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ORIGINAL ARTICLE

Hesperetin is a potent bioactivator that activates SIRT1-AMPK signaling pathway in HepG2 cells

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Abstract

Sirtuin 1 (SIRT1) is a deacetylase enzyme that plays crucial roles in controlling many cellular processes and its downregulation has been implicated in different metabolic disorders. Recently, several polyphenols have been considered as the effective therapeutic approaches that appear to influence SIRT1. The main goal of this study was to evaluate the effect of hesperetin, a citrus polyphenolic flavonoid, on SIRT1 and AMP-activated kinase (AMPK). HepG2 cells were treated with hesperetin in the presence or absence of EX-527, a SIRT1 specific inhibitor, for 24 h. Resveratrol was used as a positive control. SIRT1 gene expression, protein level, and activity were measured by RT-PCR, Western blotting, and fluorometric assay, respectively. AMPK phosphorylation was also determined by Western blotting. Our results indicated a significant increase in SIRT1 protein level and activity as well as an induction of AMPK phosphorylation by hesperetin. These effects of hesperetin were abolished by EX-527. Furthermore, hesperetin reversed the EX-527 inhibitory effects on SIRT1 protein expression and AMPK phosphorylation. These findings suggest that hesperetin can be a novel SIRT1 activator, even stronger than resveratrol. Therefore, the current study may introduce hesperetin as a new strategy aimed at upregulation SIRT1-AMPK pathway resulting in various cellular processes regulation.

Keywords Hesperetin · Polyphenol · SIRT1 · AMPK · HepG2

Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
AMPK	AMP-activated protein kinase
HST	Hesperetin
LKB1	Liver Kinase B1
LXRs	Liver X receptors
NAD^+	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyl transferase
NF-κB	Nuclear factor κB
PGC-1α	PPAR-γ co-activator 1α
PMF	Polymethoxylated flavones
PPAR	Peroxisome proliferator-activated receptor
RSV	Resveratrol
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
SREBPs	Sterol regulatory element-binding proteins
STACs	Sirtuin activating compounds
TNF-α	Tumor necrosis factor α.

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Introduction

SIRT1, mammalian orthologous of the yeast Sir2 protein, is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase with critical regulatory roles in various biologic

processes. Sirtuin family are lysine modifiers that remove the acetyl group from lysine residues of histones or a series of non-histone proteins [25, 27]. Different molecular mechanisms regulate SIRT1 catalytic activity [28]. SIRT1 activity is modulated in response to the levels of NAD^+ which is the SIRT1 substrate. Additionally, SIRT1 is sensitive to the energetic state of cells. When cells encounter energy deficiency, the AMP/ATP ratio is elevated and AMPK, an important cellular energy sensor, is activated subsequently [7, 19]. Activated AMPK increases the expression and activity of nicotinamide phosphoribosyl transferase (NAMPT), resulting in the elevation of NAD^+ concentration [35] which further stimulates SIRT1 activity. On the other hand, activation of SIRT1 leads to deacetylation of key lysine residues on liver kinase B1 (LKB1). This, in turn, enhances LKB1 kinase activity and leads to the phosphorylation/activation of AMPK. Taken together, SIRT1 and AMPK are activated in a bi-directional pathway by each other [14].

SIRT1 has a pivotal role in inflammatory processes, oxidative stress, apoptosis, and in general, increases mammalian cell survival and lifespan. SIRT1 also regulates cellular energy, blood glucose, insulin sensitivity, and lipid balance. SIRT1 mediates PGC-1α (PPARγ co-activator 1α) activation, which enhances fatty acid oxidation, thereby improving glucose homeostasis and promoting insulin sensitization [12]. Moreover, SIRT1 deacetylation regulates hepatic lipogenesis and cholesterogenesis through SREBPs (sterol regulatory element-binding proteins) inactivetion and LXRs (liver X receptors) activation [20]. Downregulation of SIRT1 has been demonstrated to be a contributing factor in liver diseases especially non-alcoholic fatty liver disease, and therefore augmentation of SIRT1 function has been suggested as an appropriate mechanism to ameliorate these disorders [14]. Inflammation and oxidative stress are the common features of many liver disorders, regardless of the cause of the disease [9]. On the other hand, dysregulation of metabolism is the prerequisite of various metabolic disorders especially fatty liver disease. For instance, suppression of lipolytic enzymes including AMPK and PPARα (peroxisome proliferator-activated receptor-alpha), and elevation the enzymes involved in lipogenesis and cholesterogenesis such as SREBPs have been reported as causative factors [37].

Citrus flavonoids are polyphenolic compounds with potent biological properties against several diseases. There is strong evidence supporting the anti-inflammatory, anti-hypertensive, insulin-sensitizing, lipid-lowering, cardio and hepatoprotective properties of citrus flavonoids [2]. The flavanones, hesperetin (HST) and naringenin, and the polymethoxylated flavones (PMF), such as nobiletin and tangeretin are the major subgroups of flavonoids [29]. Hesperidin is the main flavonoid found abundantly in orange fruit and juice [6]. HST is the aglycone metabolite of hesperidin which has better bioavailability. Hesperidin bioavailability is dependent on the glycosides hydrolysis by colonic microflora which converts it to HST and is then absorbed from the end part of intestine [30].

Recently, HST has been shown to be able to exert protective effects against metabolic disorders by modulating both inflammation and metabolic procedures. HST exerts its antioxidant and anti-inflammatory activity through prevention of ROS formation, NF-κB and iNOS inhibition [36]. Protective effects of HST against fatty liver disease and diabetes are attributed to its lipid-lowering efficacy by the reducing of HMG-CoA reductase and ACAT (acyl-CoA:cholesterol acyltransferase) activities [21], and stimulating glycogenolysis and glycolysis or inhibiting the transport of glucose into the cells [10]. Jayaraman et al. showed that the oral intake of HST resulting in lowering of serum triglycerides, cholesterol and glycaemia which improved diabetic condition in rats [18]. LDL-receptor elevation via HST leads to lower plasma LDL cholesterol that may provide cardio-protective effects [4].

During recent years, numerous findings have proposed that polyphenols could influence cellular function by acting as SIRT1 activators. SIRT1 activation is an interesting remedial approach to prevent or ameliorate the progression of many disorders, and therefore, STACs (sirtuin activating compounds) be used in researches and recommended as supplements. The first STAC discovered for SIRT1 by Howitz KT et al. was resveratrol (RSV) [15]. RSV, a polyphenolic compound found in red wine, is a well-known SIRT1 activator [3, 5, 16]. However, STACs that are now being studied may be more efficient than RSV. The effect of HST on SIRT1 regulation has not been yet established. Considering the evidence from previous studies, and the involvement of SIRT1 and HST on some similar cellular procedures, we hypothesized that the beneficial effects of HST might be due to the activation of SIRT1. Therefore, we examined the efficacy and potency of HST on SIRT1 activity and expression compared to RSV as a positive control. This study investigated our hypothesis that HST stimulates SIRT1 which is associated with AMPK phosphorylation.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin were purchased from Gibco (Carlsbad, CA, USA). 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT), nicotinamide (NAM), and HST were purchased from Sigma–Aldrich (St. Louis, MO, USA). EX-527 and HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal antibodies against SIRT1, total AMPK and phospho

(p)AMPK (Thr172), and HRP-conjugated goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA, USA). Hybrid-R™ total RNA isolation Kit was obtained from GeneAll Biotechnology (Seoul, Korea). High-capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Foster City, CA, USA). SIRT1 activity assay kit (fluorometric) was obtained from Abcam (Cambridge, UK). The SuperSignal West Pico PLUS chemiluminescent substrate was obtained from Thermo Fisher Scientific (Inc. Waltham, MA, USA).

Cell culture and treatment

HepG2 cells were obtained from the Iranian Biological Resource Center (Tehran, Iran) and maintained in DMEM medium supplemented with 10% (*v*/*v*) FBS, and 1% penicillin-streptomycin at 37 °C under a 5% CO_2 atmosphere. The treatments were added when the cells achieved approximately 70–80% confluence. HST, RSV, and EX-527 were dissolved in culture grade DMSO to prepare stock solutions. Cells were treated at the final concentration of 50 and 10 μM of RSV and EX-527, respectively. The final concentration of DMSO did not exceed 0.1%. NAM was prepared fresh before each use at 10 mM concentration with sterile ddH_2O. Cells were treated with treatments for 24 h.

Assessment of cell viability

HepG2 cells were seeded in 96 multiwell culture plates at 2×10^4 cells/well. The following day fresh medium containing 0, 1, 10, 25, 50, 100, and 200 μM HST was added. For combined treatments, cells were treated with HST, RSV, EX-527, and NAM, either alone or in combination groups. After 4 h of incubation with MTT at a final concentration of 50 μg/well, the resulting violet crystals were dissolved in DMSO. The absorbance of the solubilized formazan was measured at 540 nm with a microplate reader (BioTek Instruments, Inc. Winooski, VT, USA). The relative cell viability was reported as the percentage of absorbance compared to the control group.

SIRT1 deacetylase activity assay

The nuclear extract of cells (4×10^6) culturing in 25 cm^2 flasks were prepared according to the manufacturer's instructions. Protease inhibitor-free lysis buffer was used for cell lysate preparation. Nuclear samples containing ~2 μg of protein were used for each reaction. The lysyl deacetylase activity of SIRT1 was measured using a SIRT1 fluorometric kit, containing a fluorescence-labeled acetylated substrate peptide which is deacetylated by SIRT1 and emitted fluorescence intensity was read using a microplate fluorometer (BioTek Instruments, Inc. Winooski, VT, USA) at an excitation of 340 nm and emission of 440 nm and normalized to total protein.

SIRT1 gene expression by RT-PCR

Total RNA was extracted from 1×10^6 cells by GeneAll kit. The cDNA synthesis kit was used for reverse transcription experiments. RT-PCR was performed using SYBR green PCR Master Mix (TAKARA, Tokyo, Japan) according to the manufacturer's instructions by the ABI StepOnePlus Detection System (Applied Biosystems, Foster City, CA, USA). Relative levels of gene expression were quantified by the ΔΔCt method. The ΔCt value was normalized to the corresponding value of β-actin as the housekeeping gene. The following primer sequences were employed for PCR amplification: SIRT1 sense-5′-CAGTGGCTGGAACA GTGAGA-3′ and antisense 5′-CTGATTACCATCAA GCCGCC-3′, β-actin sense 5′-TCCTTCCTGGGCAT GGAGT-3′ and antisense 5′-ACTGTGTTGGCGTA CAGGTC-3′, (SinaClon Bioscience Co, Iran).

Western blot analysis

1. HepG2 cells were lysed using RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 100 mM PMSF, 2 mM EDTA, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate combined with protease and phosphatase inhibitor cocktail. Protein concentration of each sample was determined by the bicinchoninic acid (BCA) assay (BCA Protein Assay Kit, Thermo Fisher Scientific Inc. USA). Equal amounts of protein samples (40 μg) were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham, Buckinghamshire, UK), followed by blocking with 5% (*w*/*v*) bovine serum albumin (BSA) in Tris-buffered saline and incubating with primary antibodies overnight at 4 °C. After incubation with HRP-conjugated secondary antibody for 1 h at RT (room temperature), protein bands were visualized using an ECL detection reagent and Chemiluminescence Imaging System (Fusion FX, Vilber Lourmat), then were quantitated by densitometric analysis using ImageJ software (NIH, Bethesda, USA).

Statistical analysis

The results are expressed as the mean ± SEM from at least three independent experiments. GraphPad Prism 6.0 (San Diego, CA, USA) was employed to analyze data. Statistical analyses were performed using analysis of variance (ANOVA) followed by Tukey's post-hoc test for comparison various

groups with each other's. Results were considered statistically significant at a probability value of $p < 0.05$.

The effect of HST and other compounds on cell viability

HepG2 cells were treated by increasing concentrations (0–200 μM) of HST. As shown in Fig. 1a, the cell viability was slightly decreased in a dose-dependent manner, but none of the used concentrations reduced cell viability by 50%. HST at 100 and 200 μM concentrations reduced the cell viability to 84.01 ± 2.47 and 81.08 ± 2.51%, respectively, compared to control. So, the present results suggest that the half maximal inhibitory concentration (IC50) of HST is more than 200 μM. Therefore, HST was used at two concentrations 25 and 100 μM for subsequent experiments.

The effect of EX-527 and NAM on cell viability were examined in combination with HST and/or RSV. The concentrations of RSV, EX-527, and NAM were selected based on separate MTT results (data not shown). As indicated in Fig. 1b, RSV at 50 μM caused a significant increase in viability ($p < 0.01$). EX-527 (survival rate > 95.64 ± 2%) and NAM (survival rate > 101.08 ± 1.96%) showed no significant effects compared to untreated control group. In addition, EX-527 or NAM did not significantly change the viability after co-treatment with either HST and/or RSV compared to their controls (cells that were not treated with these inhibitors). Overall, the concentrations of compounds were selected at their lowest toxic doses based on cell viability for subsequent assessments.

HST-mediated SIRT1 and AMPK activation

In order to assess the effect of HST on SIRT1, cells were treated with HST at 25 and 100 μM and RSV at 50 μM as the positive control. As shown in Fig. 2a, SIRT1 mRNA expression was not changed under the effect of HST. There was only a marginal increase by HST at 25 μM, while it was robustly increased by RSV compared to control. The SIRT1 enzyme activity was subsequently analyzed. As depicted in Fig. 2b, HST caused a 1.91 ± 0.02 and 1.29 ± 0.04-fold increase in SIRT1 activity at 25 and 100 μM concentrations, respectively. RSV also increased SIRT1 activity by 1.58 ± 0.01-fold. These data showed that both HST and RSV are able to effectively stimulate SIRT1 deacetylase activity, although HST at the concentration of 25 μM activated SIRT1 more potently compared to RSV ($p < 0.01$).

Western blot results showed that HST notably increased SIRT1 protein levels (~ 120 kDa) with the maximal effect at 25 μM concentration (1.49 ± 0.05). Similarly a significant effect at 100 μM (1.15 ± 0.02). RSV also increased SIRT1 protein level (1.3 ± 0.04), although a significant difference was observed RSV vs. HST at 25 μM ($p < 0.05$, Fig. 2c). Since activation of SIRT1 is accompanied by AMPK phosphorylation, and the stimulatory effect of RSV on AMPK has been investigated [31], we examined the effect of HST on AMPK. As expected, AMPK phosphorylation, an indicator of AMPK activation, was significantly enhanced by RSV (1.37 ± 0.04). Similarly, cells incubated with HST revealed an enhanced phosphorylated level of AMPK (25 μM: 1.39 ± 0.03 and 100 μM: 1.14 ± 0.04 of control, respectively) (Fig. 2d) indicating the efficiency of HST on AMPK phosphorylation, possibly through SIRT1 activation.

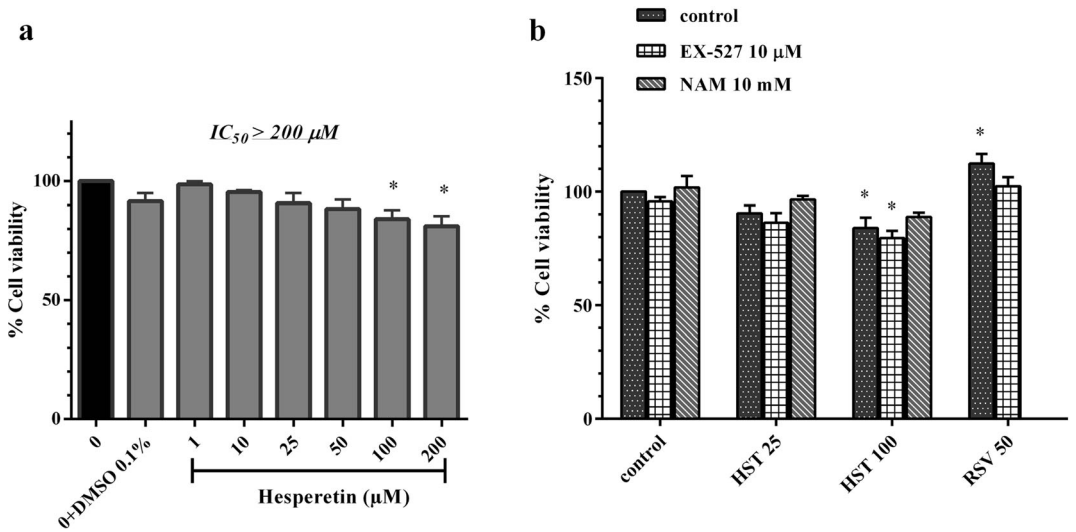

Fig. 1 HepG2 cell viability by exposure to HST and other compounds. Cells were treated with intended components during 24 h and cell viability was determined by MTT assay. **a** Dose-dependent effect of HST in HepG2 cells. **b** Cell viability under combined treatment conditions. Results are expressed as means ± SEM of three independent experiments, statistically significant at $^*p < 0.05$ compared to the untreated control (the first bar)

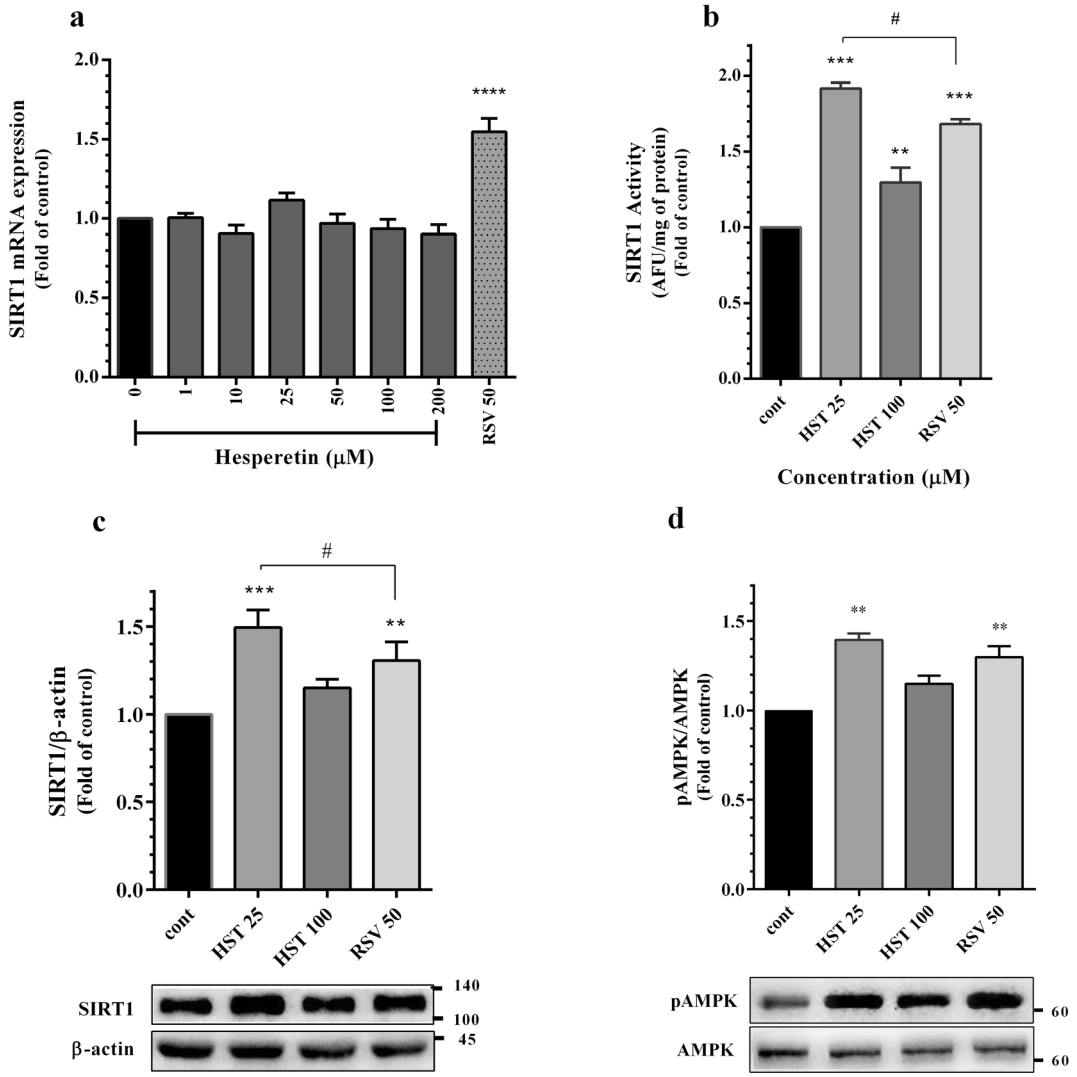

Fig. 2 The effect of HST on SIRT1 and AMPK. HepG2 cells were incubated with the indicated concentrations of HST and RSV during 24 h. **a** mRNA expression of SIRT1. **b** Nuclear extracts of cells were analyzed for SIRT1 activity which was declared as fluorescence intensity at Ex/Em = 340/440. **c** Cell lysis and immunoblotting for SIRT1 and **d** phosphorylated AMPK were scanned and the densitometric quantification normalized by β-actin and total AMPK. Results are expressed as means ± SEM of three independent experiments. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$ compared to control. #$p < 0.05$, ##$p < 0.01$ HST vs. RSV

HST acts in a SIRT1-dependent manner

To further confirm the activation of SIRT1 and AMPK by HST, SIRT1 inhibitors EX-527 and NAM were employed. HST and RSV effectively upregulated SIRT1 protein level and these positive effects were considerably blocked by EX-527 (HST: 1.42 ± 0.02; HST + EX-527: 1.02 ± 0.04; and RSV: 1.26 ± 0.05; RSV + EX-527: 0.92 ± 0.06 of control, respectively). As shown in Fig. 3a and b, EX-527 inhibited HST function ($p < 0.001$) more strongly than RSV ($p < 0.05$). Additionally, in comparison of the cells that were only treated with EX-527 and the cells that were co-treated with both EX-527 and HST or RSV, it was observed that HST could reverse EX-527 inhibition ($p < 0.01$). On the contrary, the inhibitory effect of EX-527 was not changed by RSV suggesting that HST has more impact on SIRT1 compared to RSV.

Furthermore, EX-527 significantly inhibited both HST- and RSV-induced AMPK phosphorylation in HepG2 cells (Fig. 3c, $p < 0.05$). The inhibitory effects of EX-527 on AMPK phosphorylation was reversed by both HST and RSV when compared to the cells that were only treated with EX-527 ($p < 0.0001$, $p < 0.001$, respectively). These results indicate that HST-mediated AMPK phosphorylation is involved in SIRT1 activation.

Our results showed that NAM did not alter the pAMPK level (1.0 ± 0.06, Fig. 4c), and unexpectedly increased the SIRT1 protein level (1.2 ± 0.02. Fig. 4a, b). Besides, the elevation of SIRT1 and pAMPK levels by HST was not abolished by NAM (SIRT1; HST: 1.43 ± 0.03; HST + NAM: 1.41 ± 0.06; and pAMPK; HST; 1.43 ± 0.05; HST + NAM: 1.28 ± 0.04 of control, respectively).

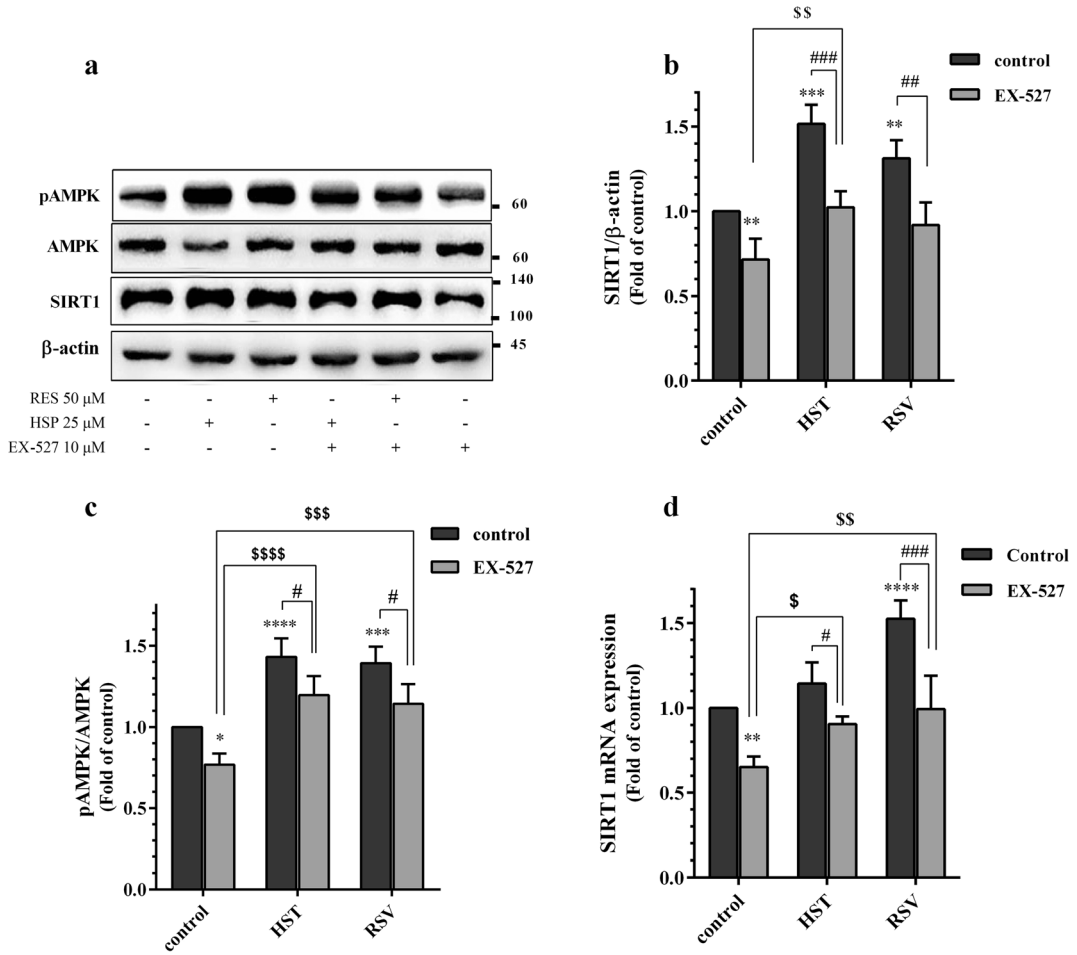

Fig. 3 The effects of EX-527on HST. HepG2 cells treated with HST 25 μM, RSV 50 μM, EX-527 10 μM in single or co-treated groups during 24 h. **a** A representative Western blot image. **b** Quantitative densitometric analysis of SIRT1 protein was normalized by β-actin. **c** Densitometric values of pAMPK were normalized by total AMPK. **d** Relative gene expression of SIRT1. Data are presented statistically significant as mean ± SEM of three independent experiments at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ compared to control. $\#p < 0.05$, $\#\#p < 0.001$, $\#\#\#p < 0.0001$ vs. treatment alone. $\$p < 0.05$, $\$\$p < 0.01$, $\$\$\$p < 0.001$, $\$\$\$\$p < 0.0001$ vs. EX-527

Moreover, we found that SIRT1 mRNA level was significantly decreased by EX-527 (0.65 ± 0.03, Fig. 3d) while increased by NAM (1.28 ± 0.06, Fig. 4d). EX-527 also significantly reduced SIRT1 mRNA in HST and RSV treated groups ($p < 0.05$ and $p < 0.0001$, respectively). Altogether, the alteration in SIRT1 mRNA level was consistent with its protein level by both EX-527 and NAM. The comparison of the two SIRT1 inhibitors EX-527 and NAM showed notable results. As shown in Fig. 4e, f, EX-527 reduced the level of SIRT1 and pAMPK, whereas NAM had the opposite effect, and this may be because NAM could not act as a SIRT1 inhibitor under the condition of our study.

Discussion

The current study represents for the first time that hesperetin (HST) stimulates SIRT1 strongly, and consequently causes AMPK activation. Our findings showed that in addition to increase in SIRT1 protein level, HST acts as a SIRT1 activator. In support of this notion, HST at the concentration of 25 μM provoked SIRT1 activity ~ 2-fold over the basal level. The upregulation of SIRT1 protein followed a similar pattern and the same concentration of HST had the maximal inducing effect on its protein level. There is accumulating evidence that SIRT1 is required for the beneficial effects of RSV [23, 31]. Under our conditions, RSV increased both SIRT1 protein and activity, however, with a significantly lower efficacy compared to HST. Recently, SIRT1 has been the focus of numerous studies influencing the treatment of different diseases [3, 5]. In addition, similar clinical effects of HST and RSV (antiatherogenic effects, lipid metabolism regulation, and amelioration of metabolic dysregulation) have been reported by previous studies [4, 14, 18, 34]. This led us to suggest that beneficial aspects of HST may be SIRT1-mediated. Thus, we hypothesize that HST may be an interesting agent for the treatment of several disorders.

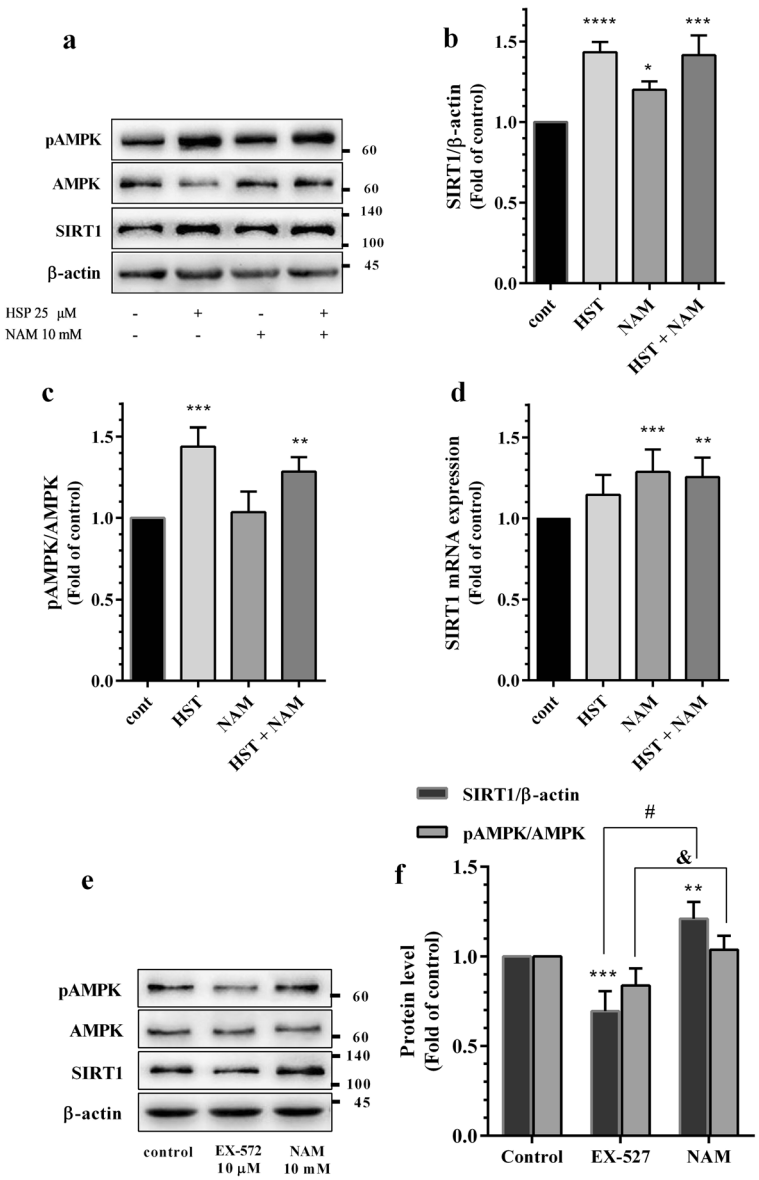

Fig. 4 The effects of NAM on HST. HepG2 cells treated with HST 25 μM and NAM 10 mM during 24 h. **a** A representative Western blot image. **b** Densitometric quantification of SIRT1 normalized by β-actin. **c** Densitometric quantification of pAMPK were normalized by total AMPK. **d** Quantitative comparison of SIRT1 mRNA expression compare to control. **e** A representative Western blot image of comparison between EX-527 and NAM and **f** the densitometric quantification of SIRT1 and pAMPK were normalized by β-actin and total AMPK levels. Data are presented statistically significant as mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared to control. #$p < 0.0001$ and $p < 0.05$

Additionally, we found that none of the HST concentrations significantly altered SIRT1 gene expression. This finding may be relevant to the mechanism of SIRT1 regulation. Consistent with earlier studies, SIRT1 can be modulated by post-transcriptional modifications such as mRNA stability promotion that increases its protein levels [1]. Furthermore, SIRT1 protein undergoes post-translational modifications, since it is a target of various effectors that regulate its protein level or activity [11, 22]. Indeed SIRT1 protein levels as well as activity is not necessarily corresponding to its mRNA level [33]. Thus, HST induces SIRT1 activity and protein expression, although it does not affect the mRNA of SIRT1. But, what is true about HST effect on SIRT1 regulatory status remains to be examined.

Our study was initially assumed to consider both low and high concentrations of HST. Although HST augmented SIRT1 protein level and activity at the concentrations of 100 μM, it was not as robust as the lower concentration. This suggests that HST may exert different responses in various doses. Morin et al. defined the biphasic dose responses for nobiletin which activated SREBP at low doses and inhibited it at higher doses, whereas hesperetin did not show the inhibitory effect at high doses (up to 200 μM) [29]. Ramful et al. also reported the retardation of free-radical-induced hemolysis by citrus fruit extracts in a *U*-shaped dose-response [32]. Therefore, it seems likely that HST has greater effective functions on SIRT1 at lower concentrations (25 μM), so HST 100 μM was ignored for further analysis.

In the present study, we showed that the phosphorylation of AMPK was increased by HST and RSV. Hou et al. showed that resveratrol-mediated SIRT1 activation modulated AMPK phosphorylation [14]. This result suggests that

the positive effects of HST on SIRT1 is accompanied by an induction of AMPK phosphorylation. However, the direct involvement of SIRT1 in AMPK phosphorylation was verified by SIRT1 inhibition. EX-527 is a specific SIRT1 inhibitor which selectively and completely inhibits SIRT1 activity at the concentration of 10 μM [13]. Our results showed that the stimulatory effect of HST on SIRT1 was blocked by EX-527 and it was more significant as compared to RSV-mediated SIRT1 elevation. On the other hand, HST effectively reversed the inhibitory effect of EX-527, which it was not seen by RSV. Therefore, it is reasonable to propose that HST could stimulate SIRT1 more strongly than RSV. Furthermore, SIRT1 inhibition by EX-527 attenuated the HST-induced AMPK phosphorylation, further confirming the contribution of SIRT1 in AMPK activation. In total, these findings postulate that HST can induce phosphorylation of AMPK in a SIRT1-dependent manner.

In addition to EX-527, we also used nicotinamide (NAM) because several studies have shown its inhibitory effect on SIRT1 [8, 14]. However, controversial findings indicate that NAM is able to stimulate SIRT1 protein and activity [24, 26]. Consistent with the latter, we found that NAM was capable to increase SIRT1 protein. The dual role of NAM has been attributed to various factors such as the concentration used, cellular location, and treatment duration [17]. Most studies reported the inhibitory effect of NAM in a short time after treatment [8], while our results obtained in a long period after treatment. This may be explained by the result of Li et al. that reported NAM acts as a predominant precursor for NAD^+ biosynthesis and SIRT1 protein elevation [24].

In conclusion, the present study has supposed the first evidence of the stimulatory effect of HST on SIRT1 and AMPK. Thus HST might be considered as a novel SIRT1 activator with possible therapeutic value relevant to disorders with SIRT1 involvement, such as metabolic and other age-related diseases.

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Authors' contributions HSH conducted research, analyzed data or performed statistical analysis, and wrote the paper. MZ conducted research and edited paper. RM designed research. MHGH conducted research. DI conducted research. AKH conducted research. SHSH conducted research. SHKH had primary responsibility for final content. MN designed research and edited the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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