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Overexpression of SIRT1 Inhibits Corticosterone-Induced Autophagy

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Abstract—Individuals continuously confronted with various stresses in modern life generate high levels of cortisol (corticosterone in rodents), the major glucocorticoid secreted by adrenal gland when hypothalamic–pituitary–adrenal axis is activated. Chronic stress can induce constant release of glucocorticoid and cause many serious health problems, such as mental disorders, cardiovascular diseases and autoimmune diseases. Many studies have suggested the neurotoxic effect of corticosterone is mediated through increased oxidative stress and apoptosis. Although SIRT1 has been shown to be protective against conditions such as DNA damage and oxidative stress through autophagy regulation, the exact role of SIRT1 and autophagy in corticosterone-induced stress is still unclear. By utilizing a cellular stress model of exposing cells to corticosterone, our study found that there were a dose-dependent decrease in SIRT1 and an increase in LC3B II/I expressions with increasing concentrations of corticosterone. In combination with SIRT1 overexpression and knockdown plasmids, the regulation of SIRT1 expression *in vitro* demonstrated that SIRT can inhibit corticosterone-induced autophagy and enhance cell apoptosis. These findings might help us better understand the role of SIRT1 and autophagy activation in chronic stress.

Key words: hypothalamic–pituitary–adrenal axis, corticosterone, chronic stress, SIRT1, autophagy.

INTRODUCTION

Corticosterone (CORT) is a glucocorticoid released by the adrenal glands in rodents and birds (cortisol in humans) following the activation of hypothalamic–pituitary–adrenal (HPA) axis by stress (Cain and Cidlowski, 2017; Filipović et al., 2017). CORT can increase the blood pressure and energy metabolism rate, which in turn plays a fundamental role in the ability of the body to cope with emergencies (Spiers et al., 2015). The hyperactivity of HPA axis induced by chronic stress can result in the constant secretion of CORT, which can cause psychological and immune alterations. This, in turn, can result in diseases such as depression, diabetes and autoimmune disease. Exogenous treatment with glucocorticoid and excessive endogenous glucocorticoid found in Cushing's disease culminate in cerebral atrophy, cognitive impairment and neuropsychiatric disorders such as depression and anxiety (Belanoff et al., 2001; McEwen, 2007; Judd et al., 2014). The underlying mechanism might include diminished dendritic branching, myelinogenesis and synaptic connections between neurons, suppressed glutamate neurotransmission and decreased neurotrophic factors like BDNF in key brain regions such as hippocampus and prefrontal cortex (Brown, 2009; Tata and Anderson, 2010; Popoli et al., 2011). The dysregulation of HPA axis is also implicated in the pathology of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (Du and Pang, 2015). Several studies have demonstrated the correlation between elevated plasma glucocorticoid levels and AD pathology (Csernansky et al., 2006; Baglietto-Vargas et al., 2013). High levels of circulating cortisol can accelerate Aβ deposition and tau phosphorylation (Green et al., 2006). Treatment with the glucocorticoid receptor antagonist Mifepristone can alleviate the molecular pathological changes associated with Aβ deposition and tau phosphorylation and also relieve the symptoms such as cognitive impairment (Baglietto-Vargas et al., 2013). Both *in vivo* and *in vitro* studies have shown that CORT could enhance cell apoptosis and neurotoxicity by

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Abbreviations: CORT, corticosterone; HPA, hypothalamic–pituitary–adrenal; ROS, reactive oxygen species; AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; Sirtuin, silent mating type information regulation 2 homolog; NAD, nicotinamide adenine dinucleotide; PPARγ, peroxisome proliferator-activated receptor γ; PGC1-α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; FOXO, forkhead box protein O; Atg5, autophagy-related protein 5; LC3, microtubule-associated proteins 1 light chain 3; PE, phosphatidylethanolamine; VSC 4.1 cells, ventral spinal cord 4.1 cells; EGFP, enhanced green fluorescent protein.

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increasing oxidative stress and generation of reactive oxygen species (ROS) (Behl et al., 1997; Gao et al., 2015; Spiers et al., 2015; Filipović et al., 2017).

Silent mating type information regulation 2 homolog 1 (SIRT1) is an NAD (nicotinamide adenine dinucleotide)-dependent histone deacetylase with a wide range of substrates besides histones. This family of proteins is highly conserved in organisms. The longevity protein, Sir2 (SIRT1 homolog), was first identified in yeast (Michan and Sinclair, 2007). While the role of SIRT1 in lifespan extension is still not clear, it has been implicated in multiple cellular activities, such as transcriptional silencing, cell survival and maintenance of energy and metabolic homeostasis (Kim et al., 2016). These functions are mediated through interactions with different proteins. By deacetylation of transcription factors such as PPARγ (peroxisome proliferator-activated receptor γ) and PGC1-α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), SIRT1 can regulate hepatic gluconeogenesis and insulin secretion (Picard et al., 2004; Nemoto et al., 2005). SIRT1 can also influence cell fate in response to oxidative stress through deacetylation of p53, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and FOXO (forkhead box protein O) (Luo et al., 2001; Brunet et al., 2004; Yeung et al., 2004; Solomon et al., 2006).

Autophagy is a cellular bulk degradation process that assists cells in clearing damaged cytosolic organelles and misfolded proteins. During starvation, it assists in generating energy from the breakdown of products (Rabinowitz and White, 2010). Both SIRT1 and autophagy are involved in calorie restriction and age-related pathologies, with several studies showing that SIRT1 can directly regulate autophagy through deacetylating proteins like autophagy-related protein 5 (Atg5) and FOXO1 during nutritional deprivation and oxidative stress (Lee et al., 2008; Rabinowitz and White, 2010; Ou et al., 2014a). Atg5 and microtubule-associated proteins 1 light chain 3 (LC3) are both key proteins involved in the elongation of autophagosome membrane, which makes them good markers for studying autophagy level. LC3 is the mammalian homolog of yeast Atg8, and its most studied isoform is LC3B. The newly translated LC3 protein is cleaved by Atg4 protease at its C-terminus and becomes LC3-I. After the initiation of autophagy, the exposed C-terminal glycine of LC3-I is conjugated to phosphatidylethanolamine (PE) by a series of ubiquitination-like reactions, resulting in the formation of membrane-bound LC3B-II. Thus, the expression ratio of LC3-II to LC3-I is an important indicator of autophagy. The ubiquitin-binding protein p62 can directly bind to LC3B, and be degraded in autophagosome along with its associated ubiquitinated proteins. Thus, the degradation of p62 can also be used as a protein marker of autophagy.

SIRT1 has been shown as a cellular guardian against diverse oxidative stress and DNA damage (Luo et al., 2001; Haigis and Guarente, 2006). However, the role of SIRT1 in CORT induced stress has not yet been deciphered. Ventral spinal cord (VSC 4.1) cell is a motor neuron hybrid cell line generated by the fusion of embryonic rat ventral spinal cord neuron with mouse N18TG2 neuroblastoma cell, and has both neuronal markers (such as neurofilament-H, synaptophysin, neuron-specific enolase) and cholinergic marker (i.e. choline acetyltransferase) (Smith et al., 1994; Ferguson and Subramanian, 2016). This study was aimed at elucidating the connection between SIRT1 and autophagy in CORT-induced stress. In doing so, we used a cell stress model by exposing VSC 4.1 cells to high levels of CORT and overexpressed or knocked down SIRT1 *in vitro*. We found that SIRT1 can inhibit CORT induced autophagy and enhance apoptosis, which would shed light on the connection between SIRT1 and autophagy in CORT induced stress.

EXPERIMENTAL PROCEDURES

Materials

VSC 4.1 cells were purchased from Shanghai Cell Institute of Chinese Academy of Sciences. CORT was purchased from Selleckchem. Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, JP). Primary antibodies: anti-SIRT1 antibody (1:1000, Cell Signaling Technology), anti-Atg5 antibody (1:1000, Abcam), anti-p62 antibody (1:200, Abnova), anti-LC3B antibody (1:1000, Novus), anti-GAPDH antibody (1:1000, Cell Signaling Technology). Secondary antibodies: HRP-conjugated goat anti-rabbit IgG (1:5000, Bioker), HRP-conjugated goat anti-mouse IgG (1:8000, EarthOX). jetPRIME Transfection Reagent was purchased from Polyplus Transfection (Illkirch, FR). Plasmid encoding human_SIRT1-P2A-EGFP (enhanced green fluorescent protein) and its EGFP control vector were purchased from OBiO Technology (Shanghai, CN). SIRT1-specific shRNA and control shRNA were purchased from Hanbio Biotechnology (Shanghai, CN). Bestar qPCR RT and Bestar SybrGreen qPCR mastermix kit were obtained from DBI Bioscience (Ludwigshafen, DE). *In Situ* Cell Death Detection Kit was supplied by Roche (Basel, CH). VECTASHIELD Antifade Mounting Medium with DAPI was purchased from Vector laboratories (Burlingame, CA).

Cell Culture and CORT Treatment

Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum and 1% penicillin–streptomycin antibiotic at 37 °C in a highly humidified atmosphere containing 5% CO_2. Medium was changed every 2 days and cells were passaged upon reaching 80% confluence. CORT was dissolved with DMSO into 160 mM stocking solution and diluted with culture medium to obtain working concentration. Same amount of DMSO was used as vehicle control. Cells seeded in 96-well plates were incubated with different concentrations of CORT (25–600 μM) for 8, 12, 24, and 48 h in order to find the appropriate damage condition.

Cell Viability Assay

Cell survival rates were measured using CCK-8. Experiments were performed according to manufacturer's

instruction. Briefly, cells seeded in 96-well plates were incubated with culture medium containing 10% CCK-8 solution at 37 °C for 3 h. The absorbance at 562 nm was then measured. Experiments were repeated three times in triplicate. Cell viability was calculated with the formula: Cell survival rate (%) = [(At – Ab) / (Ac – Ab)] × 100%; where At = Absorbance value of test group, Ab = Absorbance value of blank group, Ac = Absorbance value of control group.

Western Blot Analysis

After transfection or CORT treatment, cells were collected with ice-cold RIPA buffer containing protease inhibitor cocktail and sonicated in an ice bath. The samples were then centrifuged at 12,000 ×*g* for 30 min at 4 °C. Supernatant was transferred to a new Eppendorf tube and the protein concentration was determined using BCA protein assay kit. Samples were adjusted to the same concentration (2 μg/μL) and denatured with 5 × SDS loading buffer in boiling water for 10 min. Equal amounts of protein (20 μg) were loaded onto each lane and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at a constant voltage of 200 V until the loading dye reached the bottom of the gel. The gels were transferred onto PVDF membranes with a constant current of 300 mA for 70 min. The membranes were blocked with 5% skim milk at room temperature for 3 h, and incubated with 5% BSA-TBST diluted primary antibodies at 4 °C overnight. After washing three times with TBST, the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 2.5 h. Protein bands were detected with enhanced chemiluminescence (ECL) detection kit. The quantitative evaluation of these bands was then carried out with Image Lab software. Optical density of each band was normalized with that of corresponding internal control. All experiments were performed thrice.

Transfection

Cells were seeded in six-well plates at 2 × 10^5 cells per well the day before transfection. Expression vectors encoding either SIRT1 or SIRT1 shRNA and their control vectors were introduced into cells using jetPrime transfection reagent according to the manufacturer's instruction. For CORT treatment, cells were exposed to the indicated concentrations of CORT 24 h after transfection.

RT-qPCR

Total RNA was extracted using ice-cold Trizol reagent according to the manufacturer's instruction and reversely transcribed into cDNA using Bestar qPCR RT kit. 2 μL 5× RT buffer, 0.5 μL RT Enzyme Mix, 0.5 μL Primer Mix and 1 μg RNA were mixed and adjusted to a final volume of 10 μL with RNase free water. The following procedure was performed: 15 min at 37 °C and 5 min at 98 °C. Quantitative PCR was performed using Bestar SybrGreen qPCR mastermix kit. 10 μL 2× SYBR GREEN Mix, 0.5 μL Forward primer (10 μM), 0.5 μL Reverse primer (10 μM), and 100 ng cDNA were mixed and added to ddH_2O to a final volume of 20 μL. The following conditions were used: predenaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 30 s. Melting curves were generated for quality control. Primers used are shown in Table 1. Relative expression level of target gene was determined by $2^{-\triangle\triangle Cq}$ method and expressed as fold change in expression of experiment group in relation to control group. β-Actin was used as internal control. Experiments were performed in triplicate and repeated three times.

Table 1. Primers used for RT-qPCR.

Gene	Primer	5′-3′
Human_Sirt1	Forward	TAGGCGGCTTGATGGTAATC
	Reverse	TCTGGCATGTCCCACTATCA
Mouse_Sirt1	Forward	TTGTGAAGCTGTTCGTGGAG
	Reverse	GGCGTGGAGGTTTTTCAGTA
Atg5	Forward	AACTGAAAGAGAAGCAGAACCA
	Reverse	TGTCTCATAACCTTCTGAAAGTGC
p62	Forward	GCTGCCCTATACCCACATCT
	Reverse	CGCCTTCATCCGAGAAAC
β-actin	Forward	CTGTCCCTGTATGCCTCTG
	Reverse	ATGTCACGCACGATTTCC

TUNEL Staining

Cell apoptosis rate after CORT treatment was detected using *In Situ* Cell Death Detection Kit. Experiments were performed according to manufacturer's instructions. Briefly, cells were cultured on coverslips in six-well plates, including two additional wells for positive and negative controls. Cells were fixed at room temperature for 1 h after washing thrice with PBS. Then, cells were permeabilized using 0.1% TritonX-100 in 0.1% sodium citrate for 2 min on ice. DNase I (1000 U/mL) was added to the positive control and incubated for 10 min. Label solution (50 μL) was added to the negative control. Enzyme solution (50 μL) was mixed with label solution (450 μL). Each sample was incubated with TUNEL reaction mixture (50 μL), with the exception of the negative control, at 37 °C for 1 h. Samples were embedded with anti-fade mounting medium containing DAPI before

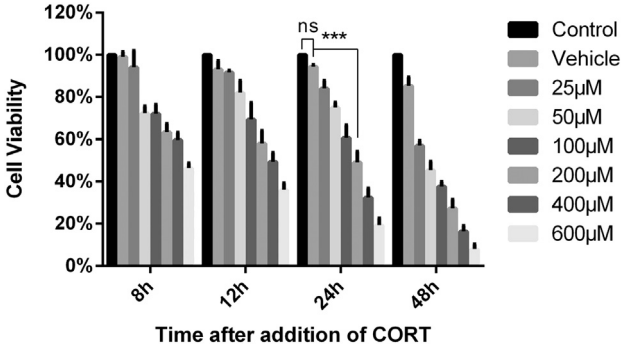
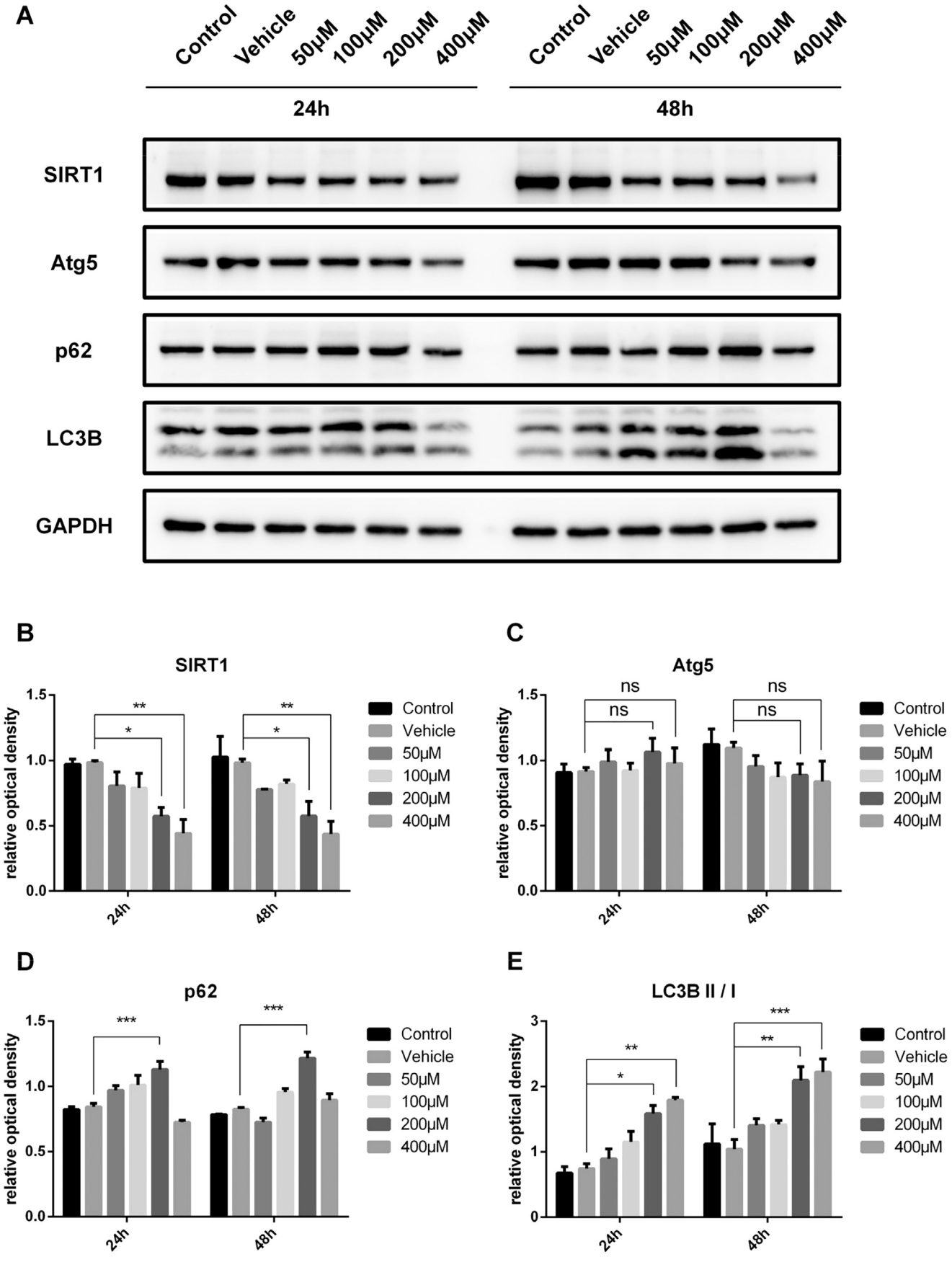


Fig. 1. Effect of different concentrations of CORT on cell viability.Cell viability was measured with CCK-8 to determine the appropriate damage condition. Data were presented as mean ± SEM (n = 3). ***P < 0.001, two-way ANOVA with Tukey's multiple comparisons test. ns, not significant; CORT, corticosterone.


A
Control
Vehicle
50μM
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GAPDH
B
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1.5
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24h
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Control
Vehicle
50μM
100μM
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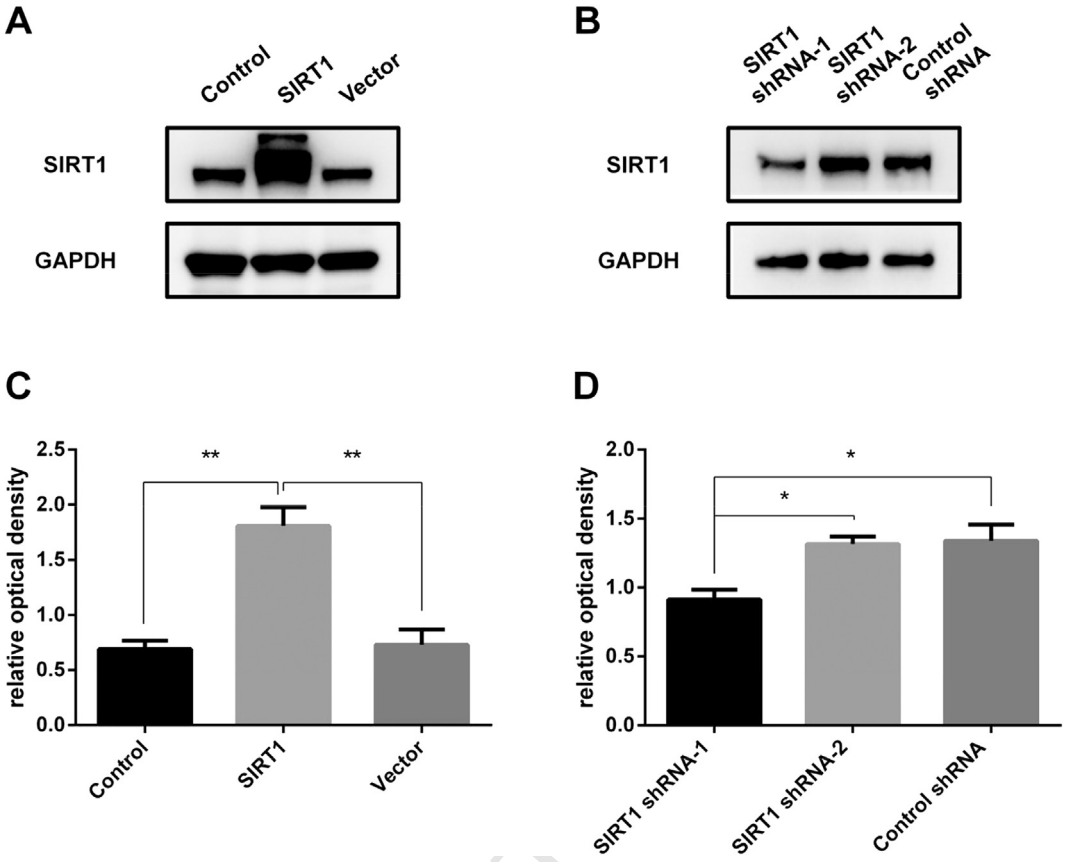

Fig. 3. Overexpression and knockdown of SIRT1 *in vitro*.(A) Western blot analysis of VSC 4.1 cells transfected with SIRT1 overexpression (SIRT1) and EGFP control (Vector) plasmids. Control group was not transfected. The upper band in the SIRT1 overexpression group corresponds to SIRT1-EGFP fusion protein. (B) Western blot analysis of VSC 4.1 cells transfected with plasmids encoding different SIRT1 shRNA or their control vector. (C) and (D) were quantified results of (A) and (B) respectively. Data were expressed as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, one-way ANOVA with Tukey's multiple comparisons test.

being analyzed under a fluorescence microscopy. TUNEL-Positive cells in five randomly selected fields of each sample in three experiments were quantified with Image J software.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis and graphs were made using Graphpad Prism 6. Differences between two groups were tested by an unpaired t-test. One-way ANOVA and two-way ANOVA followed by Tukey's post hoc test were used to determine statistical significance between three or more groups. $P < 0.05$ was deemed statistically significant, with $P < 0.01$ deemed as highly statistically significant.

RESULTS

The Effect of CORT on VSC 4.1 Cell Viability

In order to define the dose dependent sensitivity of VSC 4.1 cells to CORT, we exposed them to different concentrations of CORT from 25 μM to 600 μM for 8 h, 12 h, 24 h and 48 h. The cell viability was gradually decreased with increasing exposure time and concentration of CORT (Fig. 1). We observed about 50% decrement in cell viability after treatment with 200 μM CORT at 24 h. This was then chosen for subsequent experiments.

CORT Treatment Can Reduce SIRT1 Expression and Induce Autophagy

Considering the protective role of both SIRT1 and autophagy in oxidative stress, we analyzed the expression of SIRT1 and autophagy related proteins after different concentrations of CORT treatment for 24 h and 48 h (Fig. 2A). We found that the expression of SIRT decreased (Fig. 2B), while LC3B II/I was significantly increased when cells were exposed to 200 μM and 400 μM of CORT for 24 h and 48 h compared with the vehicle group (Fig. 2E). p62 expression was positively correlated with LC3B II/I when treated with 200 μM CORT for 24 h (Fig. 2D), which might because of the blockage of autophagic degradation or its transcriptional

Fig. 2. CORT treatment reduces SIRT1 expression and induces autophagy.(A) Protein expressions after different concentrations of CORT exposure. Quantification levels of (B) SIRT1, (C) Atg5, (D) p62 and (E) LC3B II/I. Data were presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA with Tukey's multiple comparisons test. ns, not significant.

upregulation. Atg5 expression was not altered by CORT treatment (Fig. 2C).

Overexpression of SIRT1 Can Inhibit CORT Induced Autophagy

In order to ascertain the connections between SIRT1 downregulation and autophagy initiation following CORT exposure, we first overexpressed and then knocked down SIRT1 expression respectively. As confirmed by the western blot analysis (Fig. 3A, B), SIRT1 expression was increased by more than two-folds after being transfected with the overexpression plasmid (Fig. 3C). We further designed two different SIRT1-specific shRNA constructs. One construct significantly reduced SIRT1 expression and was chosen for subsequent experiments (Fig. 3D). 24 h after transfection, cells were exposed to 200 μM CORT for 24 h, and proteins were extracted for western blotting (Fig. 4A, 5A). In line with our previous results, CORT treatment significantly decreased SIRT1 expression and enhanced LC3B II/I level (Fig. 4B, C); however, autophagy was restored to control levels after overexpression of SIRT1 (Fig. 4C). Neither SIRT1 overexpression nor CORT treatment affected Atg5 expression (Fig. 4D). Although p62 expression was increased following CORT administration, the addition of SIRT1 did not affect its expression (Fig. 4E), indicating that SIRT1 might not have an effect on autophagosome degradation. (See Fig. 6.)

Inhibition of SIRT1 Reinforced CORT Induced Autophagy

We further demonstrated our hypothesis through silencing SIRT1 in CORT treated VSC 4.1 cells (Fig. 5A). Although CORT did not induce further decrement in SIRT1 expression after shRNA transfection (Fig. 5B), its low expression level was sufficient to relieve its inhibition of autophagy as indicated by the further increase of LC3B II/I level (Fig. 5C). Atg5 was not affected by either SIRT1 knockdown or CORT treatment (Fig. 5D). The increase of p62 was also not affected by SIRT1 knockdown (Fig. 5E).

CORT Treatment Increased p62 mRNA Level

RT-qPCR was employed to examine whether the increase of p62 expression was due to enhanced transcription and if SIRT1 affected this process. Plasmid transfection significantly altered SIRT1 mRNA levels; however, CORT treatment only decreased SIRT1 mRNA in overexpression group, which may have been due to the effect of CORT on exogenous plasmid expression (Fig. 6A, B). p62 mRNA was increased by CORT but neither SIRT1 overexpression nor knockdown (Fig. 6C, D). Also, Atg5 mRNA was not affected by either transfection or CORT exposure (Fig. 6E, F).

SIRT1 Enhanced CORT Induced Apoptosis

We carried out TUNEL staining to detect apoptosis rate after different concentrations of CORT treatment for 24 h. A dose-dependent increase of apoptosis was observed with increasing concentrations of CORT, and 200 μM CORT induced significant apoptosis when compared to the vehicle group (Fig. 7A, B). In order to detect whether the inhibition of CORT-induced autophagy by SIRT1 overexpression affected apoptosis, cells transfected with overexpression (SIRT1-EGFP) or control (EGFP) plasmids were exposed to 200 μM CORT for 24 h and fixed for TUNEL staining. Cells double positive for TUNEL and EGFP are indicated in merged and magnified images with white arrowheads (Fig. 8A). There was a significant difference in apoptosis rate between cells transfected with SIRT1 and EGFP control, which suggests that reduced autophagy by SIRT1 overexpression culminates in apoptosis increment (Fig. 8B).

DISCUSSION

CORT is an important stress hormone released following HPA axis activation and regulates our responses to unexpected and stressful events. It mobilizes energy stores and stimulates gluconeogenesis, thus preparing the body for “fight or flight” responses. However, modern people are constantly exposed to chronic stressors such as exhaustive work schedules, which in turn trigger a prolonged surge of glucocorticoid levels. This results in higher risks of cardiovascular diseases, mood disorders and autoimmune diseases (de Kloet et al., 2005). Hypercortisolemia can cause memory loss and learning impairments and has been associated with several mood disorders such as depression and anxiety (De Kloet et al., 1988, Brown et al., 1999, Manji et al., 2001). High levels of exogenous CORT may reproduce the symptoms of neuronal atrophy seen in patients with mental illness, which can be remedied by adrenalectomy (Manji et al., 2001). Exposure to excess CORT caused both reversible and irreversible changes in cognition and hippocampal structure (Brown et al., 1999; Sapolsky, 2000). One underlying mechanism of CORT-induced apoptosis in neurons is through ROS generation (Spiers et al., 2015). The increased metabolic rate by CORT culminates in overproduction of free radicals as by-products of normal cellular metabolism. This, in turn, can cause disruption of cellular redox balance (Du et al., 2009). Although SIRT1 has been evidenced to protect cells against apoptosis and promote their survival under oxidative stress and starvation, its specific role in CORT-induced stress has not been investigated.

In this study, we found that CORT had a dose and time dependent effect on cell viability that reduced to around 50% after 200 μM CORT treatment for 24 h (Fig. 1). A dose-dependent decrease of SIRT1 and an increase of LC3B II/I expression were observed with increasing concentrations of CORT, suggesting that SIRT1 decrement may facilitate the initiation of autophagy (Fig. 2). Also, the decreased expression of p62 is a good indicator of autophagic flux as it is involved in the degradation of ubiquitinated proteins by autolysosomes (Bjørkøy et al., 2005; Serhiy et al., 2007). However, p62 did not show inversed expression pattern in comparison with LC3B II/I level (Fig. 2). Several studies have reported the increment of both p62 expression and autophagic flux to be a result of either autophagic degradation inhibition or transcriptional upregulation of p62 (Colosetti et al., 2009; Zheng et al., 2011; Sahani et al.,

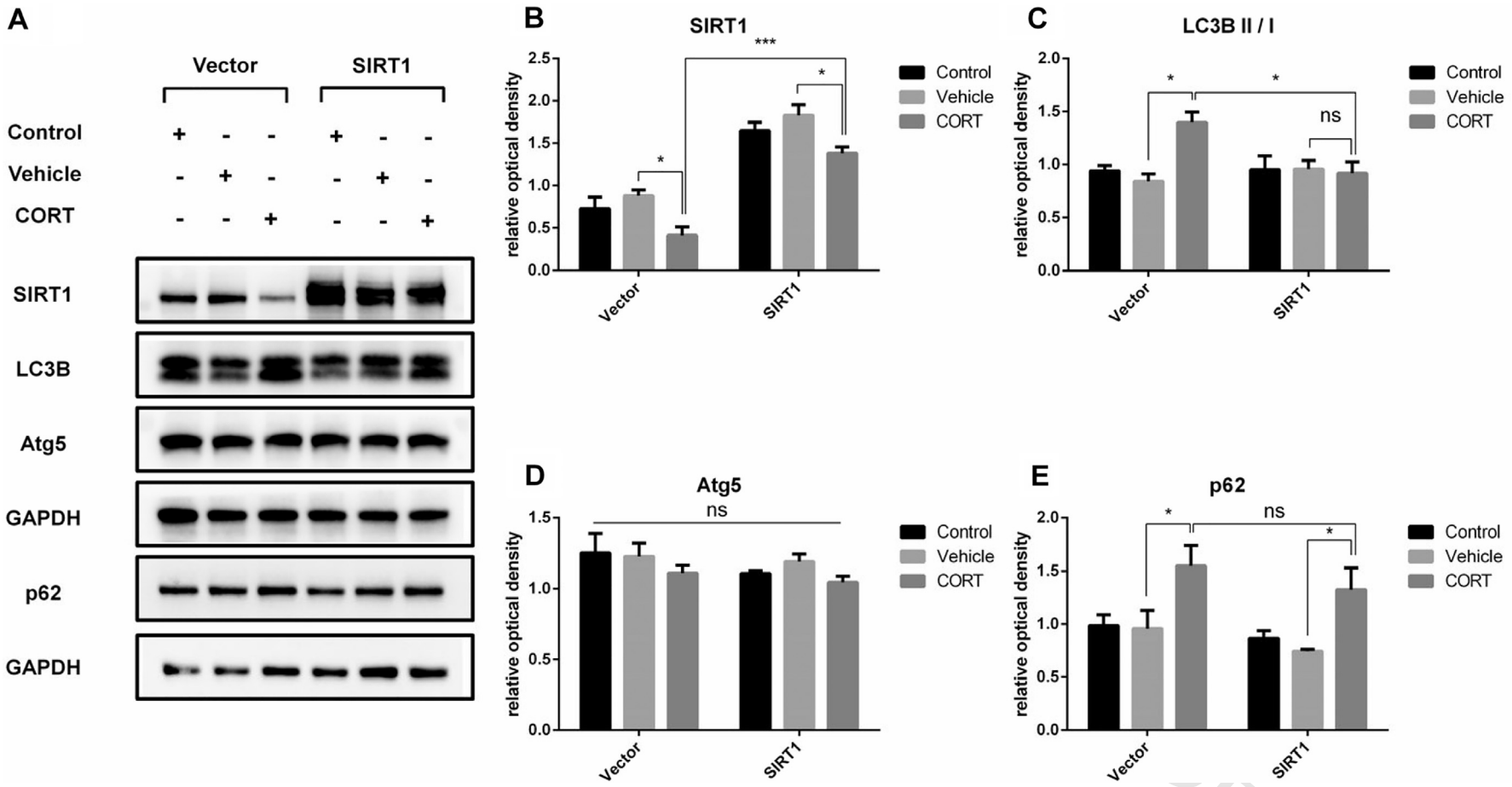

Fig. 4. Overexpression of SIRT1 inhibits CORT induced autophagy.(A) Western blot analysis of VSC 4.1 cells treated with 200 μM CORT (CORT), or culture medium containing the same amount of solvent (Vehicle), or normal culture medium (Control) after transfection. (B), (C), (D) and (E) were quantified results of SIRT1, LC3B II / I, Atg5 and p62 respectively. Data were expressed as mean ± SEM (n = 3). *P < 0.05, ***P < 0.001, two-way ANOVA with Tukey's multiple comparisons test. ns, not significant; CORT, corticosterone.

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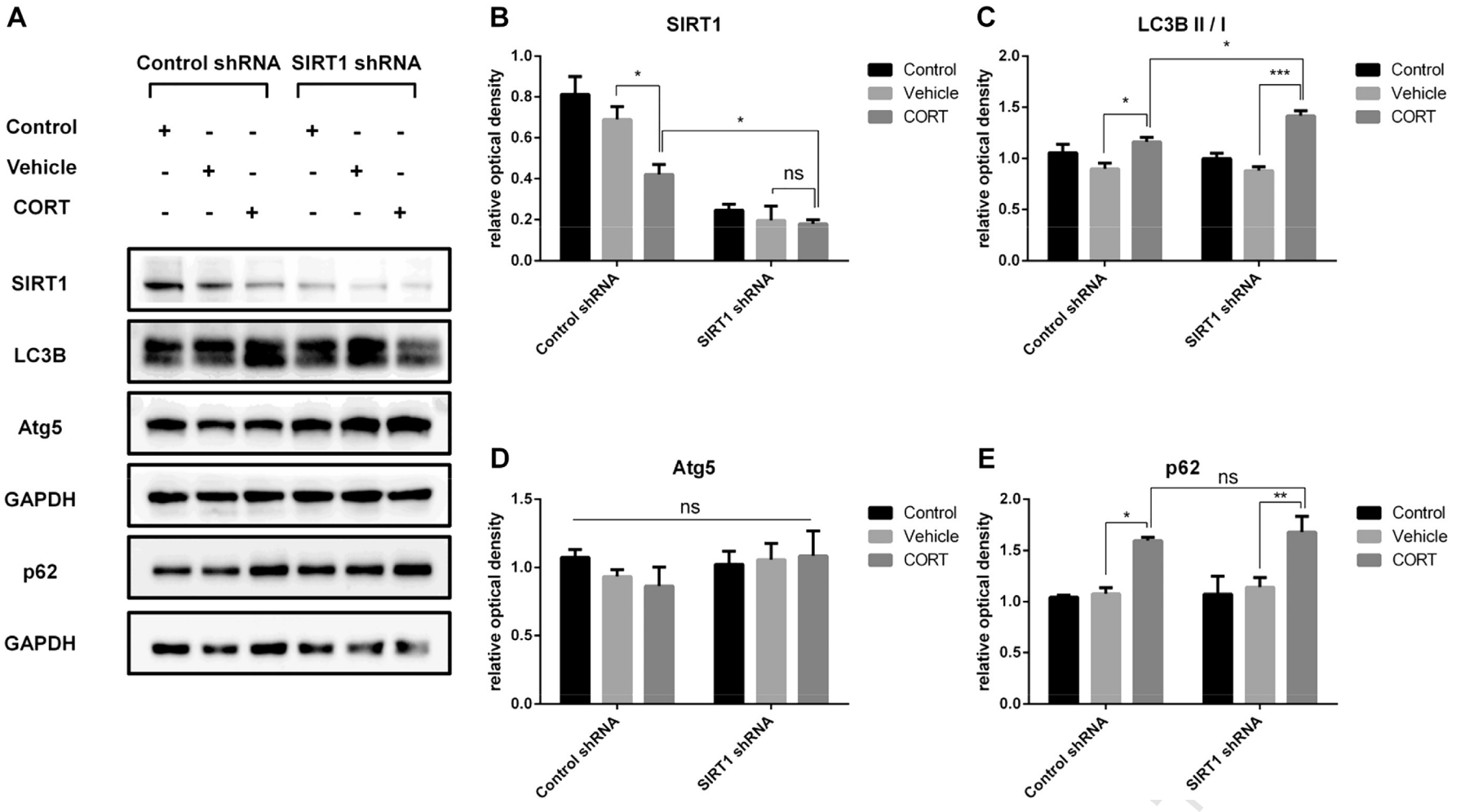

Fig. 5. Knockdown of SIRT1 reinforces CORT induces autophagy.(A) Western blot analysis of CORT treated VSC 4.1 cells after silencing of SIRT1. (B), (C), (D) and (E) were quantified results of SIRT1, LC3B II/ I, Atg5 and p62, respectively. Data were expressed as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA with Tukey's multiple comparisons test. ns, not significant; CORT, corticosterone.

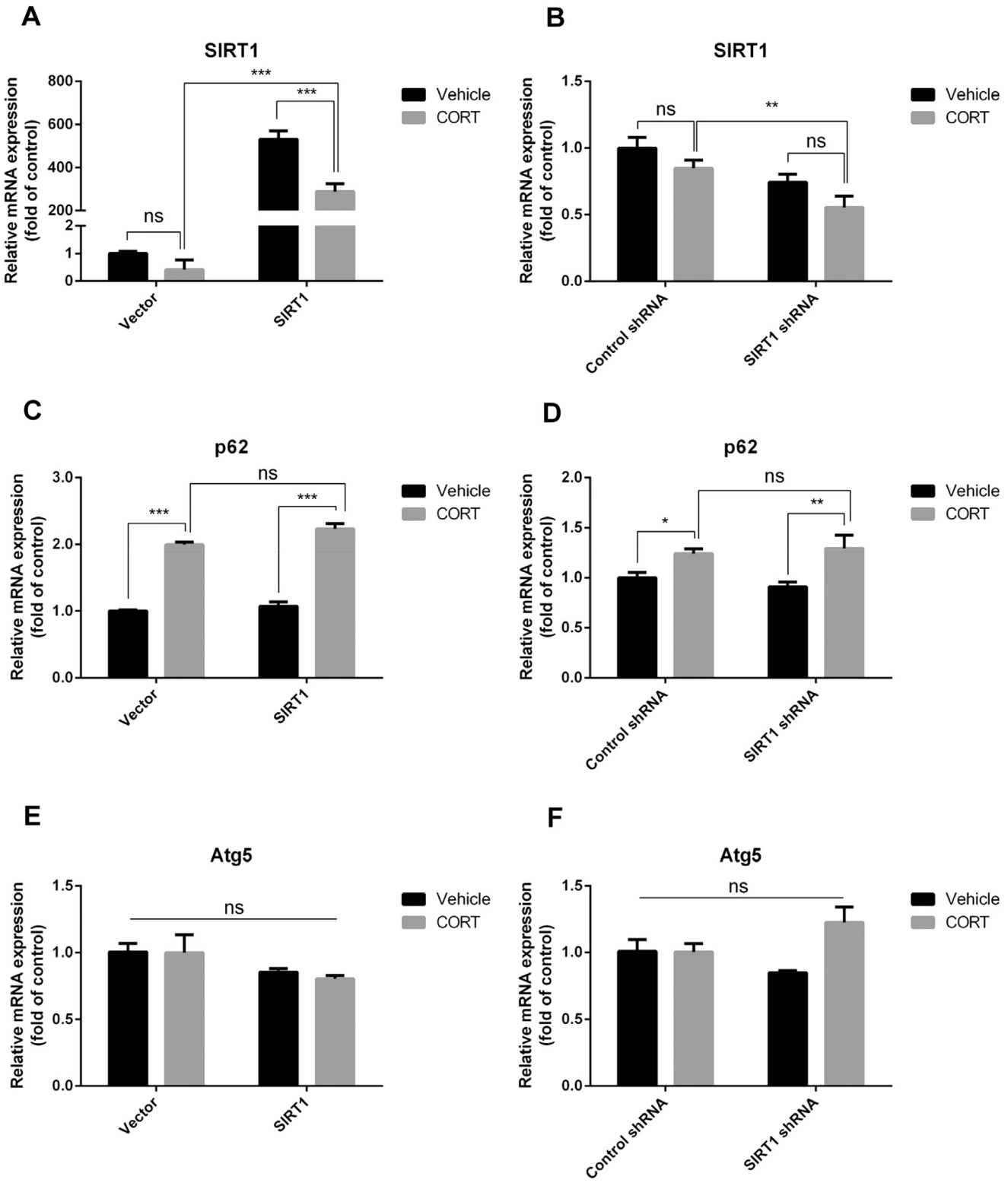

Fig. 6. mRNA alterations after transfection and CORT treatment.mRNA levels of SIRT1 (A) and (B), p62 (C) and (D), Atg5 (E) and (F) were measured with quantitative PCR and normalized to the expression levels of β-actin. The results were presented as fold changes relative to control (Vehicle + Vector group). Data were expressed as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA with Tukey's multiple comparisons test. ns, not significant; CORT, Corticosterone.

2014). We further confirmed that the increase of p62 expression was, at least in part, due to its transcriptional enhancement by the CORT exposure (Fig. 6).

It has been substantiated that SIRT1 can deacetylate Atg5 or FOXO1 and initiate autophagy during starvation (Lee et al., 2008; Hariharan et al., 2010), or mediate

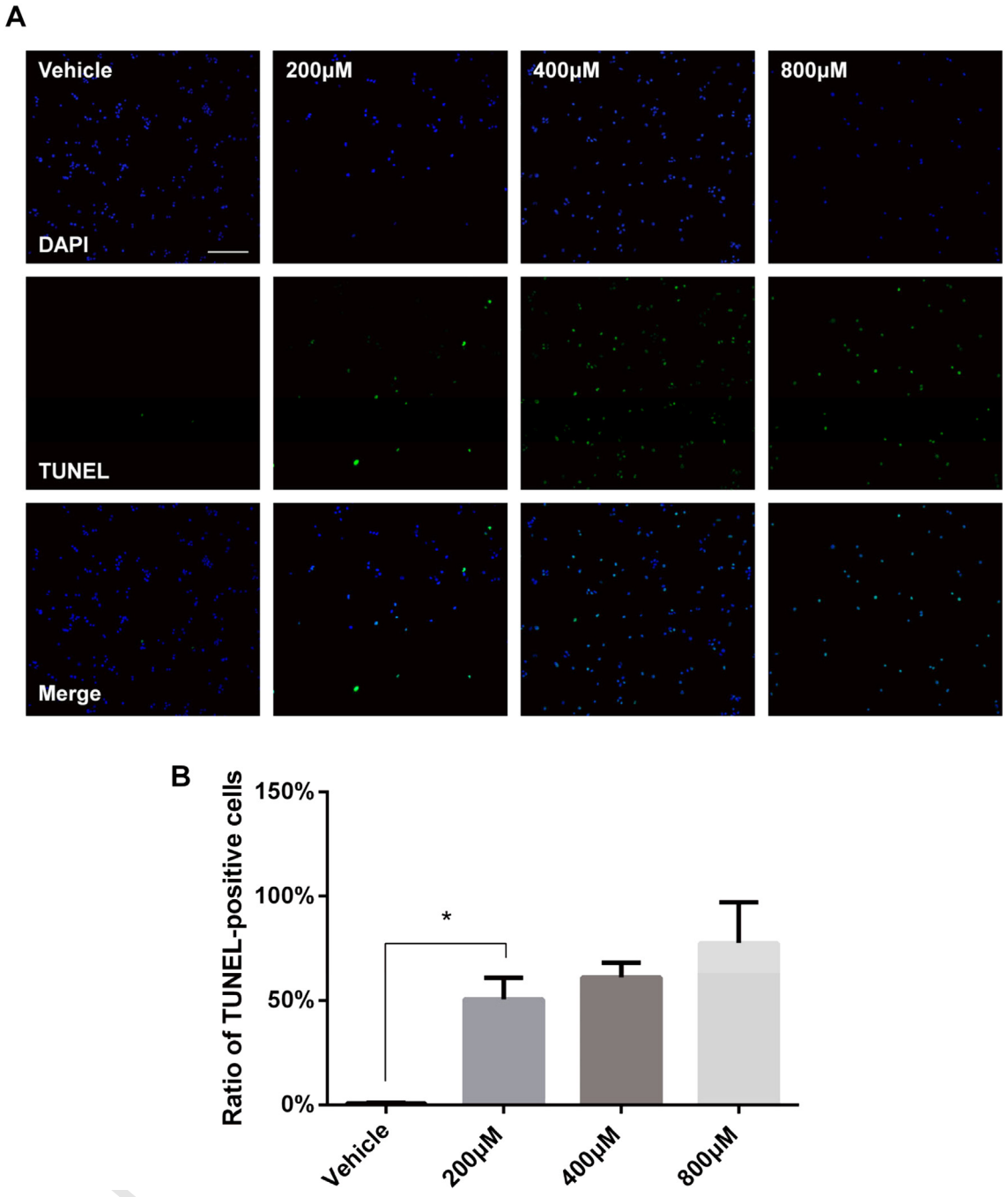

Fig. 7. Dose-dependent effect of CORT on apoptosis.(A) Representative TUNEL staining images of cells treated with different concentrations of CORT. (B) Quantification of TUNEL-positive cells. Data were expressed as mean ± SEM (n = 3). *P < 0.05, one-way ANOVA with Tukey's multiple comparisons test. Scale bar: 200 μm.

oxidative stress-induced autophagy through the mTOR pathway (Ou et al., 2014b). In this study, we found that CORT-induced autophagy was suppressed by SIRT overexpression and elevated by its knockdown with no change in Atg5 expression (Figs. 4, 5). These results indicate that SIRT1 may inhibit autophagy in CORT induced-stress through a different signal pathway. SIRT1 inhibition by Sirtinol, a class III HDAC inhibitor, has been reported to enhance autophagy in human breast cancer cells (Jing et al., 2012). That said, further studies are needed to elucidate the mechanism by which SIRT1 inhibits autophagy during CORT treatment.

We then performed TUNEL assay and found that cell apoptosis rate was positively correlated with CORT levels (Fig. 7). Our result also suggests that overexpression of SIRT1 promotes CORT-induced apoptosis (Fig. 8). The crosstalk between apoptosis and autophagy is complex. These two processes can be independently regulated under

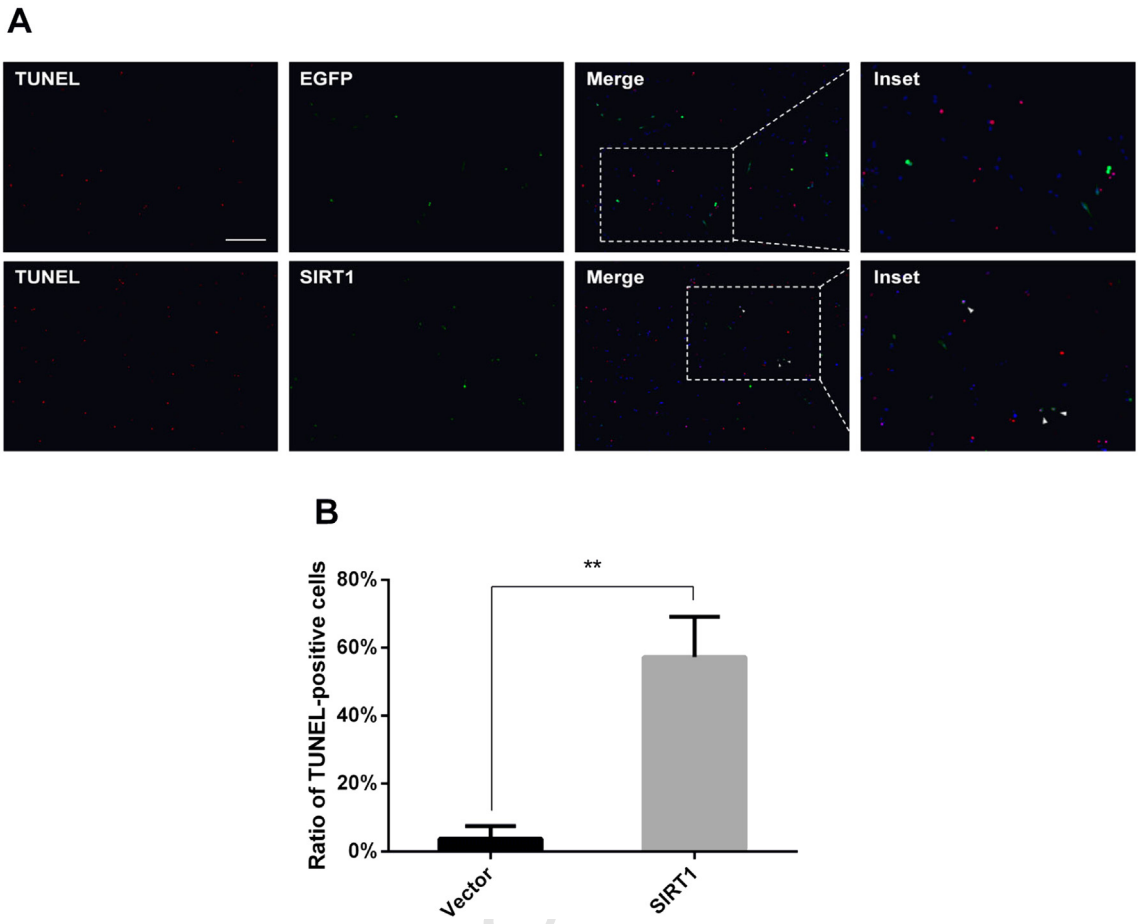

Fig. 8. Overexpression of SIRT1 promotes CORT-induced apoptosis.(A) Representative TUNEL staining images of cells transfected with SIRT1-EGFP or EGFP control plasmids followed by 24 h of 200 μM CORT treatment. Insets in merged images were magnified in the right panel. Cells double positive for TUNEL and EGFP are indicated with white arrowheads. (B) Percentage of TUNEL-positive cells in EGFP-positive cells was quantified. Data were expressed as mean ± SEM (n = 3). $^{**}P < 0.01$, t test. Scale bar: 200 μm.

certain conditions, whereas in other cellular settings, they may have common upstream regulators (Maiuri et al., 2007; Eisenberg-Lerner et al., 2009). The potential exacerbation of cell apoptosis following the inhibition of autophagy by SIRT1 warrants further investigation.

In conclusion, our study revealed that overexpression of SIRT1 may inhibit CORT-induced autophagy culminating in increased apoptosis, which can be reversed by the knock-down of SIRT1. This may help us better understand the role of SIRT1 and autophagy in CORT-induced stress.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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