

Protection effect of gatrodin on learning and memory ability in vascular dementia by promoting autophagy flux via Ca²⁺/CaMKII signal pathway

Ting ting chen

Guizhou Medical University

Xue zhou

Guizhou Medical University

Yi ni xu

Guizhou Medical University

Xiao ying wu

Guizhou Medical University

Quan xiang

Guizhou Medical University

Ling yun fu

Guizhou Medical University

Xiao xia hu

Guizhou Medical University

Ling tao

Guizhou Medical University

Xiang chun shen (✉ shenxiangchun@126.com)

Guizhou Medical University <https://orcid.org/0000-0003-4116-5058>

Research

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Abstract

Background: Vascular dementia is a common and frequently-occurring disease in the process of human aging. Although the current treatment can delay the deterioration of the disease, it has not a great breakthrough in improving cognitive impairment. Therefore, exploring the potential key molecular targets of VD provide promising strategy for prevention and treatment. **Methods:** vascular dementia rats were reproduced by permanent middle cerebral artery occlusion (pMCAO) and anoxic injury of HT22 cells were induced by Cobalt Chloride (CoCl₂, 200μM). The ability of spatial learning and memory was assessed by morris water maze (MWM) test. Histological analysis was performed by HE staining and immunohistochemical staining. The effects of gatrodin on autophagy flux and calcium signal in vascular dementia rats and HT22 cells during hypoxia injury were detected by Western blotting and immunofluorescence. Furthermore, intracellular Ca²⁺ levels were quantified using a Ca²⁺ quantification kit and were also measured by flow cytometric estimation of Fluo-4 AM. **Results:** Gastrodin significantly reversed cognitive deficits in vascular dementia rats. The results of immunohistochemical analysis and western blot confirmed that gatrodin could attenuate the levels of LC3, p62 and phosphorylated CaMKII in hippocampus of VD rats. In addition, gatrodin was similar to the early autophagic inhibitor (3-BDO) ameliorating CoCl₂-induced autophagic flux dysfunction and p62 knockdown by siRNA also promoting autophagic flux patency, but the late autophagy inhibitor (CQ) weakened the improvement effect of gatrodin. Furthermore, gatrodin markedly inhibited CoCl₂-induced autophagic flux dysfunction by inhibiting [Ca²⁺]i-dependent CaMKII. **Conclusion:** Gastrodin is a potential promising candidate for VD by improving autophagy flux dysfunction via increasing lysosome acidification and autophagosome-lysosome fusion mediated by CaMKII-regulated suppression of p62 signaling. **Keywords:** Gastrodin, Vascular dementia, Neuron injury, Autophagic flux, Ca²⁺, CaMKII

Background

The incidence of dementia is increasing with the aging population, and vascular dementia is one of the most common types[1]. At present, The patients with VD are accompanied by severe neurological symptoms and signs, such as bedridden, paralysis and loss activities of daily living, which gradually worsen over time [2]. It is widely applied in clinic for VD such as cholinesterase inhibitor, calcium channel blockers and Meijingang, etc, however those are not achieved the expected therapeutic effects on VD patients [3]. Therefore, exploring the potential key molecular targets of VD provide promising strategy for prevention and treatment, and novel pharmacological mechanism for its development of specific and efficient therapeutic drugs.

VD is an acquired intelligence impairment syndrome characterized by learning and memory impairment caused by cerebrovascular diseases [4]. Chronic cerebral hypoperfusion leads to insufficient blood and oxygen supplying to the brain and disturbance of energy metabolism, which results to cerebral infarction, finally neurons decrease and the loss of the material basis for the completion of brain function [5, 6]. This is a common pathophysiological mechanism forming the decline of cognitive ability in vascular dementia [7]. Learning and memory function is an advanced neurophysiological activity of the brain, it

has become an important indicator of drug intervention in vascular dementia animals in clinical and experimental research [8]. Hippocampal tissue is the central nervous structure involved in learning and memory and is very sensitive to the damage of ischemia and hypoxia, and the degree of injury is also the most serious in the chronic cerebral hypoperfusion [9, 10].

Autophagy is specific way to degrade long-life protein and damaged organelles, and it is also an important way for cells to adapt to adverse environment, maintain steady state and promote survival [11]. Three commonly autophagy biomarkers are LC3 (marker molecule for autophagosomes), p62 (recognition receptor for degradation of ubiquitinated proteins and organelles) and lysosomal associated membrane protein type-2 (LAMP-2, detection of fusion of autophagosomes and lysosomes) [12, 13]. There are two splicing forms of LC3 protein in cells: LC3I and LC3II. The ratio of LC3-II to LC3-I is proportional to the number of autophagosomes, which reflects the autophagic activity to some extent [14]. LAMP-2 is a protein commonly expressed in the central nervous system. Lack of LAMP-2 especially in the hypothalamus and CA3 region of the hippocamps can cause significant inflammation and lysosomal/autophagic disorders, characterized by the accumulation of autophagic vesicles and neuronal degeneration [15]. As a shuttle protein transporting lysosome and proteasome-degrading ubiquitinated proteins, p62 has been highlighted on a variety of diseases such as PD, HD, and AD [16]. When autophagy deficiency occurs in the brain, neurons in almost all regions are accompanied with polyubiquitin and p62 accumulation, suggesting that p62 plays a key role in neurons [17]. In the early stage of neurodegenerative diseases, the activation of autophagy accelerates the removal of the denatured protein and delay the development of the disease [18]. Excessive reactive oxygen species induce mitochondrial damage accumulate a large number of dysfunctional proteins and damaged organelle after cerebral ischemia-reperfusion or hypoperfusion which activate autophagy and regulate cell homeostasis [19]. It has been reported that autophagy is activated in the hippocampus of VD rats, which maybe involve in the pathogenesis of VD. Inhibition of autophagy can reduce the severity of hippocampal injury induced by VD, thus producing beneficial neuroprotective effect [20, 21]. Increasing a line evidences suggest that regulation of autophagy maybe a promising target to improve neuronal injury in patients with vascular dementia.

It is well known that intracellular calcium homeostasis is involved in neuron development and normal physiological function. As the second messenger, Calcium is regulate a host of cellular functions such as proliferation, growth, differentiation, and death [22, 23]. Notably, Ca^{2+} plays an essential role as a pro-autophagic signal, which can trigger autophagy by phosphorylating ULK1 with an activator site or inhibition of mTORC1 [24]. The calcium agonist can promote autophagy by increasing the intracellular calcium concentration [25]. The response of Ca^{2+} signal are induced by a family of multifunctional Ca^{2+} /calmodulin-dependent protein kinases (CaMKs), among which CaMKII is a player in synaptic plasticity and memory formation [26]. Phosphorylated CaMKII improves learning and memory ability by promoting synaptic transmission, however, the accumulating evidences confirmed that the expression of phosphorylated CaMKII protein was abnormally increased in VD rats [27]. As we known, regulating CAMK signaling pathway to control the homeostasis of apoptosis and autophagy can ultimately reduce the

neurodegenerative processin striatum [28]. CaMKII can phosphorylation Beclin 1 directly at Ser90, thus promoting ubiquitination of Beclin 1 and activating autophagy in neuroblastoma cell [29]. These data suggestthat excessive activation of calcium signaling pathways may cause abnormal changes in the autophagy process.

Gastrodin (GAS) is one of the bioactive ingredients from Gastrodiae Rhizoma (a Chinese herbs named TianMa), which are widely applied to prevent and ameliorate central nervous system diseases in Chinese Medicine [30]. GAS has been shown potent effects on vascular dementia by targeting multiple pathways as follows: attenuating amyloid and tau levels, inhibiting autophagy and apoptosis of hippocampus neurons, and reducing inflammation [30]. However, it is unclear that GAS ameliorates the learning and memory impairment in rats with vascular dementia involving in regulation the autophagy via the $\text{Ca}^{2+}/\text{CaMK}\zeta$ signal.

We, therefore, evaluated the effect of GAS in improving autophagy dysfunction of neurons in vitro and in vivo. Additionally, GAS attenuates CoCl_2 -induced autophagic flux inhibition and the formation of autophagosomes in HT22 cells, and its upstream Ca^{2+} and CaMKII events. The results suggest that GAS improve the autophagy dysfunction of neurons via $\text{Ca}^{2+}/\text{CaMK}\zeta$ signal and become a promising candidate against VD induced by cerebral ischemic disease.

Materials And Methods

Reagents and antibodies

GAS (SMB00313), CoCl_2 (232696) and calcium ionophore (A23187) were purchased from Sigma-Aldrich (St Louis, MO, USA). GAS and CoCl_2 were separately dissolved in phosphate buffered solution (PBS) to prepare the original solution of 100mM, which was stored at -20°C. Chloroquine diphosphate (CQ; HY-17589) and 3BDO (HY-U00434) were purchased from MedChemExpress. Fluo-4AM (S1060) were obtained from Beyotime Biotechnology, Inc. (Nanjing, China). Primary antibodies against β -actin (66009-1-Ig, 1:10000), LC3 (14600-1-AP, 1:1000) , p62 (18420-1-AP, 1:1000) and LAMP2 (66301-1-Ig, 1:1000) were purchased from Proteintech (Wuhan, China). Primary antibody against p-p62 (Ab211324) was from Abcam (Cambridge, MA, USA). Primary antibodies against CaMKII (#4436, 1:1000) and p-CaMKII (#12716, 1:1000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Goat anti-Rabbit IgG(H&L)-HRP (BS13278, 1:10000) and Goat anti-Mouse IgG(H&L)-HRP (BS12478) were obtained from Bio-World. Goat PAb to MS IgG Alexa Fluor®488 (Ab150113, 1:200) and Goat PAb to Rb IgG Alexa Fluor®488 (Ab150077, 1:200) were purchased from Abcam. A fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (A0516, 1:200) was purchased from Beyotime Biotechnology.

Animals and treatments

Adult health male Sprague-Dawley rats (SPF grade, Certificate No.SCXK2018-0001, Grant No.1800157) weighing 260±20g were approved by the Guizhou Medical University Experimental Animal Center. All animal were maintained in temperature (22-26°C)- and humidity (40%-70%)-controlled pathogen-free rooms under a 12- h:12-h light/dark cycle. All manipulations with experimental animals were approved by the Animal Ethics Committee of Guizhou Medical University. Focal cerebral ischemia injury was reproduced induced by modifying previous MCAO[31]. In brief, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were separated with bluntness, and then the ECA was ligated, the suture (0.26 mm; Beijing Xinong BioTechnologies Co, Ltd, China) from CCA to ICA eventually occludes the middle cerebral artery (MCA). The sham-operated rats received all surgical protocol without the suture inserted. Zea-Longa scoring [31] was used to evaluate neurological deficits after 12 hours of ischemia. The rats with a score of 1 and 2 were selected as model rats.

Triphenyltetrazolium chloride (TTC) staining was measured the infarct volumes after 24h hours of ischemia. Rats were sacrificed after CO₂ inhalation and rapidly took out and cut into 2-mm coronal sections which was incubated in 0.2% TTC at 37 °C for 30 min to visualize in infarctions and then photographed with a digital camera at designning time points. The model rats were randomly divided into 3 groups and given intragastrically with GAS (2.5 mg kg⁻¹, 5mg kg⁻¹) or an equal volume of distilled water, respectively; the sham operated rats were randomly divided into 2 group and were treated with GAS (5mg kg⁻¹) or an equal volume of distilled water once a day for 8 weeks.

Morris Water Maze Test (MWM)

The ability of spatial learning and memory was assessed by morris water maze (MWM) test. The water maze consists of a large circular pool (120 cm in diameter, 50 cm in height) filled with white nontoxic powder. The pool is divided into four equal quadrants and a hidden circular platform of 20-cm diameter was located in the center of the target quadrant, followed by seting up the experimental procedure to start the experiment. The system automatically records the trajectory of a rat's swim when the rats were placed in water from the pool edge. The rats did not find the platform within 120 seconds were directed by the researcher to the platform and allowed to rest on it for at least 20s. In 5 days, the space exploration experiment began, and the system automatically recorded the movement trajectory of the rats within 120 seconds.

Hippocampal morphology observation

The brain tissue was fixed in 4% paraformaldehyde for 24 hour, embedded in paraffin and sliced to analyze Hippocampal morphology via H&E and changes in LC3 levels. The brain tissue sections were deparaffinized, and antigen retrieval was achieved by microwave heating in 0.01 M citrate buffer for 20 minutes. The sections were sequentially incubated with 3% H₂O₂ for 15 minutes, goat serum was blocked for 30 minutes, and primary antibody LC3 was added dropwise and incubated overnight at 4°C. The secondary antibody was incubated for 30 minutes, and then the horseradish-labeled streptavidin working solution was added dropwise and incubated for 20 minutes. Finally, it was stained with DAB (ZLI-9018, Zhongshan Golden Bridge Biotechnology, Beijing, China) substrate and counterstained with hematoxylin,

and photographed using a light microscope (DMI8, Leica, Germany). The area of immunofluorescence positive area of hippocampal tissue was counted by image analysis software Image J (National Institutes of Health, Bethesda, MD, USA).

Cell lines and cell culture

HT22 were purchased from Shanghai Zhongqiaoxinzhou Biotech (Shanghai, China; passage number: ZQ0476) which maintained in Dulbecco's modified Eagle medium (DMEM™Gibco, Thermo Fisher Waltham, CA, USA) supplemented with 10% (FBS; Gibco), streptomycin (100 µg/mL; Gibco), and penicillin (100 U/mL; Gibco) and incubated at 5% CO₂ and 37 °C.

MTT assay

MTT was assessed the viability of HT22 Cells. The HT22 cells were seeded into 96-well plates and cultured until they reached 70% confluence. The cells were pre-incubated with the indicated concentrations of GAS (200µM) for 1 h, and then exposed to the CoCl₂ (200µM) for 24 h. And then, the medium was discarded, and the 200µL MTT(0.5mg/ml) was supplied in each well incubating 3h. The supernatant was removed, 150µl dimethyl sulfoxide (DMSO) was added and mixed thoroughly. Optical density (OD) values were detected at 490 nm with microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The cell survival rate (%) was calculated using the following equation:

$$\text{cell-viability inhibition}(\%) = [(OD_{\text{Control}} - OD_{\text{Treated}})/OD_{\text{Control}}] \times 100.$$

Western blot analysis

The total protein samples from tissues or cells were extracted by lysing in lysis buffer(R0010, Solarbio) containing phosphatase inhibitor cocktail (P1260, Solarbio) and protease inhibitor cocktail (P0100, Solarbio). Protein samples (30 µg), protein concentration quantified using a bicinchoninic acid protein assay kit (PC0020, Solarbio) and detected via a microplate spectrophotometer (Thermo FisherScientific), were fractionated on a 10% or 12% SDS - PAGE. After electrophoretic transfer to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), the membrane was blocked with 5% bovine serum albumin (A8020, Solarbio) for 1.5 h and subsequently probed overnight with the appropriate antibodies at 4°C, as described in reagents above.

Measurement of intracellular Ca²⁺ level

Intracellular Ca²⁺ levels were quantified using a Ca²⁺ quantification kit (Abcam, ab112115) following the standard manufacturer's protocol. Fluorescence was determined using a microplate spectrophotometer at Ex/Em = 540/590 nm (Varioskan LUX, Thermo). Cytosolic Ca²⁺ levels were also measured by flow cytometric estimation of Fluo-4 AM. The cells were collected and loaded with 5µM Fluo-4 (Beyotime, ab145254) for 30min at 37 °C , and then resuspended with 500 µl phosphate-buffered saline. The

fluorescence signal was recorded using a flow cytometer at Ex/Em =488/525 nm and analyzed by NovoExpress software (NovoCyte, ACEA Biosciences, SanDiego, CA, USA).

Gene silencing

CaMKII siRNA (forward, 5'-GCGGAGGAAACAAGAAGAATT-3'; reverse, 5'-UUCUUCUUGUUUCCUCGCTT-GTC-3'). and p62 siRNA (forward, 5'-GACGAUGACUGGACACAUUTT-3'; reverse, 5'-AAUGUGUCCAGUCAUCGUU-3') were constructed by GenePharma (Shanghai, China). According to protocol of manual instructions, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect siRNA.

Immunofluorescence microscopy

The HT22 were seeded on coverslips from a 6-well plate with treated in experimental design. After the designing time pointes, and the cell was operated as following: fixed cells with 4% paraformaldehyde for 12 min, permeabilized for 10 min using 0.2% Triton X-100, closed with goat serum (SL038, Solarbio). After, the cells were incubated with LC3 (1:200), P62 (1:50) and LAMP2 (1:200) overnight at 4°C, and then followed by incubation with secondary antibodies for 1h and label with DAPI (BD5010, Bioworld) for 40 min. Finally, the images were observed with DMi8 fluorescence microscope and Leica X software at 800× magnification (DMi8, Leica, Germany) .

Statistical analysis

All data are expressed by mean ± SEM using GraphPad Prism®5.0 (La Jolla, CA, USA) software. The experimental data were obtained from three or more independent experiments, and differences between groups were analyzed by one-way ANOVA followed by post hoc Tukey multiple comparisons. The differences between the two experimental groups were compared by Student's *t* test . *P*< 0.05 indicates that the difference is statistically significant.

Results

GAS ameliorated cognitive dysfunction in VD model rats

Chronic cerebral hypoperfusion leads to insufficient blood and oxygen supplying to the brain and disturbance of energy metabolism, which results to cerebral infarction, finally neurons decrease and the loss of the material basis for the completion of brain function. GAS ameliorates cognitive dysfunction of VD rats with the morris water maze behavior experiment. Firstly, the VD model was reproduced by the Zea Longa method and determined to be successful with the Zea Longa score and TTC staining (Supplementary Fig. S1). Compared with the normal group, there was no significant change in the cognitive function of the animals in the simple GAS group, which indicated that GAS had no effect on the cognitive function of the normal animals. The navigation test showed that the escape latency of VD rats was longer than sham-operated rats. In contrast, the escape latency of GAS-treated rats was significantly decreased compared with the VD rats (Fig. 1a-c). The probe trial showed that the VD rats treated with GAS

spend more time in the target quadrant and more frequently crossed platform(Fig.1d-e), which indicated improvement effect of GAS on the reduction of learning and memory function in VD rats. The pathological changes of VD are mostly secondary to the occlusion of the trunk of the cerebral artery, such as the occlusion of the middle cerebral artery lead to a large area infarction of the ipsilateral frontal, parietal and occipital lobe. Most of the lesions are in the bilateral or left cerebral hemispheres [4]. Our results showed that there was a significant collapse in the temporal lobe and parietal lobe of the telencephalon in VD rats whereas the GAS attenuated the lesion (Fig. 1f). H&E staining of hippocampal tissues of VD rat showed neuron disorders, loss and degeneration and necrosis/pyknosis of the nucleus and light coloration of the cytoplasm (Fig. 1g). However, these pathological characteristics were attenuated by GAS administration (Fig. 1g).

GAS reverses suppression of Autophagy flux and hyper-phosphorylation of CaMKII in VD model rats.

Autophagy is widely accepted in neuronal homeostasis involved in the occurrence and development of VD [21]. The effects of GAS on the expression of LC3 and p62 was detected in hippocampal tissue of vascular dementia rats. The results of western blot show that LC3 and p62, biomarker of autophagy induction, increased simultaneously in the hippocampus of VD rats, indicating inhibition of autophagosome degradation(Fig. 2a-b). However, the up-regulated LC3 and p62 were suppressed by treatment with GAS which suggested GAS activating downstream of autophagy to promote autophagosome degradation and autophagic flow. Similarly, immunohistochemical results confirmed that GAS decreased the expression of LC3 compared with VD rats, especially in the CA1 regions of the hippocampus (Fig. 2c-d) .Calcium/calmodulin-dependent protein kinase II (CaMKII) belonging to the CaMK family is a serine/threonine protein kinase as an pivotal calcium signal molecule, which is the main mediator of physiologically excitatory glutamate signal [22, 28, 32]. CaMKII has demonstrated a critical role in autophagy regulation [29]. Although the total protein of CaMKII is unchanged in the hippocampus of vascular dementia rats, the phosphorylated CaMKII protein is significantly increased. However, GAS can reduce the phosphorylation level of CaMKII (Fig. 2a-b).

GAS reduces CoCl₂-induced autophagosome accumulation in HT22 cell

Many studies have confirmed that autophagy in the hippocampus of VD rats is activated, and autophagy is involved in the pathogenesis of VD[21, 33]. Cobalt chloride (CoCl₂), as a common chemical reagent, is widely regarded as a classical stimulator of hypoxia-ischemic disease [34]. MTT assay showed that the activity of HT22 cells significantly decreased after exposure to 200μM or higher CoCl₂. However, pre-incubation with GAS (200μM) for 1 hour and then exposure to 200μM CoCl₂ for 24 hours could significantly increase the activity of the cells (Fig. 3a-b).

To further explore the role of CoCl₂ in autophagy, we incubated HT22 cells with different concentrations of CoCl₂ for 24h. Expressions of LC3 was significantly increased in a dose-dependent manner (Fig. 3d), suggesting an increase in the number of autophagic vacuoles, which may be due to elevation of autophagosome formation or suppression of autophagy degradation.In addition to LC3, the level of p62,

as an autophagy substrate which can be attached to LC3 and ubiquitinated substrates, and then integrated into autophagosomes and degraded in autophagolysosomes [17, 35], are significantly up-regulated (Fig. 3d-e). A large amount of aggregated p62 is phosphorylated at a particular amino acid site under the action of protein kinase, which are linked to the ubiquitinated protein and the LC3/ Atg8, respectively, and finally enter the autophagy lysosomes to complete the degradation of the ubiquitinated substrate [36]. CoCl₂ exposure significantly enhanced the phosphorylation level of p62 compared with the control group (Fig. 3d-e). This result suggests that the increase in autophagic vacuoles may be due to inhibition of degradation. Western blotting results showed that the protein expression of LC3, p62 and p-p62 were significantly reduced administered with GAS (Fig. 3f-g), indicating that GAS may reduce the accumulation of autophagosomes by decreasing the formation of autophagic vacuoles or promoting the autophagic flux. To further validate the present results, immunofluorescences of LC3 and p62 protein were assayed by commercial kits. Exposure CoCl₂ to HT22 cells, the number of LC3 and p62 puncta was significantly increased. However, GAS treatment significantly reversed these CoCl₂-mediated changes in autophagy-marker levels (Fig. 3h-k).

GAS ameliorates CoCl₂-induced autophagic flux inhibition in HT22 cells

Autophagic flux is a dynamic process in which these steps occur continuously in the cell. If there is an obstacle in any step, it will not be able to complete its biological function [37]. The effect of GAS on autophagy flux was evaluated by combining with lysosomal inhibitor chloroquine (CQ). Treatment with CQ alone induces an increase in LC3, p62 and p-p62 levels in HT22 cells. Moreover, CoCl₂-induced accumulation of LC3-II, p62 and p-p62 was further increased by CQ treatment. Pre-incubated with GAS could significantly alleviate accumulation of LC3-II and p62 in CQ and CoCl₂-induced suppression of autophagy (Fig. 4a-b). These results suggest that the increase of LC3II, p62 and p-p62 induced by CoCl₂ is attributed to the inhibition of autophagy flux and the enhancement of autophagosome formation. GAS alleviates CoCl₂-induced autophagic flux inhibition and the formation of autophagosomes in HT22 cells. These influences of GAS were further confirmed by examining the effect of autophagy early stage inhibitor 3-BDO with CoCl₂-induced LC3-II, p62 and p-p62 accumulation in HT22 cells. As expected, 3-BDO alone inhibited the increment of LC3-II, p62 and p-p62 induced by CoCl₂, and further down-regulation occurred when GAS was combined with 3-BDO (Fig. 4c-d).

The fusion of autophagosomes and lysosomes is the key to degradation of autophagosomes and their contents and this process is an important downstream event of autophagy flux [37]. p62 siRNA can improve autophagy by reducing the accumulation of p62 aggregation and promoting autophagy flux [38]. Therefore, knockdown of p62 by siRNA suppressed upstream event of autophagy flux. p62 expression was assessed in HT22 cells transfected with three different p62 siRNAs: siRNA (624), siRNA (1313), and siRNA(1072). The experimental results show that siRNA (1072) clearly reduced p62 expression, so that siRNA (1072) was used in subsequent experiments (Fig. 4e-f). CoCl₂ could significantly reduce the expression of LAMP-2 protein in HT22 cells, indicating some interference in autophagosome-lysosome fusion. In contrast, GAS or p62-siRNA alone could significantly increase the expression of LAMP2 protein,

while GAS combined with p62-siRNA further increased the expression of LAMP-2 (Fig. 4g-i). GAS reduces the accumulation of p62 and LC3 aggregation through autophagy lysosome pathway to improve autophagy dysfunction.

GAS alleviates CoCl₂-induced increase in intracellular Ca²⁺ abundance and CaMKII activation

The Ca²⁺/calmodulin-dependent kinase (CaMK) family has been recognized as a key mediator in living organisms and various pathophysiological processes. CaMKII is activated in the presence of Ca²⁺ and calmodulin (CaM) [39]. The activation of Ca²⁺/CaMKII signal pathway improve the learning and memory impairment caused by hypoperfusion [40]. We further explored the effect of administered GAS and/or BAPTA/AM or calcium ionophore in HT22 cells on the intracellular Ca²⁺ and phosphorylation of CaMKII induced following CoCl₂ treatment. The results indicated that Ca²⁺ abundance significantly increased from exposure to CoCl₂ for 12h, and the peak time at 24 h (Fig. 5a), and CaMKII phosphorylation was increased in a dose-dependent manner (Fig. 5b-c). Ca²⁺ sensitive fluorescence indicator Fluo-4AM and flow cytometry confirmed that GAS and intracellular Ca²⁺ chelator BAPTA-AM could inhibit the increase of intracellular Ca²⁺ and CaMKII phosphorylation (Fig. 5d-h). Interestingly, the combination of GAS with BAPTA/AM attenuated CoCl₂-triggered [Ca²⁺]_i increase and the p-CaMKII more potently than GAS or BAPTA-AM alone in HT22 cells (Fig. 5i-j), which means the inhibition of GAS on the increase of CaMKII phosphorylation induced by CoCl₂ depending on the level of [Ca²⁺]_i. These influences of GAS were further confirmed by calcium ionophore attenuating the inhibitory effect of GAS on the increase of intracellular Ca²⁺ and CaMKII phosphorylation induced by CoCl₂ in HT22 cells (Fig. 5k-l).

GAS alleviates CoCl₂-induced suppression of autophagy flux by stimulating [Ca²⁺]_i-dependent CaMKII phosphorylation in HT22 cells.

Ca²⁺ is considered to be a crucial regulator of autophagy and calcium signal is closely related to the occurrence and development of autophagy [41]. Therefore, we further explore the mechanism of whether GAS can attenuate CoCl₂-induced inhibition autophagy flux by regulating [Ca²⁺]_i-dependent CaMKII phosphorylation in HT22 cells. Co-treatment with GAS and BAPTA-AM significantly inhibited CoCl₂-induced increase of LC3, p62 and p-p62 compared to GAS or BAPTA-AM alone (Fig. 6c-d). In addition, Western blot and immunofluorescence suggested that the inhibitory effects of GAS on CoCl₂-induced LC3, p62 and p-p62 upregulation were reduced by calcium ionophore (Fig. 6a-b, 6e-f). These data indicated that GAS ameliorated CoCl₂-induced autophagic flux inhibition by reducing extracellular Ca²⁺ influx.

CaMKII is a general integrator of Ca²⁺ signaling [42]. We then confirmed that GAS improves CoCl₂-induced autophagic flux dysfunction by regulating CaMKII. Treatment with KN93—a CaMKII inhibitor—suppresses CoCl₂-induced increase of protein expressions of LC3II, p62 and p-p62, however, there is no significant difference compared with KN93 combining with GAS (Fig. 6g-h). To further corroborate the

role of CaMKII in GAS regulating autophagy, CaMKII was knocked down and its protein expression was down-regulated by about 70% in HT22 cells transfected with CaMKII-siRNA (379) compared to non-specific siRNA-infected HT22 cells (Fig. 6 i-j). Interestingly, knocking down CaMKII could significantly inhibit the increase of LC3, p62 and p-p62 induced by CoCl₂, but no further down-regulation occurred when knocking down CaMKII combined with GAS (Fig. 6 k-l). The fusion of autophagosomes and acid lysosomes is an important downstream event of autophagy flux [43]. Our results showed that CoCl₂ exposure reduced LAMP2 expression, suggesting that CoCl₂ hinders the fusion of autophagosomes and acid lysosomes. GAS significantly reversed CoCl₂-mediated changes in lysosomal markers, which was consistent with the results after knockout of the CaMKII-siRNA. In addition, transfection of CaMKII-siRNA and then administration of GAS further reduced LAMP2 expression (Fig. 6 k, m). Co-localization of LC3⁺ and LAMP2 is often used to evaluate the fusion of autophagosomes and lysosomes. CoCl₂ increased the LC3⁺ puncta in HT22 cells and weakened the extents of co-localization between LAMP2 and LC3, GAS could increase the expression of LAMP2 and reduce the accumulation of LC3⁺ puncta. However, knocking down CaMKII enhanced the co-localization of LAMP2 and LC3 promoted by GAS (Fig. 6n-o). Collectively, these results demonstrate that GAS alleviates CoCl₂-induced suppression of autophagy flux by via inhibiting CaMKII-regulated phosphorylation of p62 and accumulation of autophagosomes.

Discussion

The role of autophagy was prevented the accumulation of abnormal cytoplasmic proteins in neurons, whereas the damage of autophagy may lead to neurodegeneration, characterized by extensive neuronal loss [44, 45]. At the same time, excessive autophagy reduce the survival rate of neurons and inhibition of autophagy delay the process of neurodegeneration [46], suggesting that drug intervention the autophagy process may provide a novel strategy for dementia. VD is a chronic cerebrovascular syndrome with vascular brain tissue damage as the main pathological manifestation, which impairs learning and memory by altering neural networks at the physiological, molecular and synaptic levels. However, it is unclear the autophagy process of neurons in VD. Accumulating evidences have been reported that autophagy was widely activated in the hippocampus of rats with VD [20], which suggests autophagy involving in the pathogenesis of VD. However, some studies have found that there are superabundant autophagy vacuoles in the axons of neurons in patients with vascular dementia [47], which may reflect the enhancement of autophagy induction, obstruction of late lysosome degradation or decrease of autophagy initiation rate in autophagy pathways. The current results show that the learning and memory ability is impaired in VD rats. Furthermore, excessive autophagy is associated with the progression of VD rats induced by MCAO, which is characterized by a significant increase in LC3 and p62 in the hippocampus. These results suggest that the damage of hippocampal neurons caused by excessive autophagy, which may be one of the causes of learning and memory impairment in VD rats.

GAS, a phenolic glycoside isolated from the traditional Chinese medicine *Gastrodiae Rhizoma*, has widely concentrated on potential in the prevention and treatment of VD [30]. The previous data confirmed that GAS have multiple pharmacological properties such as antioxidant, anti-inflammatory and anti-apoptotic

activities. Recently, GAS could improve the learning and memory ability of VD rat model induced by 2VO [48]. GAS has a therapeutic effect on BCCAO-induced VD by targeting the formation of A β -related proteins and inhibiting autophagy and apoptosis of hippocampal neurons [49]. At present, we confirmed that GAS could enhance the learning and cognitive impairment of VD rats, reverse the hyperphosphorylation of CaMKII and the abnormal upregulation of autophagy biomarker proteins LC3 and p62 in the hippocampus of VD rats. These results mean that reducing excessive autophagy and down-regulating the phosphorylation level of CaMKII may be an important molecular mechanism of GAS in preventing VD. However, LC3 and p62 increase indicate that autophagy is promoted in the early stage and inhibited in the later stage (binding to lysosomes) or blocked the degradation of autophagy lysosomes. GAS could improve cognitive impairment in VD rats involving in multiple mechanisms, and exact regulation mechanism on excessive autophagy in VD rats needs to be further studied.

Autophagy is the self-protection mechanism of cells, which is beneficial to the growth and development of cells. However, excessive autophagy may lead to metabolic stress, degradation of cell components, cell death and so on [11]. Additionally, as an important metabolic activity, autophagy plays an important role in maintaining neuronal survival, clearing senescent cells and misfolded proteins under stress such as ischemia and hypoxia [45-47]. It is widely known that LC3 and p62 are two frequently used autophagy biomarkers. The accumulated autophagosomes is proportional to the content of LC3-II or the ratio of LC3-II/LC3-I, LC3 reflects the autophagy activity of cells to some extent [11, 12]. Additionally, autophagy is a highly dynamic and multi-stage process. Because of the changes of autophagy activity, it is necessary to evaluate the autophagic flow as following: the dynamic changes of autophagosome formation, fusion of autophagosome and lysosome, substrate degradation, and so on [50]. CoCl₂ has been used as a chemical compound to simulate hypoxia in vivo and in vitro [51]. At present, we confirm that GAS reduced CoCl₂-induced autophagic flux inhibition and the formation of autophagosomes via promoting lysosomal acidification and autophagosome-lysosome fusion in HT22 cells.

Intracellular free calcium (Ca²⁺) , as secondary messenger, plays a complex signal transduction role in the brain, especially in neurodegenerative diseases [40, 52]. The impaired intracellular Ca²⁺ regulatory system results in synaptic dysfunction, damaged plasticity and neuronal degeneration [52]. The depletion of energy induced by cerebral blood flow in VD patients induces the accumulation of free calcium in the cells [53]. The increase of cytoplasmic Ca²⁺ could enhance the binding of Ca²⁺-CaM complex to the regulatory domain of CaMKII, increase the activity of CaMKII, and then activate autophagy [26, 41]. Hippocampus and cerebral cortex are the structural basis of spatial learning, CaMKII is the molecular basis of spatial learning and memory [54]. There are 28 different subtypes of CaMKII, among which the threonine 286 residue controls the self-inhibition. When the site is phosphorylated, CaMKII is permanently activated and the long-term synaptic pathway is enhanced the ability of learning and memory [55]. The current research results show that autophagy lysosomal disorders induced by CoCl₂ may require both Ca²⁺ increase and CaMKII activation .GAS suppressed CoCl₂-induced phosphorylation CaMKII of (Ser286) in Ca²⁺-dependent manner, and may improve CoCl₂-induced autophagy lysosome dysfunction by

inhibiting $\text{Ca}^{2+}/\text{CaMKII}$ pathway (Fig. 7). However, the detailed molecular mechanism of GAS in improving autophagy dysfunction needs to be further studied.

p62 protein is located at the site of autophagosome formation and can bind to autophagosome localization protein LC3 and ubiquitin protein. Therefore, p62 is a recognition receptor for ubiquitin protein and organelle degradation [17, 36]. The decrease of p62 level leads to neuropathological changes, including excessive accumulation of tau and A β proteins, and even neuronal apoptosis [56]. p62 has different phosphorylation sites and is in a dynamic equilibrium between different phosphorylation states. When the inhibition of autophagy leads to the intracellular accumulation of p62, p62 phosphorylation occurs at specific amino acid sites under the action of protease (the main phosphorylation sites are Ser403 and Ser351) and directly activates other signaling pathways, such as NF- κ B signaling, Nrf2 activation, apoptosis [57-59]. GAS decreased p62 levels and p62 phosphorylation at Ser349 as well as the phosphorylation level of CaMKII. Additionally, KN93 (a CaMKII inhibitor) and CaMKII knockdown could not further decrease in total p62 and phosphorylated p62 (Ser349) levels incubated with GAS. Therefore, these results suggested that GAS may reduce the level of p62 by regulating CaMKII levels.

Conclusions

To the best known of our knowledge, gastrodin significantly reversed cognitive deficits in vascular dementia rats and reduced the accumulation of p62 and LC3 aggregation through autophagy lysosome pathway to improve autophagy dysfunction. Furthermore, the present results indicated that gastrodin inhibited the level of CaMKII phosphorylation by reducing the increase of intracellular calcium ion induced by CoCl_2 . Gastrodin combining with KN93 or CaMKII knockdown did not affect decreasing levels of LC3 and p62 compared with gastrodin only treatment. Taken together, it is the first time to clarify that gastrodin attenuates autophagic flux dysfunction by regulating $\text{Ca}^{2+}/\text{CaMKII}/\text{p62}$ signal pathway to ameliorate cognitive impairment in vascular dementia.

Abbreviations

GAS: gastrodin; VD: Vascular dementia; pMCAO: permanent middle cerebral artery occlusion; CoCl_2 : Cobalt Chloride; MWM: morris water maze; TTC: Tetrazolium Chloride; CQ: Chloroquine diphosphate; LAMP-2: lysosomal associated membrane protein type-2; CaMKII: Calcium/Calmodulin Dependent Protein Kinase;

Declarations

Acknowledgements

Not applicable.

Authors' contributions

TTC, XZ and YNX designed the study, completed the experiment. XYW, QX, LYF and XXH analyzed the data and wrote the paper. XCS and LT conceptualized the project as well as guide the design of the experiment. All the authors read and approved the final manuscript.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this article

Ethics approval and consent to participate

The Animal Ethics Committee of Guizhou Medical University approved all the animal experiments described in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹The State Key Laboratory of Functions and Applications of Medicinal Plants (The high efficacy application of natural medicinal resources engineering center of Guizhou Province and The high educational key laboratory of Guizhou province for natural medicinal Pharmacology and Druggability), School of Pharmaceutical Sciences,Guizhou Medical University, University Town, Gui'an New District, Guizhou 550000, China.

²Department of Pharmacognosy (The Key Laboratory of Optimal Utilization of Natural Medicine Resources and The union key laboratory of Guiyang City-Guizhou Medical University), School of Pharmaceutical Sciences, Guizhou Medical University, University Town, Gui'an New District, Guizhou 550000, China.

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Additional File Legends

Additional file 1: Figure S1 Establishment of a rat model of vascular dementia. **a** Representative images of “Longa scoring”. **b** Tetrazolium Chloride (TTC) staining in rat brain tissue.

Figures



Figure 1

Effect of GAS on the cognitive impairment in VD model rats. a Typical swimming tracks of VD rat in Morris water maze test. b Mean daily escape latency was examined. c The escape latency of VD model rats was significantly longer than sham group. After 8 weeks of GAS treatment, the escape latency of rats was significantly shortened in the probe trial. d The number of times to cross the target quadrante in the probe trial. e Percentage time spent in the target quadrant in the probe trial. f Representative photographs of dissected brain. g Observation of morphological changes in hippocampal neurons by H&E staining $\times 50$, $\times 200$. Scale bars: 200 μ m or 50 μ m. Data represent the mean \pm SEM. ##P< 0.01 versus sham, *P< 0.05, **P< 0.01 versus model.

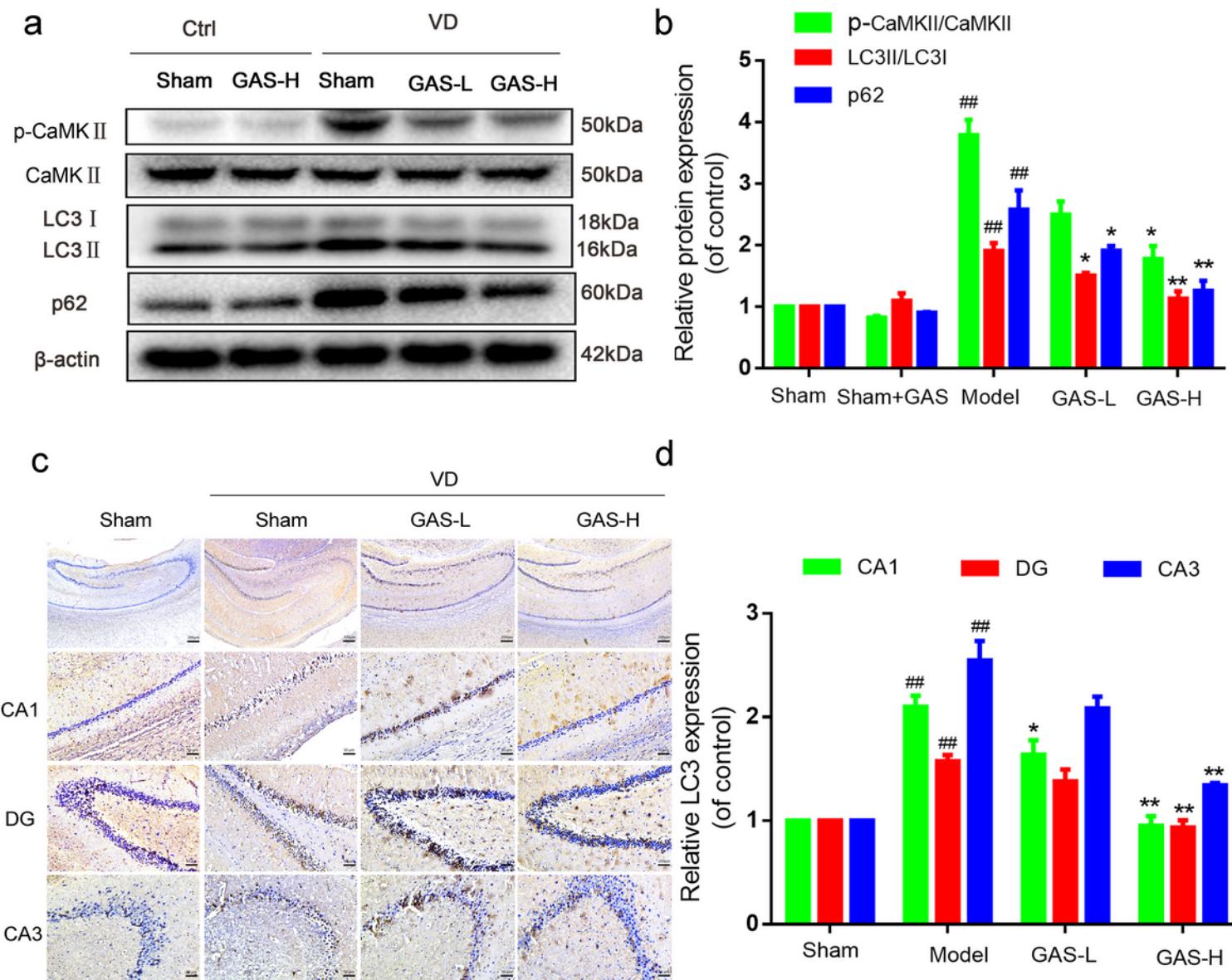


Figure 2

GAS reverses suppression of Autophagy flux and hyper-phosphorylation of CaMKII in VD model rats.
a The protein extract of hippocampal tissue was analyzed by Western blotting with LC3, p62, CaMKII, and p-CaMKII. Protein levels were quantified and normalized to β -actin. Data represent the mean \pm SEM. ##P<0.01 versus sham, *P<0.05, **P<0.01 versus model. **b** Representative images of hippocampal tissue sections immunostained with LC3 antibodies ($\times 50$, $\times 200$). Scale bars: 200 μ m or 50 μ m.

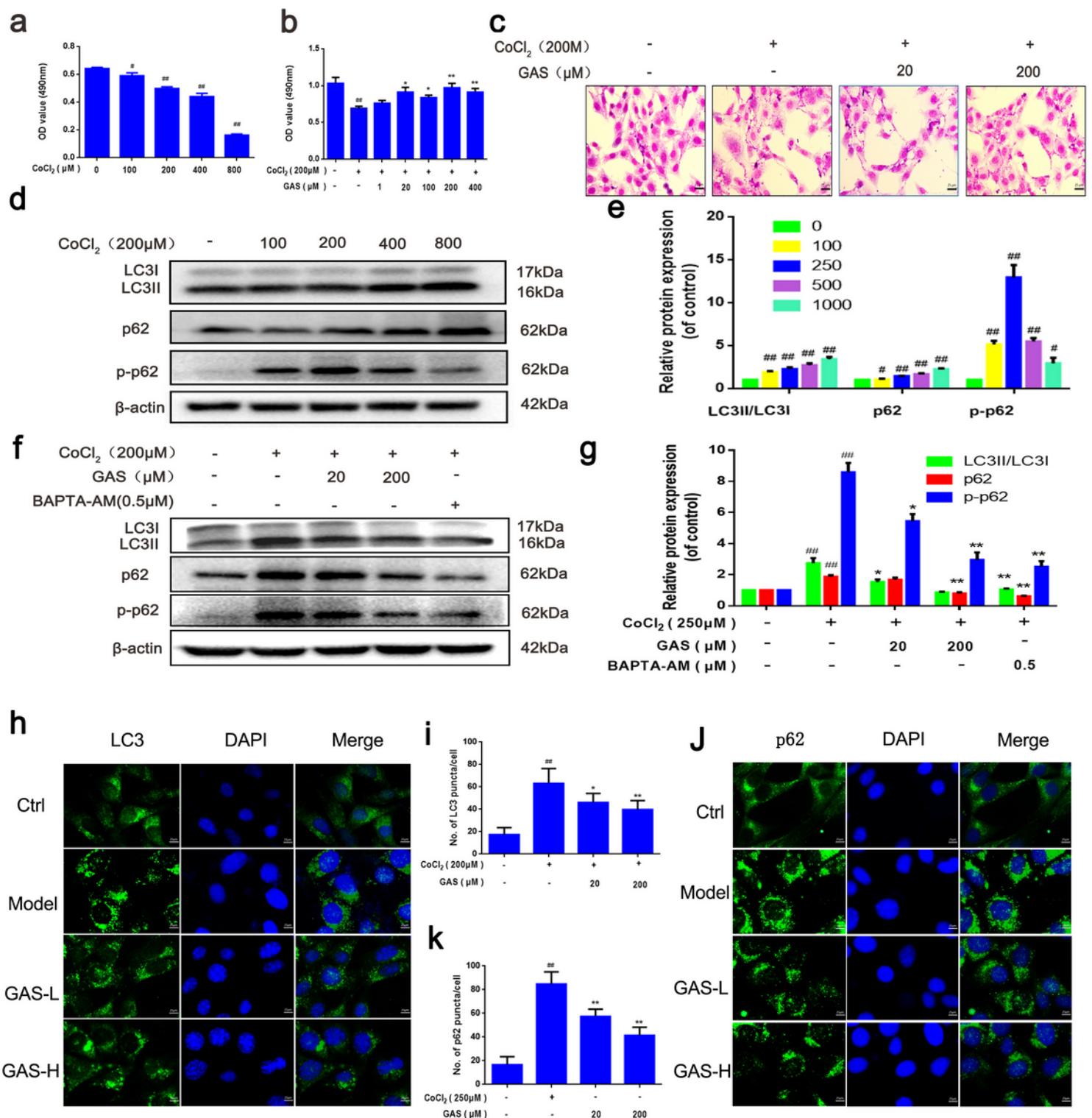


Figure 3

GAS reduces CoCl₂-induced autophagosome accumulation in HT22 cells. a HT22 was incubated with different concentrations of CoCl₂ for 24 hours, and the cell viability was detected by MTT assay (n=6). b Incubation with different concentrations of gastrodin 1h, cells were then treated with CoCl₂ (200 μ M) for 24 h. Cell viability was assessed via an MTT assay (n=6). c Representative Giemsa staining of HT22 cells (magnification, 200 \times ; n = 3) d Immunoblots showed levels of LC3, p62, p-p62 in HT22 cells treated with

various concentrations of CoCl₂ (0, 100, 200, 400 and 800 μM) for 24 h. β-actin was used as loading control. e HT22 cells were pretreated with GAS and BAPT-AM (0.5μM) for 1h and then exposure to CoCl₂ (200μM) for 24 h. LC3, p62 and p-p62 expression was detected by Immunoblots. β-actin was used as loading control. f-g Immunofluorescence analysis revealing modulation of LC3 in HT22 cells with or without gastrodin for 24 h (n = 3). Autophagosome was visualized as green puncta by using a Leica DMIRB at 800× magnification. In each independent experiment, 5 visual field cells were randomly selected and quantified and expressed as mean ± SEM. Immunofluorescence staining of p62 in HT22 cell. h-k Immunofluorescence analysis of p62 in HT22 cell with or without gastrodin treatment for 24 h (n = 3). Data represent the mean ± SEM. ##P< 0.01 versus control, *P< 0.05 versus CoCl₂.

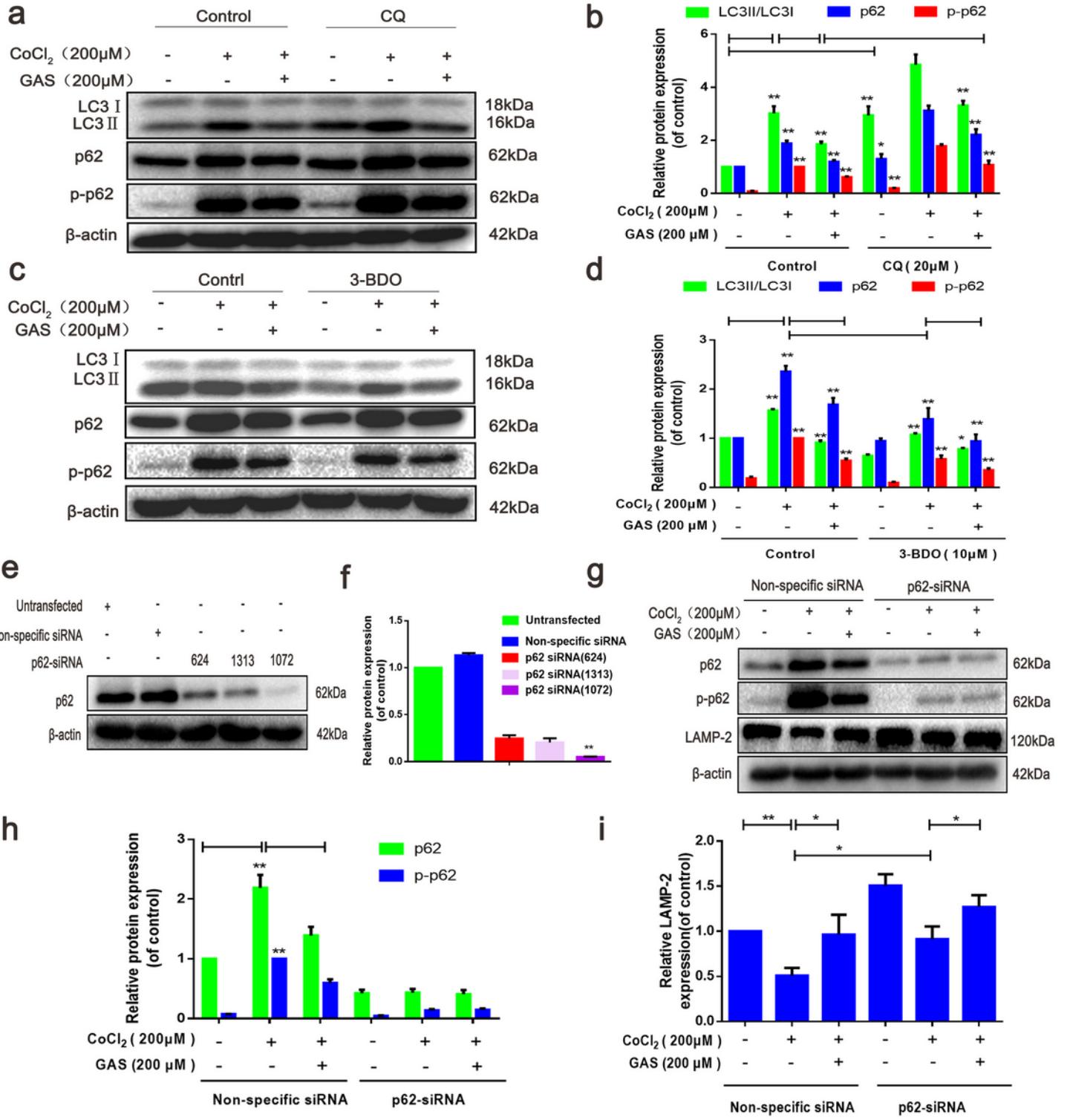


Figure 4

GAS ameliorates CoCl₂-induced autophagic flux inhibition in HT22 cells. a-b Levels of LC3, p62 and phosphorylated p62 (Ser349) in HT22 cells treated with CQ were assessed with or without gastrodin (200 μM) for 24h (n = 3).c-d Levels of LC3, p62 and phosphorylated p62 (Ser349) in HT22 cells treated with 3-BDO were assessed with or without gastrodin (200 μM) for 24 h (n = 3).e-f Detection of p62 siRNA transfection efficiency by western blot. g-i Levels of LC3, p62 and phosphorylated p62 (Ser349) in HT22

cells transfected with nonspecific or p62 siRNA were evaluated with or without gastrodin (200 μ M) for 24 h ($n = 3$). The experimental results were normalized to β -actin levels and are showed fold changes relative to control cells. Data represent the mean \pm SEM from three independent experiments. ##P< 0.01 versus control, *P< 0.05 versus CoCl₂.

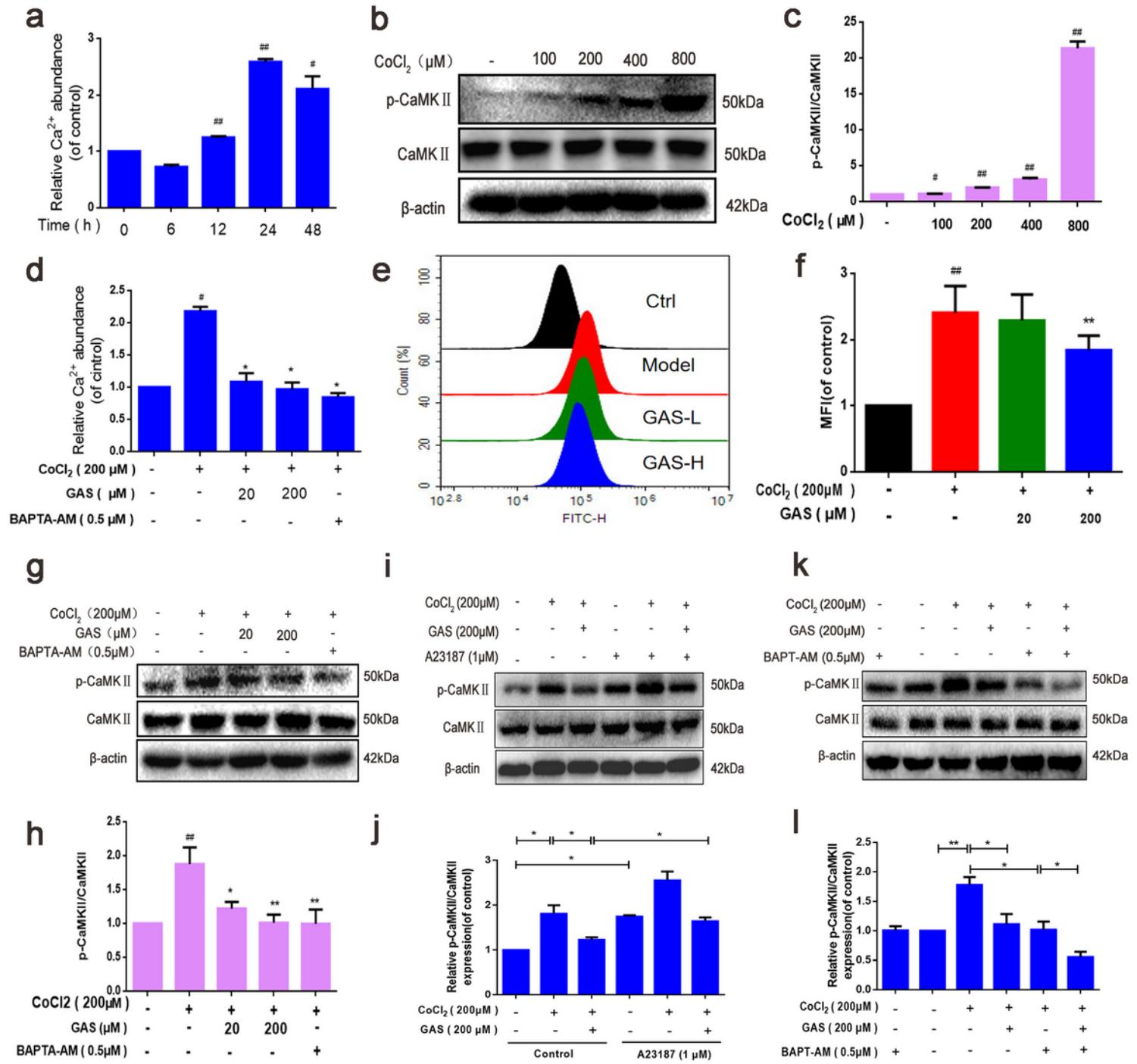


Figure 5

GAS alleviates CoCl₂-induced increase in intracellular Ca²⁺ abundance and CaMKII activation. a After HT22 cells were treated with CoCl₂ for different time, the content of Ca²⁺ was detected by commercial calcium quantitative kit. b-c After HT22 cells were treated with different doses of CoCl₂ for 24 hours, the phosphorylated CaMKII increased in a dose-dependent manner. β-actin was used as loading control. d HT22 cells were pretreated with gastrodin and BAPT-AM (0.5 μM) for 1 h and then plated on CoCl₂ (200 μM) for 24 h, the content of Ca²⁺ was detected by commercial calcium quantitative kit. e-f HT22 cells were pretreated with GAS for 1 h and then exposure to CoCl₂ (200 μM) for 24 h, cytosolic Ca²⁺ levels were measured by flow cytometry. g-h Gastrodin, similar to calcium chelator (BAPTA-AM), can reduce the level of phosphorylation of CaMKII. i-l Levels of CaMKII and phosphorylated CaMKII (Ser249) in HT22 cells treated with calcium chelator (BAPTA-AM) or calcium ionophore (A23187) were detected with or without gastrodin treatment (200 μM) for 24 h (n = 3). The experimental results were normalized to β-actin levels and are showed fold changes relative to control cells. Data represent the mean ± SEM from three independent experiments. ##P < 0.01 versus control, *P < 0.05 versus CoCl₂.

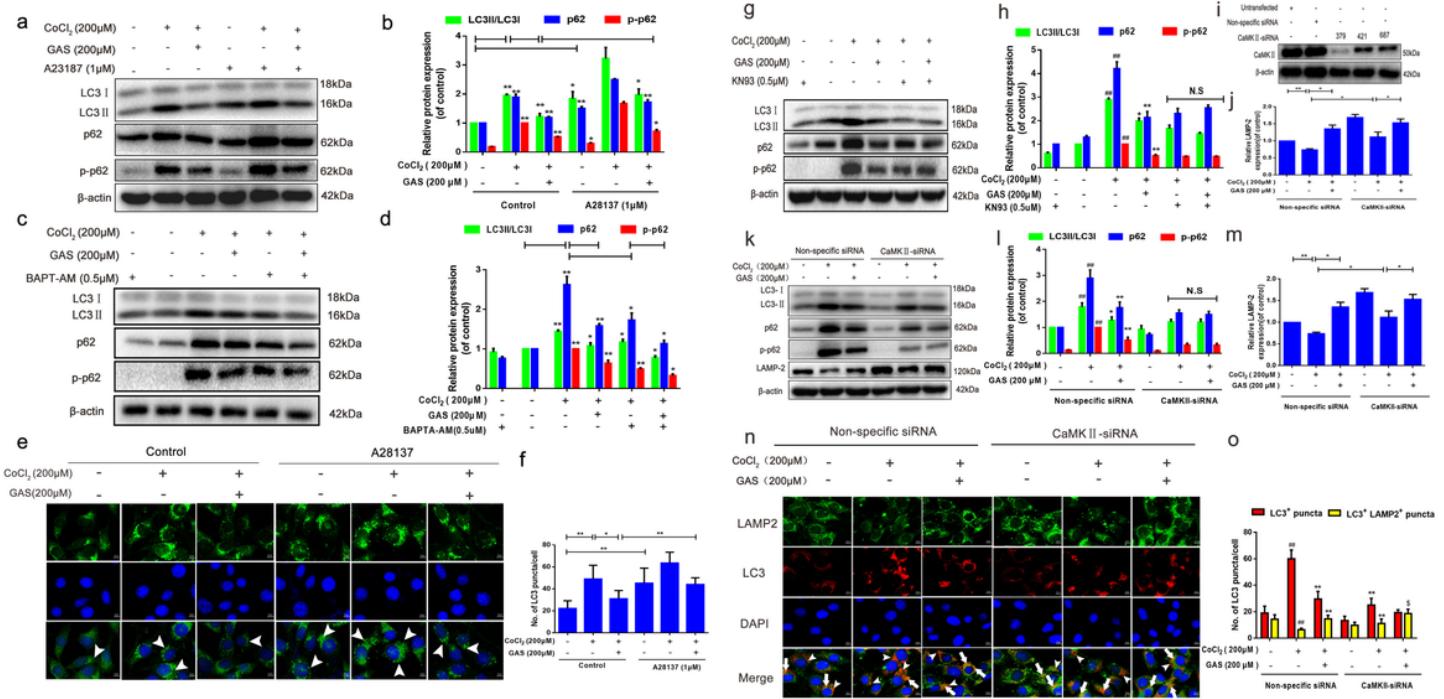


Figure 6

GAS alleviates CoCl₂-induced suppression of autophagy by stimulating [Ca²⁺]-dependent CaMKII phosphorylation in HT22 cells. a-d Levels of LC3, p62, and phosphorylated p62 (Ser349) in HT22 cells treated with calcium chelator (BAPTA-AM) or calcium ionophore (A23187) were assessed with or without gastrodin treatment (200 μM) for 24 h (n = 3). e-f Immunofluorescence analysis revealing modulation of LC3 in HT22 cells were treated with calcium ionophore (A23187) combining with or without gastrodin (200 μM) for 24 h (n = 3). Autophagosome was visualized (green puncta) by using a Leica DMIRB at 800× magnification. In each independent experiment, 5 visual field cells were randomly selected and quantified and expressed as mean ± SEM. g-h Levels of LC3, LAMP-2, p62 and phosphorylated p62

(Ser349) in HT22 cells treated with KN93 were assessed with or without gastrodin treatment (200 μ M) for 24 h (n = 3). i-j Detection of CAMKII-siRNA transfection efficiency by Western Blot. k-m Levels of LC3, LAMP2, p62 and phosphorylated p62 (Ser349) in HT22 cells transfected with nonspecific or CaMKII-siRNA were evaluated with or without gastrodin treatment (200 μ M) for 24 h (n = 3). The experimental results were normalized to β -actin levels and are showed fold changes relative to control cells. n-o Coimmunostaining of LC3 with LAMP2 in HT22. LC3+ puncta (green puncta) and LC3+LAMP2+ puncta (yellow puncta) was visualized by using a Leica DMIRB at 800 \times magnification. In each independent experiment, 5 visual field cells were randomly selected and quantified. Data represent the mean \pm SEM from three independent experiments. ##P< 0.01 versus control, *P< 0.05 versus CoCl₂, \$P<0.05 versus CoCl₂ + CaMKII-siRNA.

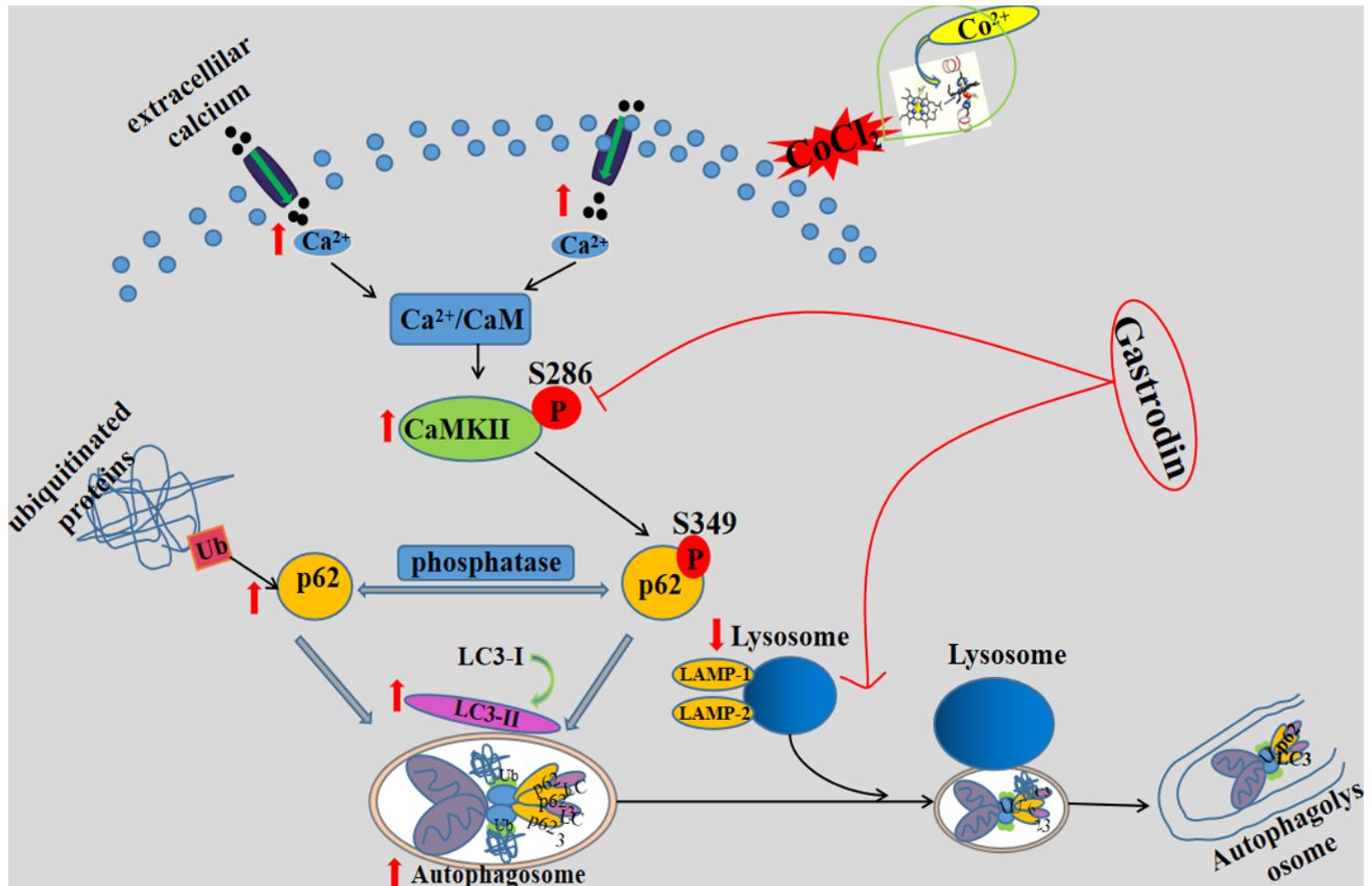


Figure 7

A schematic diagram of the possible mechanism of gastrodin improving cognitive impairment in vascular dementia rats by promoting autophagy flux through Ca²⁺/CaMKII signal pathway.

Supplementary Files

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- figureS1.tif