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RESEARCH PAPER

Activation of M_3 cholinoceptors attenuates vascular injury after ischaemia/reperfusion by inhibiting the Ca^{2+}/calmodulin-dependent protein kinase II pathway

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BACKGROUND AND PURPOSE

The activation of M_3 cholinoceptors (M_3 receptors) by choline reduces cardiovascular risk, but it is unclear whether these receptors can regulate ischaemia/reperfusion (I/R)-induced vascular injury. Thus, the primary goal of the present study was to explore the effects of choline on the function of mesenteric arteries following I/R, with a major focus on Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) regulation.

EXPERIMENTAL APPROACH

Rats were given choline (10 $mg \cdot kg^{-1}$, i.v.) and then the superior mesenteric artery was occluded for 60 min (ischaemia), followed by 90 min of reperfusion. The M_3 receptor antagonist, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), was injected (0.12 $\mu g \cdot kg^{-1}$, i.v.) 5 min prior to choline treatment. Vascular function was examined in rings of mesenteric arteries isolated after the reperfusion procedure. Vascular superoxide anion production, CaMKII and the levels of Ca^{2+}-cycling proteins were also assessed.

KEY RESULTS

Choline treatment attenuated I/R-induced vascular dysfunction, blocked elevations in the levels of reactive oxygen species (ROS) and decreased the up-regulated expression of oxidised CaMKII and phosphorylated CaMKII. In addition, choline reversed the abnormal expression of Ca^{2+}-cycling proteins, including Na^+/Ca^{2+} exchanger, inositol 1,4,5-trisphosphate receptor, sarcoplasmic reticulum Ca^{2+}-ATPase and phospholamban. All of these cholinergic effects of choline were abolished by 4-DAMP.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that inhibition of the ROS-mediated CaMKII pathway and modulation of Ca^{2+}-cycling proteins may be novel mechanisms underlying choline-induced vascular protection. These results represent a significant addition to the understanding of the pharmacological roles of M_3 receptors in the vasculature, providing a new therapeutic strategy for I/R-induced vascular injury.

LINKED ARTICLES

This article is part of a themed section on Chinese Innovation in Cardiovascular Drug Discovery. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-23

Abbreviations

4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DHE, dihydroethidium; I/R, ischaemia/reperfusion; IP_3R, inositol 1,4,5-trisphosphate receptor; NAC, N-acetyl-L-cysteine; NCX, Na^+/Ca^{2+} exchanger; PLB, phospholamban; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SNP, sodium nitroprusside

Tables of Links

TARGETS
GPCRs[a]
M_3 receptors
Enzymes[b]
SERCA 2, sarcoplasmic reticulum Ca^{2+}-ATPase
Ion channels[c]
NCX1, Na^+/Ca^{2+} exchanger
Ligand-gated ion channels[d]
IP_3R, inositol 1,4,5-trisphosphate receptor

LIGANDS
4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide
5-HT
ACh
Caffeine
Choline
Darifenacin
KN-93
L-NAME, N^G-nitro-L-arginine methyl ester
Phenylephrine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

Introduction

The restoration of blood supply following acute ischaemia leads to cellular damage that is referred to as ischaemia/reperfusion (I/R) injury. In some instances, I/R not only interferes with the beneficial effects of reperfusion therapy but also exacerbates organ dysfunction and structural damage (Kalogeris *et al.*, 2012). I/R injury is a widespread pathology that occurs in many clinical situations, including myocardial infarction, shock and transplantation (Banz and Rieben, 2012). A number of clinical and experimental studies have clearly demonstrated that injury to vascular endothelial cells is the first key step in the progression of I/R (Wang *et al.*, 2013) and that both oxidative signalling and Ca^{2+} homeostasis are involved in the modulation of vascular function (Hong *et al.*, 2012; He *et al.*, 2013). Gandhirajan *et al.* (2013) found that Ca^{2+} oscillations triggered by reactive oxygen species (ROS) could eventually lead to endothelial dysfunction. In fact, among the various molecular mechanisms that contribute to I/R injury, increases in intracellular levels of Ca^{2+} and ROS play crucial roles in vascular dysfunction and are closely related with the cellular processes that mediate signal transduction, metabolism and cell death (Kalogeris *et al.*, 2012).

The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is initially activated by the binding of the Ca^{2+}/calmodulin complex to its regulatory domain (Hudmon and Schulman, 2002). Emerging evidence indicates that CaMKII may autophosphorylate at $Thr^{286/287}$ or oxidize at $Met^{281/282}$ in the absence of Ca^{2+}/calmodulin and become constitutively active (Erickson *et al.*, 2008). Moreover, Bell *et al.* (2014) have suggested that the subcellular environment during I/R creates suitable conditions for the rapid activation of CaMKII; this type of environment is triggered by Ca^{2+} overload and oxidative stress (Erickson, 2014). As a result, CaMKII has emerged as an attractive therapeutic target for the treatment of I/R injury (Ling *et al.*, 2013). Interestingly, down-regulation of the CaMKII pathway directly attenuated vascular abnormalities in the mesenteric bed, in a model of diabetes (Yousif *et al.*, 2003). Thus, pharmacological inhibitors of CaMKII may be an effective strategy with which to treat I/R-induced vascular dysfunction.

There are five distinct subtypes of muscarinic ACh receptors – M_1, M_2, M_3, M_4 and M_5 receptors (Harvey, 2012). A growing body of evidence has demonstrated that the activation of M_3 receptors by choline results in cardioprotective actions against hypertrophy, arrhythmias and myocardial infarction via a variety of mechanisms (Pan *et al.*, 2012; Wang *et al.*, 2012a,b). M_3 receptors also favourably influence responses to ACh in most blood vessels (Lamping *et al.*, 2004). This finding is consistent with the fact that the deletion of M_3 receptors reduces the coronary vasodilation response to ACh in mice (Beny *et al.*, 2008). Recent studies from our laboratory have shown that vagal stimulation is associated with protective effects on vascular function and against structural changes following regional cardiac I/R and that this action is partly mediated by M_3 receptors (Zhao *et al.*, 2013). However, the influence of these receptors on I/R-induced vascular injury following their activation by choline remains unclear. Therefore, the primary aim of the present study was to determine whether the activation of M_3 receptors by the selective agonist choline would exert beneficial effects on vascular function after I/R. In addition, the potential regulatory actions of the ROS-mediated CaMKII pathway and the changes in the roles that Ca^{2+}-cycling proteins play after being elicited by choline were evaluated.

Methods

Animals and experimental design

All animal care and experimental procedures were approved by the Ethical Committee of Xi'an Jiaotong University. Studies with animals are described in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 178 animals were used in the experiments described here.

The animals used in the present study were obtained from the Experimental Animal Center of Xi'an Jiaotong University. Male adult Sprague Dawley rats (8–10 weeks old) were housed under controlled conditions at a room temperature of 24°C in a 12 h light/dark cycle with *ad libitum* access to water and food.

Rats were anaesthetized with pentobarbital sodium (40 mg·kg^{-1}, i.p.) and treated with heparin (200 IU, i.v.) prior to surgery. After the initial preparation and surgical procedures, the rats were allowed 30 min to stabilize. All of the animals were randomly assigned to the following experimental groups using a random number table (i) sham-operated group (undergoing abdominal surgery without I/R); (ii) I/R group; (iii) I/R + choline (Ch) group; and (iv) I/R + Ch + 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) group. In groups 3 and 4, the rats were treated with choline (10 mg·kg^{-1}, i.v.) 10 min prior to occlusion of the superior mesenteric artery. In group 4, 4-DAMP (0.12 μg·kg^{-1}, i.v.) was injected 5 min prior to choline treatment. The dosage and timing for the choline and 4-DAMP were chosen on the basis of previous studies (Pan *et al.*, 2012; Wang *et al.*, 2012a). The present study also included a set of vascular function experiments in which darifenacin (0.1 mg·kg^{-1}, i.v.), which is a different type of M_3 receptor antagonist, was injected 5 min prior to treatment with choline. In groups 2, 3 and 4, I/R was induced by occlusion of the superior mesenteric artery after all of the drugs were administered, as previously described (Bi *et al.*, 2013). The experimental protocols are set out in Figure 1.

Preparation of the mesenteric artery and measurement of isometric tension

The animals were killed at the end of the experiment and the mesenteric vascular bed was excised and immediately immersed in cold oxygenated Krebs-Henseleit solution (KHS; in mM: NaCl, 119; KCl, 4.7; KH_2PO_4, 1.2; $NaHCO_3$, 25; $MgCl_2$, 1; D-glucose, 11 and $CaCl_2$, 2.5). Briefly, the mesenteric artery was gently isolated and cleaned of adherent fat and tissue and arterial rings, approximately 2 mm long were cut, under a dissection microscope. Special care was taken to avoid stretching and endothelial damage.

For isometric tension recording, the arterial rings were suspended in a tissue bath and mounted on a pair of stainless steel pins that were fixed to an isometric force displacement transducer (Beijing Aeromedicine Engineering Research Institute, Beijing, China) and connected to a Taimeng BL-420F biotic signal collection and analysis system (Taimeng Instruments, Chengdu, China). The organ chamber was bubbled with 95% air and 5% CO_2 and filled with 2 mL KHS at 37°C. After mounting, the vessels were first stretched to an initial resting tension of 4.9 mN and were then allowed equilibrate for 90 min. The bath solution was replaced every 15 min during this resting period. At the end of the equilibration period, each arterial segment was repeatedly contracted with high-K^+ KHS (obtained by the equimolar replacement of NaCl with KCl in the KHS) until maximal and reproducible contractions were obtained.

Evaluation of vascular reactivity

The contraction studies were conducted using rings of the mesenteric artery, with intact endothelium. Rings were stimulated with KCl (5–100 mM) or 5-HT (10^{-9}–10^{-4} M), cumulatively added to the chamber until a maximal response was achieved. In another set of experiments, after pre-contractions with an equi-effective concentration of phenylephrine (10 μM; the tension was similar in all groups in response to phenylephrine; Martinez-Revelles *et al.*, 2008), the dilator responses of the mesenteric arteries to cumulative

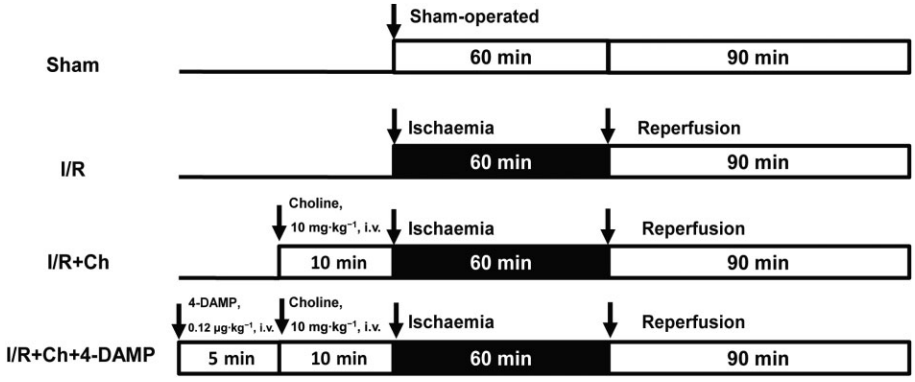

Figure 1

Experimental protocols. In the sham-operated group, the superior mesenteric artery was only separated, whereas in the remaining three groups, the I/R protocol consisted of 60 min of artery occlusion followed by 90 min of reperfusion.

concentrations of ACh (10^{-10}–10^{-5} M) were measured to assess the endothelium-dependent relaxations. To exclude the influence of endogenous NO, the vasorelaxation responses to sodium nitroprusside (SNP; 10^{-10}–10^{-5} M) were carried out in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) and after 30 min pre-incubation.

The role of the ROS was evaluated by constructing concentration-response curves to ACh after 30 min of incubation with N-acetyl-L-cysteine (NAC; 10 μM), a ROS scavenger. To evaluate the influence of CaMKII on the function of blood vessels, the concentration-response curves to KCl, 5-HT, ACh and SNP were obtained in the presence of the CaMKII inhibitor KN-93 (10 μM) after 30 min of incubation. Between the assessments of each concentration-response curve, the segments were washed with fresh KHS and then equilibrated for 45 min in KHS.

In additional experiments, Ca^{2+} was removed from the KHS. For this purpose, 0 mM Ca^{2+} solution was prepared by substitution of $CaCl_2$ with 1 mM EGTA to standard KHS. To determine the effect of choline on the contraction induced by caffeine in Ca^{2+}-free KHS, the rings were exposed to Ca^{2+}-free KHS for 20 min before contractions to caffeine (30 mM) were obtained.

Assessment of vascular superoxide anion production

The present study used dihydroethidium (DHE; Beyotime Biotech) to examine the level of superoxide anion in the vessel wall, as previously described (Colucci *et al.*, 2013). Briefly, fresh tissue sections were embedded in Tissue-Tek OCT (Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen and then cut into serial 30 μm sections using a cryostat microtome (HM500 OM, Microm International, Walldorf, Germany). The sections were then settled on poly L-lysine-coated glass slides (Boster, Wuhan, China). The unfixed cryosections were immersed in 4% paraformaldehyde for 10 min at room temperature and were subsequently incubated for 30 min at 37°C in Krebs-HEPES buffer (in mM: Na-HEPES, 20.0; NaCl, 99.01; KCl, 4.69; KH_2PO_4, 1.03; $NaHCO_3$, 25.0; $MgSO_4$, 1.20; $CaCl_2$, 1.87 and glucose, 11.1; pH 7.4) containing 2 μM DHE in a light-tight humidified container. The sections were then washed twice with PBS and stored in the dark. The tissue samples from all the groups were processed and imaged in parallel. Finally, the sections were examined using an inverted fluorescence microscope (TE-2000U, Nikon, Tokyo, Japan) and the fluorescence intensity of each image was estimated with Image-Pro Plus 5.0 (Media Cybernetics; Silver Spring, MD, USA).

Immunofluorescent staining

In the present study, indirect immunofluorescent staining was conducted in essentially the same manner as previously described (Luo *et al.*, 2013). Briefly, 14 μm thick cryosections were washed in PBS and pre-incubated in normal goat serum (Boster) for 1 h. The proteins were localized using the primary antibodies anti-nitrotyrosine (1:100, Millipore, Billerica, MA, USA) and anti-ox-CaMKII (1:100; Millipore) and were then incubated overnight in a humidified chamber at 4°C. Next, the sections were washed three times for 5 min each and were then incubated for 2 h at 37°C with the appropriate FITC-conjugated secondary antibodies (1:200, Zhongshan Goldenbridge Biotechnology, Beijing, China). The slides were then washed and visualized with a fluorescence microscope (TE-2000U) using the same amount of exposure in each case. To evaluate the specificity of the immunostaining, the tissue preparations were processed as described earlier without primary antibodies; there was no staining in the vessel wall under these conditions. All of the images were analysed with Image-Pro Plus 5.0 (Media Cybernetics).

Western blot

For the Western blot procedure, samples of frozen vascular tissue (2–3 pooled vessels per sample) were homogenized in an ice-cold RIPA buffer (Beyotime Biotech) containing a protease and phosphatase inhibitor cocktail (Sigma). The protein concentrations were quantified using a bicinchoninic acid protein assay kit (Beyotime Biotech) and samples with approximately 30 μg total protein were resolved by electrophoresis on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore). After blocking in 5% milk for 1 h at room temperature, the membranes were incubated overnight at 4°C in an antibody dilution buffer with the appropriate primary antibody: phosphorylated CaMKII (p-CaMKII; Thr^{286}, 1:1000 dilution, Signalway Antibody, Pearland, TX, USA), oxidized CaMKII (ox-CaMKII; $Met^{281/282}$, 1:1000 dilution, Millipore), CaMKII, Na^+/Ca^{2+} exchanger (NCX)1, phospholamban (PLB; 1:200, Santa Cruz Biotechnology, Dallas, TX, USA), inositol 1,4,5-trisphosphate receptor (IP_3R; 1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a; 1:500 dilution, Fantibody, New York, NY, USA) or SERCA2b (1:200 dilution, Millipore). The housekeeping protein GAPDH (1:5000 dilution, Sinopept, Beijing, China) was used to normalize the data. The membranes were washed extensively prior to incubation with HRP-linked anti-goat IgG, anti-mouse IgG or anti-rabbit IgG (1:5000 dilution, Signalway Antibody and Proteintech Group, Chicago, IL, USA). After being washed, the proteins were detected using an ECL-Plus reagent (Millipore) and the graphs were analysed with Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA).

Evaluation of serum ACh levels

At the completion of the reperfusion procedure, arterial blood samples were rapidly obtained from each rat with an arterial catheter and a syringe. The serum was separated by centrifugation at 956× *g* for 10 min at 4°C and the serum level of ACh was analysed using a commercially available kit according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China) at an absorbance of 550 nm using a microplate spectrophotometer.

Data analysis

All of the sample size estimates in the present study were made using previous data from our laboratory (Bi *et al.*, 2013) and all of the experiments were performed by researchers who were unaware of the experimental treatments and procedures. The contractile force to KCl and 5-HT were expressed as an increase in the tension (mN) developed per unit tissue

weight (mg) of the mesenteric rings, as previously described (Matsumoto *et al.*, 2010). The vasodilator responses to ACh and SNP were measured as the percentage of the contraction induced by phenylephrine (10 μM). E_{max} and the half maximal effect (EC_{50}) were derived from the concentration-response curves constructed by sigmoidal curve fitting using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA).

Data are expressed as the means ± SEM and were assessed with a two-tailed Student's *t*-test or a one-way ANOVA followed by Tukey's *post hoc* test using GraphPad Prism software version 5.0 (GraphPad Software). The Kolmogorov–Smirnov test was used to determine whether the continuous data were normally distributed; if a variable had a skewed distribution, log transformations were conducted prior to analysis. Multiple experiments performed with different ring segments from a single animal were averaged and counted as a single experiment. For the Western blot analyses, *n* indicates the number of samples (2–3 pooled vessels per sample). $P < 0.05$ was considered to be statistically significant.

Materials

The present study utilized ACh, Ch chloride, 5-HT, phenylephrine, SNP, caffeine, KN-93 (Sigma, St Louis, MO, USA), 4-DAMP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), darifenacin (MedChem Express, Princeton, NJ, USA), L-NAME and NAC (Beyotime Biotech, Haimen, China). All of the other reagents and solvents used in the experiments were of analytical grade and dissolved in distilled water. All of the drug concentrations are expressed as the final molar concentration within the organ chamber.

Results

Effects of choline on I/R-induced vascular dysfunction in mesenteric arteries

The present study examined the effects of choline on the vasoconstrictor responses to KCl (5–100 mM) and 5-HT (10^{-9}–10^{-4} M) using endothelium-intact mesenteric artery rings. There were no significant differences in the response to KCl between the sham-operated and I/R group ($P > 0.05$; Figure 2A) and the responses were not altered by treatment with choline or 4-DAMP ($P > 0.05$; Figure 2A). There were no significant differences in the EC_{50} values among the groups (Supporting Information Table S1). However, the contractile responses to 5-HT were greater in rings from the I/R group than in the sham-operated group ($P < 0.05$; Figure 2B). The $-\log EC_{50}$ values of the sham-operated and I/R groups were similar (Supporting Information Table S2). Treatment with choline decreased the maximum vasoconstrictor responsiveness to 5-HT of the I/R + Ch group relative to the untreated I/R rats ($P < 0.05$; Figure 2B and Supporting Information Table S2), but these effects of choline were abolished by pretreatment with 4-DAMP ($P < 0.05$; Figure 2B). The $-\log EC_{50}$ values in the I/R + Ch and I/R + Ch + 4-DAMP groups were not different ($P > 0.05$ vs. I/R + Ch; Supporting Information Table S2). However, these protective effects of choline were also abolished by darifenacin (Supporting Information Tables S1–2).

The effects of choline on endothelium-dependent and endothelium-independent vasodilation responses are shown in Figure 2C and D. Following preconstriction with phenylephrine (10 μM), the relaxation responses to a range of concentrations of ACh or SNP were determined. The ACh-induced relaxation response was markedly weaker in the I/R group than in the sham-operated group ($P < 0.05$; Figure 2C), but this change was prevented by the administration of choline, 10 min prior to the I/R procedure ($P < 0.05$; Figure 2C). However, the protective effects of choline were reversed by the M_3 receptor antagonists 4-DAMP ($P < 0.05$; Figure 2C) and darifenacin (Supporting Information Table S3), which suggested that the protective effects of choline were mediated by M_3 receptors. The $-\log EC_{50}$ values for all of the groups were comparable (Supporting Information Table S3). The vasorelaxation responses of the sham-operated and I/R groups to SNP were similar ($P > 0.05$; Figure 2D) and neither of the responses of these groups were modified by treatment with choline or 4-DAMP *in vivo* ($P > 0.05$; Figure 2D; Supporting Information Table S4). No significant differences were observed in $-\log EC_{50}$ values of these groups (Supporting Information Table S4). Overall, the present findings demonstrated that the protective effects of choline were similarly diminished by pretreatment with 4-DAMP or darifenacin (Supporting Information Tables S3–4), which further confirmed the involvement of M_3 receptors in the regulation of vascular function.

Endothelial function in response to NAC

To determine the contribution of ROS to vascular function, the effects of NAC (10 μM) on the vasodilator responses to ACh were assessed. NAC is an antioxidant that can neutralize intracellular ROS (Kawagishi and Finkel, 2014). In the sham-operated group, the response of arterial rings to ACh was not affected by incubation with NAC *in vitro* ($P > 0.05$; Figure 3A), but in the rings from the I/R group, NAC normalized the relaxation response to ACh ($P < 0.05$; Figure 3B). In the choline-treated group, NAC failed to modify the relaxation response to ACh ($P > 0.05$; Figure 3C), but as expected, NAC clearly normalized ACh-induced relaxation in the presence of 4-DAMP ($P < 0.05$; Figure 3D).

Choline treatment inhibits I/R-induced superoxide anion generation and protein nitration

In the present study, DHE staining of the vessels was performed to evaluate and localize vascular superoxide anion production. These data revealed a marked increase in the production of superoxide anion in the mesenteric vessels of the I/R group compared with those from the sham-operated group ($P < 0.05$; Figure 4A). The enhanced level of superoxide anion was attenuated by treatment with choline ($P < 0.05$) and this attenuation was abolished by pretreatment with 4-DAMP ($P < 0.05$).

To determine the effects of choline on I/R-induced increase in peroxynitrite formation, we assessed protein nitration in the mesenteric artery sections using specific anti-nitrotyrosine antibodies in an immunofluorescence procedure. The I/R group exhibited significantly higher levels of protein nitration ($P < 0.05$; Figure 4B), but this increase was

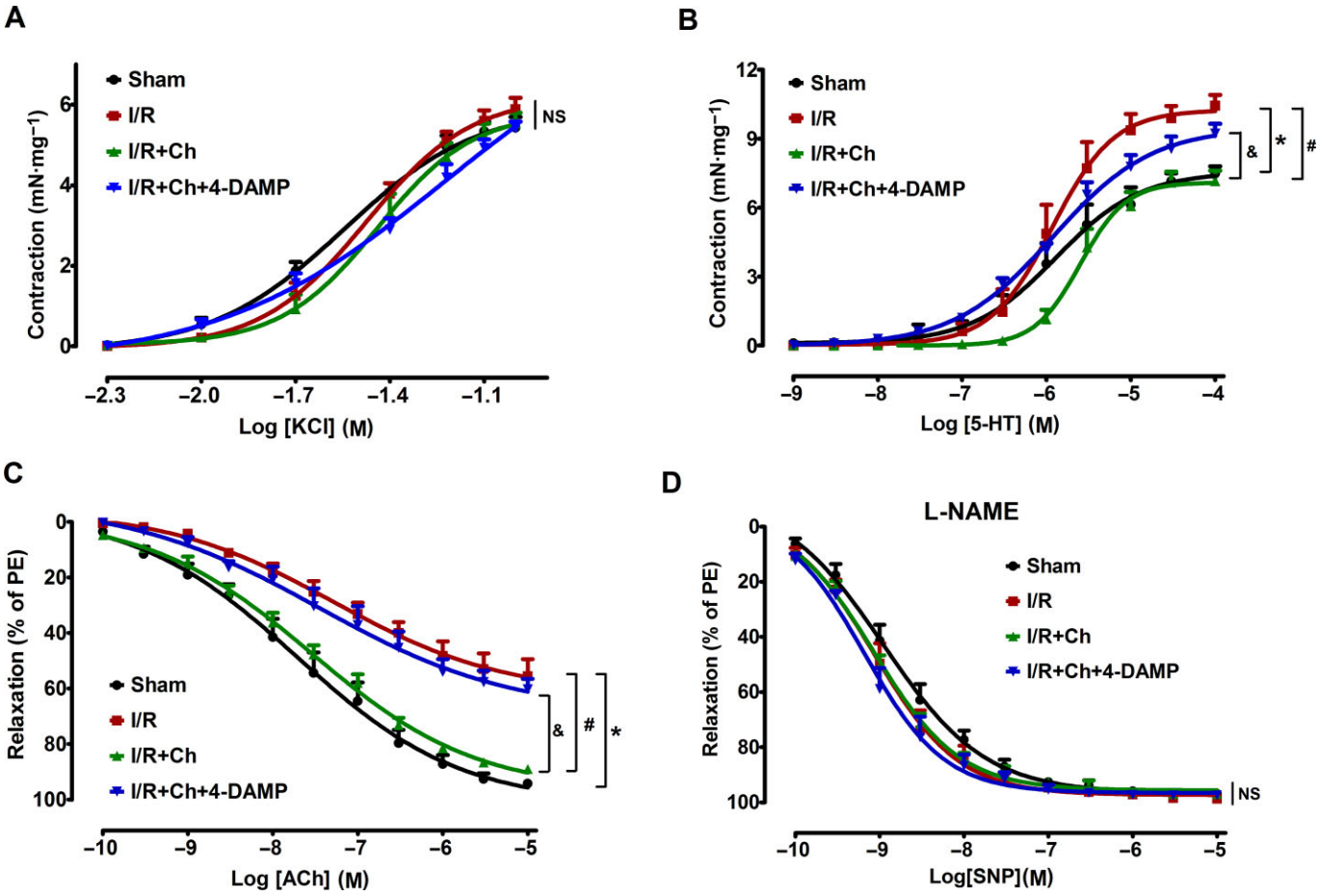

Figure 2

Treatment *in vivo* with choline prevents I/R-induced vascular dysfunction. Concentration-response curves to KCl (A), 5-HT (B), ACh (C) and SNP (D) in the mesenteric arteries of the sham-operated, I/R, I/R + Ch and Ch + 4-DAMP groups. Values are presented as the means ± SEM of eight (A, B, C) and six (D) rats per group. *$P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the I/R group; $^{\&}P < 0.05$ versus Ch-treated group; NS, non-significant.

diminished following choline treatment ($P < 0.05$). However, the beneficial effects of choline were attenuated by the administration of 4-DAMP ($P < 0.05$), confirming the results obtained using DHE.

Choline attenuates ROS-induced CaMKII activation

CaMKII is an important mediating factor following I/R injury and may be a possible downstream target for oxidative stress (Luczak and Anderson, 2014). To determine whether choline would affect the activation of CaMKII, ox-CaMKII and p-CaMKII in vascular tissues, these levels were measured using immunoblotting. There were increases in ox-CaMKII and p-CaMKII protein levels in the I/R group ($P < 0.05$; Figure 5A), but these changes were attenuated by choline treatment ($P < 0.05$). However, the effects of choline were reversed by 4-DAMP ($P < 0.05$). To determine whether ROS mediated the activation of CaMKII, NAC (150 mg·kg^{-1}, i.v.) was injected immediately prior to occlusion (Takhtfooladi *et al.*, 2013). The expression of ox-CaMKII and p-CaMKII were similar in the sham- and NAC-treated animals ($P > 0.05$; Figure 5B).

Next, ox-CaMKII levels in the mesenteric vessels of the rats were assessed by immunofluorescence using an antibody against oxidized Met$^{281/282}$ of CaMKII. There was an increase in ox-CaMKII immunofluorescence induced by I/R ($P < 0.05$; Figure 6), but this was independently prevented by treatment with either choline ($P < 0.05$) or NAC ($P < 0.05$). The effects of choline were reversed by 4-DAMP ($P < 0.05$).

Influence of CaMKII on vascular function in mesenteric arteries after I/R

To investigate the contribution of the CaMKII pathway in mesenteric arteries, the effects of an inhibitor of this enzyme, KN-93 (10 μM), on vascular function were investigated. The vasoconstrictor responses to KCl in arterial rings from the sham-operated and I/R groups did not significantly differ after treatment of KN-93 (Figure 7A; Supporting Information Table S1). Likewise, there were no significant differences in

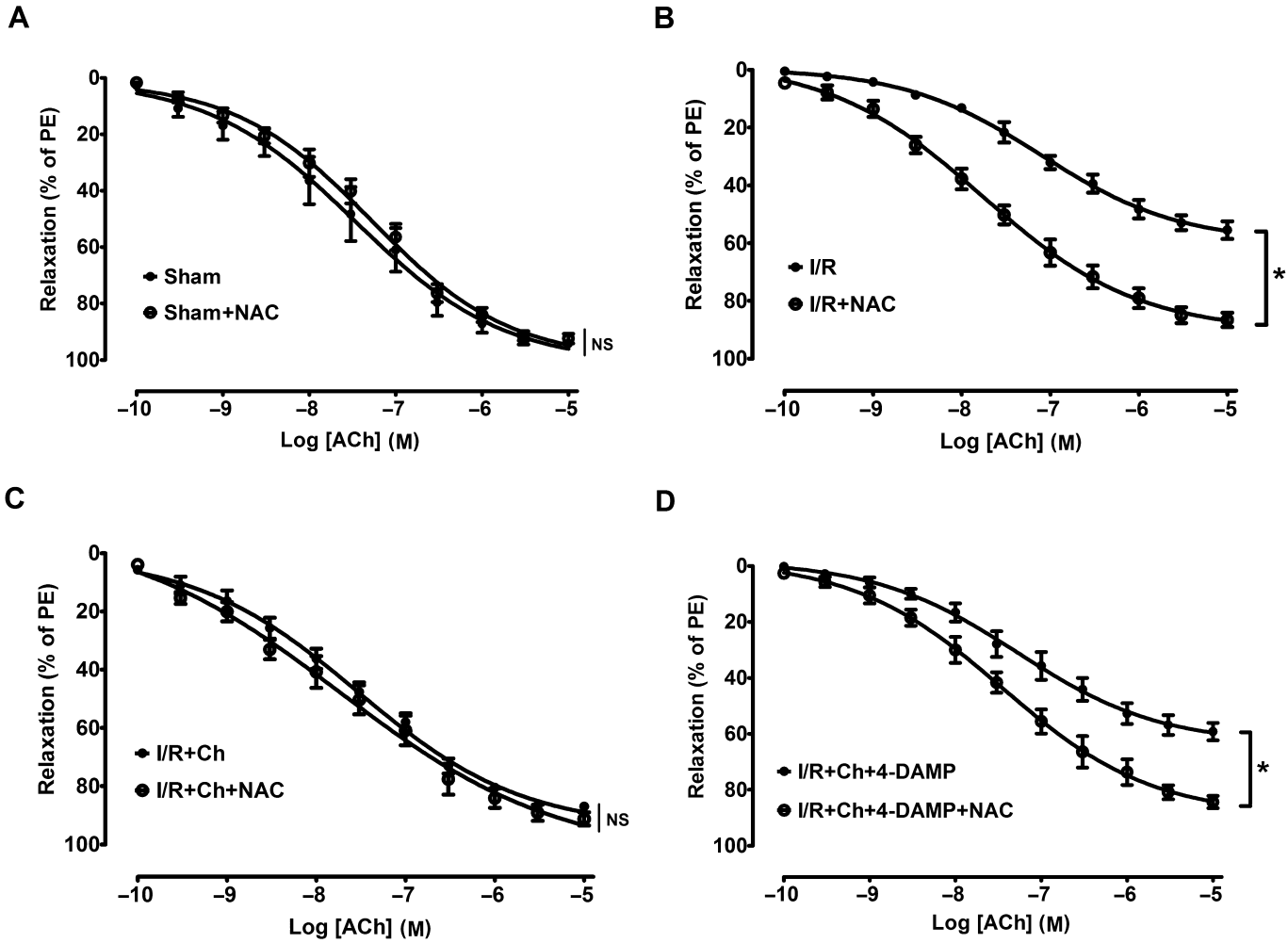

Figure 3

The contribution of ROS to vascular function. Effects of the ROS scavenger NAC (10 μM) on the relaxation responses to ACh in the mesenteric artery rings of the sham-operated (A), I/R (B), I/R + Ch (C) and Ch + 4-DAMP (D) groups. Data are presented as the means ± SEM of eight rats per group. *$P < 0.05$ versus respective control; NS, non-significant.

EC_{50} values among any of the groups (Supporting Information Table S1). However, incubation with KN-93 significantly attenuated the 5-HT-induced vasoconstriction responses in the arterial rings from I/R animals ($P < 0.05$; Figure 7B; Supporting Information Table S1), without affecting the corresponding $-\log EC_{50}$ values ($P > 0.05$; Supporting Information Table S2). In the sham-operated group, KN-93 failed to modify the contractions induced by 5-HT ($P > 0.05$; Figure 7B) or the the $-\log EC_{50}$ values ($P > 0.05$; Supporting Information Table S2).

Furthermore, arterial rings from I/R rats, treated with KN-93 *in vitro* exhibited a reversal of the I/R-induced changes in the vasodilator response to ACh ($P < 0.05$, Figure 7C). In the sham groups, the relaxation responses to ACh were not modified by KN-93 ($P > 0.05$; Figure 7C) and no significant differences were seen among the $-\log EC_{50}$ values of the groups (Supporting Information Table S3). Likewise, there were no significant changes in the responses to SNP, an endothelium-independent vasodilator, when it was applied in conjunction with KN-93 (Figure 7D; Supporting Information Table S4) and there were no significant differences among the $-\log EC_{50}$ values of the groups (Supporting Information Table S4).

Effects of I/R and Ch treatment on Ca^{2+} cycling proteins

To determine the mechanisms underlying the modification by choline of vascular function after I/R, the expression of Ca^{2+}-handling proteins in arterial rings was investigated (Figure 8). The protein levels of the NCX1 and IP_3R were significantly up-regulated in samples from the I/R group ($P < 0.05$; Figure 7A and B), but choline significantly reduced the increased expression of these proteins ($P < 0.05$). However, this choline-induced attenuation was inhibited by 4-DAMP ($P < 0.05$).

Western blot analysis of PLB levels revealed that PLB expression found in arterial rings from the I/R group was elevated compared with that found in rings from the

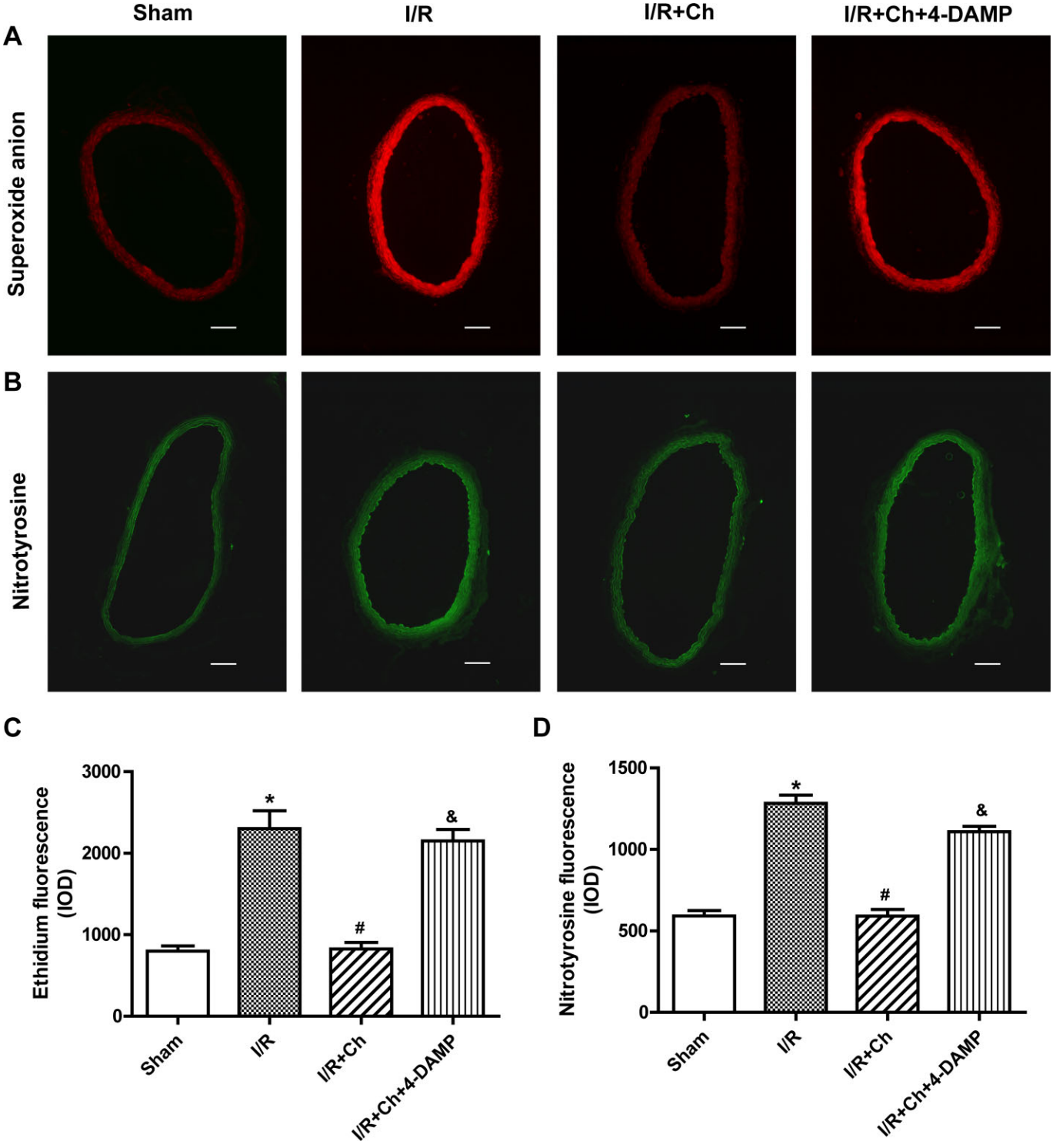

Figure 4

Treatment *in vivo* with choline inhibited I/R-induced oxidative stress and protein nitration in the mesenteric sections. Superoxide anion levels were determined using DHE staining (red). Protein nitration levels were determined by fluorescence of nitrotyrosine (green). Representative fluorescence photomicrographs (A, B) and quantification (C, D) of the microscopic sections of rat mesenteric arteries of the sham-operated, I/R, I/R + Ch and Ch + 4-DAMP groups. Scale bar = 200 μm. Data are presented as the means ± SEM of six rats per group. $^{*}P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the I/R group; $^{\&}P < 0.05$ versus the I/R + Ch group.

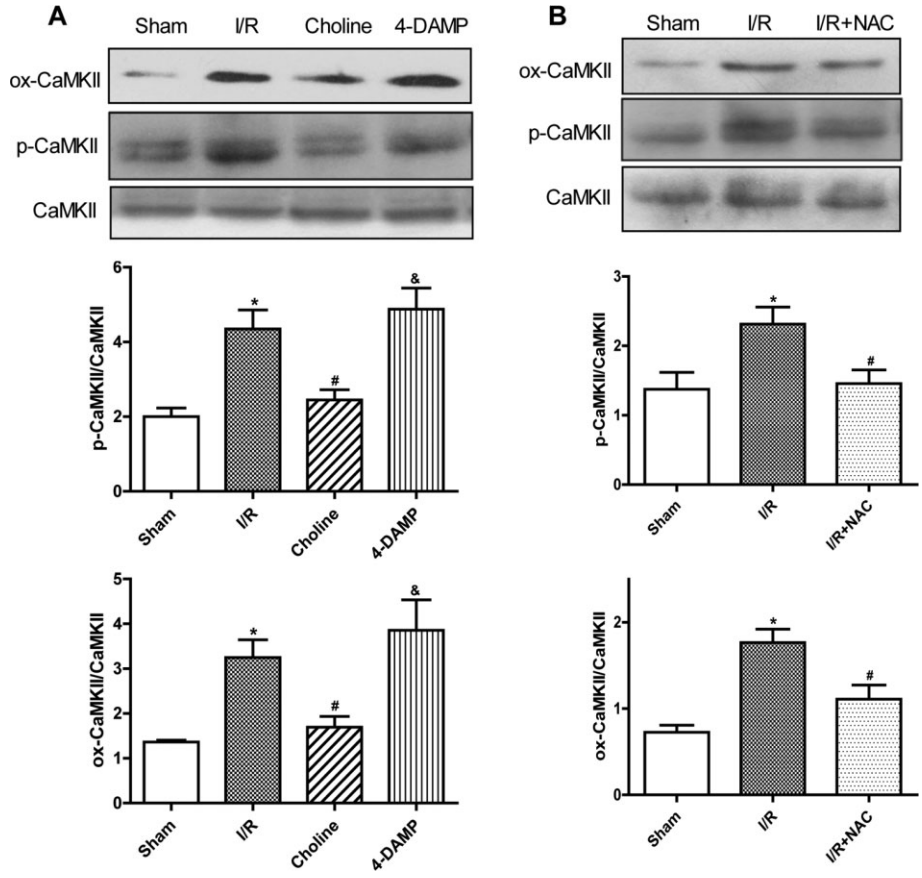

Figure 5

Treatment *in vivo* with choline reduced the expression of ox-CaMKII and p-CaMKII. (A) Effects of I/R and Ch on the activation of CaMKII in mesenteric arteries. (B) Effects of NAC on the activation of CaMKII. Representative Western blots of ox-CaMKII (top), p-CaMKII (middle) and total CaMKII (bottom). Oxidized and phosphorylated protein levels are presented relative to total protein levels. Data are presented as the means ± SEM for each group ($n = 6$). *$P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the I/R group; $^{\&}P < 0.05$ versus the I/R + Ch group.

sham-operated group ($P < 0.05$), but was restored after pretreatment with choline ($P < 0.05$). However, the beneficial effects of choline were partly attenuated by pretreatment with 4-DAMP ($P < 0.05$; Figure 8C). The levels of SERCA protein expression (SERCA2a and SERCA2b) were decreased by I/R ($P < 0.05$; Figure 8D and E). Choline treatment *in vivo* resulted in complete normalization of SERCA protein levels ($P < 0.05$) and this effect was reversed by 4-DAMP ($P < 0.05$).

Discussion

The present study demonstrated that choline treatment *in vivo* prevented I/R-induced vascular dysfunction *in vitro* via inhibition of the ROS-mediated CaMKII pathway and improvement in the expression of Ca^{2+}-cycling proteins. Our results showed that (i) activating M_3 receptors by choline down-regulated ROS production and CaMKII activity in a rat model of I/R; (ii) the ROS-activated CaMKII pathway is required for vascular dysfunction, based on the observation that the latter was restored in the presence of NAC or KN-93; (iii) choline treatment reversed the abnormal expression of Ca^{2+}-cycling proteins, including NCX1, IP_3R, SERCA and PLB; (iv) the protective effects of choline were abolished by 4-DAMP. These novel findings may help provide new insights into the mechanisms underlying the choline-induced protection of vascular function.

A substantial amount of evidence has indicated that endothelial dysfunction is a common symptom following I/R injury (Martinez-Revelles *et al.*, 2008; He *et al.*, 2013). As in previous studies, the present findings observed an attenuated vasodilator response to ACh that was indicative of endothelium-dependent impairments. Vascular dysfunction refers to the impaired relaxation and exaggerated contractile responses in the context of I/R (Martinez-Revelles *et al.*, 2008; Szadujkis-Szadurska *et al.*, 2010). The present study also observed an enhanced constrictor response to 5-HT after I/R, which is in agreement with previous reports (Borer *et al.*, 2013; Zhao *et al.*, 2013). It is possible that this finding is related to the up-regulation of 5-HT receptors in smooth muscle cells during ischaemia (Johansson *et al.*, 2012). Thus, the present findings suggest that I/R-induced vascular dysfunction involves both the endothelial and smooth muscle layers.

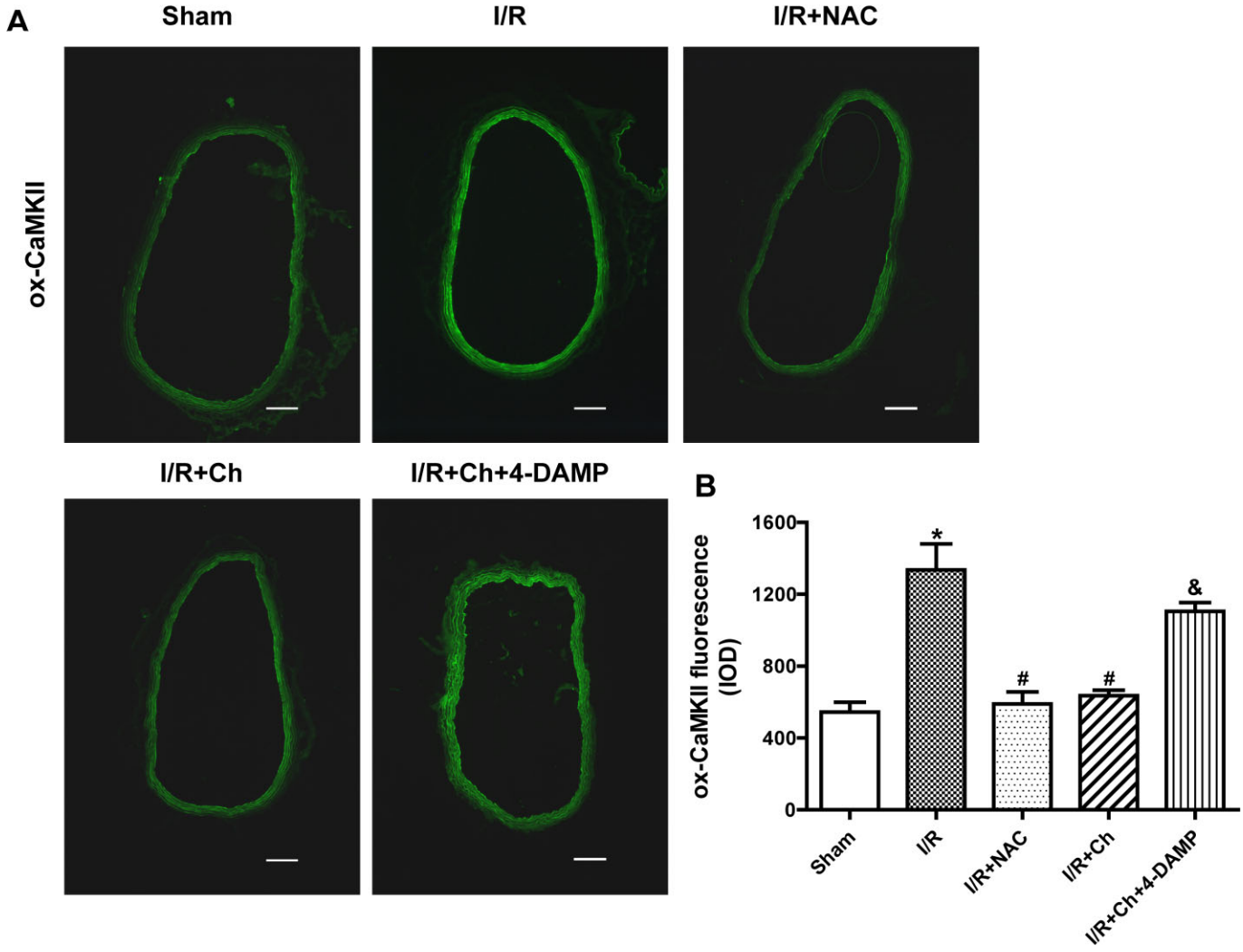

Figure 6

Representative example of ox-CaMKII immunofluorescence and quantification (green signal) in the mesenteric arteries of the sham-operated, I/R, I/R + Ch and Ch + 4-DAMP groups. Scale bar = 200 μm. Data are presented as the means ± SEM of six rats per group. $^{*}P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the I/R group; $^{\&}P < 0.05$ versus the I/R + Ch group.

The ROS are important factors in the pathogenesis of I/R injury (Wang *et al.*, 2013). In the present study, there was a marked elevation in superoxide anion production in the mesenteric arteries of rats that underwent I/R. The fact that NAC reversed the impairment in ACh-induced vasorelaxation following I/R further supports the contribution of ROS to vascular dysfunction. Superoxide rapidly scavenges NO and leads to protein nitration via the generation of peroxynitrite, which is associated with vascular disease (Herranz *et al.*, 2013). In the present study, the expression of nitrotyrosine in vessels taken from I/R rats was greater than those of sham rats, which indicates an enhancement in oxidative stress following I/R. Due to the short half-life of nitrotyrosine *in vivo*, its expression in blood vessel rings may more accurately reflect the level of vascular oxidative stress than free nitrotyrosine in the circulatory system (Tabrizi-Fard *et al.*, 1999).

The kinase CaMKII is a critical sensor of oxidative stress in the cardiovascular system (Luczak and Anderson, 2014) and the inhibition of CaMKII activity (via KN-93 or CaMKII-directed siRNA) produced cardioprotective effects in angiotensin II-treated cardiomyocytes (Zhao *et al.*, 2011) and I/R rats (Ma *et al.*, 2009). The present study demonstrated that both ox-CaMKII and p-CaMKII were elevated in mesenteric arteries subjected to I/R. However, these levels were normalized after pretreatment with NAC, which indicates that CaMKII functioned as a downstream signal following oxidative injury. The present study also showed that the inhibition of CaMKII attenuated I/R-induced abnormalities in vascular function. When mesenteric arteries from the I/R group were incubated with KN-93, there was a restoration of the vasoconstrictor response to 5-HT and the vasodilator response to Ach, further supporting the essential role of CaMKII in the vasculature.

Because it serves as a link between ROS and Ca^{2+} signals in subcellular domains (Luczak and Anderson, 2014), CaMKII is considered to be a master regulator of the Ca^{2+}-cycling proteins that lead to mitochondrial dysfunction and subsequent

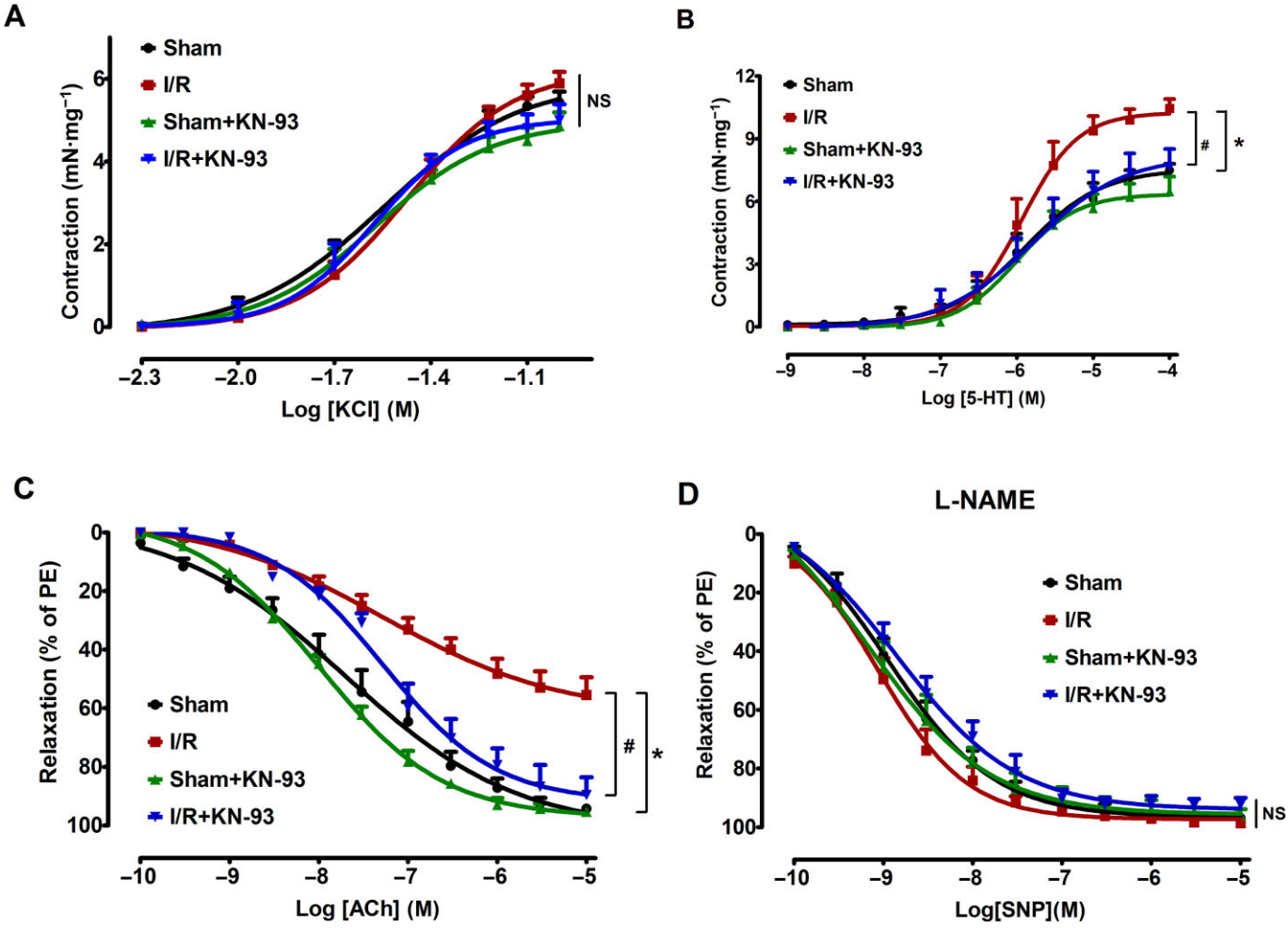

Figure 7

Role of CaMKII on vascular function. Effects of CaMKII inhibitor KN-93 (10 μM) on the concentration-response curves to KCl (A), 5-HT (B), ACh (C), and SNP (D) in the mesenteric artery rings of the sham-operated (black), I/R (red), sham + KN-93 (green) and I/R + KN-93 (blue) groups respectively. Data are presented as the means ± SEM of six rats per group. *$P < 0.05$ versus the sham-operated group; #$P < 0.05$ versus the I/R group; NS, non-significant.

cell death (Roe *et al.*, 2013; Bell *et al.*, 2014). Ca^{2+}-cycling proteins such as NCX, IP_3R, PLB and SERCA regulate intracellular Ca^{2+} concentrations. The up-regulated expression of NCX is thought to be a major contributing factor to Ca^{2+} overload under conditions of ischaemia and reperfusion (Wang *et al.*, 2012a; Ma *et al.*, 2014). The specific ablation of NCX mitigated I/R injuries via a decrease in Ca^{2+} influx (del Monte *et al.*, 2004). Therefore, Paillard *et al.* (2013) proposed that the Ca^{2+} release mediated by IP_3R participates in the process of Ca^{2+} overload during hypoxia-reoxygenation. In addition, I/R increases the activity of PLB, which is a regulatory protein of SERCA, and decreases SERCA protein levels (Guo *et al.*, 2013; Wu *et al.*, 2013), which leads to a reduction of sarcoplasm reticulum Ca^{2+} reuptake and an increased cytoplasmic Ca^{2+} overload. Furthermore, it is well known that SERCA2b is the primary isoform in vascular tissues (Szewczyk *et al.*, 2007). Emerging evidence suggests that SERCA2a gene transfer to vascular smooth muscle cells and endothelial cells is a novel potential treatment for cardiovascular diseases (Hadri *et al.*, 2013; Lipskaia *et al.*, 2013). The present findings demonstrated that I/R up-regulated NCX1, IP_3R and PLB at the protein level and down-regulated the expression of SERCA2a and SERCA2b. These changes may be related to the impaired vascular function observed in the present study. As expected, the changes in the levels of Ca^{2+}-cycling proteins were ameliorated by treatment with choline. In addition, the present study has shown that the contraction induced by high concentrations of caffeine was decreased following choline treatment (Supporting Information Fig. S1), which suggests that the vascular protection afforded by choline was attributed, at least in part, to its salutary effects on Ca^{2+} regulation.

Recently, it has been proposed that the activation of M_3 receptors by choline induced delayed preconditioning in a rat model of ischaemic insult (Zhao *et al.*, 2010). In the present study, the M_3 receptor antagonist 4-DAMP abolished the protective effects of choline, which indicates that these receptors were key players in the responsiveness of mesenteric arteries.

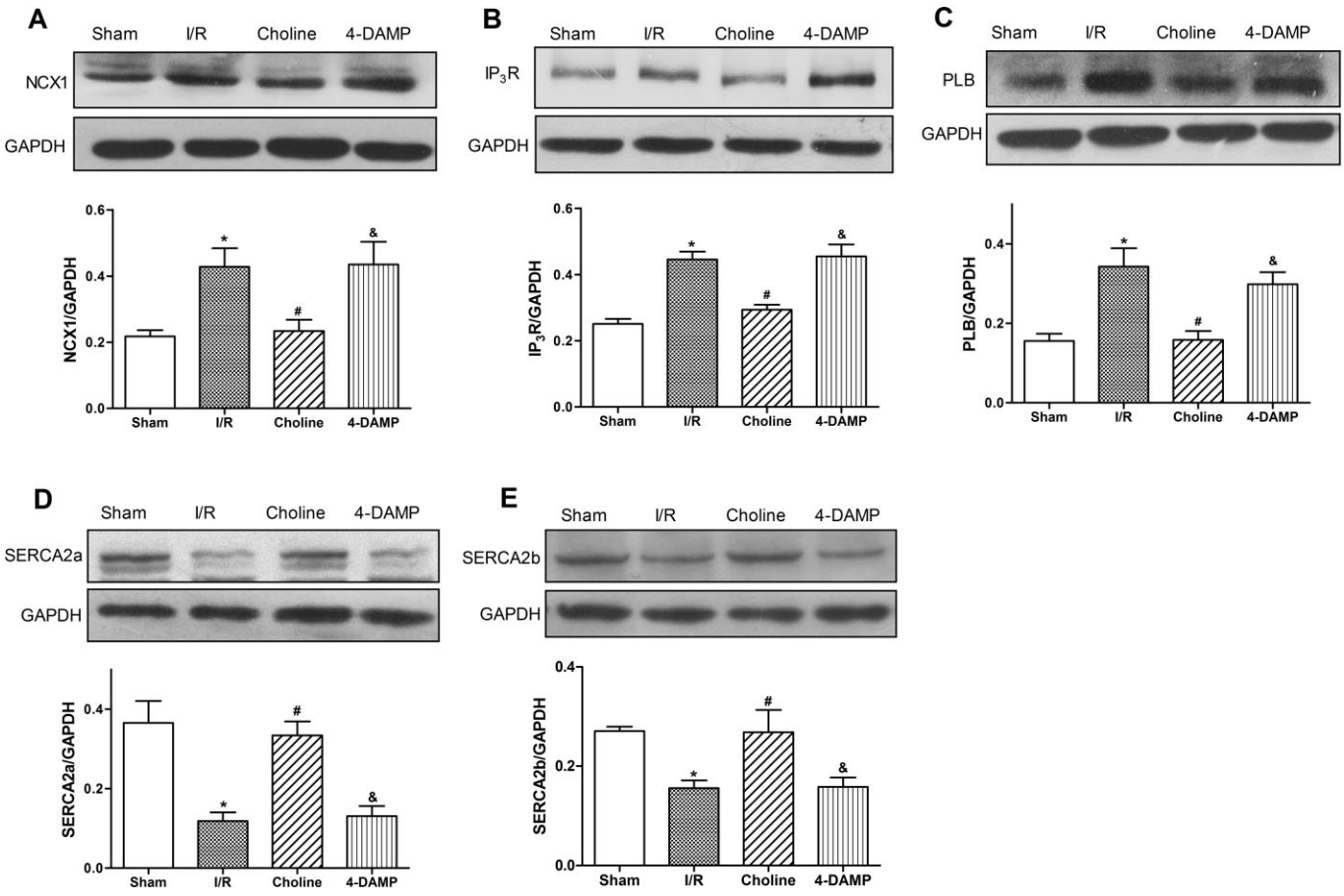

Figure 8

Effects of treatment *in vivo* with choline on expression of Ca^{2+}-cycling proteins. The protein expression of NCX1(A), IP_3R (B), PLB (C), SERCA2a (D) and SERCA2b (E) in the mesenteric arteries of the sham-operated, I/R, I/R + Ch and Ch + 4-DAMP groups respectively. The bar graphs summarize the Western blot data; values are presented as the means ± SEM for each group ($n = 6$). *$P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the I/R group; $^{\&}P < 0.05$ versus the I/R + Ch group.

Similarly, clinical findings have shown that M_3 receptors play a major role in vasomotor functions in several different human vascular beds (Pesic *et al.*, 2002; Attina *et al.*, 2008). The findings of Norel *et al.* (1996) support the notion that the ACh-induced relaxation response is mediated by M_1 receptors and M_3 receptors in the human pulmonary artery. These results highlight the important role that the activation of M_3 receptors plays in clinical situations. In addition, choline uptake via choline transporters is used for the synthesis of the neurotransmitter ACh, which releases vasoactive mediators via the activation of muscarinic receptors in vascular tissues and controls vascular responsiveness (Harvey, 2012). In the present study, choline treatment restored I/R-induced decreases in serum ACh levels (Supporting Information Fig. S2), which suggests that there may be a choline transport and uptake system that exists in blood vessels. The existence of such a system would result in an enhanced release of endogenous ACh that would act on mesenteric arteries.

It should be noted that 4-DAMP was used as an antagonist of M_3 receptors in the present study. Although the effects of 4-DAMP on nicotinic ACh receptors could not be excluded in the present experiment (Lawoko *et al.*, 1995), it is worth noting that specific agonists and antagonists towards M_3 receptor subtypes are not currently commercially available and choline and 4-DAMP have been widely used to characterize the function of M_3 receptors. Recent studies have demonstrated that the administration of choline (10 mg·kg^{-1}) 10 min prior to occlusion in rats results in a marked decrease in ischaemia-induced infarct size and arrhythmias, but this ameliorative effect is abolished by the presence of 4-DAMP (0.12 μg·kg^{-1}; Liu *et al.*, 2008; Pan *et al.*, 2012; Wang *et al.*, 2012a). Thus, based on our own preliminary experiments and the published data, a 10 mg·kg^{-1} dose of choline was administered 10 min prior to ischaemia surgery and a 0.12 μg·kg^{-1} dose of 4-DAMP was given 5 min prior to choline pretreatment in the present study.

In summary, the present experiments have provided convincing evidence that the administration of choline exerts a protective influence against I/R-induced vascular dysfunction in the peripheral vessels of rats. These effects are most likely to depend on the stimulation of M_3 receptors, which in turn, lead to inhibition of the ROS-mediated CaMKII pathway, as well as an improvement of the levels of Ca^{2+}-cycling proteins (Figure 9). These findings represent a significant addition to

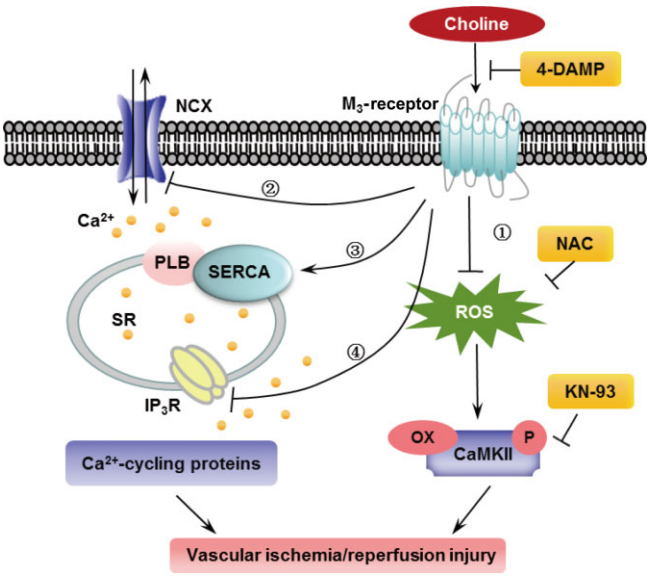

Figure 9

Proposed scheme of the mechanisms underlying the vascular protective effects of M_3 receptors against I/R injury in the present study. ① The stimulation of M_3 receptors with choline inhibits ROS-mediated CaMKII activation; ② choline reduces the protein levels of NCX; ③ choline ameliorates the balance between SERCA and PLB; and ④ choline depresses the expression of IP_3R. SR, sarcoplasmic reticulum.

our understanding of the pharmacological roles of M_3 receptors in the vasculature. The M_3 receptors may represent a novel target for drug discovery for the prevention or alleviation of I/R-induced vascular injury.

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Author contributions

X.-Z. L., W.-J. Z. and Z.-H. Z. designed the research study. X.-Z. L., X.-Y. B., X. H. and M. X. performed the research. X.-Z. L., M. Z. and X.-J. Y. analysed the data. X.-Z. L., X.-Y. B. and W.-J. Z. wrote the paper. W.-J. Z. acquired funding for the research. All authors read and approved the final manuscript.

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13183

Table S1 E_{max} and EC_{50} values for KCl-induced contraction of mesenteric rings from male SD rats.
Table S2 E_{max} and $-\log EC_{50}$ values for 5-HT-induced contraction of mesenteric rings from male SD rats.
Table S3 E_{max} and $-\log EC_{50}$ values for ACh-induced relaxation of mesenteric rings from male SD rats.
Table S4 E_{max} and $-\log EC_{50}$ values for SNP-induced relaxation of mesenteric rings from male SD rats.
Figure S1 Effects of I/R and choline treatment on the contractions induced by 30 mM caffeine in mesenteric arterial rings. Values are presented as the means ± SEM for each group (n = 6).*P < 0.05 versus the sham-operated group, $^{\#}P$ < 0.05 versus the I/R group; $^{\&}P$ < 0.05 versus the I/R + choline group (ANOVA followed by Tukey's test).
Figure S2 Effects of I/R and choline treatment on the serum ACh level. Values are presented as the means ± SEM for each group (n = 6). *P < 0.05 versus the sham-operated group; $^{\#}P$ < 0.05versus the I/R group; $^{\&}P$ < 0.05 versus the choline treated group (ANOVA followed by Tukey's test).