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

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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 and metformin, less specific activators of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1), can effectively attenuate acute liver injury. The aim of this experimental study was to elucidate 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 a toxic dose of APAP. At the end of cultivation, medium samples were collected for biochemical analysis of alanine-aminotransferase 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 a 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.

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

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INTRODUCTION

Liver diseases are a significant cause of morbidity and mortality and account for approximately 2 million deaths per year worldwide[1]. In addition, liver injury is a major 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]. 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 (ref.[5]). Of these, 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]. AILI is also a major clinical problem in a number of Western countries[6]. Despite the clinical significance of human APAP toxicity, the only one existing approved antidote is N-acetylcystein (NAC). Unfortunately, NAC therapy has some limitations[7]. For this reason, APAP overdose in rodents is frequently used as a hepatotoxic model to test the hepatoprotective potential of herbal and other compounds[8-10].

Experimental studies on resveratrol[11,12], silymarin[13], curcumin[14], and quercetin[15] 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, anti-oxidant, anti-aging, and anti-carcinogenic properties that may be pertinent to chronic diseases and/or longevity in humans. Resveratrol, among others, has been described[16] 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[17,18-20]. However,

there is still an open question whether resveratrol can activate SIRT1 directly or indirectly through AMPK or act independently[21].

In addition, some hepatoprotective effect has been also demonstrated for metformin, the first-line pharmacological treatment in type 2 diabetes mellitus. Several animal studies have 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-1 expression, resulting in hepatoprotection against oxidative stress[6,22,23]. Although the fundamental mechanism of metformin has not been fully clarified its dual activating effect on AMPK/SIRT1 should be also taken into 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[24,25]. Other studies, however, showed that metformin reduces lipid accumulation by SIRT1 stimulation independently of AMPK (ref.[26]) or by acting primarily through AMPK independent of SIRT1 when increasing SIRT1 activity simultaneously[27].

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 of 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[28]. 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 such 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 , obesity and tumorigenesis[29,30]. 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[18,19,31,32]. Therefore, we focused our research 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.[12]) to 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 individual role of AMPK and SIRT1 in APAP-induced hepatotoxicity. Under the set experimental conditions, the effect of the selective agonists and/or antagonists in their mutual novel combinations have not been evaluated before.

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). The rats were maintained under standard conditions (12-h light/dark cycle, 22±2 °C ambient temperature and 50±10% relative humidity), provided with tap water and fed a 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[33]. 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[34]. Unattached cells were removed 3 h 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 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 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.[35]).

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[34].

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.[11]). 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[36]. 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.

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.

SIRT1 activity assay

Deacetylase activity of SIRT1 enzyme was determined in lysed hepatocytes using commercial fluorometric SIRT1 Assay Kit (Sigma-Aldrich) as described previously[12].

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 h 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[15].

Statistical analysis

Data are summarized as means ± SEM (standard error of the mean) from at least 3 independent *in vitro* experiments.

The data were tested for normality and the significance of differences between the groups was assessed by a 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

In another set of experiments, 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 EX-527+APAP combination. 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 lev-

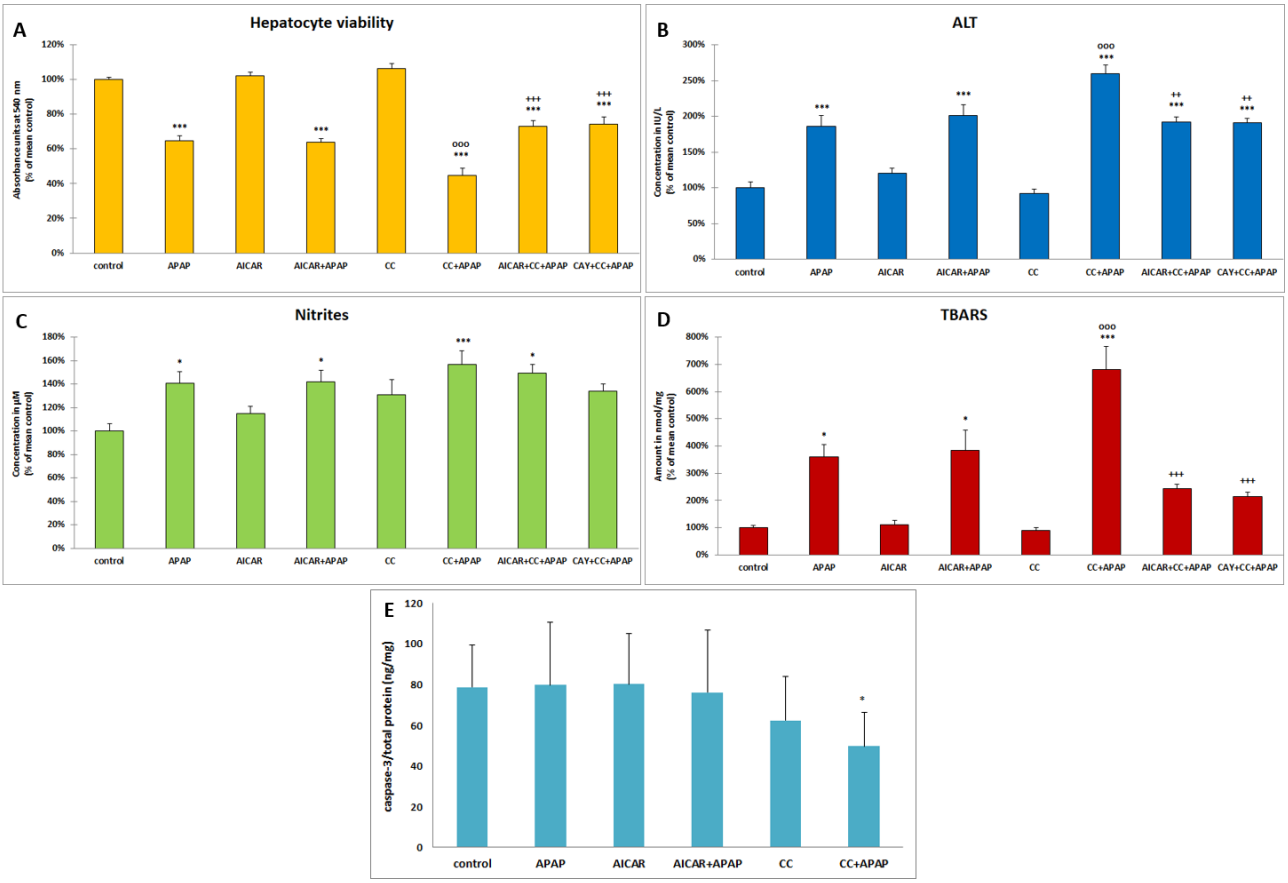

Fig. 1. Effects of specific AMPK modulators (activator – AICAR and inhibitor - Compound C, CC) and specific activator of SIRT1 (CAY10591/CAY) in *in vitro* acetaminophen (APAP)-induced hepatotoxicity **A.** on hepatocyte viability, **B.** and levels of: medium alanine aminotransferase (ALT), **C.** medium nitrites (NO_2^-), **D.** cell lysate thiobarbituric acid reactive substances (TBARS), **E.** caspase-3 proenzyme after 24 h 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.

els (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 h 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 h, 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).

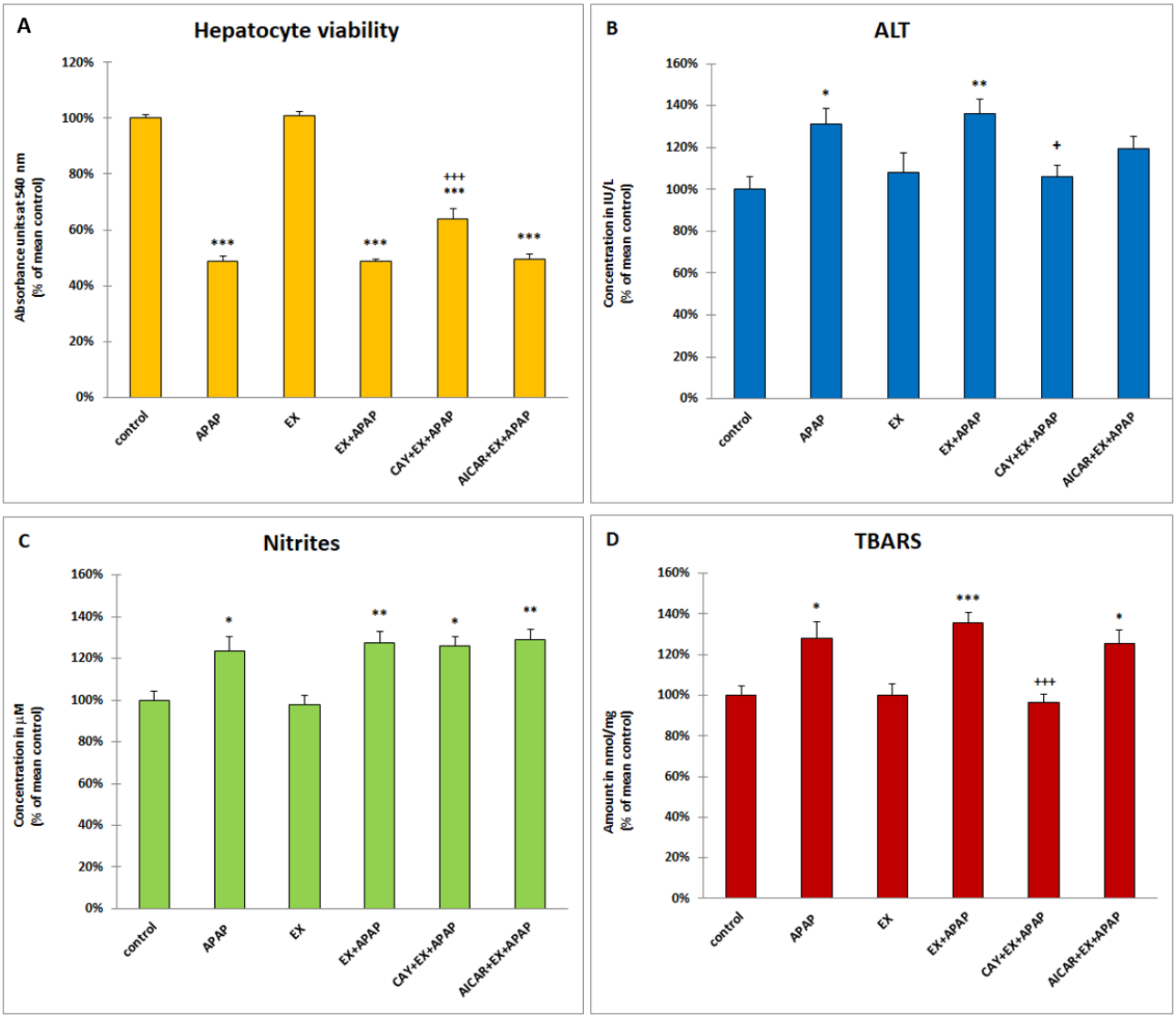

Fig. 2. Effects of specific SIRT1 modulators (activator – CAY10591/CAY and inhibitor – EX-527/EX) and AMPK activator (AICAR) in acetaminophen (APAP)-induced hepatotoxicity on **A.** hepatocyte viability, **B.** alanine aminotransferase (ALT) release, **C.** nitrite (NO_2^-) production, **D.** thiobarbituric acid reactive substances (TBARS) formation in *in vitro* experiments after 24 h. 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.

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[37,38]. APAP-raised oxidative stress and mitochondrial dysfunction also play a key role in the pathogenesis of acute APAP-induced liver injury[39]. 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 adenosine triphosphate (ATP) production[7]. Newer possible pharmacotherapeutic interventions including metformin should alternate its mechanisms of action, mainly targeting mitochondrial dysfunction[6]. The ambiguous role of AMPK and SIRT1 in metformin-induced hepatoprotection is still debated[24-27]. Similarly, our own[11,12] and other[40] earlier studies have shown that resveratrol as another less specific SIRT1 activator can effectively attenuate acute liver injury[11,12,40]. In addition, it was reported that resveratrol has also the ability to activate AMPK and this activation is much faster than that generally required for SIRT1 activation even in hepatic cell models and that the salutary effect of resveratrol is crucially dependent on AMPK activation[38,40-42]. Moreover, SIRT1 and AMPK have been shown to play many similar roles and share a numberof target molecules[32].

For these reasons, the current study focused on investigating 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

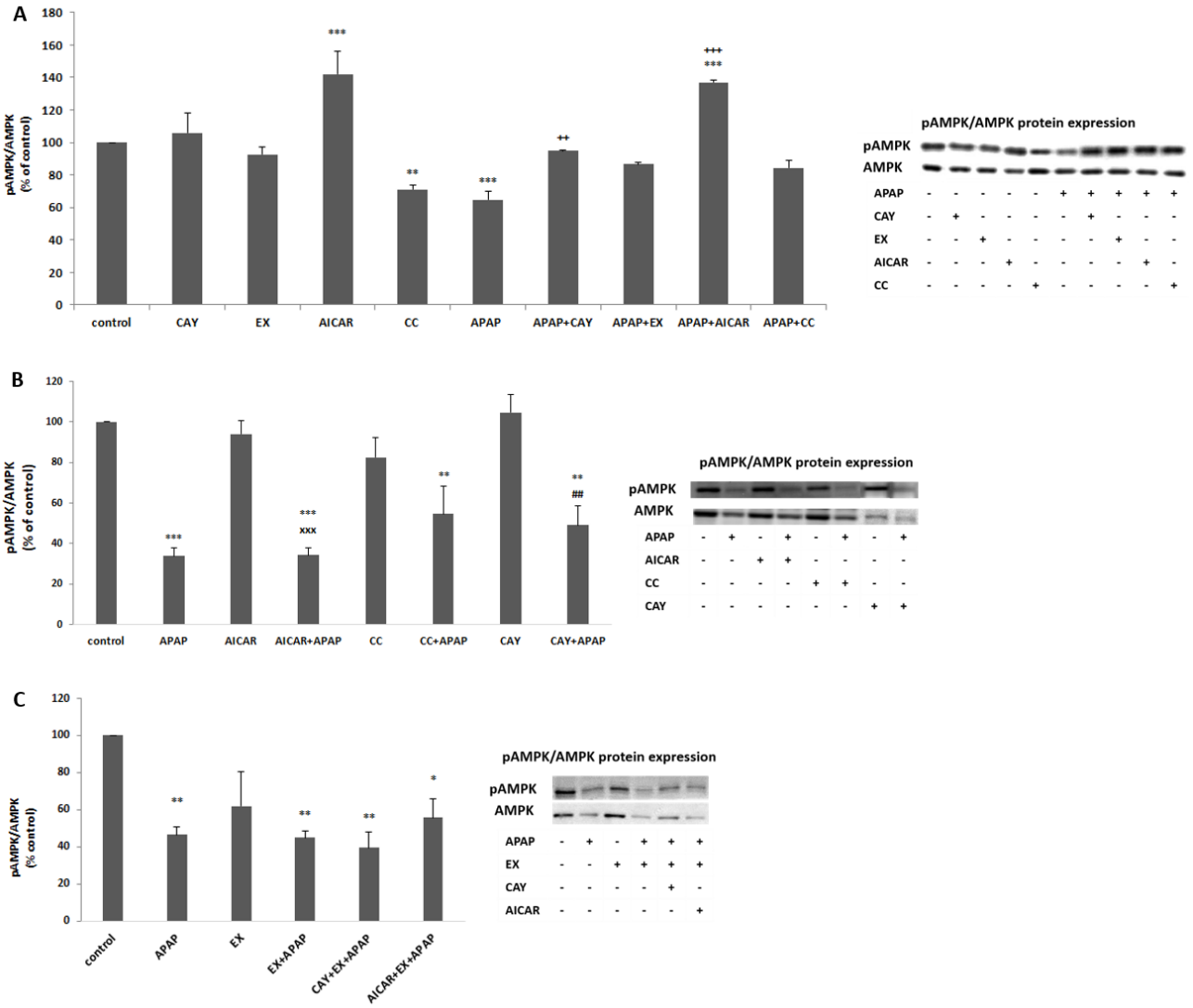

Fig. 3. Effects of acetaminophen (APAP), specific modulators of AMPK (activator – AICAR and inhibitor – Compound C, CC) and SIRT1 (activator – CAY10591/CAY and inhibitor EX-527/EX) on AMPK activity in cultured primary rat hepatocytes after **A.** 4 h, **B,C.** 24 h. Activity of AMPK was calculated as 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.

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. APAP toxicity is composed of multi-stages and multi-signaling pathways, including mitochondrial malfunction, which yields oxidative stress and excess superoxide free radicals, resulting in DNA fragmentation and finally hepatocyte necrosis[6,43]. Our data confirm 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, Jaeschke and Ramachandran[44] reported that it is not apoptosis but oncotic necrosis which is the form of cell death in APAP-induced liver injury both *in vitro* and *in vivo*[44].

Our current results demonstrated 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.[45]. 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

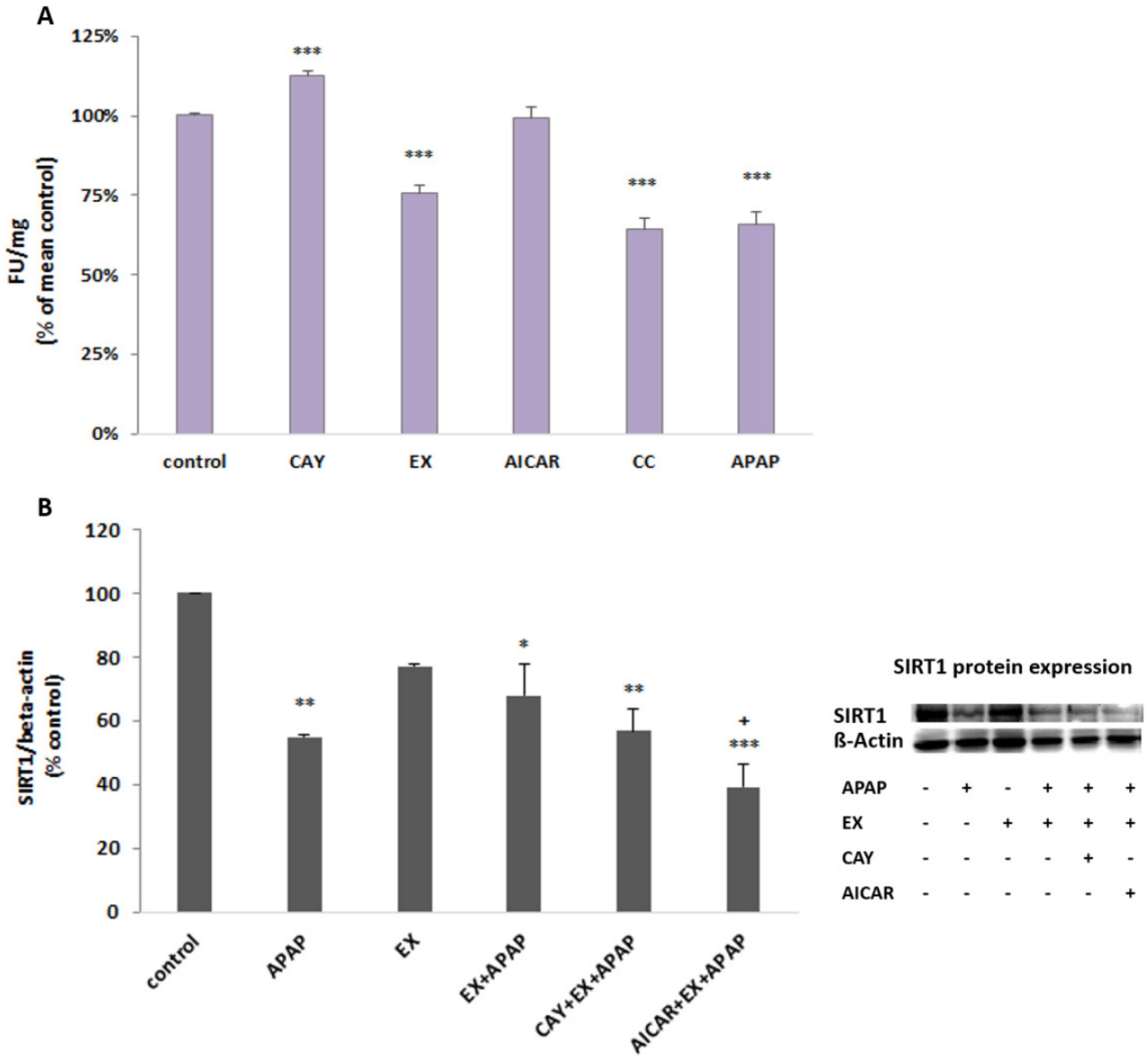

Fig. 4. Effects of acetaminophen (APAP), specific modulators of SIRT1 (activator – CAY10591/CAY and inhibitor – EX-527/EX) and AMPK activator (AICAR) on: **A.** SIRT1 activity after 4 h, **B.** SIRT1 protein expression after 24 h; both in cultured primary rat hepatocytes.
Quantitative data of fluorescence 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.

to cellular stress and damage which could be prevented and reversed by AICAR(ref.[45]). AMPK sets in motion alterations in mitochondrial biogenesis and function that could increase the efficiency of a cell to give rise to and weaken oxidative stress and other potentially harmful cellular events[42]. It was found that APAP-induced liver injury causes hepatocyte depletion of ATP by inhibiting mitochondrial function. Hwang et al.[46] observed that APAP administration reduces active phosphorylation of the Thr172 active site of AMPK, following a model resembling that observed with ATP loss. They hypothesized that ATP depletion caused by APAP may correlate with inhibition of AMPK activity. Further explanation could be the duration of time after administration of APAP (ref.[46]).

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 used 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 Jiang et al.[47] who revealed that treatment with CC aggravated APAP-induced hepatotoxicity in mice by inactivating AMPK and abolished the hepatoprotective effect of a medicinal mushroom (*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[47,48]. Furthermore, it was shown that inhibition of AMPK activity by CC or by transfection with a dominant negative form of AMPK nearly entirely suppressed autophagy in hepatocytes[49]. This 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[50]. The activation of AMPK inhibits mTOR and thus increases autophagy[51]. When autophagy was enhanced by treatment with rapamycin, APAP-induced necrosis was significantly inhibited in cultured primary hepatocytes and mouse liver[52]. As autophagy and apoptosis are interrelated and play an important role in liver injury[53], 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 observed that pretreatment with AICAR (aminoimidazole-4-carboxamide riboside), adenosine analog that selectively activates AMPK, and SIRT1 synthetic activator, CAY10591, significantly increased the AMPK activity already after 4 h of hepatocyte incubation with APAP. However, AMPK activity was not influenced or only slightly increased with AICAR and CAY10591, respectively, after 24 h. One explanation of this time-dependent trend could be the short half-life of AICAR in cells[54] 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 (ref.[20,42]). 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[37]. 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)/manganese superoxide dismutase (MnSOD) pathway, and PGC-1α, and then diminish the expression of inflammatory factors[46,55]. 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[38,56]. Interestingly, this relationship was confirmed in the publication of Rada et al.[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-h 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[12]. The same trend was also reported in the publication of Rada et al.[2] where SIRT1 protein levels were decreased in the liver of humans and mice in APAP-induced liver injury. By stimulating the SIRT1-Farnesoid X receptor (FXR) pathway, APAP hepatotoxicity was also reduced[43]. In addition, earlier studies reported that liver specific SIRT1 deficiency caused an increase in ROS production[57,58]. 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[59]. In agreement with that, our results demonstrate 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 a role SIRT1 plays in the process of hepato-protection we also performed experiments with EX-527. EX-527 is broadly used as a SIRT1 inhibitor both *in vitro* and *in vivo* with high potency and significant isoform selectivity. 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[60]. Our current data show 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 against APAP-induced hepatotoxicity[21]. Above that, hypoxia, nutrient deprivation, DNA damage, and oxidative stress have been reported to control SIRT1 expression at both transcriptional and post-translational levels[18]. We hypothesized that the addition of AICAR in the combination with EX-527+APAP could increase the expression of SIRT1 protein which was not, however, 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 possible mechanism for explaining how AMPK could activate SIRT1 is through an indirect increase in cellular NAD^+ levels[42]. Reciprocally, this is consistent with our results demonstrating that CC alone significantly reduced SIRT1 activity.

The above discussion highlights that AMPK and SIRT1 signaling pathways are interrelated on the one hand and that they can also work, at least partially, independently to exert their hepatoprotective properties as demonstrated by our results. Hence, differentiated pharmacologic modulation of AMPK and SIRT1 activity could be a future major step in the understanding of DILI.

CONCLUSIONS

By using an *in vitro* experimental model of drug-induced acute hepatotoxicity, we found 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 in agreement with the published 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 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 an SIRT1 inhibitor (EX-527). Taken together, our results from *in vitro* experiments originally suggest that hepatoprotective effects of SIRT1 against APAP toxicity is related to the change in its catalytic activity rather than SIRT1 protein expression and could be at least partially independent of AMPK activity. Thus, 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.

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