Caspofungin suppresses brain cell necroptosis in the ischemic stroke rat via up-regulation of Pellino3

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Research Article

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Abstract

Purpose

Pellino3, an ubiquitin E3 ligase, prevents the formation of the death-induced signaling complex in response to TNF-α via targeting receptor-interacting protein kinase 1 (RIPK1), and bioinformatics analysis predicts the interaction between Pellino3 and caspofungin, a common antifungal drug used in clinic. This study aims to explore the effect of caspofungin on brain injury in ischemic stroke and the underlying mechanisms.

Methods

The SD rat brains (or nerve cells) were subjected to 2h-ischemia (or 8h-hypoxia) plus 24h-reperfusion (or 24h-reoxygenation) to establish the I/R (or H/R) injury model. The cerebral injury was assessed by the methods of triphenyltetrazolium chloride (TTC) staining and Hematoxylin & eosin (H&E) staining. The correlations among caspofungin, Pellino3 and necroptosis in I/R-treated brain or H/R-treated nerve cells were evaluated by biochemistry, molecular and gene overexpression assays.

Results

The I/R-treated brain showed the injuries (increase in neurological deficit score and infarct volume), downregulation of Pellino3, decreased ubiquitination of RIPK1 and up-regulation of necroptosis-associated proteins [RIPK1, RIPK3, mixed lineage kinase domain-like protein (MLKL), p-RIPK1, p-RIPK3 and p-MLKL]. Caspofungin treatment improved neurological function, reduced infarct volume, up-regulated Pellino3, increased RIPK1 ubiquitination and down-regulated levels of RIPK1, p-RIPK1, p-RIPK3 and p-MLKL. In PC12 cells, H/R treatment caused cellular injury (LDH release and necroptosis), downregulation of Pellino3, decreased ubiquitination of RIPK1 and up-regulation of necroptosis-associated proteins, these phenomena were reversed by overexpression of Pellino3.

Conclusion

Pellino3 has an important role in counteracting necroptosis via ubiquitination of RIPK1 and caspofungin can suppress the brain cell necroptosis in ischemic stroke via upregulation of Pellino3.

1. Introduction

Ischemic stroke is the most common type of stroke, accounting for approximately 80% of all cases which result in focal cerebral ischemia secondary to thromboembolic arterial occlusion [1, 2]. Timely restoration of the blood supply (named “reperfusion”) is a major strategy in clinic to reduce the ischemic brain injury. Besides ischemia, however, reperfusion itself can also bring damage to the brain, referring to reperfusion

injury [3]. No matter ischemia or reperfusion injury, cell death is the final destiny for the injured brain cells if the effective measures are not taken. Thus, rescue of the injured brain cells from death is critically important for the therapy of stroke.

Necrosis and apoptosis are the two major forms of cell death in the brain after stroke [4]. Recently, more and more attentions have been transferred from apoptosis to necrosis because certain necrosis can also occur in a regulated manner as apoptosis. For example, necroptosis, a novel manner of cell death with the features of either apoptosis or necrosis, is controlled in a regulated manner depending on a pathway of RIPK1/RIPK3/MLKL (Receptor-interacting protein kinase 1/ Receptor-interacting protein kinase 3/Mixed lineage kinase domain-like protein) [5]. Growing evidence has shown that necroptosis occurred in multiple ischemia-relevant diseases, such as stroke and myocardial infarction [6]. Thus, targeting necroptosis is novel strategy to rescue the injured brain cells after stroke. Although it is well known that the occurrence of necroptosis is dependent on the RIPK1/RIPK3/MLKL pathway, the mechanisms responsible for its activation in the ischemic stroke remain largely unknown.

It has been reported that RIPK1 is a potential target of Pellino3, an ubiquitin E3 ligase, and suppressing the expression of Pellino3 leads to enhanced formation of the death-induced signaling complex in response to TNF-α [7]. It is not known, however, whether Pellino3 is involved in the activation of RIPK1/RIPK3/MLKL pathway, leading to necroptosis in the stroke brain. If this is the case, Pellino3 might be a novel drug target for therapy of ischemic stroke. By using the software of Molecular Operating Environment (MOE), couple of chemicals are predicted to interact with Pellino3. Among them, caspofungin is attracted our attention in particular because it is a medicine available in clinic for treating fungal infections [8].

The study has two-fold purpose: (1) to evaluate the therapeutic effect of caspofungin on ischemic stroke; and (2) to explore whether caspofungin reduces the brain injury of stroke via targeting Pellino3 and subsequent prevention of brain cell necroptosis.

2. Material And Methods

2.1 Animals

Male Sprague-Dawley (SD) rats (250~300g) were purchased from the Laboratory Animal Center, Xiangya School of Medicine, Central South University, China. Food was withheld from the animals for 24h before the experiments, but they are free access to tap water. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH Publication, 8th edition, 2011) and the ARRIVE guidelines (Animal Research: Reporting In-Vivo Experiments). The animal protocols were approved by the Institutional Animal Care and Use Committee of Central South University.

2.2 Ischemic stroke rat model

A rat model of ischemic stroke was established by middle cerebral artery occlusion (MACO) as we described previously [9]. Briefly, the rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and then the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were surgically isolated. A nylon suture with a rounded tip (0.40 mm diameter) was inserted into internal carotid artery (ICA) through a tiny incision in ECA and gently advanced 18~20 mm past the carotid bifurcation to occlude the origin of middle cerebral artery (MCA). After 2h of ischemia, the nylon suture was withdrawn to allow reperfusion. The sham-operated rats were subjected to the same procedure except no nylon suture was inserted.

2.3 Protocols for the experiments in animals

The first set of animal experiments was designed to examine the expression of Pellino3 after stroke. The rats were randomly divided into 3 groups (n = 12 per group): (1) the control group, no treatment for rats; (2) the sham group, rats were subjected to surgical procedures but with no ischemic insult; and (3) the stroke group, the rats were subjected to 2 h-ischemia plus 24 h-reperfusion.

In the second set of experiments, the effects of caspofungin on Pellino3 expression as well as necroptosis were explored. The animals were divided into 5 groups (n=12 per group): (1) the control group; (2) the sham group; (3) the stroke group; (4) and (5) the caspofungin (low dose, L) group and the caspofungin (high dose, H) group: the rat received caspofungin (Selleckchem, USA) at 2 mg/kg and 6 mg/kg (dissolved in saline, i.m.) at 1h after the ischemia, respectively.

After 24h-reperfusion, neurological deficit score was assessed first, and then the brain tissues of 6 rats from each group were saved for infarct volume measurement, while the brain tissues of the remaining 6 rats from each group were saved for morphological, biochemical and molecular analysis.

2.4 Protocols for the experiments in cells

The well-differentiated PC12 cells (Chinese Academy of Sciences, Shanghai, China) were seeded at a density of 1×10^4 cells/cm^2 and cultured in DMEM with 10% FBS. According to the instruction provided by the cell supplier, the differentiation of PC12 cells was induced by nerve growth factor. The cells were maintained at 37°C in 95% air/5% CO2 in a humidified incubator. Under such conditions, cells grew well and exhibited spindle-shaped cell morphology similar to neuronal cells, which was consistent with morphological characteristics of well-differentiated PC12 cells. The cells were kept in the serum-free DMEM for 16h before the experiments.

To verify the anti-necroptosis effect of caspofungin via upregulation of pellino3 in the stroke rat brain, the PC12 cells were cultured in a medium without glucose under hypoxic condition (N2/CO2, 95:5), which is widely used to mimic the ischemic stroke in vitro [10]. The cells were divided into 4 groups (6 individual experiments per group): (1) The control group, cells were cultured under normoxic condition; (2) The hypoxia group, cells were cultured for 8h-hypoxia plus 24h-reoxygenation; (3) The hypoxia plus pellino3 overexpression group, cells transfected with Adenovirus-mediated pellino3 *gene* and subjected to

hypoxia; and (4) The hypoxia plus negative control group, cells transfected with Adenovirus-mediated GFP and subjected to hypoxia.

At the end of the experiments, cells and culture mediums were collected for flow cytometry, LDH release and molecular analysis.

2.5 Assessment of neurological function and infarct volume

The neurological behavioral tests were carried out after 24h reperfusion by an investigator blinded to the experimental groups using five-point neurological deficit score (0 = no deficit, 1 = failure to extend the left forepaw, 2 = decreased grip strength of left forepaw, 3 = circling to the left by pulling the tail, 4 = spontaneous circling).

After neurological behavioral tests, the rats were killed under anesthesia, and the brains were rapidly removed and sliced into 2-mm thick coronal sections with the aid of a brain matrix. The sections were stained with 1% triphenyltetrazolium chloride (TTC) for 15 min at 37 °C and scanned into a computer. The images of brain sections with TTC-staining were analyzed with the imaging software (Image J, NIH, USA). The absence or presence of infarction was determined by TTC staining.

The infarct volume (in mm^3) of each section was calculated as infarct area (in mm^2) multiplied by the section thickness (2 mm). The total infarct volume of each brain was equal to the summation of the infarct volume of all sections. To eliminate the effect of edema on the accuracy of infarct volume assay, the final infarct volume was corrected by following equation: corrected infarct volume=total infarct volume× (left hemisphere volume/right hemisphere volume). Here, left hemisphere refers to no-ischemic hemisphere of brain while right hemisphere refers to ischemic contralateral side.

2.6 Hematoxylin-eosin (HE) staining

HE staining was performed to evaluate the morphological changes in brain. Briefly, the brain tissues were fixed in 4 % paraformaldehyde and embedded in paraffin, and then they were cut into 5-μm sections. The slices underwent hematoxylin and eosin staining for 20 and 2 min, respectively. After HE staining, the results were imaged by microscope (Nikon Eclipse 80i, Japan) to evaluate the morphological changes among the experimental groups.

2.7 Overexpression of Pellino 3

The Adenoviral constructs carrying rat *pellino 3* gene (ADV1 ×Flag-*Pellino3*) (1×10^{10} pfu/mL) was purchased from GenePharma (Shanghai, China). PC12 cells were seeded in 6-well plates at 1×10^6 per well and transduced at approximately 70~80% confluence with the adenoviral vectors at MOI 8 according to a standard protocol for 24h. Afterward, the media were exchanged for fresh DMEM media to proceed the following experiments. The specificity or efficiency of Pellino 3 overexpression was evaluated by Western blot. The negative control cells were transfected with adenoviral constructs carrying GFP (Ad-GFP) (1×10^{10}pfu/mL).

2.8 Lactate dehydrogenase release and flow cytometry analysis

Culture medium was collected for analysis of lactate dehydrogenase (LDH) release (an indicator of cellular necrosis) by using a colorimetric assay kit (Beyotime, Jiangsu, China) following the protocol provided by the manufacturer. Released LDH was determined by a coupled enzymatic reaction that resulted in the conversion of a tetrazolium salt into a red color formazan by diaphorase. Briefly, 120 μL of culture medium was mixed with 60 μL of LDH work solution, then incubated at 25~30°C for 30 min. The absorbance was measured at 490 nm. The percentage of LDH release was calculated by a formula provided by the protocol.

Flow cytometry was performed to further evaluate the type of cell death. In brief, the PC12 cells were collected and centrifuged at 1000 rpm for 5 min. After washing 2 times with PBS, 200 μL of FITC-conjugated Annexin V (195 μL AnnexinV-FITC Binding Solution + 5 μL AnnexinV-FITC) were added to the cells and incubated at room temperature for 15 min in the dark. Ten μL of propidium iodide (PI) were added before flow cytometry analysis. PI^+ /Annexin V^+ cells were considered as necroptotic cells.

2.9 Western blot analysis

Brain tissues or PC12 cells were lysed in ice-cold lysis buffer containing proteinase inhibitor to obtain the proteins following a regular method. The protein concentration in homogenate was measured by BCA Protein Assay kit (Beyotime, Jiangsu, China). Protein samples (20~40μg) were separated on 8~10 % SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). The membranes were then blocked in Tris-buffered saline containing Tween-20 (TBST) with 5% skim milk powder for 1 h at room temperature and incubated with primary antibodies against Pellino 3 (Santa Cruz, California, USA), RIPK1 (Boster, Wuhan, China), RIPK3 (Biovision, CA, USA), MLKL (Abcam, Cambridge, UK), p-RIPK1 (CST, Boston, USA), p-RIPK3 (Abcam, Cambridge, UK), p-MLKL (Abcam, Cambridge, UK) or β-actin (Beyotime, Jiangsu, China) overnight at 4 °C. The PVDF membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime, Jiangsu, China) for 1 h at room temperature. Protein signals were detected by Luminata Creseendo Western HRP substrate through Molecular Imager ChemiDoc XRS System (Bio-Rad, Philadelphia, PA). Quantitative analysis of protein bands was conducted using Image J software. β-actin served as the internal control. Arbitrary optical density units of the targeting protein were normalized against control and expressed as fold change.

2.10 Co-immunoprecipitation (Co-IP) assay

Co-IP was performed to evaluate the RIPK1 ubiquitination and the interaction between RIPK1 and RIPK3 following a protocol provided by the kit supplier (Absin, Shanghai, China). Briefly, the brain tissues or PC12 cells were lysed in lysed in IP buffer supplemented with protease inhibitors. The lysates were immunoprecipitated with the anti-RIPK1 antibody (Proteintech, Wuhan, China) or rabbit IgG (Beyotime, Jiangsu, China) overnight at 4°C, and then incubated with Protein A/G Agarose beads for another 4h at 4°C. Agarose beads were washed with ice-cold washing buffer (supplied by the kit) followed by elution of bound proteins. Precipitated proteins were determined by Western blot with anti-Ub (CST, Boston, USA),

anti-K48 linked UB (CST, Boston, USA), anti-K63 linked UB (CST, Boston, USA), or anti-RIPK3 (Biovision, CA, USA) antibody. Rat IgG was used as negative control. The samples in the input group only proceeded Western blot and served as positive controls.

2.11 Statistical analysis

Prism GraphPad 6.0 was used for statistical analysis. The data were presented as mean ± SEM. Differences in measured values among multiple groups were analyzed by one-way ANOVA followed by Tukey test for comparing multiple groups. The neurological deficit scores were analyzed by Kruscal-Wallis H and Wilcoxon tests. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Downregulation of Pellino3 in the stroke rat brain

The 5-point rating scale of neurological deficit score was used to assess neurological function in the stroke rat brain. There was no significant difference in neurological function between the control and the sham group, while ischemic stroke caused an obvious increase in neurological deficit score compared with that in the sham-operated rats (Fig. 1A); consistent with the neurological deficit score, no evident infarction was seen in the control and the sham group, while a dramatic increase of infarct volume was observed in stroke rat brains (Fig. B and C). These results suggest a successfully-established ischemic stroke model in rat. Compared with the sham group, downregulation of Pellino3 was found in the stroke rat brain (Fig. D), indicating a reverse correlation between Pellino3 level and ischemic brain injury.

3.2 Caspofungin exerted effect on reduction of ischemic injury in the stroke rat brains

As the first of a new class of antifungal drug (Fig. 2A), caspofungin was able to improve the neurological function and attenuate the cerebral infarct volume in the stroke rat brain in a dose-dependent manner (Fig. 2B-D). Consistently, the pathological morphology of brain (such as cell loss and disarray) in the stroke rat brain was also reversed in the presence of caspofungin at the dose of 6mg/kg (Fig. 2E).

3.3 Caspofungin downregulated necroptosis-relevant protein levels in the stroke rat brain

Necroptosis is a type of regulated necrosis, and RIPK1, RIPK3 and MLKL are the proteins for executing the necroptosis. As displayed in Fig. 3A-C, the protein levels of RIPK1, RIPK3 and MLKL were up-regulated in the stroke rat brain compared with the sham-operated rat. Administration of caspofungin (6 mg/kg) significantly decreased RIPK1 protein level but it had little effect on RIPK3 and MLKL protein levels. We further detected the phosphorylation levels of RIPK1 (p-RIPK1), RIPK3 (p-RIPK3) and MLKL (p-MLKL), which were all up-regulated in stroke rat brains. These phenomena were reversed in the presence of caspofungin at the dose of 6mg/kg (Fig. 3D-F), suggesting that caspofungin can suppress the activation of RIPK1/RIPK3/MLKL pathway in the stroke rat brains.

3.4 Caspofungin increased the ubiquitination levels of RIPK1 via up-regulation of Pellino3 in the stroke rat brain

To elucidate the mechanisms for the effect of caspofungin on inhibiting necroptosis, the Pellino 3 protein level, RIPK1 ubiquitination level and the interaction between RIPK1 and RIPK3 were examined. As shown in Fig. 4A, the downregulation of Pellino 3 in the stroke rat brain was evidently reversed in the presence of caspofungin at a dose of 6 mg/kg (Fig. 4A). In agreement with the downregulation of Pellino 3, the total ubiquitination level of RIPK1 as well as k63 and k48 ubiquitination levels of RIPK1 were significantly decreased in the stroke rat brain; these phenomena were reversed by caspofungin (6 mg/kg) due to the restoration of Pellino 3 level (Fig. 4B, top and middle). Consistently, the interaction between RIPK1 and RIPK3 was weakened in the stroke rat brain by caspofungin (6 mg/kg) because of the restoration of RIPK1 ubiquitination at the sites of k63 and k48 (Fig. 4B, bottom).

3.5 Overexpression of pellino3 reduced hypoxic injury in PC12 cells

To verify the role of Pellino 3 in suppress of necroptosis in stroke rat brain via ubiquitination of RIPK1, PC12 cells were cultured under hypoxia condition to mimic the ischemic stroke in vitro. Consistent with the findings in stroke rat brains, downregulation of Pellino3 was observed in the hypoxia-treated PC12 cells concomitant with an increase in LDH release (Fig.5A and B). Transfection of the Adenovirus-mediated *pellino3* gene successfully overexpressed pellino3 protein in hypoxia-treated PC12 cells accompanied by reduction of LDH release (Fig.5A and B). The flow cytometry analysis also showed that the hypoxia treatment markedly caused the cell death because the Annexin-V and PI positive cells were evidently increased, which were attenuated via overexpression of Pellino 3 (Fig. 5C-F). The Ad-GFP did not show such effects.

3.6 Overexpression of Pellino3 decreased the necroptosis-relevant protein levels in hypoxia-treated PC12 cells

In agreement with the results in vivo, the levels of necroptosis-relevant proteins (RIPK1, RIPK3, MLKL, p-RIPK1, p-RIPK3 and p-MLKL) were increased in hypoxia-treated PC12 cells. Similar to the effects of caspofungin, overexpression of Pellino 3 reduced the levels of RIPK1, p-RIPK1, p-RIPK3 and p-MLKL, but it did not significantly affect the levels of RIPK3 and MLKL (Fig. 6 A-E).

3.7 Overexpression of pellino3 increased the ubiquitination levels of RIPK1 in hypoxia-treated PC12 cells

In accordance with the results in the animals, the total ubiquitination level of RIPK1 as well as k63 and k48 ubiquitination levels of RIPK1 were significantly decreased in the hypoxia-treated PC12 cells; these phenomena were reversed by overexpression of pellino3 (Fig.7 top and middle). Consistently, the interaction between RIPK1 and RIPK3 was enhanced in the hypoxia-treated PC12 cells due to the decreased ubiquitination of RIPK1, which was reversed under the condition of Pellino3 overexpression, supporting the anti-necroptosis effect of caspofungin via upregulation of Pellino3.

4. Discussion

In this study, by using the ischemic stroke animal and cell models, we explored the effect of caspofungin on ischemic brain injury and the potential mechanisms. The two major findings of this study are summarized as follows: (1) the protein levels of Pellino3 were downregulated in the stroke rat brains in vivo or in the hypoxia-treated PC12 cells in vitro concomitant with the decreased ubiquitination of RIPK1, meanwhile, the levels of necroptosis-associated proteins (RIPK1, RIPK3 and MLKL) were increased while the interaction between RIPK1 and RIPK3 was enhanced; and (2) Up-regulation of Pellino3 with caspofungin or overexpression of Pellino3 mitigated the stroke brain injury in vivo or the hypoxic injury in the PC12 cells due to reduction of RIPK1/RIPK3/MLKL-dependent necroptosis. To the best of our knowledge, we have identified a novel function of Pellino 3 in regulating the RIPK1/RIPK3/MLKL pathway for the first time, and we have also demonstrated for the first time that caspofungin reduces the stroke brain injury via suppression of necroptosis due to up-regulation of Pellino3.

For many years, necrosis is thought as a random, passive cell death without definable mediators. Although necrosis is a major type of brain cell death in patients suffered ischemic stroke [11], few drugs are designed to specifically target it. Recently, more and more studies have demonstrated that certain necrosis, such as necroptosis, ferroptosis, pyroptosis and cyclophilin D-mediated necrosis, actually occurs in a programmed fashion, referring to regulated necrosis [12]. Among them, necroptosis is the most understood one, which is triggered by RIPK1/RIPK3/MLKL signaling pathway. Assembly of RIPK1 and RIPK3 through a series of RIPK1 and RIPK3 auto- and trans-phosphorylation events leads to the formation of necrosome complex followed by the recruitment of MLKL and creation of a supramolecular protein complex at the plasma membrane, which is responsible for executing the necroptosis [13]. The studies from other labs and ours have repeatedly demonstrated that brain cell necroptosis occurred in ischemic stroke animal models and targeting necroptosis significantly reduced the cerebral ischemic injury in vivo and hypoxic injury in the cultured nerve cells [14-17].Since necroptosis is mediated by RIPK1/RIPK3/MLKL signaling pathway, most of the current interventions thus focus on RIPK1, RIPK3 or MLKL. For example, necrostatin-1 can reduce infarct volume and improve neurological scores in the ischemic stroke animals via inhibition of RIPK1 while GSK’872 can prevent brain cell necroptosis in stroke rats via targeting RIPK3 [18, 19]. Recently, we have reported that both ponatinib, a tyrosine kinase receptor inhibitor for therapy of chronic myelogenous leukemia (CML), and ligustroflavone, an ingredient from natural herb named common privet, can protect the rat brain from ischemic injury via targeting RIPK1 and/or RIPK3 [14, 20]. Among these RIPK1/RIPK3 inhibitors or regulators, the clinical prospect for necrostatin-1, GSK’872 and ligustroflavone remain unclear. Although ponatinib is available in clinic for treating CML, its side effects on cardiovascular system pose a major concern. Thereby, it is necessary to identify novel targets for prevention of necroptosis in the stroke rat brain.

There is evidence that Pellino3 might be a potential target for inhibition of brain cell necroptosis in ischemic stroke because it can prevent formation of the death-induced signaling complex in response to TNF-α via targeting RIPK1 [7]. Based on this finding, we predict that Pellino3 may have a role in regulation of RIPK1/RIPK3/MLKL-dependent necrosis in ischemic stroke. In the present study, the results showed a

reverse correlation between Pellino3 level and ischemic brain injury in stroke rat concomitant with an increase in the levels of necroptosis-associated proteins (RIPK1, RIPK3 and MLKL). These results indicate that Pellino3 may negatively control the RIPK1 protein levels via its ubiquitin ligase activity. Consistently, with the down-regulation of Pellino3 in the stroke rat brain, the ubiquitination level of RIPK1 was decreased, leading to a decrease in RIPK1 degradation and the enhanced interaction between RIPK1 and RIPK3 and similar situations were observed in the hypoxia-treated PC12 cells. To provide the direct evidence for its role in suppression of RIPK1, overexpression of Pellino3 was carried out in PC12 cells. We have found that overexpression of Pellino3 increased the ubiquitination level of RIPK1 in hypoxia-treated PC12 cells concomitant with a decrease in the levels of RIPK1, p-RIPK1, p-RIPK3 and p-MLKL and the weakened interaction between RIPK1 and RIPK3, supporting that Pellino3 promotes the degradation of RIPK1 via ubiquitination and the down-regulation of Pellino3 in the stroke brain accounts, at least in part, for the activation of RIPK1/RIPK3/MLKL pathway.

Due to the role of Pellino3 in counteracting the activation of RIPK1//RIPK3/MLKL pathway, it is reasonable to speculate the Pellino3 might be a novel drug target for treating ischemic stroke via prevention of necroptosis. With the help of the bioinformatics analysis, we have identified that caspofungin could be a regulator for Pellino3. Caspofungin is a long-standing antifungal agent [8]. Recently, Itoh K et al have demonstrated that caspofungin is able to suppress zymosan-induced cytokine and chemokine release in THP-1 cells [21], revealing the additional pharmacological action of caspofungin besides its antifungal activity. Based on this report as well as the potential interaction between caspofungin and Pellino3, we hypothesize that caspofungin may have beneficial effect on ischemic stroke. Expectedly, in the present study, the results really showed that caspofungin improved the neurological function and reduced the infarct volume in the stroke rat brain. Further experiments revealed that caspofungin increased the ubiquitination levels of RIPK1 via up-regulation of Pellino3 in the stroke rat brain, resulting in downregulation of RIPK1, p-RIPK1, p-RIPK3 and p-MLKL, and the weakened interaction between RIPK1 and RIPK3. These results are in agreement with those of Pellino3 overexpression in hypoxia-treated PC12 cells.

In summary, this study has identified a novel function of Pellino3 in counteracting necroptosis via ubiquitination of RIPK1 and provided evidence for the first time that caspofungin is able to suppress the brain cell necroptosis in ischemic stroke via upregulation of Pellino3. Our study may lay a basis on extension of the clinical indications for caspofungin in treating patients with ischemic stroke.

Declarations

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Conflicts of interest

The authors declare no conflicts of interest.

Availability of data and material

Data are available upon request.

Code availability

Not applicable.

Author contributions

Peng Jun, Luo XiuJu and Zhang XiaoJie conceived and designed the research. Zhang YiYue, Tian Jing and Peng ZiMei conducted experiments. Zhang YiYue and Hu ZhongYang analyzed data. Zhang YiYue, Luo XiuJu and Peng Jun wrote the manuscript. All authors read and approved the manuscript.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Consent to participate and publication

We state here that all authors have seen and approved the final version of the manuscript being submitted.

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Figures

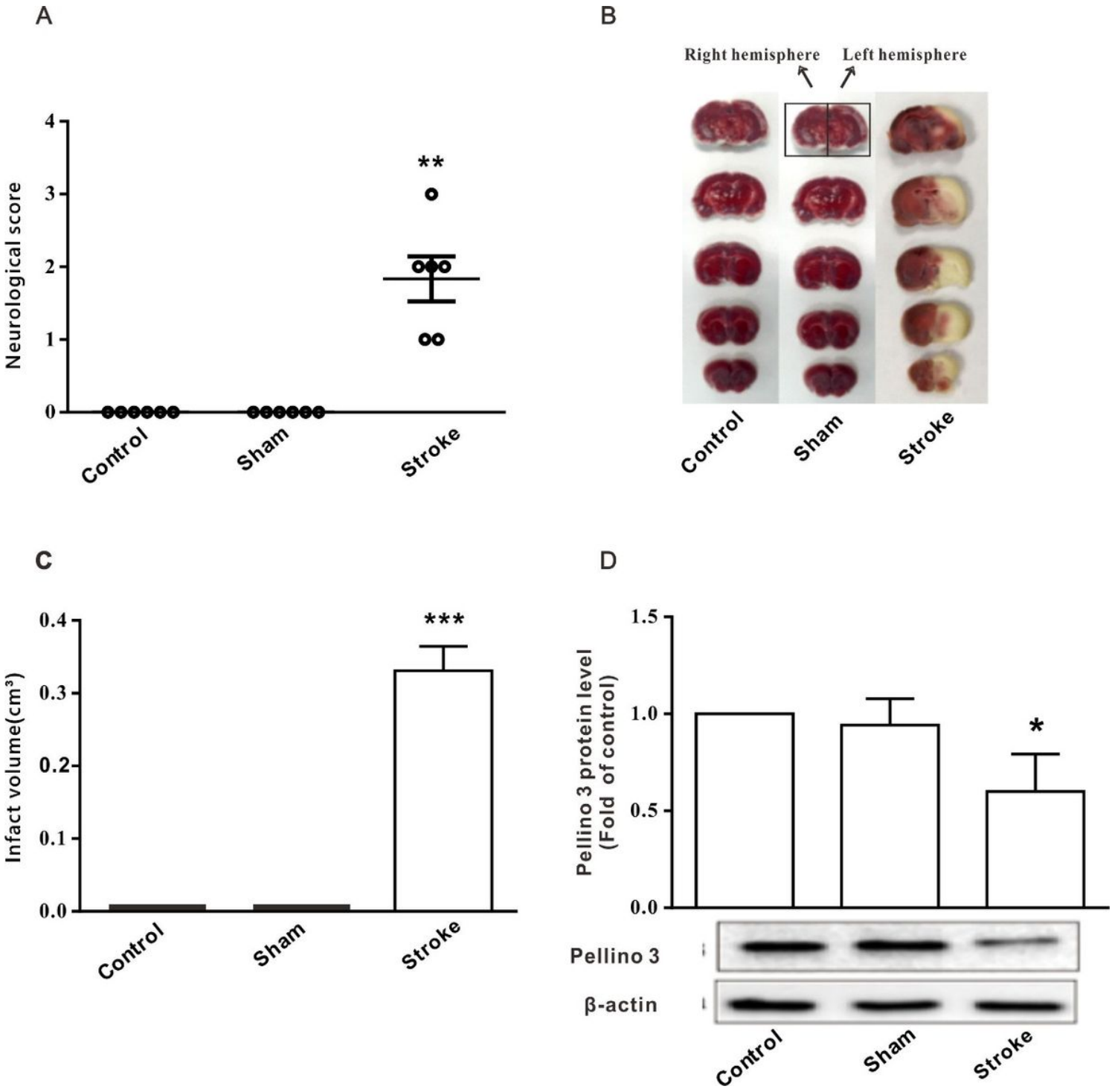

Figure 1

Downregulation of Pellino3 in the stroke rat brain A. Neurological deficit score (n=6 per group). B. Representative images of triphenyltetrazolium chloride (TTC)-stained brain tissue. C. Infarct volume (n =6). D. Pellino3 protein expression. Top: optical density for the protein blot of Pellino3 vs β-actin, which were normalized by the control; bottom, the representative images of Western blot results (n=6 per group). All values were expressed as means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 vs Sham.

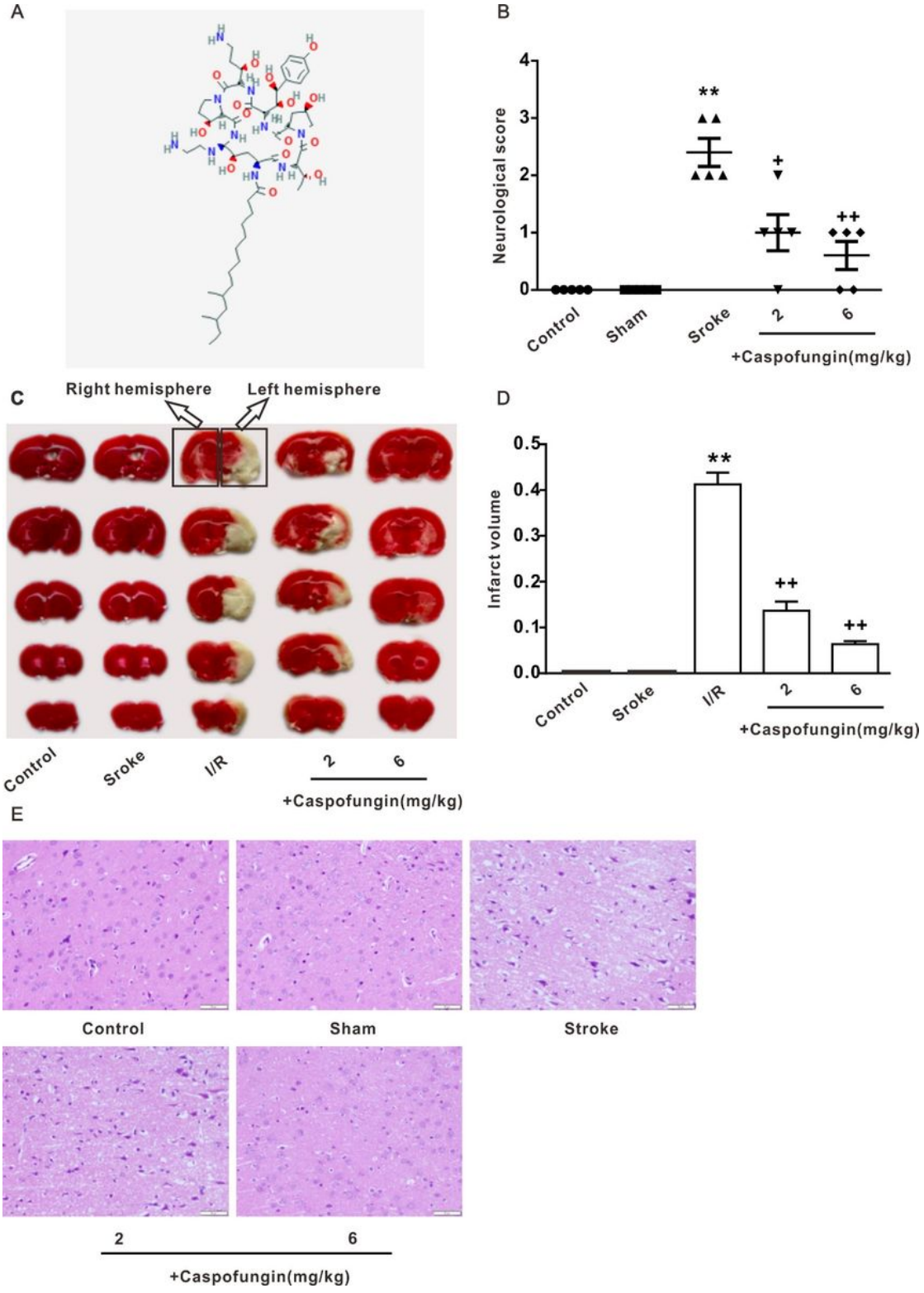

Figure 2

Effect of caspofungin on brain injury in the stroke rats A. Chemical structure of caspofungin (molecular formula: C52H88N10O15, molecular weight: 1093.31). B. Neurological deficit score (n=6 per group). C. Representative images of triphenyltetrazolium chloride (TTC)-stained brain tissue. D. Infarct volume (n =6 per group). E. Representative images of hematoxylin-eosin (HE) staining. All values were expressed as means ± S.E.M. + caspofungin: stroke + caspofungin. *P<0.05, **P<0.01 vs Sham; +P<0.05, ++P<0.01 vs Stroke.

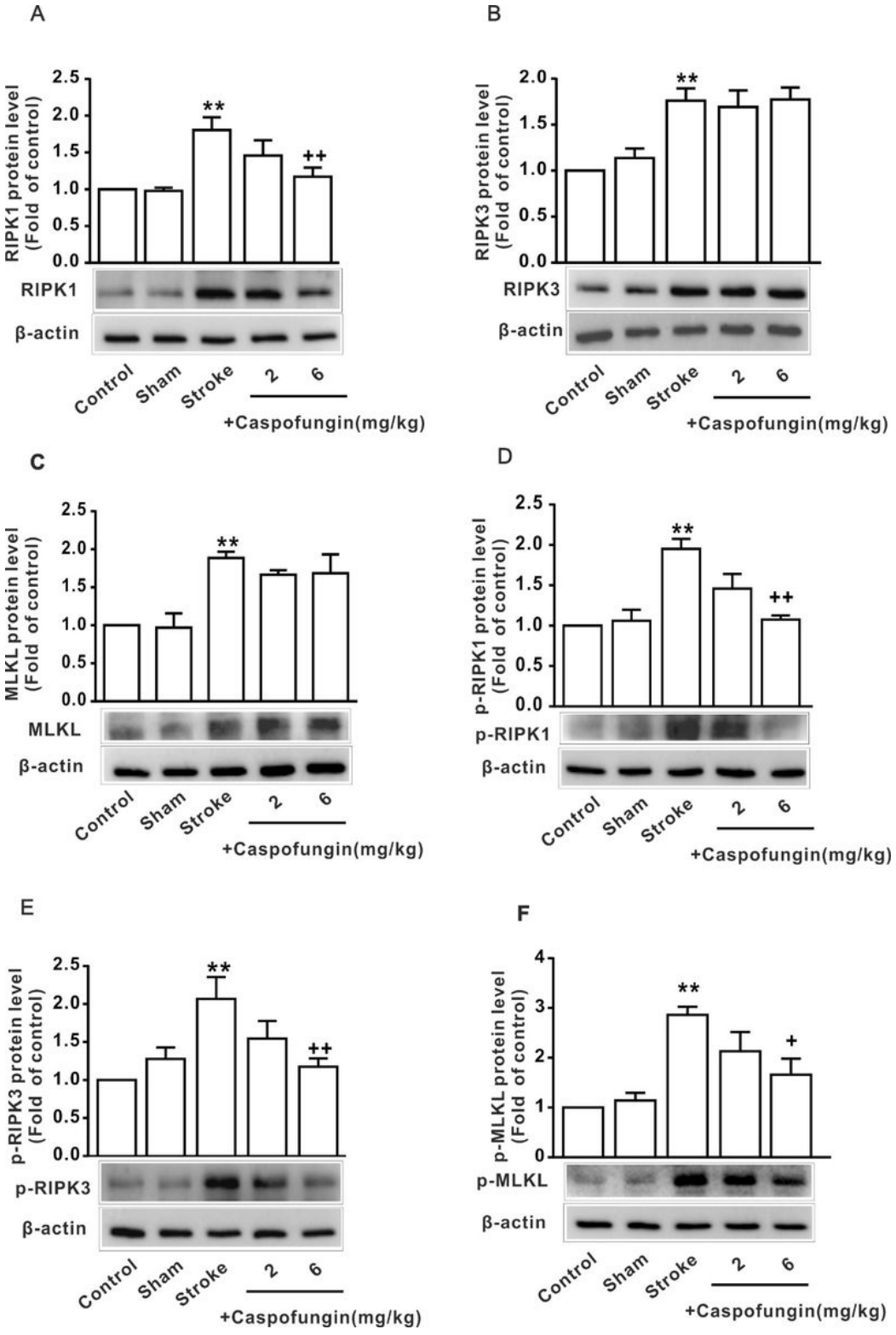

Figure 3

Effect of caspofungin on necroptosis-associated proteins in the brain of stroke rats Top: optical density for the protein blot of RIPK1 (A), RIPK3 (B), MLKL(C), p-RIPK1(D), p-RIPK3(E) or p-MLKL (F) against β-actin, which were further normalized by the control; bottom, the representative images of Western blot results (n=5 per group). All values were expressed as means ± S.E.M. + caspofungin: stroke + caspofungin. *P<0.05, **P<0.01 vs Sham; +P<0.05, ++P<0.01 vs Stroke.

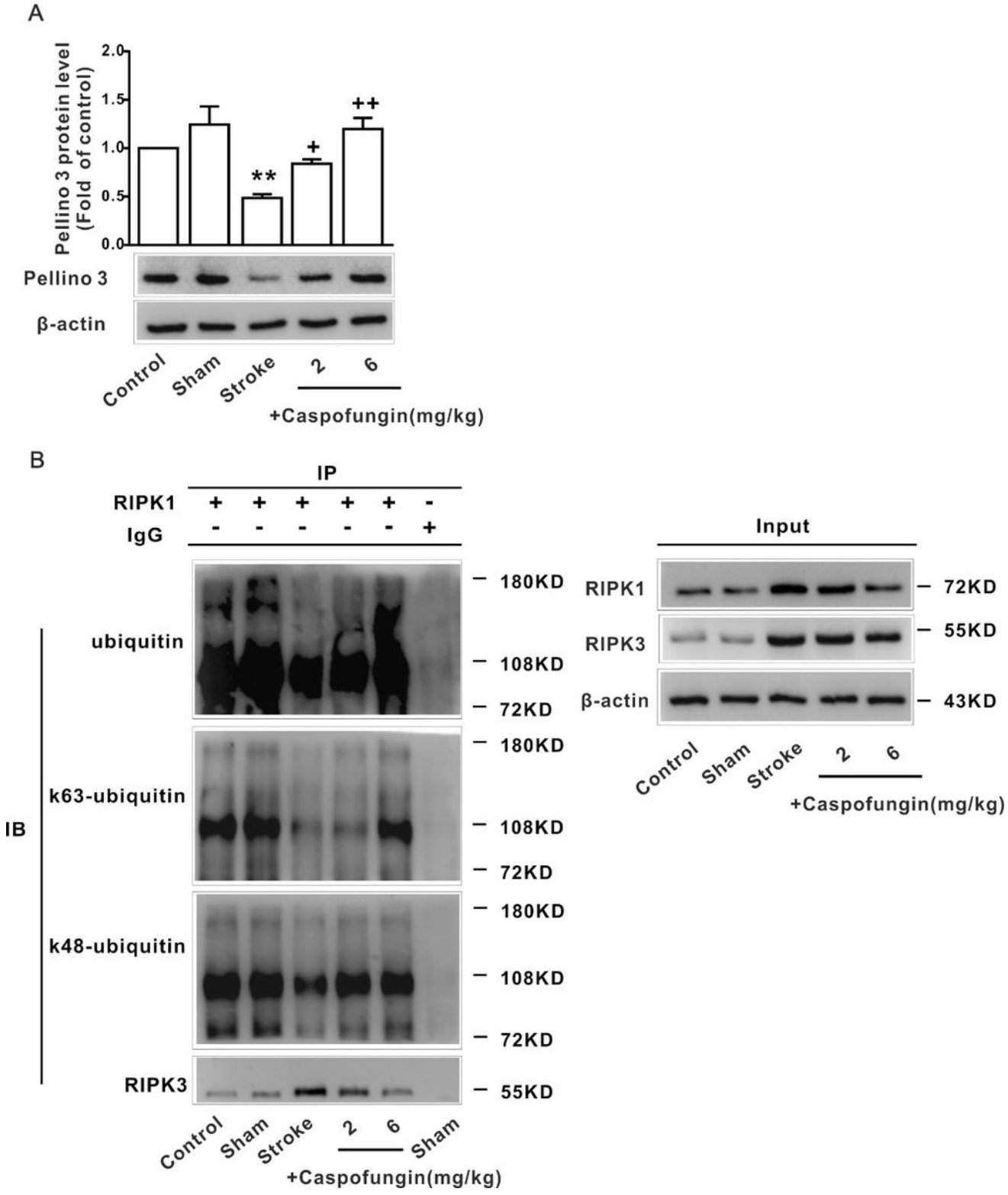

Figure 4

Effect of caspofungin on the ubiquitination levels of RIPK1 and the interactions between RIPK1 and RIPK3 in the brain of stroke rats A. Pellino3 protein expression. Top: optical density for the protein blot of Pellino3 vs β-actin, which were normalized by the control; bottom, the representative images of Western blot results (n=5 per group). B. Representative images of Co-immunoprecipitation assay for RIPK1 ubiquitination assay and the interaction between RIPK1 and RIPK3 in stroke rat brain. All values were expressed as means ± S.E.M. + caspofungin: stroke + caspofungin. **P<0.01 vs Sham; +P<0.05, ++P<0.01 vs Stroke.

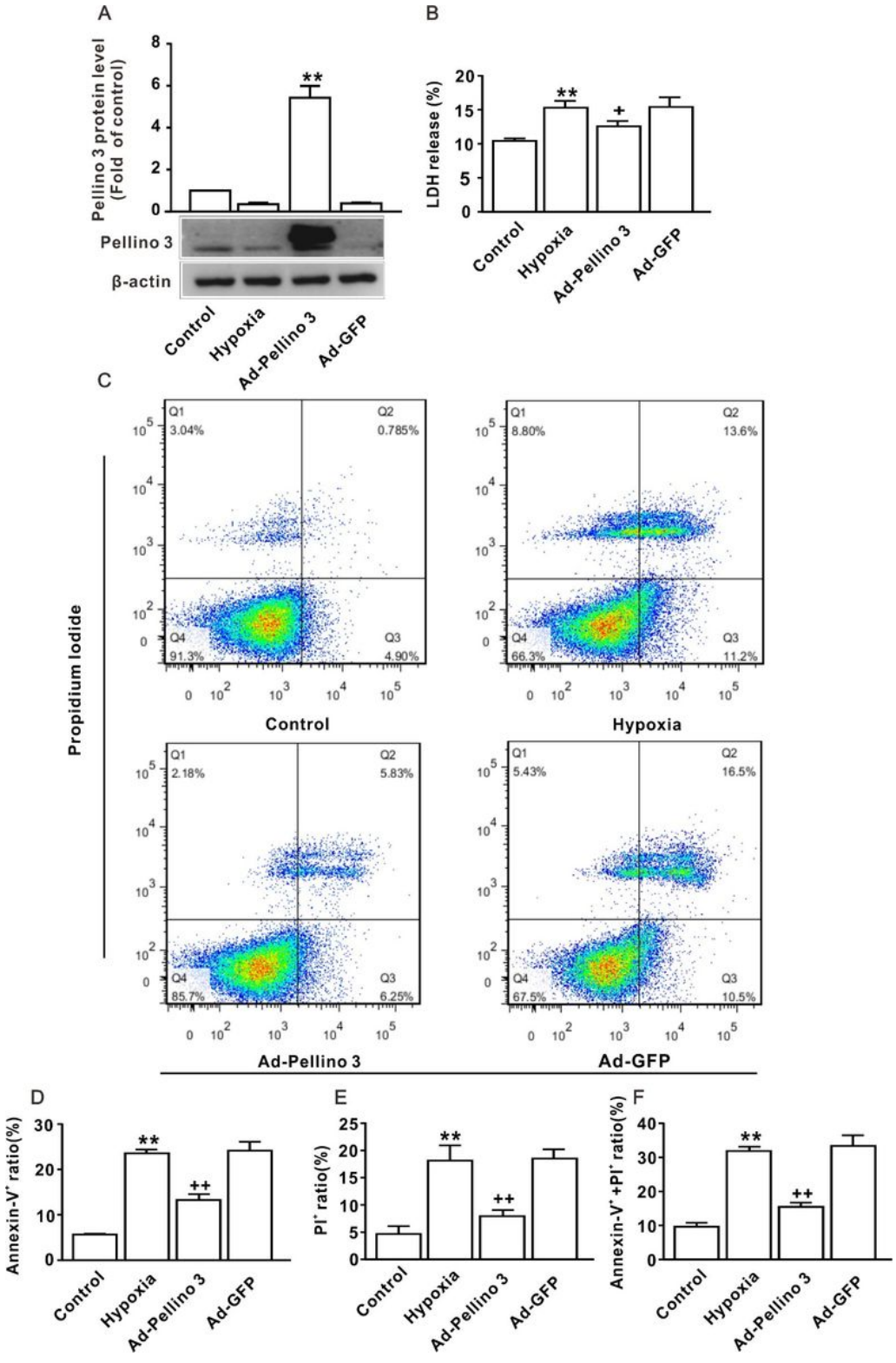

Figure 5

Overexpression of Pellino3 protected PC12 cells from hypoxic injury A. Pellino3 protein expression. Top: optical density for the protein blot of Pellino3 vs β-actin, which were normalized by the control; bottom, the representative images of Western blot results (n=3 per group). B. LDH release (n=6). C. Representative images of flow cytometry for necroptosis assay. D. Percentage of Annexin V+ cells. E. Percentage of PI+ cells. F. Percentage of Annexin V+/PI+ cells. All values were expressed as means ± S.E.M. Ad-Pellino3: hypoxia+ Ad-Pellino3; Ad-GFP: hypoxia+ Ad-GFP. **P<0.01 vs control; +P<0.05, ++P<0.01 vs hypoxia.

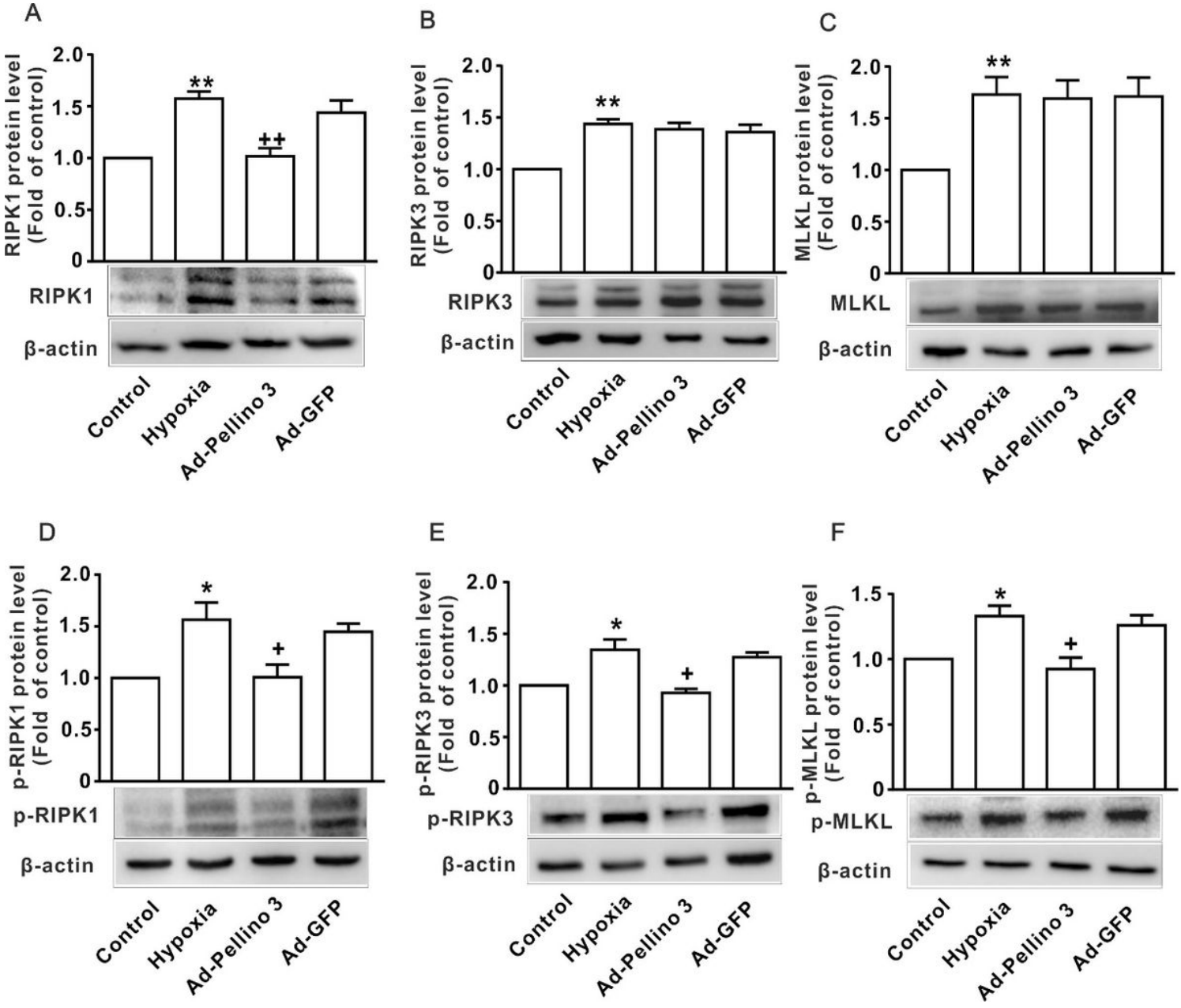

Figure 6

Overexpression of Pellino3 decreased necroptosis-associated proteins in hypoxia-treated PC12 cells Top: optical density for the protein blot of RIPK1 (A), RIPK3 (B), MLKL(C), p-RIPK1(D), p-RIPK3(E) or p-MLKL (F) against β-actin, which were further normalized by the control; bottom, the representative images of

Western blot results (n=3 per group). All values were expressed as means ± S.E.M. Ad-Pellino3: hypoxia+ Ad-Pellino3; Ad-GFP: hypoxia+ Ad-GFP. *P<0.05, **P<0.01 vs control; +P<0.05, ++P<0.01 vs hypoxia.

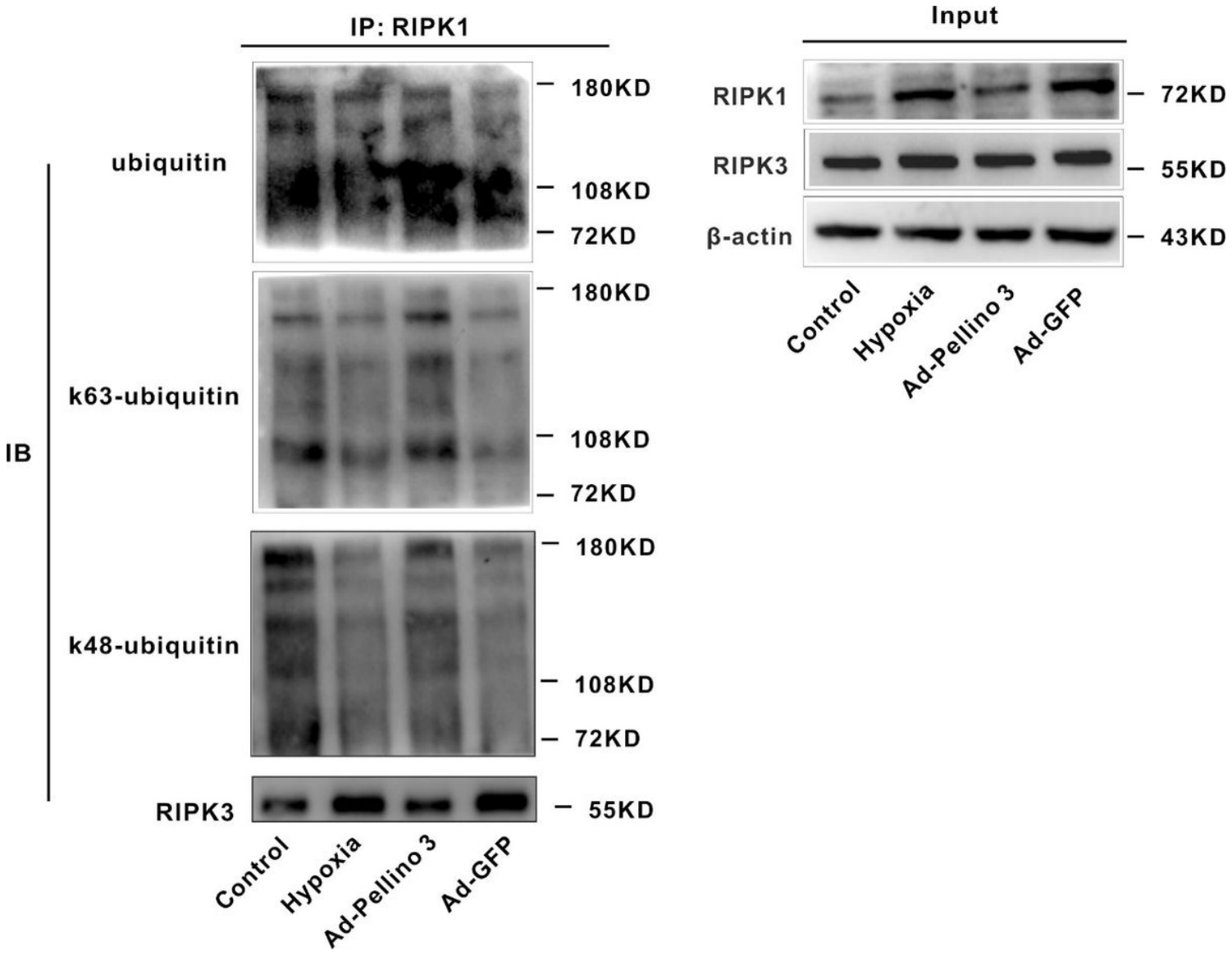

Figure 7

Overexpression of Pellino3 restored the ubiquitination of RIPK1 and weakened the interactions between RIPK1 and RIPK3 in hypoxia-treated PC12 cells. Representative images of Co-immunoprecipitation assay for RIPK1 ubiquitination and the interaction between RIPK1 and RIPK3 in hypoxia treated PC12 cells. Ad-Pellino3: hypoxia+ Ad-Pellino3; Ad-GFP: hypoxia+ Ad-GFP.