ORIGINAL RESEARCH

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Novel Role of Carbon Monoxide in Improving Neurological Outcome After Cardiac Arrest in Aged Rats: Involvement of Inducing Mitochondrial Autophagy

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***Background*—**Dysfunctional mitochondria are associated with neurological injury after cardiac arrest (CA). Although carbon monoxide (CO) has shown various potential therapeutic effects in preclinical tissue injury models, its mechanism of action in CA remains unclear. We sought to investigate the effects of a novel CO-releasing molecule on cerebral mitochondrial dysfunction and neurological injury after CA.

***Methods and Results*—**Male Sprague-Dawley rats aged 20 to 22 months were subjected to 6-minute asphyxia CA before receiving CO treatment. Survival, neurologic deficit scores, neuronal death, mitochondrial function, and autophagy were evaluated after the return of spontaneous circulation. Results showed that CO post-treatment increased 3-day survival rate from 25% to 70.83% and reduced neurologic deficit scores. CO also ameliorated CA-induced neuronal apoptosis and necrosis in the cerebral cortex and improved cerebral mitochondrial function by reducing reactive oxygen species, reversing mitochondrial membrane potential depolarization, and preventing cytochrome C release. Furthermore, CO increased mitochondrial autophagy by inducing mitochondrial accumulation of PINK1 (PTEN-induced putative kinase 1) and Parkin. Downregulation of PINK1 with genetic silencing siRNA abolished CO-afforded mitochondrial autophagy.

***Conclusions*—**Taken together, our results indicate, for the first time, that CO treatment confers neuroprotection against ischemic neurological injury after CA possibly by promoting mitochondrial autophagy. **(*J Am Heart Assoc*. 2019;8:e011851. DOI: 10.1161/JAHA.118.011851.)**

Key Words: cardiac arrest • mitochondria • mitochondrial autophagy • neuroprotection

Sudden cardiac arrest (CA) is a major cause of death worldwide.[1] Despite great improvements in resuscitation techniques, the survival rate in patients with CA remains low.[2] Poor neurological outcome is a leading problem after the return of spontaneous circulation (ROSC), which is responsible for significant post-CA mortality and morbidity.[2] Although mild hypothermia has been confirmed clinically to improve neurological outcome, it has shown limited benefit, with at least 80% of treated patients continuing to have adverse outcomes.[3,4] Therefore, Pharmacological medications should be developed to provide neuroprotection for patients with CA.

Mitochondrial dysfunction plays a causative role in the pathogenesis of global ischemia-reperfusion (IR) after CA.[5] In this regard, pathological alterations in mitochondrial function have been observed in neurological injury in models of CA.[6,7] Abnormal mitochondria not only compromise energy metabolism of the cell but also produce excessive reactive oxygen species (ROS) and release proapoptotic factor, which may ultimately result in cell death. Consistently, preservation of mitochondrial function through mitochondria-targeted therapy has been shown to prevent neuronal cell death, protect brain tissues, and maintain significant neurological function in cerebral IR injury.[8–10]

Under pathophysiological conditions, dysfunctional mitochondria are usually identified and removed by autophagosomes. This process is termed mitochondrial autophagy or mitophagy,[11] which is critical for maintaining mitochondrial homeostasis and cellular viability. Several regulators are involved in the process of mitochondrial autophagy. PINK1 (PTEN-induced putative kinase 1) and Parkin are important contributors that lead to the removal of damaged mitochondria

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Clinical Perspective

What Is New?

- Carbon monoxide treatment confers neuroprotection against ischemic neurological injury secondary to cardiac arrest by augmenting mitochondrial autophagy activation.
- PTEN-induced putative kinase 1–Parkin mediates augmented mitochondrial autophagy induced by carbon monoxide treatment.

What Are the Clinical Implications?

- Carbon monoxide treatment is a potential neuroprotectant for patients with cardiac arrest.
- This study provides a basis for finding the strategy of treating neurological injury after cardiac arrest.

via autophagy.[12,13] Normally, PINK1 is degraded and maintained at a very low level by proteolysis in mitochondria. Once mitochondria become damaged and depolarized, PINK1 would rapidly accumulate on the mitochondrial outer membrane and subsequently recruit the mitochondrial translocation of Parkin to initiate the process of mitochondrial autophagy.[12,13]

Carbon monoxide (CO) is known as a toxic gas that interferes with oxygen delivery to the tissues because its high affinity for hemoglobin can form carboxyhemoglobin.[14] However, as a product of heme oxygenases, endogenous CO has potent protection against inflammatory, apoptotic, and proliferative effects.[15] Transitional metal carbonyls, CO-releasing molecules (CORMs), have recently been used to deliver CO without significantly altering the formation of carboxyhemoglobin, which has been shown to be an effective alternative to the CO gas.[16] Consequently, CORMs have received increased attention as a potential therapeutic agent. A ruthenium-based water-soluble CO carrier, tricarbonylchoro (glycinato)ruthenium (II) known as CORM-3, can rapidly release defined amounts of CO and show biological activities in attenuating myocardial IR injury, reducing inflammatory response, and inhibiting platelet aggregation and arterial thrombosis without significant toxicity.[17–19]

The role of CO in the mitochondrial autophagy that is responsible for mitochondrial quality control in the brain after CA remains unclear. In this study, we investigated whether exogenous CO attenuates neurological injury and prevents brain mitochondrial dysfunction by inducing mitochondrial autophagy in an established rat model of CA.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Animal Preparation

Experiments were performed on aged male Sprague-Dawley rats (20–22 months of age, 550–600 g) purchased from the Experimental Animal Center of Soochow University (Soochow, China). Animals were maintained on laboratory chow and housed in a specific pathogen-free room at a constant temperature (20–22°C) with a 12-hour light-dark cycle. Research procedures were approved by the Institutional Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals*.

CA Model

Rats were anesthetized by an intraperitoneal injection of 45 mg/kg pentobarbital sodium. The trachea was orally intubated with a 14-gauge cannula (Abbocath-T). A 23-gauge polyethylene 50 catheter (Abbocath-T) was advanced through the left femoral artery for measurement of mean arterial pressure and withdrawal of blood for arterial blood gas analysis. An additional polyethylene 50 catheter was advanced into the right atrium through the left external jugular vein for the measurement of right atrial pressure. A conventional ECG lead II and hemodynamic data were continuously recorded via a data acquisition system (DATAQ). Rectal temperature was monitored and maintained within 36.5±0.5°C using a heat lamp.

In this study, CA was established according to our previously published protocol with a slight modification.[20] An illustration of the protocol used is provided (Figure 1A). Briefly, CA was initiated in animals by endotracheal tube clamping. CA was defined as mean arterial pressure ≤25 mm Hg, which occurred ≈4 minutes after endotracheal tube clamping. After 6 minutes of CA, cardiopulmonary resuscitation was started by mechanical ventilation and precordial compression. Mean arterial pressure that increased above 60 mm Hg for a minimum of 5 minutes was defined as ROSC. Rats that failed to ROSC within 5 minutes and unable to be weaned from ventilator after 1-hour observation were excluded. After spontaneous breath began, rats were weaned from the ventilator and all catheters were removed.

Experimental Groups and Drug Administration

The present study included 4 parts (Figure 1B). In the first 3 parts of the experiment, animals were randomly divided into a CA or CA+CO group by using a random number table after achieving ROSC. Animals underwent the same procedures except that CA and resuscitation were used as the sham group. CORM-3 (Sigma-Aldrich) was dissolved in saline. Rats in the CA+CO group were administered 4 mg/kg of CORM-3 after ROSC by intravenous infusion according to the dose as

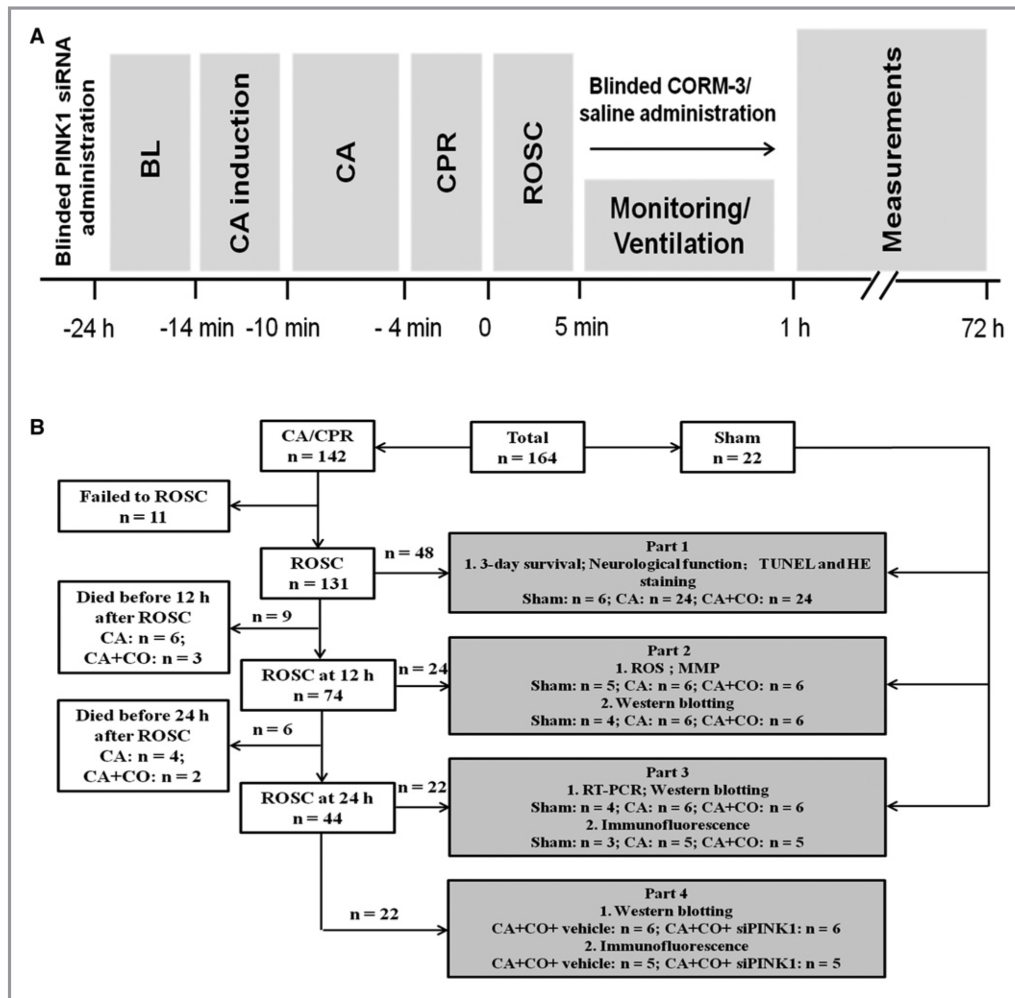

Figure 1. Experimental procedures and flow diagram of the study. **A**, Schematic of experimental procedures and groups. **B**, Flow diagram of the experimental measurements. BL indicates baseline; CA, cardiac arrest; CO, carbon monoxide; CORM-3, CO-releasing molecules-3; CPR, cardiopulmonary resuscitation; HE, hematoxylin-eosin; MMP, mitochondrial membrane potential; PINK1, PTEN-induced putative kinase 1; ROS, reactive oxygen species; ROSC, return of spontaneous circulation; TUNEL, terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling.

previously described,[21] whereas rats in the CA group received an equivalent volume of saline.

In the last part, rats were randomly assigned into 2 groups after ROSC: CA+CO+vehicle group and CA+CO+siPINK1 group. Transfection in vivo was performed as previously described.[22] PINK1 siRNA (RiboBio) and control siRNA (RiboBio) was dissolved in RNase-free H_2O with a concentration of 500 pmol/10 μL. Next, PINK1 siRNA was added to 10 μL of a transfection reagent (Entranster-in vivo, Engreen) and mixed gently for 15 minutes at room temperature. Then, the mixture was administered intracerebroventricularly using a microsyringe under the guidance of a stereotaxic apparatus after anesthesia. PINK siRNA was administered once at 24 hours before inducing CA in the CA+CO+siPINK1 group, whereas rats in the CA+CO+vehicle group received an equivalent volume of control siRNA.

Neurological Function Examination

Neurologic deficit score was used to evaluate the neurologic function at 24-hour intervals for a total of 72 hours after ROSC, which consisted of consciousness, respiration, reflex, motor sensory function, and behavior, and was conducted by 2 investigators who were blinded to group identities. The neurologic deficit score was scored from 0 (normal neurologic function) to 500 (death or brain death).[23]

TUNEL, Hematoxylin-Eosin, and Immunofluorescence Staining

Brain tissues were fixed with 4% paraformaldehyde overnight and embedded in paraffin, and 5-μm-thick sections were cut and stained with hematoxylin-eosin to assess the number of surviving neurons. Terminal deoxynucleotide transferase-

mediated dUTP-biotin nick-end labeling (TUNEL) assay (Roche) was performed using an in situ apoptosis detection kit, as per the manufacturer's instruction. Ten fields of vision (magnification of ×40) in the cerebral frontal cortex area were randomly selected from each brain tissue sample, and the viable neurons and TUNEL-stained neuronal cells were counted under an optical microscope. The cerebral cortex was selected in this study because it is closely related to functional outcomes.

For immunofluorescence, brain sections were labeled with specific primary antibodies including LC3 (diluted 1:400; Abcam) and COX4 (diluted 1:400; Abcam) overnight at 4°C, followed by the incubation with Alexa Fluor dye–conjugated secondary antibodies (1:200; Jackson ImmunoResearch) for 2 hours at room temperature. The fluorescence was detected under a fluorescent microscope (Olympus) and merged the photomicrographs by Image-Pro Plus 6.0 (Olympus).

Mitochondria Isolation

Rat brain mitochondria were isolated with the use of a mitochondria isolation kit for tissue (Abcam) according to the manufacturer's instructions. Fresh brain tissues were homogenized and centrifuged at 1000*g* for 10 minutes to collect supernatants, followed by centrifuging at 12 000*g* for 15 minutes. The final pellet was resuspended in isolation buffer and frozen at −80°C. Protein concentration was determined by BCA protein assay kit (Beyotime).

Measurement of Mitochondrial ROS

ROS production was measured using the tissue of ROS classical assay kit (GENMED) that utilized 2,7-dichlorofluorescein diacetate as the oxidative fluorescent probe. Briefly, brain mitochondria were mixed with 1 mmol/L 2,7-dichlorofluorescein diacetate and measured by fluorescent spectrophotometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The results were normalized by mitochondrial protein concentration.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was measured using the fluorescent dye JC-1 (Beyotime). JC-1 is a membrane potential-sensitive probe that accumulates in energized mitochondria and subsequently forms J-aggregates from monomers. Isolated brain mitochondria were stained with JC-1 at 37°C for 30 minutes. The fluorescence intensity of JC-1 was observed by fluorescent spectrophotometry with an emission wavelength of 485 nm and an excitation wavelength of 590 nm. The results were normalized by mitochondrial protein concentration.

Real-Time Quantitative Polymerase Chain Reaction for Mitochondrial DNA

Total DNA from brain tissues was extracted using the QIAamp DNA Mini Kit (Qiagen). Mitochondrial DNA (mtDNA) copy number was quantified by real-time quantitative polymerase chain reaction using Maxima SYBR Green/ROX qPCR Master Mix. The specific primers for mtDNA, which are complementary to the sequence of the cytochrome C oxidase subunit II gene, were 5′-AACCGAGTCG TTCTGCCAAT-3′ (forward) and 5′-CTAGGGAGGG GACTGC TCAT-3′ (reverse), respectively. The 5′-CCGCATCTTC TTGTGCAGTG-3′ (forward) and 5′-GTTCA CACCGACCTTCACCA-3′ (reverse) sequence, complementary to the GAPDH gene. The relative level of gene expression was normalized to the level of GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method.

Western Blotting Analysis

The protein samples were separated on 10% SDS-PAGE gel, and the fractionated proteins were transferred to

Table 1. Baseline Characteristics of 3 Groups

Parameters	Sham (n=6)	CA (n=24)	CA+CO (n=24)
Body weight, g	351±5.72	357±2.45	348±3.06
Heart rate, beats per min	377±13.88	381±6.33	383±7.14
Mean arterial pressure, mm Hg	127±3.67	131±1.43	129±1.63
Rectal temperature, °C	37.0±0.082	36.9±0.061	36.9±0.081
$PETCO_2$, mm Hg	33.6±1.67	34.9±0.76	34.1±1.08
PH	7.41±0.008	7.40±0.002	7.42±0.006
Lactate, mmol/L	0.9±0.049	0.9±0.029	1.0±0.018

All values are expressed as mean±SEM. CA indicates cardiac arrest; CO, carbon monoxide; $PETCO_2$, end-tidal CO_2 partial pressure.

Table 2. CA Resuscitation Outcomes Between 2 Groups

Parameters	CA (n=24)	CA+CO (n=24)
CA induction time, s	231±2.45	236±2.25
Duration of PC, s	253±4.29	245±6.33
No. of shocks	0.8±0.18	0.8±0.16
CPP in PC1, mm Hg	14.1±0.47	14.8±0.43
CPP in PC2, mm Hg	34.5±1.67	34.8±1.74

All values are expressed as mean±SEM. CA indicates cardiac arrest; CO, carbon monoxide; CPP, coronary perfusion pressure; PC, precordial compression; PC1, 1 minute after precordial compression; PC2, 2 minutes after precordial compression.

polyvinylidene fluoride membranes. After blocking the non-specific binding sites with 5% BSA and 0.1% Tween-20 in Tris buffer saline for 1 hour, the membranes were incubated with primary antibodies against LC3 (diluted 1:1000; Abcam), P62 (diluted 1:1000; Abcam), PINK1 (diluted 1:1000; Abcam), Parkin (diluted 1:1000; Abcam), Cytochrome C (Cytc, diluted 1:1000; Cell Signaling Technologies) GAPDH (diluted 1:1000; Cell Signaling Technologies), and VDAC (diluted 1:1000; Cell Signaling Technologies).The protein bands were detected using an enhanced chemical luminescence system (Cell Signaling Technologies). GAPDH was used for cytosolic protein loading control, and VDAC was used for mitochondrial protein control.

Statistical Analysis

Data are presented as mean±SEM, and statistical analyses were performed using SPSS version 21.0 (SPSS Inc) and GraphPad Prism 5.0 (GraphPad). Continuous data were analyzed with unpaired *t* tests or 1-way ANOVA followed by Tukey post hoc tests for multiple comparisons. Neurologic deficit scores were examined by 2-factor repeated-measures ANOVA. The difference in the survival rate between groups was compared by Kaplan–Meier survival analysis with log-rank test. $P<0.05$ was considered significant.

Results

CO Treatment Improves Survival and Neurological Recovery After ROSC

A total of 164 rats were prepared for the study (Figure 1B). Among them, 142 underwent CA but 11 rats failed to achieve ROSC, and the remaining 22 rats received sham operation. Nine and 6 rats were excluded from the group failed to survive for 12 and 24 hours, respectively. There were no significant differences in physiologic variable including body weight, heart rate, mean artery pressure, temperature, end-tidal CO_2

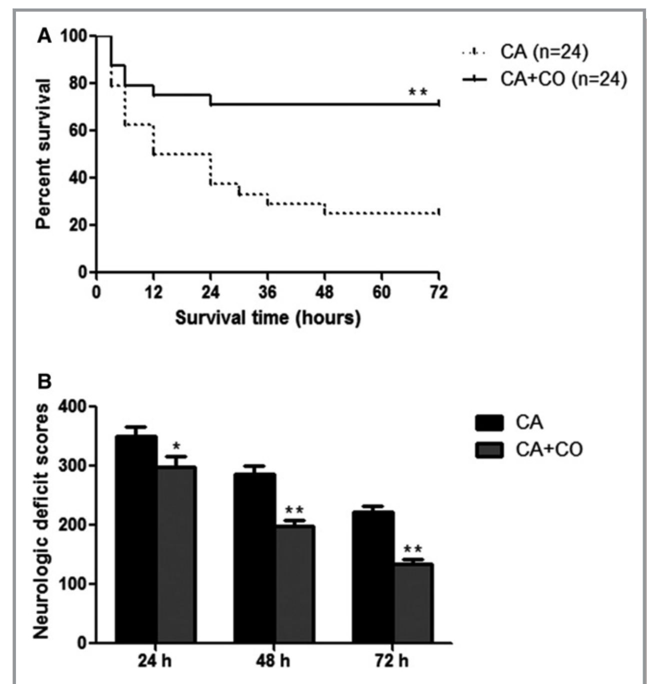

Figure 2. Carbon monoxide (CO) treatment improved survival and neurological outcome after cardiac arrest (CA). **A**, Kaplan–Meier analyses of cumulative survival of rats during 3-day follow-up after CA. **B**, Neurologic deficit scores of survived rats at 24, 48, and 72 hours after CA. *$P<0.05$ vs the CA group. **$P<0.01$ vs the CA group.

partial pressure, and blood analytical measurements among 3 groups at baseline (Table 1). For animals that successfully achieved ROSC, resuscitation outcomes were compared between the CA group and CA+CO group (Table 2).

The 3-day survival experiment showed that 25% of the rats (6/24) in the CA group survived at day 3, which was much lower than that in the CA+CO group (70.83%; 17/24) ($P<0.05$). Kaplan–Meier survival analysis showed that CORM-3 treatment significantly increased the 3-day survival time of the animals after ROSC ($P<0.05$) (Figure 2A). In addition, neurologic deficit score at 24, 48, and 72 hours after ROSC were significantly lower after CORM-3 application ($P<0.05$) (Figure 2B). These results indicate that CO treatment improves survival and neurological outcome after CA.

CO Treatment Attenuates Neuronal Death in the Cortex After CA

TUNEL assay and hematoxylin-eosin staining were performed to detect the neuronal apoptosis and necrosis in the cortex at 72 hours after ROSC. Compared with the sham group, apoptotic cells significantly increased in the CA group (12±4.2% versus 75.3±8.8%, $P<0.05$), and this increase was significantly attenuated by CO treatment (75.3±8.8% versus 40.1±7.6%, $P<0.05$) (Figure 3A and 3B). In comparison with

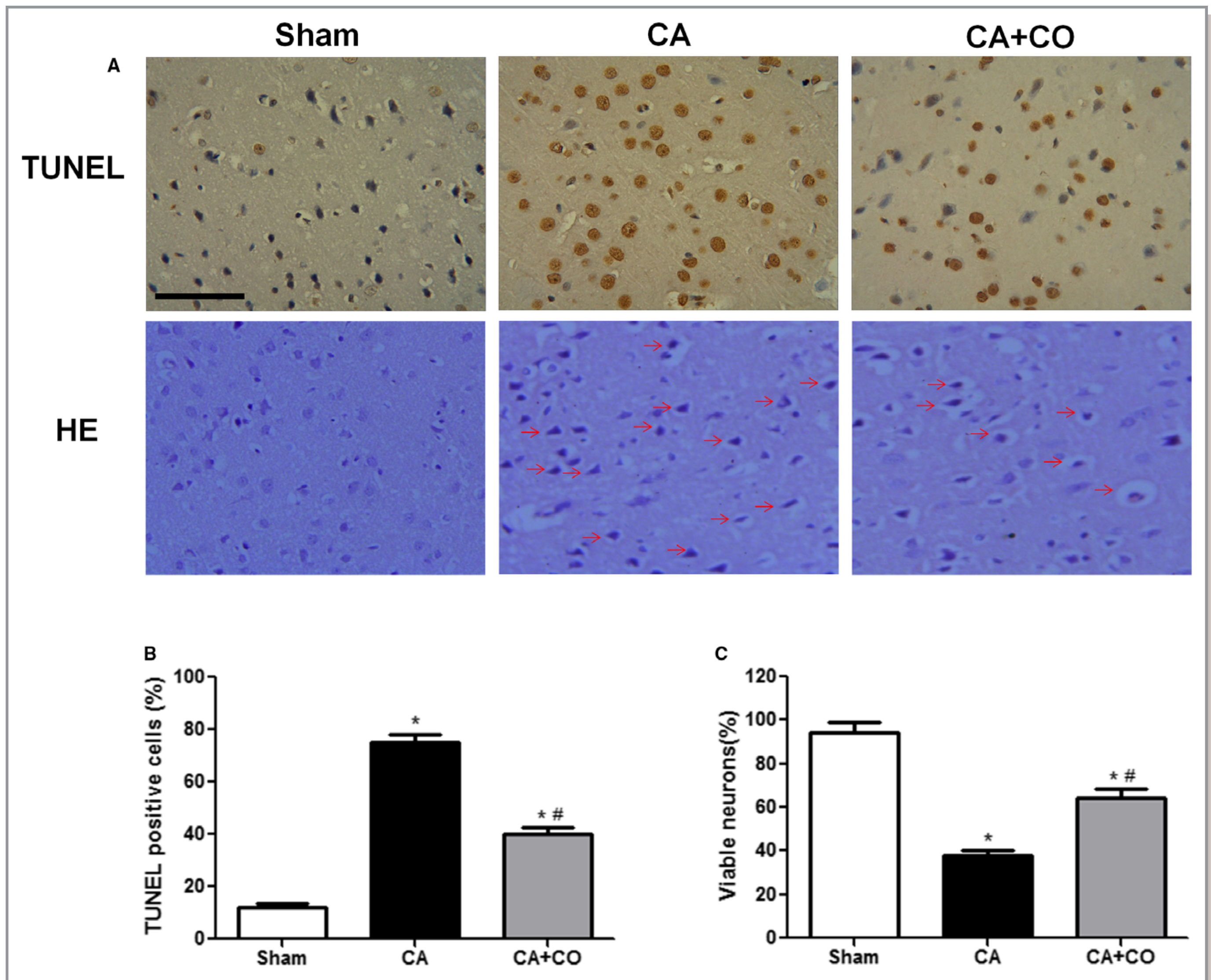

Figure 3. Carbon monoxide (CO) treatment protected neurons after cardiac arrest (CA). **A**, Representative images of terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and hematoxylin-eosin (HE) staining of the frontal cortex in 3 groups (×400). Scale bar=50 μm. Arrows indicate neuronal necrosis. **B**, Quantification of the apoptotic neurons in the frontal cortex by TUNEL staining. **C**, Quantification of the viable neurons in the frontal cortex by HE staining. Data in (**B** and **C**) are shown as mean±SEM. One-way ANOVA followed by Tukey post hoc test. *$P<0.05$ vs the sham group. $^{\#}P<0.05$ vs the CA group.

the sham group, the viable neurons in the CA group were significantly reduced (94.5±13.2% versus 38.7±7.9%, $P<0.05$), and this reduction was significantly attenuated by CO treatment (38.7±7.9% versus 64±15.3%, $P<0.05$) (Figure 3A and 3C).

CO Treatment Prevents Brain Mitochondrial Dysfunction After CA

Mitochondria are the primary source of ROS. To determine the effect of CO on mitochondrial function in the rats with CA, mitochondrial ROS production in the brain were measured. A significant decrease in mitochondrial ROS production was observed at 12 hours after ROSC, which was significantly prevented by CO treatment (Figure 4A). Loss of MMP plays an essential role in the release of apoptogenic factors to induce apoptosis.[24] The results showed that a decrease in MMP was observed in the brain at 12 hours after ROSC, and this decrease was significantly attenuated by CO treatment (Figure 4B). Compared with the sham group, apoptogenic factors cytochrome C from mitochondria was largely translocated to cytosol at 12 hours after ROSC. Moreover, CO treatment reversed CA-induced translocation of cytochrome C from mitochondria (Figure 4C and 4D),

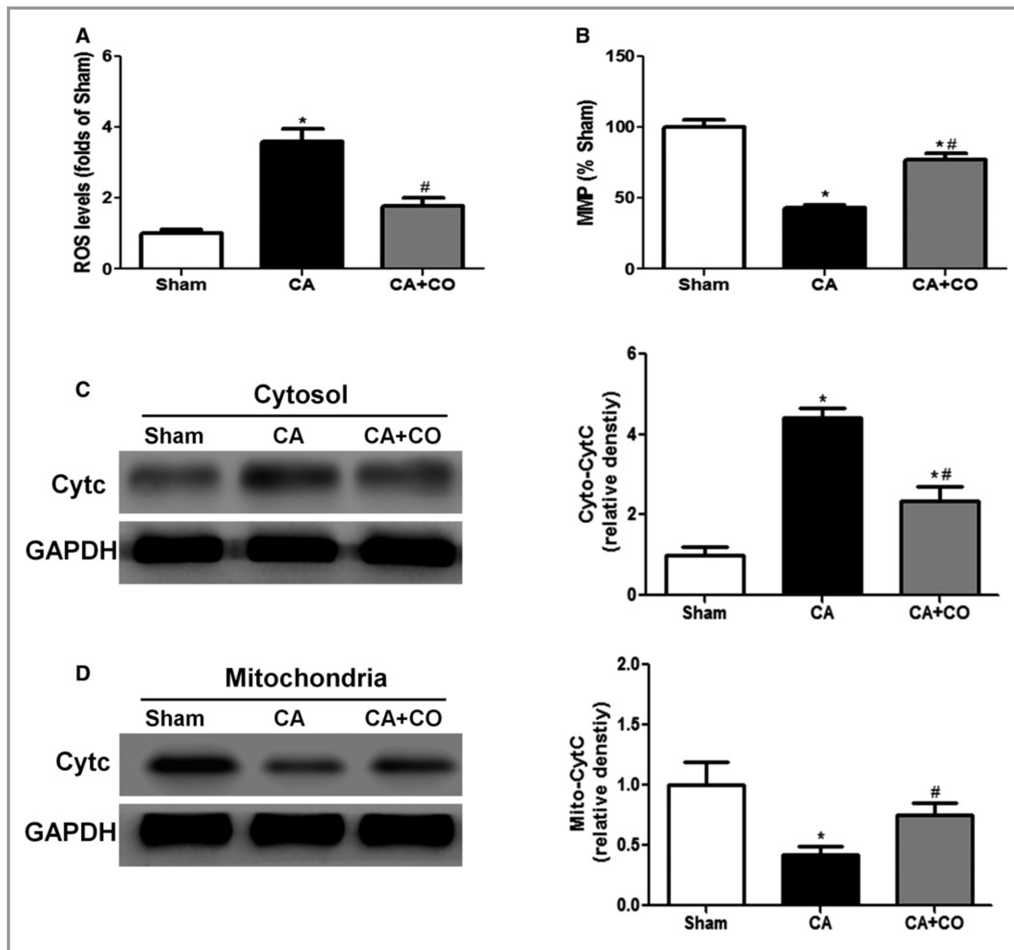

Figure 4. Carbon monoxide (CO) treatment protected brain mitochondrial function after cardiac arrest (CA). **A**, Brain mitochondria reactive oxygen species levels in the 3 groups. **B**, Brain mitochondria membrane potential levels in the 3 groups. **C**, Representative Western blot and quantitative analysis of cytosolic cytochrome C (Cytc) protein expression. Data are shown as mean±SEM. **D**, Representative Western blot and quantitative analysis of mitochondrial Cytc protein expression. Data are shown as mean±SEM. One-way ANOVA followed by Tukey post hoc test. *$P<0.05$ vs the sham group. $^{\#}P<0.05$ vs the CA group.

further supporting the protective role of CO treatment against mitochondrial injury.

CO Treatment Increased Brain Mitochondrial Autophagy After CA

Autophagy is crucial for the elimination of damaged mitochondria to preserve mitochondrial function under certain conditions.[25] Therefore, we were interested in determining the influence of CO on mitochondrial autophagy after CA. First, the mtDNA copy number was evaluated to assess the relative mitochondria number. The results showed that brain mtDNA copy number was significantly reduced at 24 hours after ROSC. Intriguingly, CO treatment led to a further decrease in the copy number of mtDNA (Figure 5A).

Then, we next evaluated the changes of LC3 and P62 proteins in mitochondrial fraction at 24 hours after ROSC in rats treated with or without CO. The results showed that the ratio of LC3 II/LC3 I was markedly increased in mitochondrial fraction at 24 hours after ROSC ($P<0.05$). Moreover, this increase was augmented in rats treated by CO ($P<0.05$) (Figure 5B and 5C). Accordingly, a significant decrease in the protein level of P62 was observed in mitochondrial fraction at 24 hours after ROSC compared with the sham group ($P<0.05$), and CO treatment augmented this decrease ($P<0.05$) (Figure 5B and 5D).

Last, we examined the number of mitochondria undergoing autophagy with double immunofluorescence staining. COX4, a marker of mitochondria, colocalizing with LC3 puncta could be obviously detected in the cortex of rats subjected to CA. Furthermore, CO treatment significantly increased the

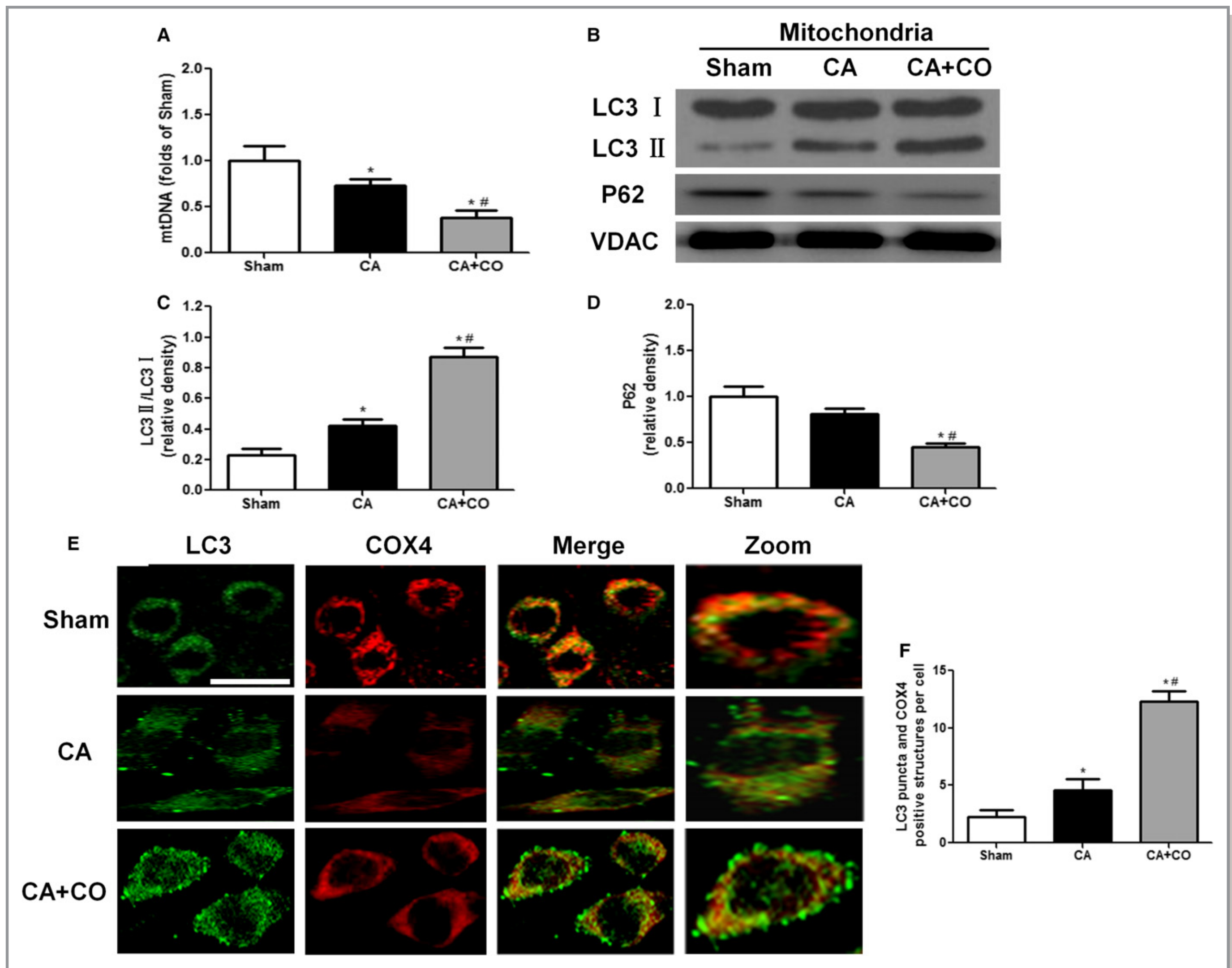

Figure 5. Carbon monoxide (CO) treatment increased brain mitochondrial autophagy after cardiac arrest (CA). **A**, Brain mitochondrial DNA (mtDNA) copy number in the 3 groups. **B**, Representative Western blot of mitochondrial LC3 I, LC3 II, P62, and voltage-dependent anion channel (VDAC) protein expression in the frontal cortex. VDAC was used as mitochondrial loading control. **C**, Quantification of Western blot analysis of mitochondrial LC3 II/LC3 I. Data are shown as mean±SEM. **D**, Quantification of Western blot analysis of mitochondrial P62. Data are shown as mean±SEM. **E**, Representative double-staining images with COX4 and LC3 in the frontal cortex. Scale bar=10 μm. **F**, Quantification of COX4-labeled mitochondria encapsulated by LC3 puncta-positive structures per cell. Data are shown as mean±SEM, n>30 cells per group. One-way ANOVA followed by Tukey post hoc test. *$P<0.05$ vs the sham group. $^{\#}P<0.05$ vs the CA group.

colocalizing of COX4 and LC3 puncta (Figure 5E and 5F), indicating that CO treatment could further increase CA-induced activation of mitochondrial autophagy.

CO Treatment Promoted PINK1 and Parkin Activation in the Brain

PINK1 accumulate on the mitochondria and then recruit translocation of Parkin from the cytosol to the mitochondria to initiate mitochondrial autophagy process in most cases. To further study the effect of CO on key players of mitochondrial autophagy, the related protein expression from mitochondrial and cytosolic fractions was detected with Western blot. The results showed that PINK1 levels in mitochondrial fraction were significantly increased at 24 hours after ROSC. Moreover, this increase was augmented in rats by CO treatment after ROSC (Figure 6A and 6B). Meanwhile, Parkin translocation from the cytosol to the mitochondria was observed at 24 hours after ROSC, and CO treatment augmented this translocation (Figure 6A, 6C and 6D).

To determine whether PINK1 contributes to mitochondrial autophagy activation induced by CO treatment, we administered PINK1 siRNA, which could specifically inhibit PINK1 expression using RNA interference (Figure 7A and 7B). As shown in

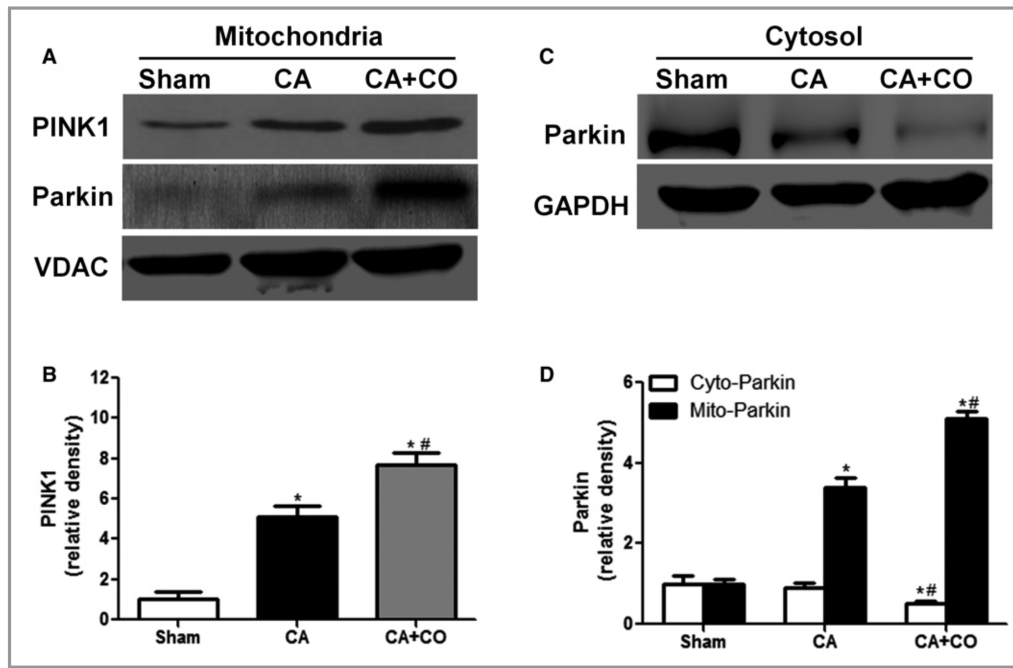

Figure 6. Carbon monoxide (CO) treatment increased brain PINK1 (PTEN-induced putative kinase 1) in the mitochondrial fraction and Parkin translocation to the mitochondria after cardiac arrest (CA). **A**, Representative Western blot of mitochondrial PINK1, Parkin, and voltage-dependent anion channel (VDAC) protein expression in the frontal cortex. VDAC was used as a mitochondrial loading control. **B**, Quantification of Western blot analysis of mitochondrial PINK1. Data are shown as mean±SEM. **C**, Representative Western blot of cytosolic Parkin and GAPDH protein expression in the frontal cortex. GAPDH was used as a cytosolic loading control. **D**, Quantification of Western blot analysis of cytosolic Parkin. Data are shown as mean±SEM. One-way ANOVA followed by Tukey post hoc test. $^*P<0.05$ vs the sham group. $^\#P<0.05$ vs the CA group.

Figure 7A, 7C, and 7D, PINK1 siRNA treatment significantly attenuated Parkin translocation to the mitochondria in the brain at 24 hours after ROSC in CO-treated rats. Furthermore, PINK1 siRNA significantly prevented CO-induced colocalizing of COX4 and LC3 puncta (Figure 7E and 7F). These results suggest that CO treatment increased brain mitochondrial autophagy by inducing PINK1 and Parkin activation after CA.

Discussion

In the present study, we found that exogenous CO liberated from CORM-3 after successful resuscitation from CA markedly improved 3-day survival, neurological outcome, and neuronal survival in rats. The neuroprotective role of CO was associated with preservation of the mitochondrial function after ROSC. The salutary impact of CO on the outcome of CA may be a result of the enhancement in mitochondrial autophagy induction. Our results are particularly promising when considering the fact that CA occurs most frequently in the elderly, a highly clinically relevant age group with CA.

Different from previous viewpoints that CO is poisonous, increasing evidence supports that it mediates protection against IR-induced injury in multiple organs and tissues.[26–28] For potential clinical applications of CO, the main scientific and technical challenges are the safe and specific manner of delivering CO. Low doses of inhaled CO was found to attenuate focal cerebral ischemic injury.[29] However, the application of gaseous CO develops several disadvantages, such as the potential exposure for partial systemic hypoxia and toxicity and the need for CO inhalation facilities and monitoring of blood oxygen levels.[14] Given these limitations, CORMs have been identified as potential candidates to facilitate the pharmaceutical release of CO by delivering it to the tissues and organs of interest. CORM-3 as a water-soluble CORM has been demonstrated to provide protective benefits in several disease models, such as myocardial infarction, thrombosis, and collagen-induced arthritis.[17–19] In this study, we treated rats with CA with CORM-3 after resuscitation and determined the therapeutic effects. A CORM-3 dose of 4 mg/kg is selected because this dose was shown to be sufficient to exert a protective effect.[21] Herein, similar beneficial results were obtained using 4 mg/kg of CORM-3 in the present study. Therefore, low doses of applied CO by CORM-3 could be recognized as a safe and feasible therapy after CA.

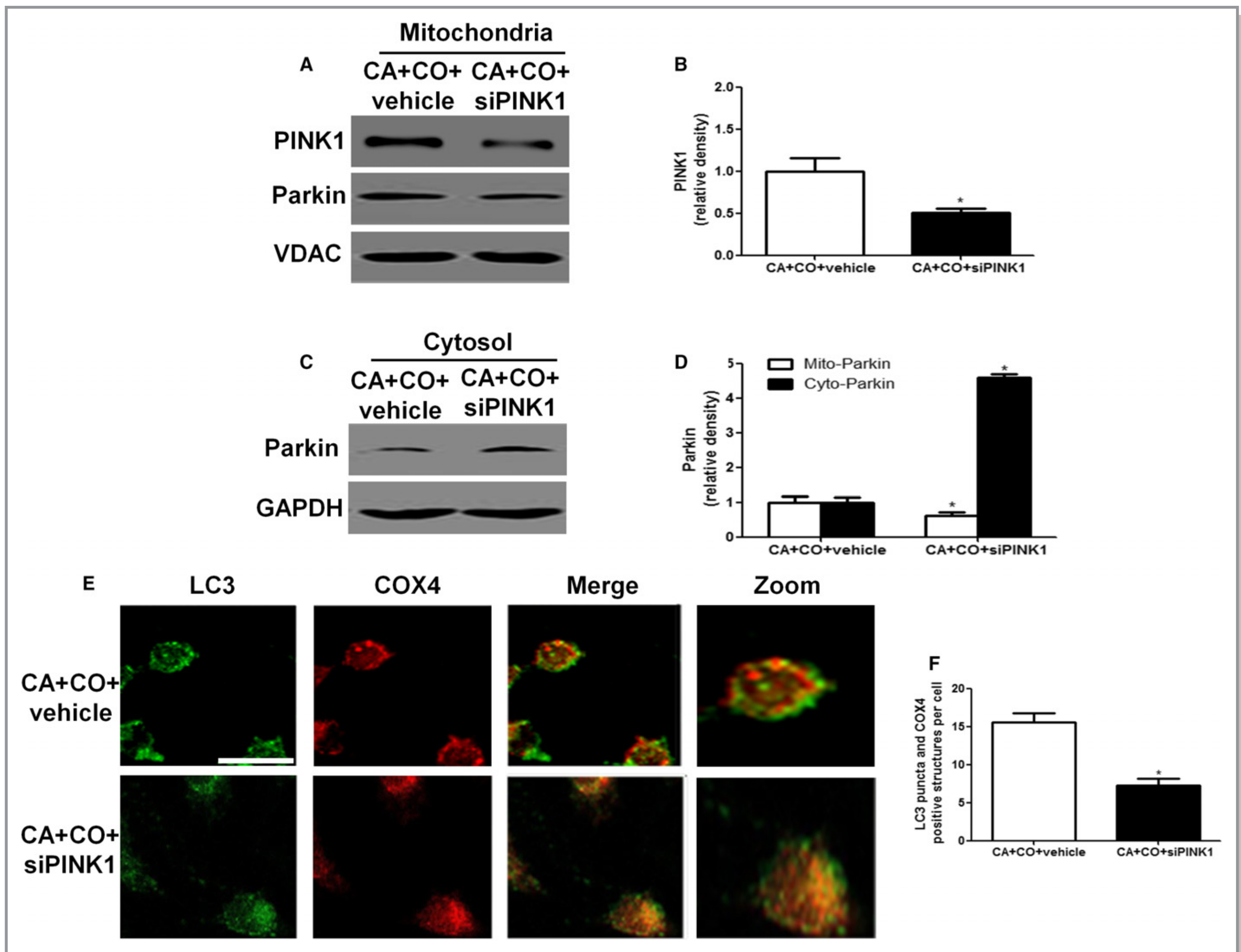

Figure 7. PINK1 (PTEN-induced putative kinase 1) siRNA reduced brain mitochondrial autophagy activation induced by carbon monoxide (CO) treatment after cardiac arrest (CA). **A**, Representative Western blot of mitochondrial PINK1, Parkin, and voltage-dependent anion channel (VDAC) protein expression in the frontal cortex. VDAC was used as a mitochondrial loading control. **B**, Quantification of Western blot analysis of mitochondrial PINK1. Data are shown as mean±SEM. **C**, Representative Western blot of cytosolic Parkin and GAPDH protein expression in the frontal cortex. GAPDH was used as a cytosolic loading control. **D**, Quantification of Western blot analysis of cytosolic Parkin. Data are shown as mean±SEM. **E**, Representative double-staining images with COX4 and LC3 in the frontal cortex. Scale bar=10 μm. **F**, Quantification of COX4-labeled mitochondria encapsulated by LC3 puncta-positive structures per cell. Data are shown as mean±SEM, n>30 cells per group. **P*<0.05 vs the CA+CO+vehicle group.

Mitochondria are intracellular organelles that are particularly abundant in the brain and serve as the repository for the biological "machinery" for the generation of adenosine triphosphate through oxidative phosphorylation.[30] The brain relies mainly on adenosine triphosphate for its function and is particularly sensitive to IR injury. This reliance has made the function of mitochondria as a target for therapy after CA. Some therapeutic interventions that target mitochondrial dysfunction in CA animal models have been proven to be beneficial. Pharmacological inhibition of mitochondrial permeability transition pore opening was found to improve short-term survival and cardiac function and attenuate multiple organ failure.[31] Recently, injection of Shenfu, a well-known traditional Chinese herbal medicine, was shown to attenuate CA-induced cerebral IR injury by modulating mitochondrial dysfunction.[32] In this study, we found that the administration of CORM-3 protected mitochondrial function by reducing ROS generation, inhibiting MMP depolarization and cytochrome C release, similar to results from previous studies.[33]

Mitochondrial autophagy is important for mitochondrial homeostasis and is essential for cells and tissues to survive via eliminating abnormal mitochondria. Studies have proven that enhancing mitochondrial autophagy attenuates cerebral IR injury. A study showed that the overexpression of hypoxia-inducible factor 1α triggered mitochondrial autophagy in primary cortical neurons subjected to oxygen-glucose

deprivation, and increased neuronal survival.[34] Methylene blue improved neurological function and reduced infarct volume and necrosis by augmenting mitochondrial autophagy after acute cerebral ischemia injury.[35] In the present study, CO treatment preserved cerebral mitochondrial function by inducing mitochondrial autophagy and therefore improved survival and attenuated cerebral IR injury after CA. However, the effects of activated mitochondrial autophagy on cerebral IR are still controversial. Opposite views support that suppressing mitochondrial autophagy shows the neuroprotective effects against cerebral IR injury.[36] Regardless, CORM-3 treatment could increase activated mitochondrial autophagy and preserved cerebral mitochondrial function, suggesting that insufficient removal of damaged mitochondria is harmful in the CA-induced ischemic brain and enhancement of mitochondrial autophagy induction exerts neuroprotective effects by CO administration.

Accumulating evidence suggests that mitochondria have become increasingly important cellular targets of CO.[37] CO has been shown to regulate autophagy in various cell types.[38–40] Herein, this is the first report that CO treatment could promote PINK1 and Parkin accumulation on the dysfunctional mitochondria and subsequently increase mitochondrial autophagy activation in an in vivo CA model. B-cell lymphoma-2 proteins such as Bcl-xL and Mcl-1 suppress mitochondrial autophagy through binding to PINK1/Parkin complexes and inhibiting Parkin translocation to damaged mitochondria.[41] CO could improve mitochondrial function (in particular oxidative phosphorylation) by enhancing B-cell lymphoma-2 expression,[42] thus B-cell lymphoma-2 may be involved in the protective mechanism of CO through increasing induction of mitochondrial autophagy after CA.

Study Limitations

There are several limitations to our study. First, one time point for mitochondrial function and autophagy studies was selected because of a limited number of animals. In addition, we mainly focused on the effect of CO on PINK1-Parkin–mediated mitochondrial autophagy. The other possible molecular pathway involved in the neuroprotection of CO cannot be absolutely excluded. Third, our study mainly focused on the frontal cortex, as it is known to have a critical role in neurological function outcome after CA, but other regions such as the hippocampus may be more vulnerable to IR injury.

Conclusions

In the present study, we demonstrate, for the first time, that CO treatment improves neurological outcome after CA and resuscitation by maintaining mitochondrial function and improving PINK1-Parkin–mediated mitochondrial autophagy.

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Disclosures

None.

References

1. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER III, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee; Stroke Statistics Subcommittee. Heart disease and stroke statistics—2016 update: a report from the American Heart Association. *Circulation*. 2016;133:e38–e360.
2. Nolan JP, Neumar RW, Adrie C, Aibiki M, Berg RA, Bbttiger BW, Callaway C, Clark RS, Geocadin RG, Jauch EC, Kern KB, Laurent I, Longstreth WT, Merchant RM, Morley P, Morrison LJ, Nadkarni V, Peberdy MA, Rivers EP, Rodriguez-Nunez A, Sellke FW, Spaulding C, Sunde K, Vanden Hoek T; International Liaison Committee on Resuscitation, Emergency Cardiovascular Care Committee, American Heart Association; Council on Cardiovascular Surgery and Anesthesia; Council on Cardiopulmonary Perioperative, and Critical Care; Council on Clinical Cardiology; Council on Stroke. Post-cardiac arrest syndrome: epidemiology, pathophysiology, treatment, and prognostication: a scientific statement from the International Liaison Committee on Resuscitation; the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; the Council on Stroke (Part II). *Int Emerg Nurs*. 2010;18:8–28.
3. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. *N Engl J Med*. 2002;346:557–563.
4. Hypothermia after Cardiac Arrest Study G. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. *N Engl J Med*. 2002;346:549–556.
5. Gazmuri RJ, Radhakrishnan J. Protecting mitochondrial bioenergetic function during resuscitation from cardiac arrest. *Crit Care Clin*. 2012;28:245–270.
6. Li Y, Tang Q, Wang P, Qin J, Wu H, Lin J, Huang Z. Dynamic changes of mitochondrial fusion and fission in brain injury after cardiac arrest in rats. *Biomed Res Int*. 2017;2017:1948070.
7. White BC, Hildebrandt JF, Evans AT, Aronson L, Indrieri RJ, Hoehner T, Fox L, Huang R, Johns D. Prolonged cardiac arrest and resuscitation in dogs: brain mitochondrial function with different artificial perfusion methods. *Ann Emerg Med*. 1985;14:383–388.
8. Christophe M, Nicolas S. Mitochondria: a target for neuroprotective interventions in cerebral ischemia-reperfusion. *Curr Pharm Des*. 2006;12:739–757.
9. Solenski NJ, diPierro CG, Trimmer PA, Kwan AL, Helm GA. Ultrastructural changes of neuronal mitochondria after transient and permanent cerebral ischemia. *Stroke*. 2002;33:816–824.
10. Kumar R, Bukowski MJ, Wider JM, Reynolds CA, Calo L, Lepore B, Tousignant R, Jones M, Przyklenk K, Sanderson TH. Mitochondrial dynamics following global cerebral ischemia. *Mol Cell Neurosci*. 2016;76:68–75.
11. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol*. 2011;12:9–14.
12. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol*. 2008;183:795–803.
13. Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, Sou YS, Saiki S, Kawajiri S, Sato F, Kimura M, Komatsu M, Hattori N, Tanaka K. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J Cell Biol*. 2010;189:211–221.

14. Queiroga CS, Vercelli A, Vieira HL. Carbon monoxide and the CNS: challenges and achievements. *Br J Pharmacol*. 2015;172:1533–1545.
15. Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov*. 2010;9:728–743.
16. Schatzschneider U. Novel lead structures and activation mechanisms for CO-releasing molecules (CORMs). *Br J Pharmacol*. 2015;172:1638–1650.
17. Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li QH, Dawn B, Motterlini R, Bolli R. Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. *Am J Physiol Heart Circ Physiol*. 2004;286:H1649–H1653.
18. Ferrandiz ML, Maicas N, Garcia-Arnandis I, Terencio MC, Motterlini R, Devesa I, Joosten LA, van den Berg WB, Alcaraz MJ. Treatment with a CO-releasing molecule (CORM-3) reduces joint inflammation and erosion in murine collagen-induced arthritis. *Ann Rheum Dis*. 2008;67:1211–1217.
19. Soni H, Jain M, Mehta AA. Investigation into the mechanism(s) of antithrombotic effects of carbon monoxide releasing molecule-3 (CORM-3). *Thromb Res*. 2011;127:551–559.
20. Li Y, Wang P, Wei J, Fan R, Zuo Y, Shi M, Wu H, Zhou M, Lin J, Wu M, Fang X, Huang Z. Inhibition of Drp1 by Mdivi-1 attenuates cerebral ischemic injury via inhibition of the mitochondria-dependent apoptotic pathway after cardiac arrest. *Neuroscience*. 2015;311:67–74.
21. Yabluchanskiy A, Sawle P, Homer-Vanniasinkam S, Green CJ, Foresti R, Motterlini R. CORM-3, a carbon monoxide-releasing molecule, alters the inflammatory response and reduces brain damage in a rat model of hemorrhagic stroke. *Crit Care Med*. 2012;40:544–552.
22. Dang B, Li H, Xu X, Shen H, Wang Y, Gao A, He W, Wang Z, Chen G. Cyclophilin A/cluster of differentiation 147 interactions participate in early brain injury after subarachnoid hemorrhage in rats. *Crit Care Med*. 2015;43:e369–e381.
23. Hendrickx HH, Rao GR, Safar P, Gisvold SE. Asphyxia, cardiac arrest and resuscitation in rats. I. Short term recovery. *Resuscitation*. 1984;12:97–116.
24. Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential (deltapsi (m)) in apoptosis; an update. *Apoptosis*. 2003;8:115–128.
25. Wang K, Klionsky DJ. Mitochondria removal by autophagy. *Autophagy*. 2011;7:297–300.
26. Wei Y, Chen P, de Bruyn M, Zhang W, Bremer E, Helfrich W. Carbon monoxide-releasing molecule-2 (CORM-2) attenuates acute hepatic ischemia reperfusion injury in rats. *BMC Gastroenterol*. 2010;10:42.
27. Caumartin Y, Stephen J, Deng JP, Lian D, Lan Z, Liu W, Garcia B, Jevnikar AM, Wang H, Cepinskas G, Luke PP. Carbon monoxide-releasing molecules protect against ischemia-reperfusion injury during kidney transplantation. *Kidney Int*. 2011;79:1080–1089.
28. Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF, Cepinskas G. Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. *Inflammation*. 2010;33:92–100.
29. Zeynalov E, Dore S. Low doses of carbon monoxide protect against experimental focal brain ischemia. *Neurotox Res*. 2009;15:133–137.
30. Blomgren K, Zhu C, Hallin U, Hagberg H. Mitochondria and ischemic reperfusion damage in the adult and in the developing brain. *Biochem Biophys Res Commun*. 2003;304:551–559.
31. Cour M, Loufouat J, Paillard M, Augeul L, Goudable J, Ovize M, Argaud L. Inhibition of mitochondrial permeability transition to prevent the post-cardiac arrest syndrome: a pre-clinical study. *Eur Heart J*. 2011;32:226–235.
32. Gu W, Hou X, Zhou H, Li C. Protective effect of shen-fu injection on neuronal mitochondrial function in a porcine model of prolonged cardiac arrest. *Evid Based Complement Alternat Med*. 2014;2014:523847.
33. Lo Iacono L, Boczkowski J, Zini R, Salouage I, Berdeaux A, Motterlini R, Morin D. A carbon monoxide-releasing molecule (CORM-3) uncouples mitochondrial respiration and modulates the production of reactive oxygen species. *Free Radic Biol Med*. 2011;50:1556–1564.
34. Gong G, Hu L, Liu Y, Bai S, Dai X, Yin L, Sun Y, Wang X, Hou L. Upregulation of HIF-1alpha protein induces mitochondrial autophagy in primary cortical cell cultures through the inhibition of the mTOR pathway. *Int J Mol Med*. 2014;34:1133–1140.
35. Di Y, He YL, Zhao T, Huang X, Wu KW, Liu SH, Zhao YQ, Fan M, Wu LY, Zhu LL. Methylene blue reduces acute cerebral ischemic injury via the induction of mitophagy. *Mol Med*. 2015;21:420–429.
36. Baek SH, Noh AR, Kim KA, Akram M, Shin YJ, Kim ES, Yu SW, Majid A, Bae ON. Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage. *Stroke*. 2014;45:2438–2443.
37. Almeida AS, Figueiredo-Pereira C, Vieira HL. Carbon monoxide and mitochondria-modulation of cell metabolism, redox response and cell death. *Front Physiol*. 2015;6:33.
38. Lee S, Lee SJ, Coronata AA, Fredenburgh LE, Chung SW, Perrella MA, Nakahira K, Ryter SW, Choi AM. Carbon monoxide confers protection in sepsis by enhancing beclin 1-dependent autophagy and phagocytosis. *Antioxid Redox Signal*. 2014;20:432–442.
39. Lee SJ, Ryter SW, Xu JF, Nakahira K, Kim HP, Choi AM, Kim YS. Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am J Respir Cell Mol Biol*. 2011;45:867–873.
40. Kim DS, Song L, Wang J, Wu H, Gou W, Cui W, Kim JS, Wang H. Carbon monoxide inhibits islet apoptosis via induction of autophagy. *Antioxid Redox Signal*. 2018;28:1309–1322.
41. Hollville E, Carroll RG, Cullen SP, Martin SJ. Bcl-2 family proteins participate in mitochondrial quality control by regulating Parkin/PINK1-dependent mitophagy. *Mol Cell*. 2014;55:451–466.
42. Almeida AS, Queiroga CS, Sousa MF, Alves PM, Vieira HL. Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: role of Bcl-2. *J Biol Chem*. 2012;287:10761–10770.