin acts to suppress β-hydroxybutyric acid-mediated inflammatory responses through activation of AMPK signaling in bovine hepatocytes. J Anim Sci 2021;99(7): Article No. skab153. Available on: https://academic.oup.com/jas/article/99/7/skab153/6275009
40. Song YM, Lee YH, Kim JW, Ham DS, Kang ES, Cha BS, Lee HC, Lee BW. Metformin alleviates hepatosteatosis by restoring SIRT1-mediated autophagy induction via an AMP-activated protein kinase-independent pathway. Autophagy 2015;11(1):46-59.
41. Nelson LE, Valentine RJ, Cacicedo JM, Gauthier MS, Ido Y, Ruderman NB. A novel inverse relationship between metformin-triggered AMPK-SIRT1 signaling and p53 protein abundance in high glucose-exposed HepG2 cells. Am J Physiol Cell Physiol 2012;303(1):C4-C13.
42. Kulkarni SS, Cantó C. The molecular targets of resveratrol. Biochim Biophys Acta 2015;1852:1114-23.
43. Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, Agarwal B, Ye L, Ramadori G, Teodoro JS, Hubbard BP, Varela AT, Davis JG, Varamini B, Hafner A, Moaddel R, Rolo AP, Coppari R, Palmeira CM, de Cabo R, Baur JA, Sinclair DA. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab 2012;15:675-90.
44. Ramachandran A, Jaeschke H. Acetaminophen Hepatotoxicity. Semin Liver Dis 2019;39:221-34.
45. Jaeschke H, Ramachandran A. Acetaminophen-induced apoptosis: Facts versus fiction. J Clin Transl Res 2020;6:36-47.
46. Kang SW, Haydar G, Taniane C, Farrell G, Arias IM, Lippincott-Schwartz J, Fu D. AMPK Activation Prevents and Reverses Drug-Induced Mitochondrial and Hepatocyte

Injury by Promoting Mitochondrial Fusion and Function. PLoS One 2016;11(10):e0165638. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0165638

47. Hwang JH, Kim YH, Noh JR, Choi DH, Kim KS, Lee CH. Enhanced Production of Adenosine Triphosphate by Pharmacological Activation of Adenosine Monophosphate-Activated Protein Kinase Ameliorates Acetaminophen-Induced Liver Injury. Mol Cells 2015;38:843-50.
48. Ke R, Xu Q, Li C, Luo L, Huang D. Mechanisms of AMPK in the maintenance of ATP balance during energy metabolism. Cell Biol Int 2018;42:384-92.
49. Jiang WP, Deng JS, Huang SS, Wu SH, Chen CC, Liao JC, Chen HY, Lin HY, Huang GJ. Sanghuangporus sanghuang mycelium prevents paracetamol-induced hepatotoxicity through regulating the MAPK/NF-?B, Keap1/Nrf2/HO-1, TLR4/PI3K/Akt, and CaMKK?/LKB1/AMPK pathways and suppressing oxidative stress and inflammation. Antioxidants (Basel) 2021;10(6): Article No. 897. Available from: https://www.mdpi.com/2076-3921/10/6/897/htm
50. Zhang J, Liang X, Li J, Yin H, Liu F, Hu C, Li L. Apigenin Attenuates Acetaminophen-Induced Hepatotoxicity by Activating AMP-Activated Protein Kinase/Carnitine Palmitoyltransferase I Pathway. Front Pharmacol 2020;11: Article No. 549057. Available from: https://www.frontiersin.org/articles/10.3389/fphar.2020.549057/full
51. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, Meijer AJ. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem 2006;281:34870-9.
52. Czaja MJ, Ding WX, Donohue TM Jr, Friedman SL, Kim JS, Komatsu M, Lemasters JJ, Lemoine A, Lin JD, Ou JH, Perlmutter DH, Randall G, Ray RB, Tsung A, Yin XM. Functions of autophagy in normal and diseased liver. Autophagy 2013;9:1131-58.
53. Morita M, Gravel SP, Hulea L, Larsson O, Pollak M, St-Pierre J, Topisirovic I. mTOR coordinates protein synthesis, mitochondrial activity and proliferation. Cell Cycle 2015;14:473-80.
54. Ni HM, Bockus A, Boggess N, Jaeschke H, Ding WX. Activation of autophagy protects against acetaminophen-induced hepatotoxicity. Hepatology 2012;55:222-32.
55. Wang K. Autophagy and apoptosis in liver injury. Cell Cycle 2015;14(11):1631-42.

56. Scudiero O, Nigro E, Monaco ML, Oliviero G, Polito R, Borbone N, D'Errico S, Mayol L, Daniele A, Piccialli G. New synthetic AICAR derivatives with enhanced AMPK and ACC activation. J Enzyme Inhib Med Chem 2016;31:748-53.
57. Zhu H, Chai Y, Dong D, Zhang N, Liu W, Ma T, Wu R, Lv Y, Hu L. AICAR-Induced AMPK Activation Inhibits the Noncanonical NF-κB Pathway to Attenuate Liver Injury and Fibrosis in BDL Rats. Can J Gastroenterol Hepatol [serial on internet] 2018: Article No. 6181432. Available from: https://www.hindawi.com/journals/cjgh/2018/6181432/
58. De Gregorio E, Colell A, Morales A, Marí M. Relevance of SIRT1-NF-κB Axis as Therapeutic Target to Ameliorate Inflammation in Liver Disease. Int J Mol Sci [serial on internet] 2020;21: Article No. 3858. Available from: https://www.mdpi.com/1422-0067/21/11/3858
59. Iside C, Scafuro M, Nebbioso A, Altucci L. SIRT1 Activation by Natural Phytochemicals: An Overview. Front Pharmacol [serial on internet] 2020;11: article No. 1225. Available from: https://www.frontiersin.org/articles/10.3389/fphar.2020.01225/full
60. Hwang JW, Yao H, Caito S, Sundar IK, Rahman I. Redox regulation of SIRT1 in inflammation and cellular senescence. Free Radic Biol Med 2013;61:95-110.
61. Yan T, Huang J, Nisar MF, Wan C, Huang W. The Beneficial Roles of SIRT1 in Drug-Induced Liver Injury. Oxid Med Cell Longev [serial on internet] 2019: Article No. 8506195. Available from: https://www.hindawi.com/journals/omcl/2019/8506195/
62. Huang J, Tian R, Yang Y, Jiang R, Dai J, Tang L, Zhang L. The SIRT1 inhibitor EX-527 suppresses mTOR activation and alleviates acute lung injury in mice with endotoxiemia. Innate Immun 2017;23:678-86.
63. Gertz M, Fischer F, Nguyen GTT, Lakshminarasimhan M, Schutkowski M, Weyand M, Steegborn C. Ex-527 inhibits Sirtuins by exploiting their unique NAD+-dependent deacetylation mechanism, Proc Natl Acad Sci 2013;110:E2772-81.

Table 1. Concentration of drugs used in primary rat hepatocyte cultures.

Drug Treatment	**Concentration**
APAP (acetaminophen)	12.5 mM*
AICAR (AMPK activator)	50 μM^{+}
CC ("Compound C" – AMPK inhibitor)	10 μM^{+}
CAY10591 (SIRT1 activator)	30 μM^{+}
EX-527 (SIRT1 inhibitor)	10 μM^{+}

Concentrations were determined by MTT test in 96-well plates as follows: *APAP effective concentration EC50 causing 50% loss of rat hepatocyte viability; $^{+}$a half of minimal significantly toxic concentration providing hepatocyte viability to be in average 100% (with tolerated ± 5%) of control group.

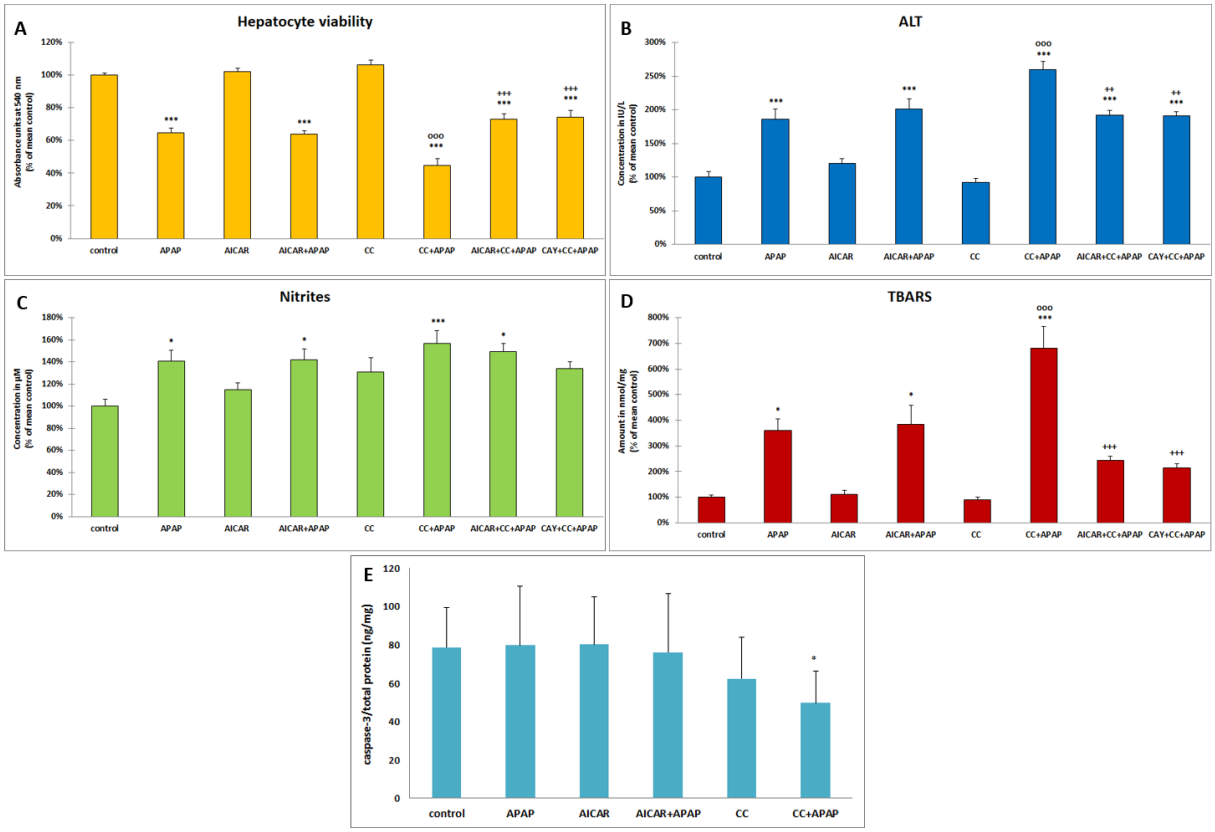

Figure 1. Effects of specific AMPK modulators (activator – AICAR and inhibitor - Compound C, CC) and specific activator of SIRT1 (CAY10591/CAY) in *in vitro* acetaminophen (APAP)-induced hepatotoxicity on hepatocyte viability (A) and levels of: medium alanine aminotransferase (ALT) (B), medium nitrites (NO_2^-) (C), cell lysate thiobarbituric acid reactive substances (TBARS) (D), and caspase-3 proenzyme (E) after 24 hours of treatment. Data are expressed as means ± SEM (n = 7-16 for A-D and n = 3 for E): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{ooo}P < 0.001$ vs. APAP; $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. CC+APAP combination.

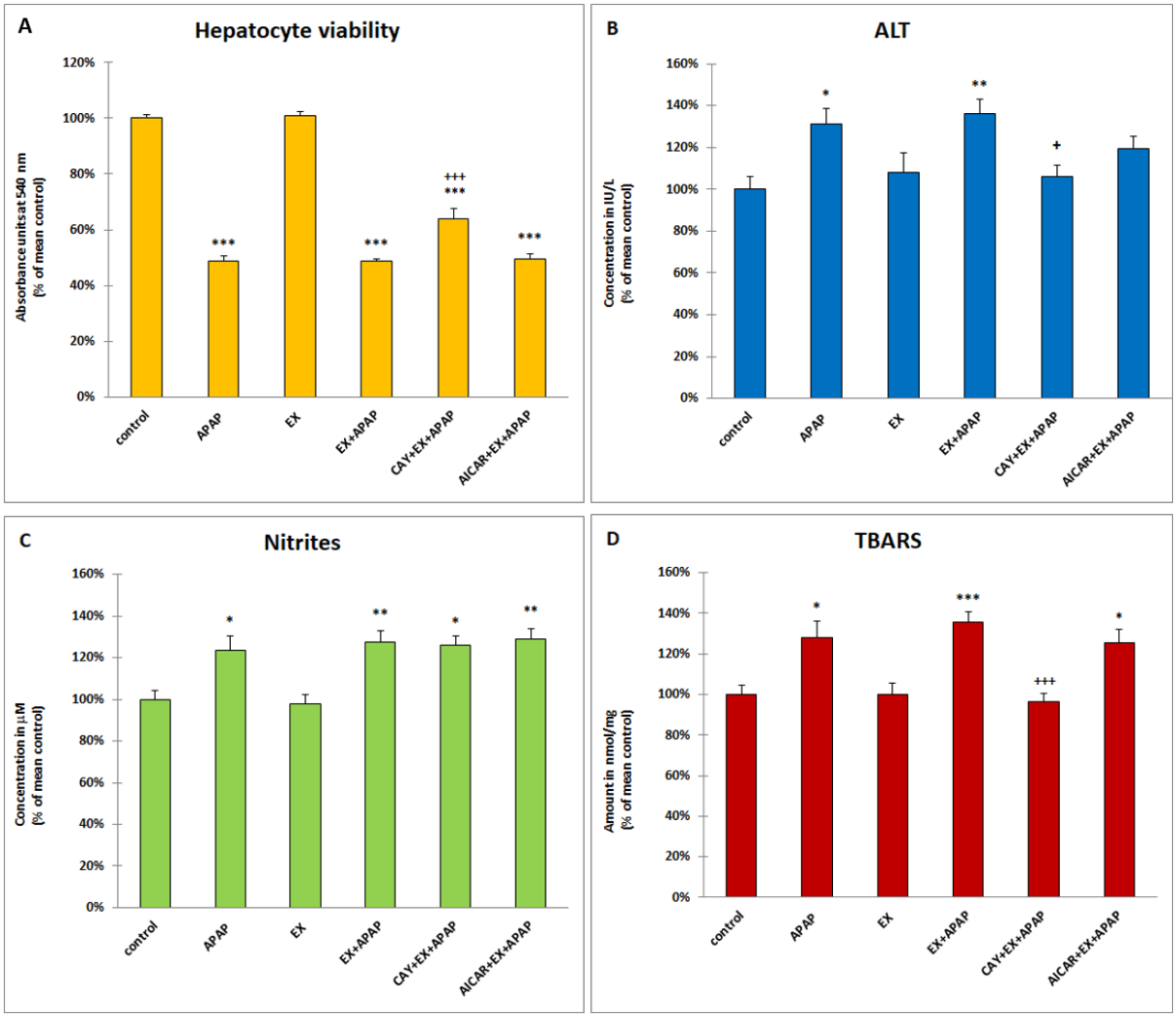

Figure 2. Effects of specific SIRT1 modulators (activator – CAY10591/CAY and inhibitor – EX-527/EX) and AMPK activator (AICAR) in acetaminophen (APAP)-induced hepatotoxicity on A) hepatocyte viability, B) alanine aminotransferase (ALT) release, C) nitrite (NO_2^-) production, and D) thiobarbituric acid reactive substances (TBARS) formation in *in vitro* experiments after 24 hours. Data are expressed as means ± SEM (n = 9-16): *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ vs. respective control; $^{+}P < 0.05$, $^{+++}P < 0.001$ vs. EX+APAP combination.

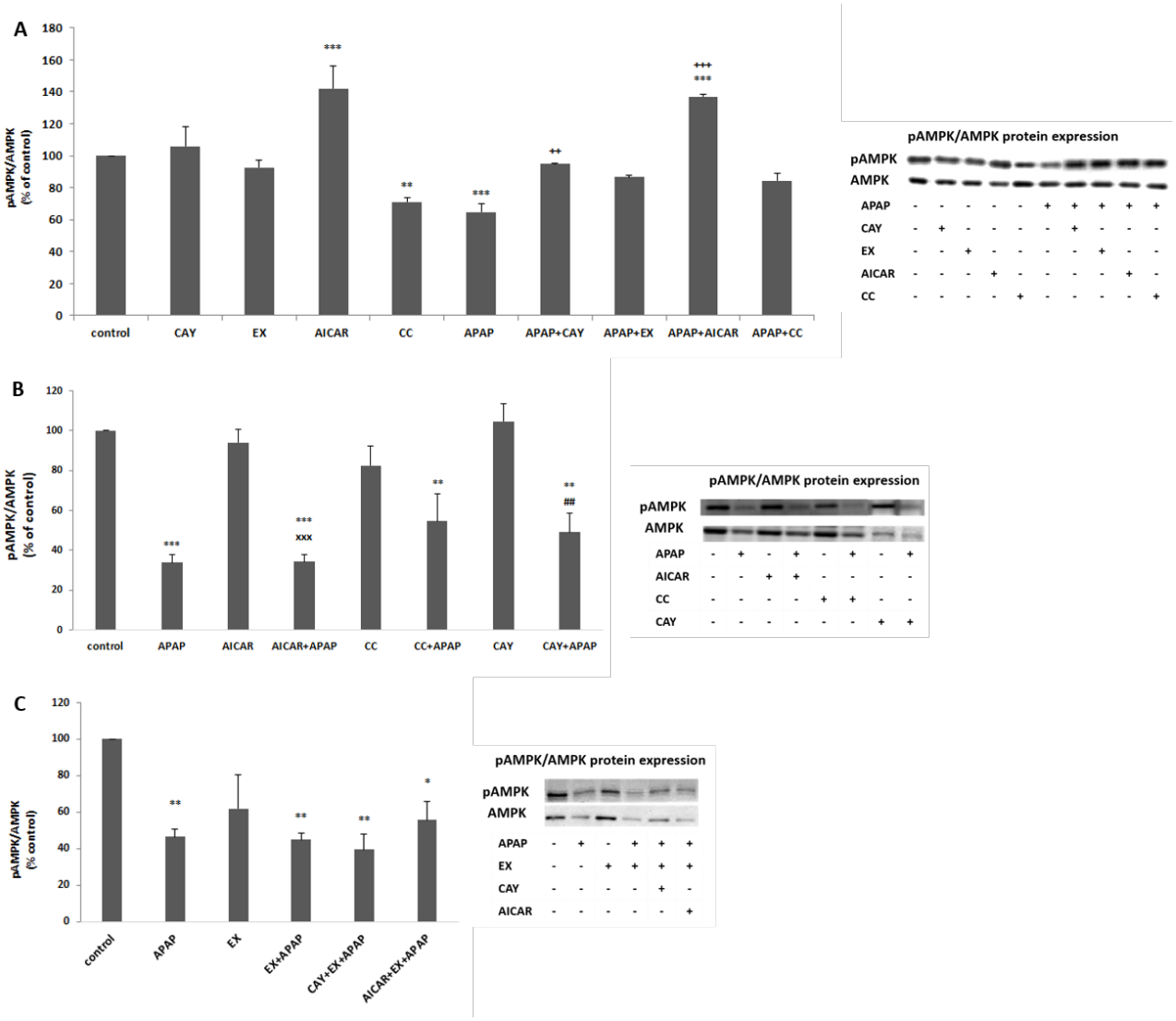

Figure 3. Effects of acetaminophen (APAP), specific modulators of AMPK (activator – AICAR and inhibitor - Compound C, CC) and SIRT1 (activator - CAY10591/CAY and inhibitor EX-527/EX) on AMPK activity in cultured primary rat hepatocytes after 4 hours (A) and 24 hours (B, C). Activity of AMPK was calculated as pAMPK/AMPK ratio of protein expression. Quantitative data of optical band densitometry (graphs) and representative Western blot images are presented. Data are expressed as means ± SEM (n = 3-5): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. respective control; $^{+}P < 0.05$; $^{++}P < 0.01$, $^{+++}P < 0.001$ APAP in combination vs. APAP alone; $^{XXX}P < 0.001$ vs. AICAR; $^{\#\#}P < 0.01$ vs. CAY.

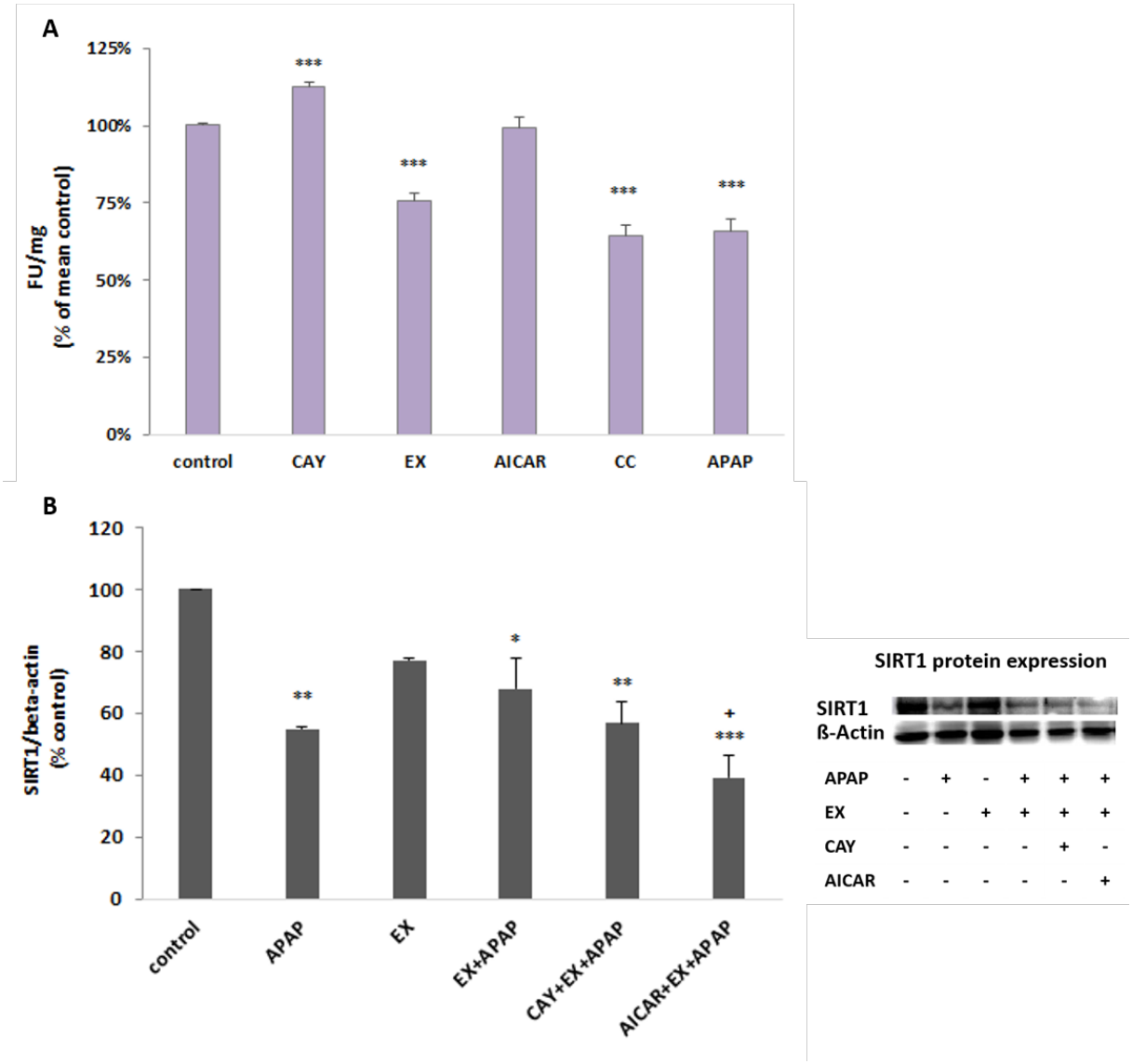

Figure 4. Effects of acetaminophen (APAP), specific modulators of SIRT1 (activator – CAY10591/CAY and inhibitor – EX-527/EX) and AMPK activator (AICAR) on: A) SIRT1 activity after 4 hours, and B) SIRT1 protein expression after 24 hours; both in cultured primary rat hepatocytes. Quantitative data of fluorescence activity (A) and optical band densitometry (B) are expressed in graphs as means ± SEM (n = 5 and 3, respectively): $^{**}P < 0.01$, $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control; $^{+}P < 0.05$ vs. EX+APAP. Representative Western blot image is also presented.