# NOS cofactor tetrahydrobiopterin contributes to anesthetic preconditioning induced myocardial protection in the isolated *ex vivo* rat heart

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Received July 4, 2019; Accepted December 4, 2019

DOI: 10.3892/ijmm.2019.4445

**Abstract.** Anesthetic preconditioning (APC) may decrease the myocardium injury nearly 50% following ischemia/reperfusion (I/R) by enhancing recovery of cardiac function, reducing myocardial enzyme release and lowering infarct size when utilized as pretreatment or posttreatment agents. I/R increases nitric oxide (NO) production through endothelial NO synthase (NOS3) and heat shock protein 90 (HSP90). The present study aimed to observe the role of BH4 availability and the association of HSP90 with NOS3 in APC-mediated cardioprotection against I/R injury. Isolated rat hearts were subjected to no-flow ischemia for 30 min and reperfusion for 120 min. Sevoflurane (3.5%) was administered for 15 min followed by a 15 min washout prior to ischemia. 2,4-Diamino-6-hydroxypyrimidine (DAHP) or sepiapterin (SP) was administered for 40 min until the onset of ischemia. The results revealed that compared with pre-ischemic basal levels, BH4 levels decreased and BH2 levels increased following I/R. BH4 levels were significantly increased and BH2 levels were significantly decreased in the APC + I/R hearts compared with the I/R group hearts. The BH4:BH2 ratio in the APC-treated hearts was also increased compared with that in the I/R group hearts. SP increased the recovery of contractile function and the production of NO, and decreased the production of superoxide anion ($O_2^{\cdot-}$) in I/R heart, but did not elicit these effects in APC-treated hearts. DAHP treatment inhibited the APC-mediated recovery of contractile function, increased $O_2^{\cdot-}$ levels and decreased NO production, but had no effect in I/R hearts. The cardioprotection of APC was demonstrated to be modulated by the BH4 precursor SP, which increased BH4 levels, or DAHP, which inhibited GTP cyclohydrolase I. Both APC and SP treatments increased the combination of HSP90 and NOS3, which improved the NOS3 activity and function. The results suggested that BH4, which servesas a cofactor for NOS, mediated the resistance of APC to I/R injury by promoting the binding of HSP90 and NOS3.

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*Key words:* anesthetics preconditioning, heart, ischemia reperfusion injury, nitric oxide synthase, tetrahydrobiopterin

## Introduction

Nitric oxide (NO) synthase, endothelial (NOS3)-derived NO is an important signaling molecule in the vascular system (1-3). The bioavailability of tetrahydrobiopterin (BH4) is critical for the catalytic activity of NOS3. The decreased effectiveness of BH4 leads to the decoupling of NOS3 and the production of reactive oxygen species (ROS) in endothelial cells (4,5). Decreased bioavailability of NO is an important factor in myocardial ischemia-reperfusion injury. The mechanism of NO reduction is associated with the lack of NOS substrate L-arginine and the cofactor BH4 leading to NOS decoupling (6).

Ischemia time-dependently decreases cardiac BH4 content and NOS3 activity, and increases NOS-derived superoxide anion ($O_2^{\cdot-}$) production, which is considered to contribute to post-ischemic endothelial dysfunction and myocardial injury (6). The decrease in BH4 bioavailability is associated with the uncoupling of NOS3 activity and the production of NOS3-dependent $O_2^{\cdot-}$. Increased resistance of the heart to ischemia is associated with the combination of heat shock protein 90 (HSP90) and NOS3 and subsequent increases in NO production (7). BH4 supplementation may restore endothelium-dependent coronary blood flow and decrease ischemia/reperfusion (I/R) injury in rat hearts (8,9), while

inhibition of BH4 synthesis may increase I/R injury (10). These results suggest that myocardial BH4 bioavailability is an important factor in cardioprotection and supports the hypothesis that BH4 may be a novel therapeutic target for the treatment of I/R injury.

Increasing evidence indicates that NOS3-derived NO is a critical mediator of anesthetic preconditioning (APC) (10-13). Under hyperglycemic conditions, the cardioprotective effects of volatile anesthetics by BH4 and HSP90-regulated NOS3 activity was abrogated (14). However, the role of BH4 availability and the association of HSP90 with NOS3 in APC-mediated cardioprotection against I/R injury remain to be elucidated. The aims of the present study were to determine: i) Whether BH4 levels were different in I/R rat hearts with or without APC, and whether increasedBH4 levels contributed to resistance to I/R injury by APC; ii) whether BH4 supplementation with BH4 precursorsepiapterin (SP) and GTP cyclohydrolase I (GCH-1) inhibitor, 2,4-diamino-6-hydroxypyrimidine (DAHP) differentially modulated resistance to I/R injury in the control (I/R alone) and APC rat hearts and iii) whether BH4 supplementation alteredthe BH4:BH2ratio, consequently affecting NOS3 activity and the association of HSP90 with NOS3 in APC-induced cardioprotection.

## Materials and methods

*Animals*. The present study was approved by the Institutional Animal Care and Use Committee of Nanjing University. Male Sprague-Dawley rats (250±50 g), aged 8-10 weeks, were purchased from the Animal Center of Suzhou University and were housed at 25˚C with 60% humidity in a 12:12 h light: Dark cycle. All rats were housed in each cage and were allowed *ad libitum* access to food and water. All procedures performed on the rats used in the present study were in accordance with the National Institute Health Guide for the care and Use of Laboratory Animals.

*Isolated heart preparation*. The isolated heart preparation was performed as previously described (8,13,15). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and decapitated when unresponsive to noxious stimulation (pinching the paw). The hearts were excised and perfused in the Langendorff mode at a perfusion pressure equivalent to 80 mmHg. Perfusate and bath temperatures were maintained at 37.2±0.1˚C using a thermostatically controlled water circulator (Lauda E100; LAUDA). Left ventricular pressure (LVP) was measured isovolumetrically with a transducer connected to a thin saline-filled latex balloon inserted into the left ventricle through the mitral valve from an incision in the left atrium. The hearts were immersed in gassed physiological buffer solution at 37.2˚C, and subjected to 30 min global ischemia followed by 120 min reperfusion. At the time points prior to ischemia or at the end of the experiments, the hearts were freeze-clamped and stored at -80˚C until use in the BH4 and BH2 analyses by high performance liquid chromatography (HPLC) or western blot analysis.

*Experimental protocols*. Each experimental treatment phase lasted for 220 min. A total of 70 rats were used. The functional parameters were stabilized for 30 min; after that the hearts were randomly divided into 7 groups with 10 hearts in each group (Fig. 1). Untreated time controls (TC; without I/R) were perfused for 220 min without drugs or ischemia (timeline A). After 40 min of vehicle perfusion, the I/R group was subjected to 30 min of global ischemia and 120 min of reperfusion; the APC group was exposed to sevoflurane (3.5%; Abbott Pharmaceutical Co., Ltd.) for 15 min followed by a 15 min washout prior to the onset of ischemia (timeline B). In the SP + I/R or SP + APC groups, SP (50 $\mu$M; cat. no. 11.225; Schircks Laboratories) was administered for 40 min prior to the onset of ischemia (timeline C). In the DAHP + I/R or DAHP + APC groups, DAHP (2.5 mM; cat. no. D19206; Sigma-Aldrich, Merck KGaA) was administered for 40 min prior to the onset of ischemia (timeline D).

*Measurement of BH4 and BH2*. The contents of BH4 and BH2 in all cardiac homogenates were determined viaHPLC as previously described (8). BH4 and BH2 were quantified via HPLC with an electrochemical detector (ESA Biosciences CoulArray® system Model 542) using a Synergi Polar-RP column (Phenomenex) eluted with argon degassed 50 mM phosphate buffer (pH 2.6). Multi-channel colorimetric detection was set between 0 and 600 mV. A separate channel was set at -250 mV in order to verify the reversibility of BH4 oxidative peak detection. The peak areas were collected at 0 and 150 mV for BH4, and 280 and 365 mV for BH2, and the data were combined to obtain a calibration curve. Intracellular concentrations of BH4 and BH2 were calculated using authentic external BH4 and BH2 standards as previously described (8). Cellular BH4 and BH2 levels were then normalized to cell protein concentrations.

*Western blot analysis*. The western blot analysiswas performed as previously described (8). The protein content of the samples was determined using a BCA assay kit (P0010; Biyuntian Biotechnology Company) and was adjusted to the same concentration. After denaturation, 20 $\mu$g of each sample was dissolved in Laemmli sample buffer (cat. no. S3401; Sigma-Aldrich, Merck KGaA) and separated via SDS-PAGE (12% gel). Following transfer to nitrocellulose membranes, membranes were blocked with 5% non-fat milk in PBS and probed with primary antibodies at 4˚C overnight and were incubated with HRP-Conjugated goat anti-rabbit secondary antibodies (1:10,000; cat. no. ab6721; Abcam) or HRP-conjugated goat anti-mouse secondary antibody (1:10,000; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The protein bands were visualized with a SuperSignal West Pico kit (cat. no. 34577; Pierce; Thermo Fisher Scientific, Inc.) and band densities were quantified using UN-SCAN-IT software (v.7.0; Silk Scientific, Inc.). Antibodies specific for GCH-1 (cat. no. ab26439, 1:2,000) and GAPDH (cat. no. ab9485, 1:2,500) used in the study were purchased from Abcam. The NOS3 (cat. no. PA3-031A; 1:1,000) and HSP90 (cat. no. MA1-10372; 1:1,000) antibodies were purchased from Invitrogen; Thermo Fisher Scientific, Inc.

*Immunoprecipitation assay*. Immunoprecipitation was performed as previously described (8). Hearts were homogenized in ice-cold lysis buffer and subjected to SDS-PAGE (12% gel). Proteins were detected using enhanced chemiluminescence

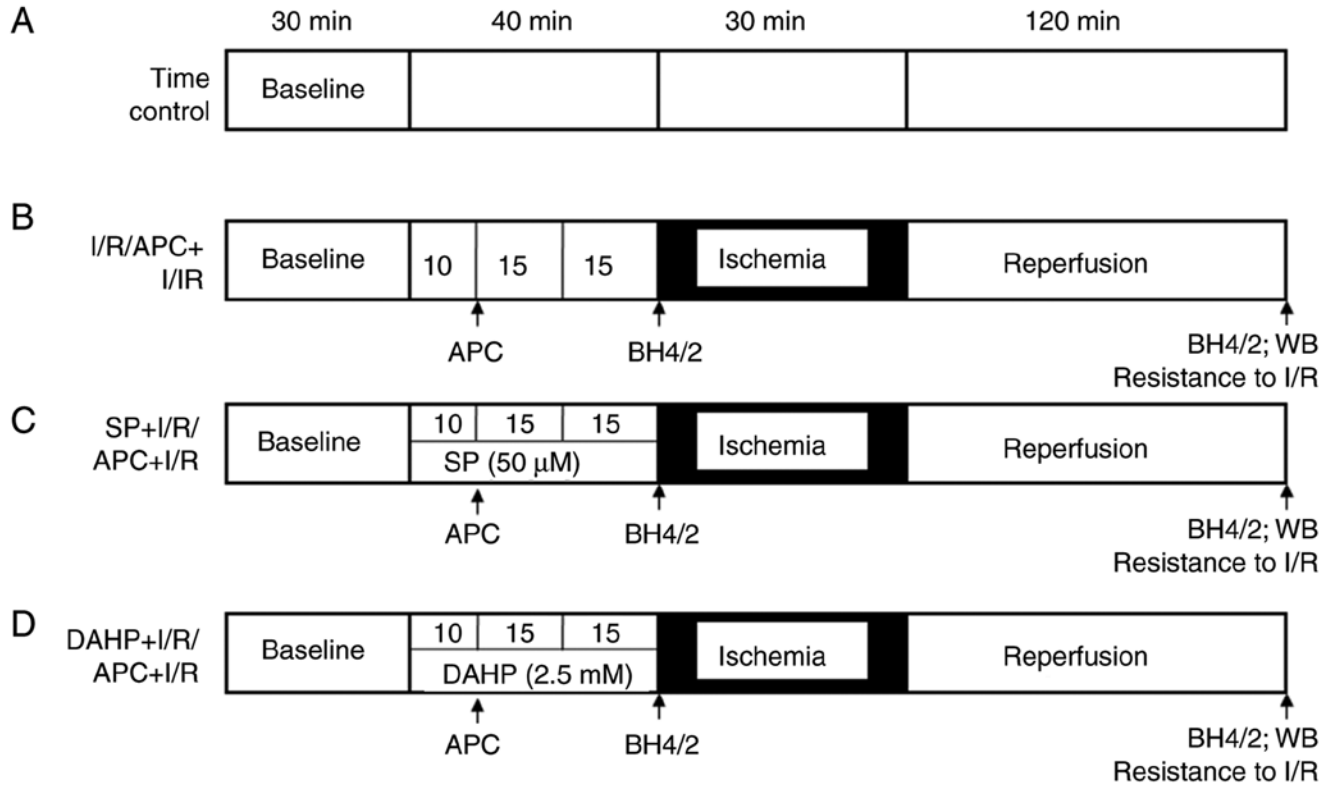


Figure 1. Schematic illustration of the experimental protocols used in all groups. (A) Timeline A represents the untreated time controls (TC, no I/R). (B) Timeline B represents the ischemia reperfusion controls (I/R) or I/R with sevoflurane treatment (APC + I/R). (C) Timeline C represents I/R with SP treatment (SP + I/R) or I/R with SP and sevoflurane treatment (SP + APC + I/R) (D) Time line D represents I/R with DAHP treatment (DAHP + I/R) or I/R with DAHP and sevoflurane treatment (DAHP + APC + I/R). BH4/2 marks denote the time points at which the tissue samples were harvested for the HPLC assay for BH4 and BH2. Resistance to I/R injury means determining functional recovery after 30 min global ischemia and 120 min reperfusion. The APC group received sevoflurane (3.5%) for 15 min followed by a 15 min washout before global ischemia. Vehicle, SP or DAHP were perfused for 40 min prior to ischemia in the presence of absence of APC. I/R, ischemia/reperfusion; APC, anesthetic preconditioning; SP, sepiapterin; DAHP, 2,4-diamino-6-hydroxypyrimidine.

detection reagents (cat. no. 34577; Pierce; Thermo Fisher Scientific, Inc.) for densitometric analysis with UN-SCAN-IT software.

*Reactive oxygen species (ROS) detection*. ROS ($O_2^{\cdot-}$) production was detected using lucigenin-enhanced chemiluminescence as previously described (8). During the first minute of reperfusion, coronary effluent (1 ml) was collected and 500 $\mu$l was immediately transferred to a 1.5 ml vial, in which 5 $\mu$l of 500 $\mu$M lucigenin (cat. no. M8010; Sigma-Aldrich; Merck KGaA) was added. The final concentration was 5 $\mu$M. The vial was placed in a luminometer (Turner BioSystems, Inc.) in order to measure chemiluminescence for 5 min in the dark. A vial containing lucigenin was used to measure background luminescence, and this background value was subtracted from each sample value.The data of $O_2^{\cdot-}$ production was expressed as relative light units.

*NO detection*. In order to assess the effects of BH4 on NOS3 coupling, at 1 min of reperfusion, 1 ml coronary effluent was collected and NO concentrations were immediately determined using a NO electrode (World Precision Instruments). The values were normalized by heart wet weight and coronary flow rate as previously described (8). The NO electrodes were calibrated using $NaNO_2$ (cat. no. 72586; Sigma-Aldrich; Merck KGaA) and KI (cat. no. 746428) +$H_2SO_4$ (cat. no. 339741) (both Sigma-Aldrich; Merck KGaA) following the manufacturer's protocol, to quantify the amount of NO produced.

*Statistics analysis*. The data are presented as the means ± standard error of the mean. In order to examine the overall differences between the groups, a two-way analysis of variance was used. The Student-Newman-Keuls multiple comparison post hoc test was used to differentiate within the groups. SPSS 19.0 (IBM Corp.) was used to perform the statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*BH4 and BH2 levels in APC and I/R rat hearts*. As presented in Fig. 2, prior to ischemia, BH4 and BH2 levels were similar in both the I/R and APC + I/R groups (Fig. 2A). The BH2 level was significantly increased and the BH4 level decreased significantly in I/R hearts when compared with the pre-ischemia baseline values. Following I/R, the BH4 levels (27.4±1.9 vs. 18.1±1.9 pmols/mg protein) were increased and BH2 levels (22.7±1.8 vs. 40.3±3.2 pmols/mg protein) were decreased in the APC + I/R hearts when compared with the I/R hearts ($P<0.05$; n=10/group). The BH4:BH2 ratio in APC + I/R rat hearts was increased 2-fold compared with the I/R hearts (1.2±0.15 vs. 0.45±0.09).

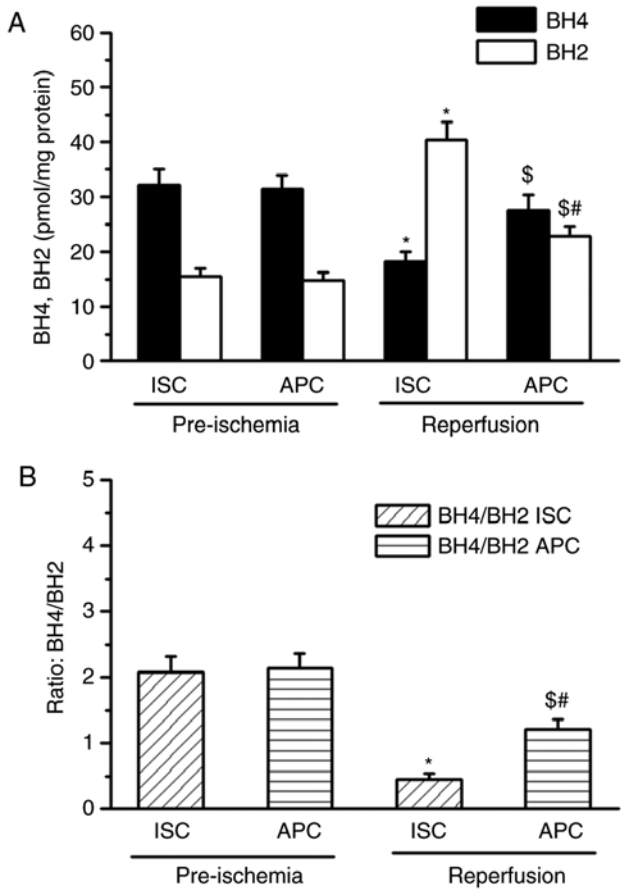


Figure 2. BH4, BH2 levels and the ratio of BH4/BH2 in rat hearts. (A) BH4 and BH2 levels and (B) BH4:BH2 ratio in the TC, APC, I/R and APC + I/R rat hearts. In the TC and APC groups, hearts were harvested after 70 min perfusion without or with APC treatment. In the I/R and APC + I/R groups, hearts were harvested after 30 min no-flow global ischemia and 120 min reperfusion. The data are presented as mean ± standard error of the mean. *P<0.05 vs. I/R pre-ischemia group. #P<0.05 vs. APC + I/R pre-ischemia group. $P 0.05 vs.I/R reperfusion group. n=10/group. TC, time control; ISC, ischemia; I/R, ischemia/reperfusion; APC, anesthetic preconditioning.

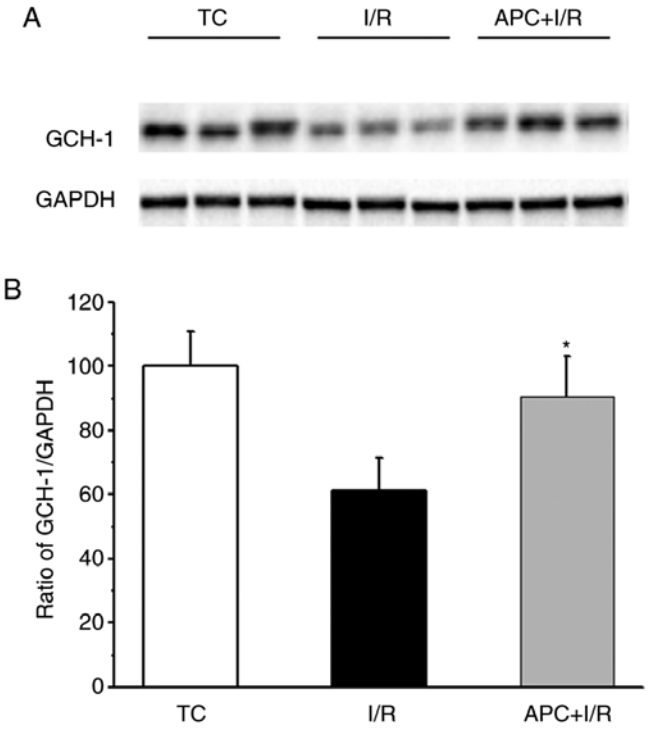


Figure 3. GCH-1 protein levels in the TC, I/R and APC + I/R rat heart homogenates. (A) GCH-1 and GAPDH were detected by western blot analysis using lysates of heart homogenates. GADPH was used as a loading protein control. (B) The densitometric analyses of the blot gel in part A are presented as GCH1/GADPH ratio for each group. Data are presented as the mean ± standard error of the mean. n=3/group). *P<0.05 vs. I/R group. GCH-1, GTP cyclohydrolase I; TC, time control; I/R, ischemia/reperfusion; APC, anesthetic preconditioning.

*GCH-1 protein levels*. Fig. 3 presents the GCH-1 contents in TC, I/R and APC + I/R heart tissues. The GCH-1 expression level in APC + I/R hearts was increased by almost 30% compared with that in I/R hearts after I/R (n=3/group).

*Supplementation of SP or DAHP on cardiac function*. In order to determine whether BH4 contributed to the increased resistance to I/R injury in APC + I/R hearts compared with I/R hearts, the present study perfused isolated hearts with either a GCH-1 inhibitor (DAHP, 2.5 mM) or a BH4 donor (SP, 50 $\mu$M) for 40 min prior to ischemia with or without APC treatment. Left ventricular developed pressure (LVDP) was measured after 120 min reperfusion, using the percentage of pre-drug and pre-ischemic values. The recovery rate of LVDP following I/R in the APC + I/R hearts (62.7±7.6 mmHg and 62.6±5.2%) was significantly increased compared with that in the I/R group (48.0±4.0 mmHg and 47.4±5.1%) (P<0.01). SP treatment significantly improved the LVDP in I/R hearts (64.5±6.7 mmHg and 64.3±6.2%), but did not increase the recovery of LVDP in APC + I/R hearts (68±6.1 mmHg and 66±5.9%; Fig. 4A). DAHP treatment decreased the recovery of LVDP in APC hearts (41.7±5.1 mmHg and 45.7±4.6%) to the values observed in the I/R group hearts (Fig. 4B).

*Supplementation of SP or DAHP on cardiac BH4 and BH2 levels*. In order to observe the changes in BH4 and BH2 levels following SP or DAHP treatment in I/R or APC + I/R hearts, BH4 and BH2 levels were measured at 40 min after perfusion with SP or DAHP (pre-ischemia) or at 120 min reperfusion. Following SP treatment and prior to ischemia in the SP + I/R hearts, the levels of BH4 (45.4±5.4 vs. 32.1±2.9 pmols/mg protein; P<0.05) and BH2 (203±19 vs. 15.5±1.4 pmol/mg protein) were significantly increased when compared with the I/R hearts (P<0.001; Fig. 5A). At 120 min reperfusion, SP treatment increased BH4 levels significantly in the SP+IR group compared with the I/R group (32.4±4.5 vs. 18.1±1.9 pmol/mg protein; P<0.05). In the APC + I/R rat hearts, DAHP treatment prior to ischemia did not affect BH4 or BH2 levels (Fig. 5B). Following I/R, DAHP treatment significantly decreased BH4 levels (21.4±2.2 vs. 27.4±2.2 pmol/mg protein) and increased BH2 levels (56.1±6.2 vs. 22.7±1.8 pmol/mg protein) in the DAHP+APC hearts compared with the APC + I/R hearts (P<0.01). After 120 min reperfusion, the BH4:BH2 ratio was decreased in DAHP+APC + I/R hearts compared with the APC + I/R hearts (P<0.01).

*Supplementation of SP or DAHP on the association of HSP90 and NOS3*. In order to observe the effects of SP or APC on the interaction between HSP90 and NOS3 in I/R rat hearts,

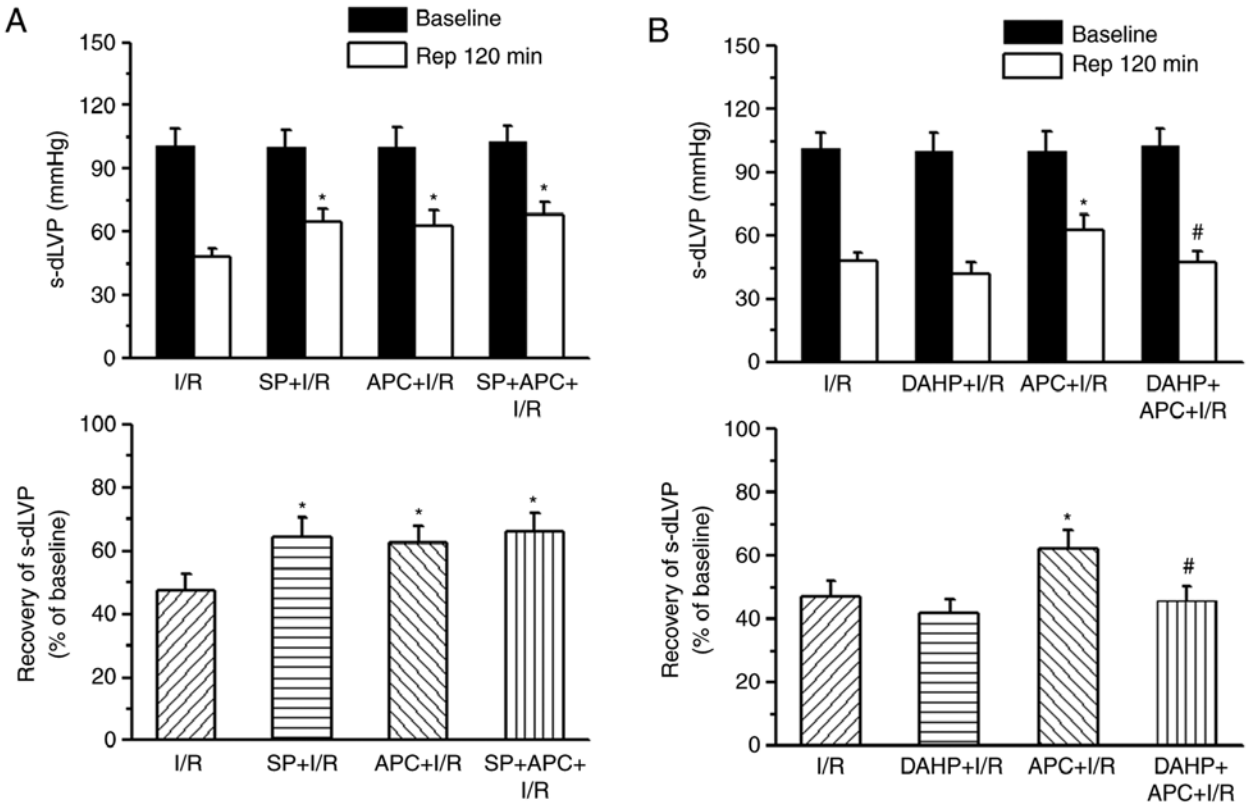


Figure 4. Resistance to myocardial I/R injury in heart tissues from I/R rats treated with SP and APC + I/R rats treated with DAHP. Isolated rat hearts were perfused with (A) SP (50 $\mu$M) or (B) DAHP (2.5 mM) for 40 min prior to 30 min no-flow global ischemia followed by 120 min reperfusion. The upper graphs in parts A and B demonstrate the absolute values for the s-dLVP (mmHg), and the lower graphs in parts A and B demonstrate the relative recovery of s-dLVP for each group based on their respective basal values. Data are presented as mean ± standard error of the mean(n=10/group). *P<0.05 vs. I/R group. #P<0.05 vs. APC + I/R group. SP, sepiapterin; DAHP, 2,4-diamino-6-hydroxypyrimidine; dLVP, developed left ventricle pressure; s-dLVP, systolic minus diastolic left ventricle pressure.

NOS3 was immunoprecipitated from the homogenates of I/R hearts following APC, SP or DAHP treatment and probed for NOS3 and HSP90 proteins (Fig. 6). The results demonstrated an increased association between HSP90 and NOS3 (>30%) in the SP+IR (IB hsp90-SR+I/R row) and APC + I/R hearts (IB hsp90-APC + I/R row) compared with the I/R group. However, this increase in association betweenHSP90 and NOS3 in the APC + I/R hearts was inhibited by treatment with DAHP (IB hsp90-DAHP+APC + I/R row).

*NO and $O_2^{\cdot-}$ levels following I/R*. The present study also measured •NO and $O_2^{\cdot-}$ production in I/R hearts following SP treatment or APC + I/R hearts following DAHP treatment (Fig. 7). The data revealed that the lucigenin chemiluminescence signal intensity, which represents $O_2^{\cdot-}$ (ROS) production, was significantly decreased in the SP + I/R and APC + I/R rat hearts, but increased in DAHP + APC + I/R rat hearts at the end of 120 min reperfusion. Following treatment with SP, the NO levels in the SP + I/R group was significantly increased compared with those in the I/R group, whereas DAHP treatment significantly decreased the NO levels in DAHP + APC + I/R hearts. APC treatment also significantly increased NO production in APC + I/R hearts compared with the I/R group.

## Discussion

The maintenance of physiological levels of NO produced by NOS3 represents a key element for vascular function (16,17). As a pteridine cofactor, BH4 is an important regulator of NOS3 function, as BH4 is required to maintain enzymatic coupling of L-arginine oxidation, to produce NO (18,19). The present study assessed the role of BH4 and the BH4 biosynthetic precursor, SP, in the mechanisms underlying resistance to I/R injury in I/R hearts with or without APC. The results revealed that: i) BH4 levels were significantly increased and BH2 levels were significantly decreased in APC + I/R hearts compared with the I/R group hearts. The BH4:BH2 ratio in the APC-treated hearts was increased 2-fold compared with that in the I/R group hearts. The recovery of cardiac function in the APC-treated hearts was consistent with the alterations of BH4 and BH2 levels, and the BH4:BH2 ratio; ii) SP supplementation increased the resistance to I/R injury in the I/R group, and GCH-1 inhibition decreased APC-induced cardiac function recovery; iii) SP supplementation increased the association between HSP90 and NOS3; while GCH-1 inhibition by DAHP decreased this association; iv) ROS ($O_2^{\cdot-}$) production decreased and NO levels were increased significantly following SP treatment in the I/R group, and ROS production increased and NO levels decreased following DAHP treatment in APC + I/R hearts at the initial time of reperfusion. The results of the present study indicate that BH4 and the ongoing synthesis of BH4 participated in the susceptibility to I/R injury in the APC + I/R and I/R group hearts. APC-induced resistance to I/R injury was mediated by the NOS3 cofactor BH4 and the elevation of HSP90-NOS3 association.

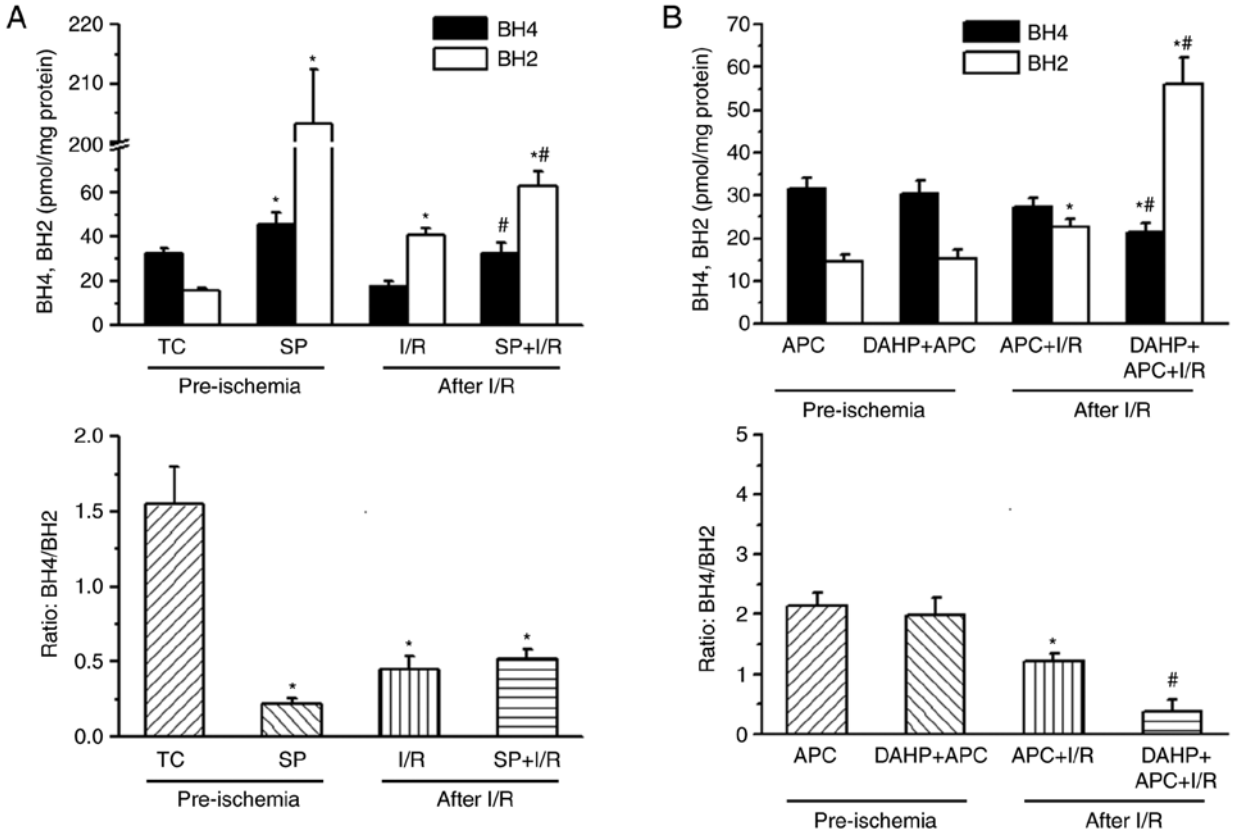


Figure 5. BH4 and BH2 levels and ratio of BH4/BH2 in (A) TC and I/R rat hearts with or without SP prior to ischemia or following reperfusion (50 $\mu$M) or (B) APC and APC + I/R hearts with or without DAHP (2.5 mM) treatment prior to ischemia or following reperfusion. The values are presented as mean ± standard error of the mean (n=10/group). *P<0.05 vs. TC or APC pre-ischemia group. #P<0.05 vs. I/R or APC + I/R reperfusion group. TC, time control; I/R, ischemia/reperfusion; APC, anesthetic preconditioning; SP, sepiapterin; DAHP, 2,4-diamino-6-hydroxypyrimidine.

In I/R-injured hearts, lowering BH4 levels may be an important cellular defect involving endothelial and cardiomyocyte dysfunction (6,20). The role of BH4 in NOS activity is important in cardioprotection. The decrease in BH4 levels not only prevents NOS3 from generating NO, but also causes NOS3 'uncoupling', resulting in NOS3 generating $O_2^{\cdot-}$ instead of NO (21). BH4 deficiency due to decreased BH4 biosynthesis or BH4 oxidation to BH2 (less catalytic activity) is known to be one of the causes of I/R injury (8). The results of the present study are consistent with these data, in that BH4 level and the BH4:BH2 ratio in APC-treated hearts were significantly increased compared with that in the I/R group, confirming that BH4 serves a crucial role in I/R injury. The importance of the BH4:BH2 ratio in determining whether BH4 and BH2 bind to NOS3 has been revealed in recent studies. As the affinity of BH4 and BH2 to NOS3 is equal, BH4 in NOS3 can be rapidly and efficiently replaced by BH2, resulting in the uncoupling of NOS3 activity, and decreased NO synthesis (2,22). APC treatment demonstrated increased BH4 and decreased BH2 levels following I/R. Therefore, in addition to decreased BH4 levels, the BH4:BH2 ratio was significantly decreased in I/R group hearts compared with that in the APC + I/R hearts, which may also lead to NOS3 uncoupling, an increase in $O_2^{\cdot-}$ production and decreased cardiac function recovery.

Increasing the availability of BH4 by modulating GCH-1 has been recognized as a novel strategy for protecting the heart during post-infarction remodeling, dilated myopathic remodeling and cardiac hypertrophy (23). Certain studies have demonstrated that the treatment of exogenous BH4 or BH4 precursor SP may promote NO production and NOS-dependent coronary flow recovery following ischemia (6,24). SP supplementation enhanced the resistance to I/R injury in the I/R group. Notably, the results of the present study demonstrated that SP did not alter the APC-induced cardioprotection observed, suggesting that the cardiac protection induced by APC had reached its maximum effect. Resistance to I/R injury by APC was abrogated to levels that were not significantly different from the I/R group when GCH-1 was inhibited with DAHP. In addition, the GCH-1 inhibitor DAHP did not affect the functional recovery of the I/R group, but SP supplementation increased the functional recovery to a level comparable with that of APC-treated hearts. This association provides evidence that BH4 levels are associated with APC-induced cardioprotection.

The present study hypothesized that BH4 serves a crucial role in NOS3 activity, NO generation, potentially with $O_2^{\cdot-}$ formation as suggested by the lucigenin chemiluminescence assay, and vascular reactivity during I/R. The increased lucigenin chemiluminescence signal intensity, representing the level of $O_2^{\cdot-}$ released, or net emission, from I/R rat hearts was significantly attenuated by SP and APC, but increased in the DAHP +APC +I/R group. Furthermore, there was a greater lucigenin chemiluminescence signal intensity from I/R alone

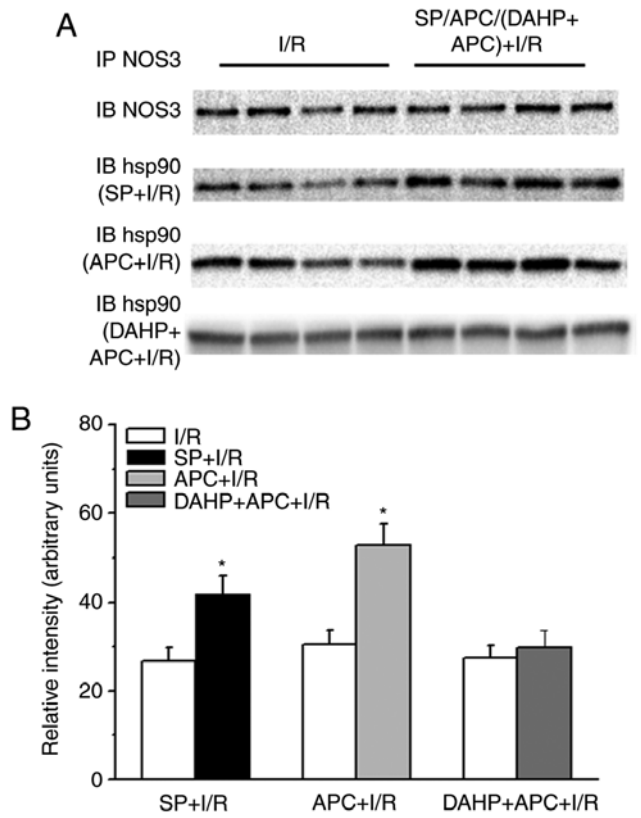


Figure 6. Western blot analysis of the association between NOS3 and HSP90 in heart homogenates from rat hearts. (A) The IB NOS3 row represents the I/R and APC + I/R heart tissues. The IB hsp90 (SP + I/R) row represents the I/R and SP + IR heart tissues. The IB hsp90 (APC + I/R) row represents the I/R and APC + IR heart tissues. The IB hsp90 (DAHP + APC + I/R) row represents the I/R and DAHP + APC + I/R heart tissues. The effects of SP (50 $\mu$M), APC (sevoflurane at 3.5%) or DAHP (2.5 mM) treatment on HSP90 association with NOS3. (B) The densitometric analyses of the blot gel in part A. n=3/group. IP, immunoprecipitation; IB, immunoblot. *P<0.05 vs. I/R group. NOS3, nitric oxide synthase, endothelial; HSP90, heat shock protein 90; I/R, ischemia/reperfusion; APC, anesthetic preconditioning; SP, sepiapterin; DAHP, 2,4-diamino-6-hydroxypyrimidine.

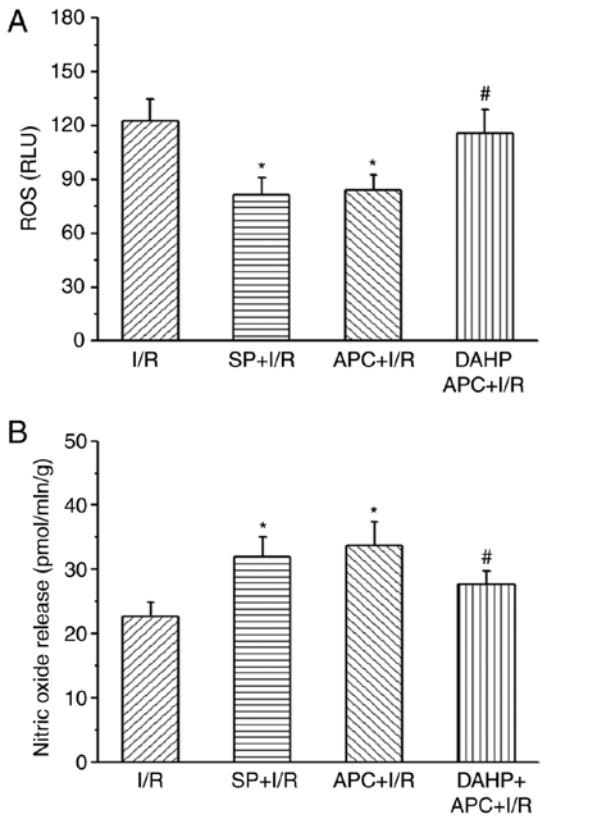


Figure 7. Lucigenin chemiluminescence signal intensity. Superoxide and NO production from hearts of I/R rats with/without SP (50 $\mu$M) treatment and APC + I/R with/without DAHP (2.5 mM) treatment at 1 min reperfusion. (A) ROS production in I/R, SP + I/R, APC + I/R and DAHP + APC + I/R groups. (B) NO levels in the I/R, SP + I/R, APC + I/R and DAHP + APC + I/R groups. The values are presented as mean ± standard error of the mean. n=6/group. *P<0.05 vs. I/R group. #P<0.05 vs. I/R + APC group. NO, nitric oxide; I/R, ischemia/reperfusion; APC, anesthetic preconditioning; SP, sepiapterin; DAHP, 2,4-diamino-6-hydroxypyrimidine.

hearts. NO release was increased in I/R alone hearts through SP supplementation, while DAHP significantly decreased NO production in APC + I/R hearts. These data confirmed that BH4 levels were associated with NOS-dependent NO production, and decreased $O_2^{\cdot-}$ generation.

A previous study has demonstrated that APC enhanced GTPCH-1 and NOS3 expression levels, and promoted the production of NO in the myocardium following reperfusion (11). In C57BL/6 mice hearts, the rate of LV pressure rise, BH4 level, $Ca^{2+}$ handling proteins and SR $Ca^{2+}$ release were decreased following I/R, which was associated with GCH1 degradation (25). In the coronary arteries of rats with heart failure, both NOS3 and GCH1 protein expression levels increased concomitantly with the decrease in ROS generation, increase of NO bioavailability and NOS3 coupling (26). A high level of GCH-1 expression in APC + I/R rats was also observed in the present study, indicating that increased GCH-1 expression is involved in volatile anesthetic-induced cardioprotection.

Previous studies have demonstrated that an increased association between HSP90 and NOS3 contributed to cardioprotection against I/R by increasing NO generation and decreasing $O_2^{\cdot-}$ production (7,27). The results of the present study also demonstrated that SP supplementation increased the association between HSP90 and NOS3, while DAHP supplementation decreased this association in APC + I/R hearts, consistent with the results of a previous study (8). These data demonstrated that BH4 improved NOS3 activity and function, which is an important mechanism underlying APC-induced cardioprotection.

In summary, the present study demonstrated that BH4 and GCH-1 differentially modulated APC-induced cardioprotection against I/R injury. BH4 biosynthesis, regulated by GCH-1, served a key role in the enhancement of NO generation during APC, and NO, in turn, conferred a cardioprotective effect. Furthermore, the differences in the BH4:BH2 ratio, which were associated with NOS3 coupling state and the increase in heart BH4 oxidation, conferred a cardioprotective effect in APC-treated hearts. These results indicated that APC may mediate resistance to I/R injury by enhancement of BH4 level and the association between HSP90 and NOS3. In addition, the combination of BH4 and HSP90 that contributes to the resistance to I/R injury may be dependent on increasing NOS3-coupled activity and function. Overall, the pharmacological targeting of the BH4-HSP90-NOS3 axis may represent a novel approach to alleviating myocardial I/R injury.

**Acknowledgements**

Not applicable.

**Funding**

The present study was supported by the Suzhou Science and Technology Development Plan (grant nos. SS201756 and SS201613); the Suzhou New District Science and Technology Project (grant no. 2017Z004); and the National Science and Technology Development Plan (grant no. NSFC 81703501).

**Availability of data and materials**

All data generated or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors' contributions**

CW, JA and AC were involved in the experimental design, data collection, data analysis and manuscript writing. SQ, LH, JS and TC performed the experiments. CW, JA and AC provided critical comments throughout the process of the present study. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Institutional Animal Care and Use Committee of Nanjing University

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors have declared that they have no competing interests.

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