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...
Carbon Monoxide and Mitochondrial Autophagy

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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.

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

Methods

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

DOI: 10.1161/JAHA.118.011851

Journal of the American Heart Association

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previously described, 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. PINK1 siRNA (RiboBio) and control siRNA (RiboBio) was dissolved in RNase-free H2O with a concentration of 500 pmol/10 μL. Next, PINK1 siRNA was added to 10 μL of a transfection reagent (Entranser-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). TUNEL, terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling.

**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 \( \times 40 \)) 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 \(-80^\circ C\). 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'-GTTCACGACCTCATCA-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.
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 CO₂ 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

**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.
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 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."
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. 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...
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

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**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.
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. 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. 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. 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. 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. 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.
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. 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. Recently, injection of Shenfu, a well-known traditional Chinese herbal medicine, was shown to attenuate CA-induced cerebral IR injury by modulating mitochondrial dysfunction. 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.

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.

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.
deprivation, and increased neuronal survival.\textsuperscript{34} Methylene blue improved neurological function and reduced infarct volume and necrosis by augmenting mitochondrial autophagy after acute cerebral ischemia injury.\textsuperscript{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.\textsuperscript{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.\textsuperscript{37} CO has been shown to regulate autophagy in various cell types.\textsuperscript{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.\textsuperscript{41} CO could improve mitochondrial function (in particular oxidative phosphorylation) by enhancing B-cell lymphoma-2 expression,\textsuperscript{42} thus B-cell lymphoma-2 expression 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.

**Sources of Funding**

This study was supported by the Soochow Science and Education Foundation for Young Scholar (No. KXBW2017008); the Soochow Municipal Science and Technology Project (No. SYS201616); the National Natural Science Foundation of China (No. 81701213); and the Jiangsu Province Natural Science Foundation (No. BK20170369).

**Disclosures**

None.

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