Mol Cell Toxicol (2019) 15:297-305
DOI 10.1007/s13273-019-0033-9

ORIGINAL PAPER

Mild NO preconditioning protects H9c2 cells against NO-induced apoptosis through activation of PI3K/Akt and PKA-dependent pathways

Hyun-Jeong Kwak[1], **Jae-Young Um**[2] **& Sang-Seob Lee**[1]

Received: 23 October 2018 / Accepted: 14 January 2019

Abstract

Backgrounds: Nitric oxide (NO) plays a key role in cardioprotection. Its role against cardioprotection is dependent on the level of NO. Although it is well known that NO preconditioning has cardioprotective effects, but its mechanism remains unsatisfactory.
Methods: To induce NO preconditioning, H9c2 cells were treated with low NO concentration and subsequently induced apoptosis by high NO. The signalling and anti-apoptotic effects of NO preconditioning were monitored by Western blotting, facs analysis.
Results: Sodium nitroprusside (SNP)-induced cytotoxicity was inhibited by low SNP (0.3 mM) preconditioning. Furthermore, low SNP phosphorylated Akt/FoxO1 in the presence of high SNP (1.5 mM), while phosphorylated Akt/FoxO1 and viability were reversed by PI3K inhibitor. Also, low NO-induced CREB phosphorylation with high NO was inhibited by PKA inhibitor, indicating that NO preconditioning protects NO-induced cytotoxicity in PKA dependently. Apoptotic inhibition with NO preconditioning was accompanied with increased Bcl-2, decreased Bax, and caspase3 activation, which was all reversed by LY294002.
Conclusion: Our results indicate that low NO preconditioning prevent subsequent high NO-induced apoptosis via Akt/PKA/CREB activation in H9c2 cells.

[1]Department of Biological Engineering, Kyonggi University, 154-42 Gwanggosan-ro Yeongtong-gu, Suwon-si, Gyeonggi-do 16227, Republic of Korea
[2]Department of Pharmacology, College of Korean Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea
Correspondence and requests for materials should be addressed to S. S. Lee (✉sslee@kyonggi.ac.kr)

Keywords: Cardioprotection, Ischemia/Reperfusion, Preconditioning, Nitric oxide

Introduction

Powerful endogenous processes protect the heart and other organs from ischemia/reperfusion (I/R) damage. The phenomenon, ischemic preconditioning (IPC) is one of the most reproducible forms of cardioprotection. Since its initial description by Murry *et al.*[1], there has been sustained interest in unravelling the underlying mechanisms of IPC with the hope of pharmacologically mimicking its beneficial effects. Moreover, this concept recently has been extended to preconditioning induced by non-ischemic stress, including NO, hydrogen peroxide and reactive oxygen species (ROS). Among these, preconditioning with NO was reported to provide cardioprotection similar to ischemic preconditioning against I/R and this was blocked by treatment with L-nitro-arginine, a nonselective NOS inhibitor[2,3]. Specifically, NO appears to be a bi-directional regulator for apoptosis, as it can either promote or inhibit apoptosis in cardiomyocyte[4-6]. For example, high levels of NO produced by inducible nitric oxide synthase (iNOS) and exogenous NO can be promoted apoptosis while basal levels of NO produced by endothelial nitric oxide synthase (eNOS) prevent cardiomyocytes from apoptosis[7,8]. Accordingly, low levels of NO have been used as preconditioning mimetics and this effect was demonstrated to be cardioprotective in *in vitro* and *in vivo* models[9-12]. However, the mechanisms underlying these effects are not completely understood, and the identification of downstream signaling is important for understanding against NO preconditioning.

A number of diverse signaling pathways have been implicated in cardioprotection. One of them appears to be PI3K/Akt pathway, which is a crucial role in the regulation of myocardial cell growth, survival and proliferation[13,14]. The phosphorylation of Akt protects myocardial I/R injury by decreasing oxidative stress, repressing the inflammatory cascade, and inhibiting apoptosis[15-17]. Experimental investigation of the *in vivo* Akt gene transfer into the rat, the MI decreased by 64% in Akt transgenic rats compared with the control group after I/R[18].

The harmful effects of persistent PKA signaling during prolonged ischemia are well recognized[19]. However, activation of PKA has also been verified to cardioprotection, where adenylyl cyclase activator or phosphodiesterase type 4 (PDE 4) inhibitor all of which cause activation of PKA, protect the heart or cardiomyocytes against subsequent ischemia[20,21]. Similarly, IPC can activate PKA and subsequently phosphorylate eNOS, thereby leading to induce cardioprotection[22].

Herein, we investigate mechanism by which low levels of NO preconditioning against high levels of NO-induced H9c2 cardiomyocytes death. Our findings provide *in vitro* evidences that cardioprotective effects of NO preconditioning are mediated through PI3K/Akt/PKA signaling pathways.

Materials & Methods

Materials

DMEM, FBS, and other cell culture reagents were purchased from GibcoBRL (GrandIsland, NY, USA). SNP, FITC-annexin V, H89 were from Sigma (St Louis, MO, USA). JC-1 was from Invitrogen (Eugene, Oregon, USA). N^6-Benzoyl-sodium salt (N^6Bz-cAMP) were obtained by Calbiochem (La Jolla, CA, USA). Antibodies against Bcl-2 (#sc-7382), Bax (#sc-7480), and β-actin (#sc-47778) were the products of Santa Cruz (Santa Cruz, CA, USA). p-Akt^{ser473} (#9271), Akt (#9272), p-FoxO1^{ser256} (#9461) and FoxO1 (#2880) were from Cell Signaling (Beverly, MA, USA). p-CREB^{ser133} (#06-519) and CREB (#06-863) were from Upstate biotechnology (Lake Placid, NY, USA).

Culture of cardiomyocytes

H9c2 embryonic rat heart-derived cells obtained from ATCC (CRL-1446) and cultured in DMEM (Invitrogen, Groningen, Netherlands) supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. Cells were grown in 100 mm dishes at 37°C in 5% CO_2 in air and sub-cultured when they reached 70% confluence.

Induction of apoptosis and MTT assay

Apoptosis was generated *in vitro* using NO donor SNP. SNP was diluted extemporaneously in PBS and then used in our experiment. Cell viability was measured using an MTT assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide by mitochondrial reductases in live cells. Sub-confluent cells in 24 well plates were incubated with low concentration of NO (SNP 0.3 mM) for 4 h before exposed to SNP (1.5 mM) for additional 24 h. After high dose SNP treatment, 100 μL/well of MTT solution (5 mg/mL in PBS) was added. And cells were incubated at 37°C for 3 h before lysis in isopropanol 0.2% HCl. Absorbance at 570 nm was determined on 200 μL of each lysate and cell viability was calculated by comparing control cells, considered as 100% viable.

Assessment of apoptosis

The apoptosis was assessed by morphological feature using phase contrast microscopy and flow cytometry analysis. Apoptotic chromosomal condensation was determined by Hoechest33258 dye. Cells were washed with PBS and prepared in slide using a Cytospin (Cytospin, Shandon, UK) and fixed with methanol. And then, 1 μg/mL Hoechest33258 in PBS was placed on slide for 15 min and washed with distilled water. Cells were examined using a fluorescence microscope and condensed chromatin or fragmented nuclei in the cells were considered to be apoptotic cells. For facs analysis, apoptotic cells were identified by double staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propridium iodide (PI) using the Annexin V-FITC apoptosis detection kit from Genzyme (Cambridge, MA) as the manufacturer's protocol. After 24 h of culture under the different treatments, cells were harvested, then washed with PBS and re-suspended in a binding buffer. Next, the cells were incubated with FITC-Annexin V (1 μg/mL) and PI (20 μg/mL) for 10 min in the dark at room temperature and immediately analyzed by fluorescent-activated cell sorter Vantage flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA). The percentage of cells with Annexin V-positive staining were calculated as an apoptotic cells.

Western blot analysis

To prepare cell lysates, the cells were rinsed twice with ice-cold PBS and solubilized in lysis buffer (20 mM Tris, pH7.9, containing 1.0% Triton X-100, 1 mM Na_3VO_4, 137 mM NaCl, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 5 mM

Na_2EDTA, 10% glycerol, 1 mM β-glycerophosphate, 0.1 g/mL ρ-nitrophenyl phosphate, and 0.2 mM phenylmethylsulfonyl fluoride). The cells were scraped, and the supernatants were collected after 10 min of centrifugation at 14,000 × g and 4°C. Protein concentrations were determined using the detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Whole cell lysate (30 μg) were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min then separated using 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk for 1 h, rinsed, and incubated with specific antibodies against Akt, p-Akt, CREB, p-CREB, Bcl-2, and Bax in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4°C. Primary antibody was removed by washing the membranes 3 times in TBS-T, and the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1 : 1,000). Following 3 times of washing in TBS-T, immunopositive bands were visualized by ECL and exposed to X-ray film (Amersham, Piscataway, NJ).

Assessment of mitochondrial membrane potential (Ψ_m)

The mitochondrial membrane potential was measured using the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolycarbocyanine (JC-1; Invitrogen, Eugene, OR). After the treatment of chemical, cells in 96 well plates were treated with JC-1 (1 μg/mL) and stained with cells for 30 min at 37 °C. Then, cells were washed with PBS in twice. The fluorescence of cell was measured by using microplate fluorescence reader (excitation at 488 nm, emission at 595 nm; FLx800; Bio-Tek Instrument).

Caspase-3 activity assay

The details of caspase-3 activity have been described previously[11]. Caspase-3 activity was measured by colorimetric caspACE™ assay kit (Promega, Madison, WI). After the treatment of cells with indicated agent, cells were harvested and the pellet was resuspended in lysis buffer. After 15 min of incubation on ice, cell lysates were centrifuged 15,000 × g for 20 min and supernatants were removed for determination of caspase-3 activity. Proteolytic reactions were performed in total volume of 100 μL reaction buffer containing 54 μL of cytosolic extracts and 2 μL DEVD-PNA. The reaction mixture was incubated at 37°C for 4 h, and the formation of *p*-nitroaniline was measured at 405 nm by an ELISA reader (Spectra Max250, Molecular Device).

Statistical analysis

Data are represented as means ± SD of more than three separate experiments. The significance of difference from the respective control for each experimental test condition was assessed by using Student's t-test for each paired experiments. P value <0.05 was regarded as a significant difference.

Results

Protective effects of low NO preconditioning against high NO-induced apoptosis in H9c2 cells

To examine whether high concentration of NO-induced cell death in H9c2 cells, we treated with various concentration of SNP (0.1-2 mM). In Figure 1A, SNP treatment decreased cell viability concentration dependently, reaching a maximum death at a concentration of 2 mM (61.7 ± 3.2% reduction of viability compared to non-treated cells, $LD_{50} = 1.19$ mM), whereas had no significant cytotoxic effect up to 0.3 mM (low SNP). Hence, unless otherwise mentioned, cells were treated with 1.5 mM SNP (high SNP) to induce cell death. Next, to examine preconditioning effect of NO, cells were pretreated with nontoxic concentration of SNP (0.1 and 0.3 mM) for 4 h, then treated with toxic 1.5 mM SNP for 24 h. Result from Figure 1B shows that low SNP preconditioning protected cells from high SNP-induced cell death. In addition, the reversal of high SNP-induced cell death by low SNP preconditioning was associated with antiapoptotic effects since NO preconditioning reduced high SNP-induced apoptosis, determined by Annexin-V and Hoechest33258 (Figure 1C and 1D).

PI3K/Akt- and PKA-dependent pathways participated in the preconditioning effect of low NO against high NO-induced apoptosis

It has been well established that Akt pathway is involved in the regulation of cellular survival[13,14]. Thus, we examined whether the Akt activation is required for NO preconditioning. As shown in Figure 2A, LY29 4002 (5 μM) treatment abolished the effect of low NO-induced preconditioning against high NO-induced cell death (Figure 2A). In addition, both phosphorylation of Akt and FoxO1 increased in both low and high SNP, while low NO preconditioning for 4 h prior to high NO resulted in a further increase of Akt and FoxO1 phosphorylation (Figure 2B), and this effect was inhibited by LY294002, implying that Akt/FoxO1 pathway is involved in NO preconditioning.

In order to further explore the mechanism, we examined whether PKA-dependent pathway is involved in the low NO preconditioning on high NO-induced apoptosis. To evaluate the role of PKA in the protec-

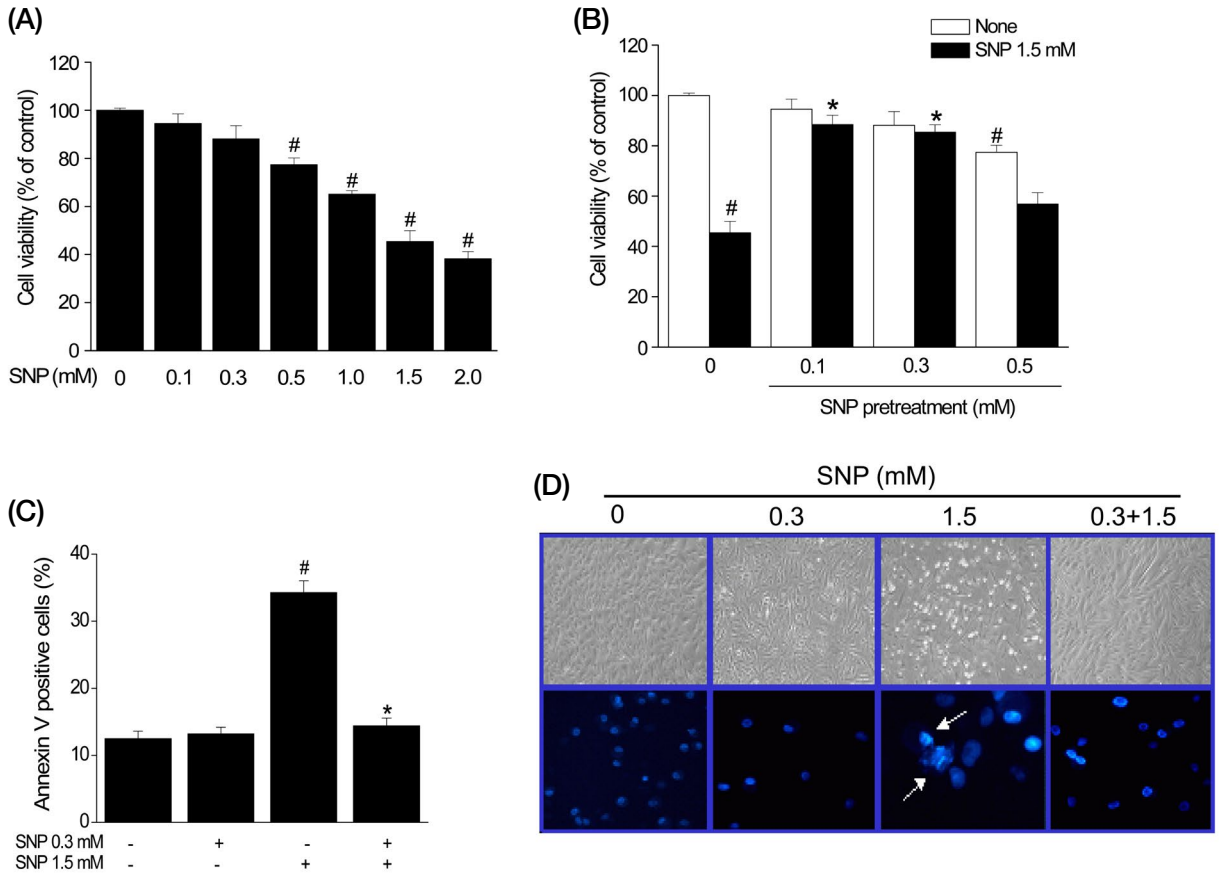

Figure 1. Low NO preconditioning prevents H9c2 cells from subsequent high SNP-induced apoptosis. Cells were exposed to different concentration of SNP for 24 h (A) or pretreated with indicated concentration of SNP (0.1-0.5 mM) for 4 h, then, incubated with 1.5 mM SNP for 24 h (B). Viability was analyzed by using MTT assay and the viability of control cells was set as 100. Cells were pretreated with 0.3 mM SNP for 4 h, followed by incubation with 1.5 mM SNP for 24 h, then the cells were washed and tested for apoptosis using FITC-annexin V assay (C), phase contrast (upper, magnification ×100) and Hoechest 33258 staining (D; bottom, magnification ×200). The arrow indicates the location of the fragmented nuclei. Data are the mean ± S.D of four experiments (each performed in duplicates). $^{\#}P<0.05$ vs. untreated cell. $^{*}P<0.05$ vs. 1.5 mM SNP-treated cell.

tive effect of NO preconditioning, we employed a specific PKA inhibitor, H89. As expected, protective effect of low NO preconditioning was abolished by H89 (5 μM) against high NO-induced apoptosis. To directly assess the involvement of PKA in NO-induced apoptosis, we examined the effect of N^6Bz-cAMP, an activator for PKA. Based on results, N^6Bz-cAMP (150 μM) mimicked the protective effect of NO preconditioning, while H-89 reversed effects of N^6Bz-cAMP. These results imply that the protection against NO-induced apoptosis require PKA signaling (Figure 2A). To confirm the involvement of PKA pathway, we examined CREB phosphorylation as an indicator of PKA activation. As shown in Figure 2C, CREB phosphorylation was increased by low NO preconditioning alone and this increase was synergistically phosphorylated in the presence of high NO treatment. Moreover, phosphorylation of CREB was completely inhibited by H89 (Figure 2C).

Akt phosphorylation by low NO preconditioning modulates CREB phosphorylation

According to our results, mechanisms of NO preconditioning are dependent on the activation of Akt/FoxO1 and PKA. Therefore, we examined whether Akt phosphorylation induced by low NO preconditioning is key player as an upstream molecule for contributing CREB activation. To test this hypothesis, cells were pretreated with LY294002 or H89 in the presence of low and high SNP. Based on the results, LY294002 blocked the CREB phosphorylation (Figure 3A), while H89 did not affect Akt phosphorylation suggesting that low NO preconditioning-induced Akt phosphorylation down-

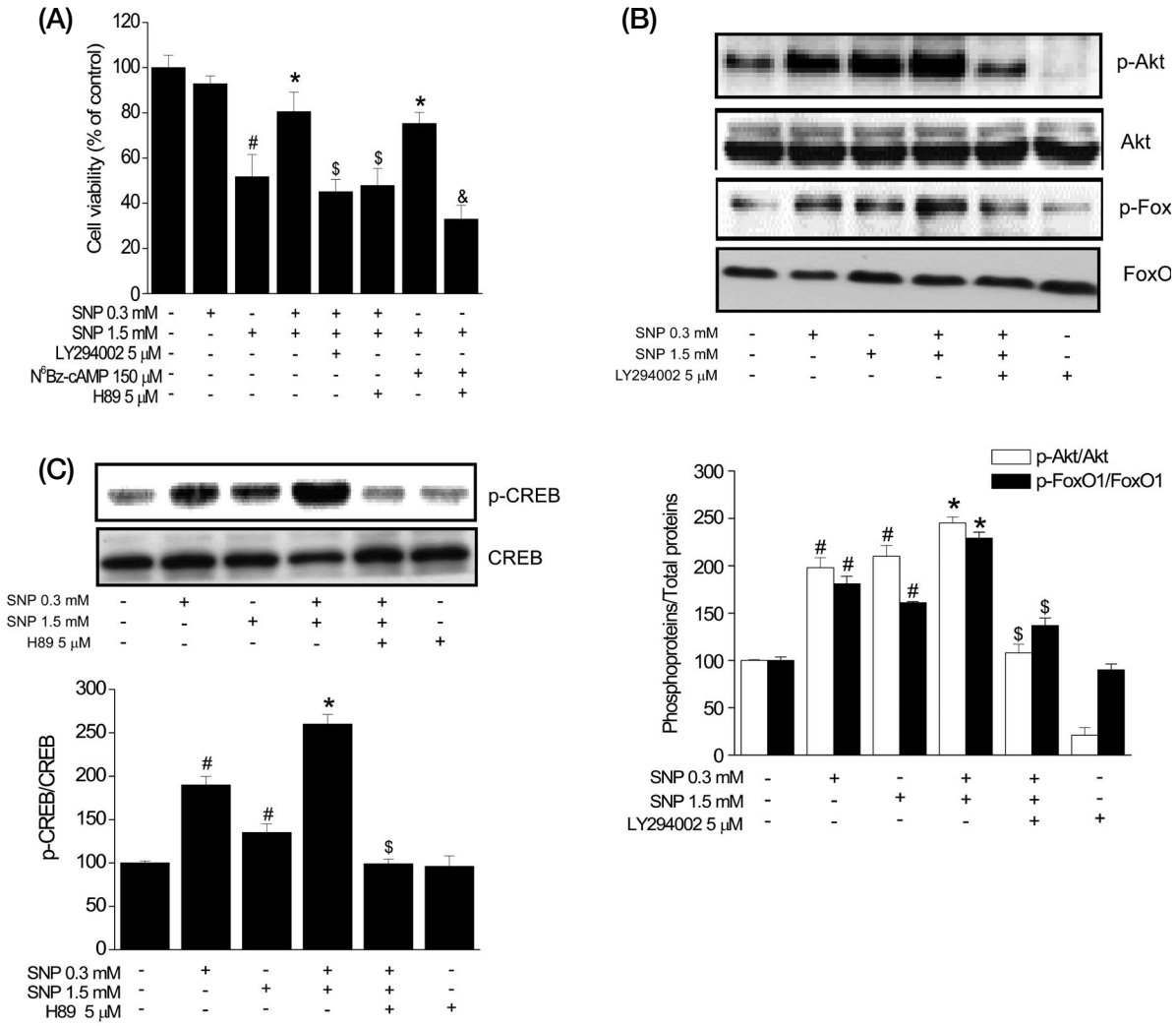

Figure 2. Roles of Akt-FoxO1-PKA signaling induced by low NO preconditioning against high SNP-induced apoptosis. H9c2 cells were preincubated with low SNP (0.3 mM) or N^6Bz-cAMP (150 μM) for 4 h in the absence or presence of LY294002 (5 μM) and H89 (5 μM), and then high SNP (1.5 mM) was treated for either 24 h (for MTT assay) or 30 min (for Western blot). Cell viability was determined by MTT assay (A) and p-Akt, Akt, p-FoxO1, FoxO1 (B) and p-CREB and CREB (C) were detected using Western blot. All phosphorylated forms were quantified by densitometry and normalized by their total forms. Data are represented as mean ± S.D. $^{\#}P<0.05$ vs. untreated cells. $^{*}P<0.05$ vs. 1.5 mM SNP-treated cells. $^{\$}P<0.05$ vs. 1.5 mM plus 0.3 mM SNP-treated cells. $^{\&}P<0.05$ vs. 1.5 mM plus N^6Bz-cAMP-treated cells. Shown immunoblots are the representative of three independent experiments.

regulates CREB phosphorylation (Figure 3B).

Low NO preconditioning modulates high NO-induced apoptosis via mitochondrial death pathway

Our previous results demonstrated that high NO-induced cell death through mitochondrial death pathway is closely related to mitochondrial membrane potential ($\Delta\Psi_m$), apoptosis-related Bcl-2 and Bax[11]. In line with previous results, we examined the effects of low NO preconditioning to modulate the mitochondrial potential and expression of apoptosis related proteins. In Figure 4A, treatment with high NO decreased the level of mitochondrial potential ($\Delta\Psi_m$) and rescued this suppression by low NO preconditioning. This protective effect of low NO preconditioning was abrogated with LY294002.

To more closely access the role of apoptotic regulation in low NO preconditioning, we checked Bcl-2 expression as an anti-apoptotic and Bax expression as a proapoptotic protein. High NO treatment profoundly decreased Bcl-2 expression and their expression was recovered by low NO preconditioning. Furthermore,

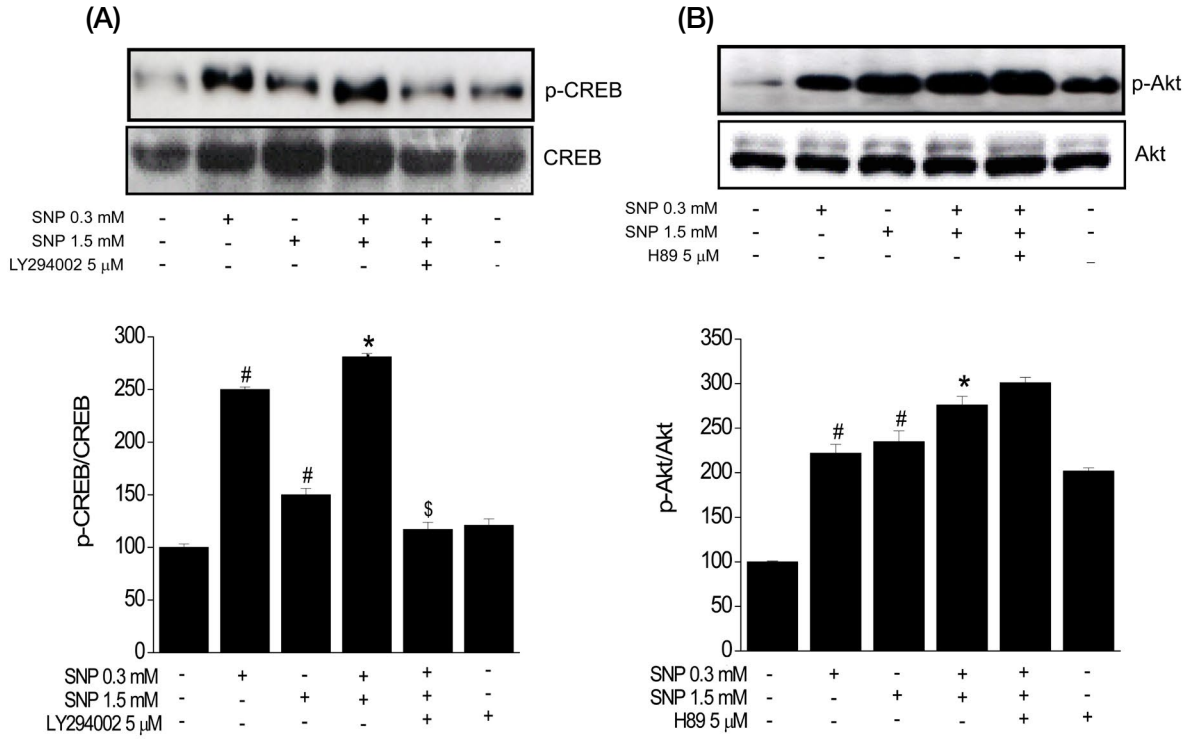

Figure 3. The effect of LY293002 and H89 on low NO preconditioning against high SNP-induced apoptosis. H9c2 cells were pretreated with LY294002 (5 μM) or H89 (5 μM) then incubated with SNP (0.3 mM) for 4 h. After incubation, high SNP (1.5 mM) was treated for 30 min to analyze protein levels. Levels of p-CREB, CREB (A) and p-Akt and Akt (B) were detected using Western blot. p-Akt and p-CREB expressions were quantified by densitometry and normalized by their total forms. Data are represented as mean ± S.D. $^{\#}P<0.05$ vs. untreated cells. $^{*}P<0.05$ vs. 1.5 mM SNP-treated cells. $^{\$}P<0.05$ vs. 1.5 mM plus 0.3 mM SNP-treated cells. Shown immunoblots are the representative of three independent experiments.

expression of Bax was significantly decreased by low NO preconditioning (Figure 4B). Likewise, caspase-3 activity was significantly increased by high SNP but this was suppressed with low SNP preconditioning (Figure 4C). However, beneficial effect of NO preconditioning disappeared after Akt pathway inhibition by LY294002. Thus, these results suggest that anti-apoptotic effect of low NO preconditioning was mediated by regulation of Bcl-2/Bax protein and caspase-3 activity via PI3K/Akt-dependent pathway.

Discussion

Nitric oxide has emerged as a signaling molecule that mediates cytoprotection during I/R in a number of organ[23-25]. In the heart, low concentration of NO decrease infarct size in MI animal models[26,27]. This well-established phenomenon, called NO preconditioning provides cardioprotective effects[2,9,10-12]. However, questions regarding these signaling pathway of NO preconditioning remain unclear, and how precisely NO preconditioning regulates cardiomyocytes protection. In the present study, preconditioning with low NO ameliorated the high NO-induced cell death, these protective effect of NO preconditioning is mediated by PI3K/Akt/PKA pathway.

Cardiomyocytes apoptosis is dominant in the pathogenesis of myocardial I/R injury[28] and prevention of apoptotic cell attenuates cardiomyocytes loss and protects I/R injury. Particularly, NO can induce cardiomyocytes apoptosis in higher concentration while exerts protective effects against I/R injury in a range of low concentration[12]. Consistent with these prior observations, our data in this study provide comprehensive evidence that low levels of NO preconditioning are necessary for the prevention of H9c2 cardiomyocytes apoptosis against high levels of NO-induced apoptosis. Accumulating studies with NO preconditioning have demonstrated a critical role of NO against I/R injury, however, the molecular mechanism of NO preconditioning remains unresolved.

PI3K/Akt pathway has been implicated as a crucial effector signaling for cardioprotection against I/R injury[15-17,29]. Activation of Akt can be both beneficial and detrimental depending on the intensity and duration of

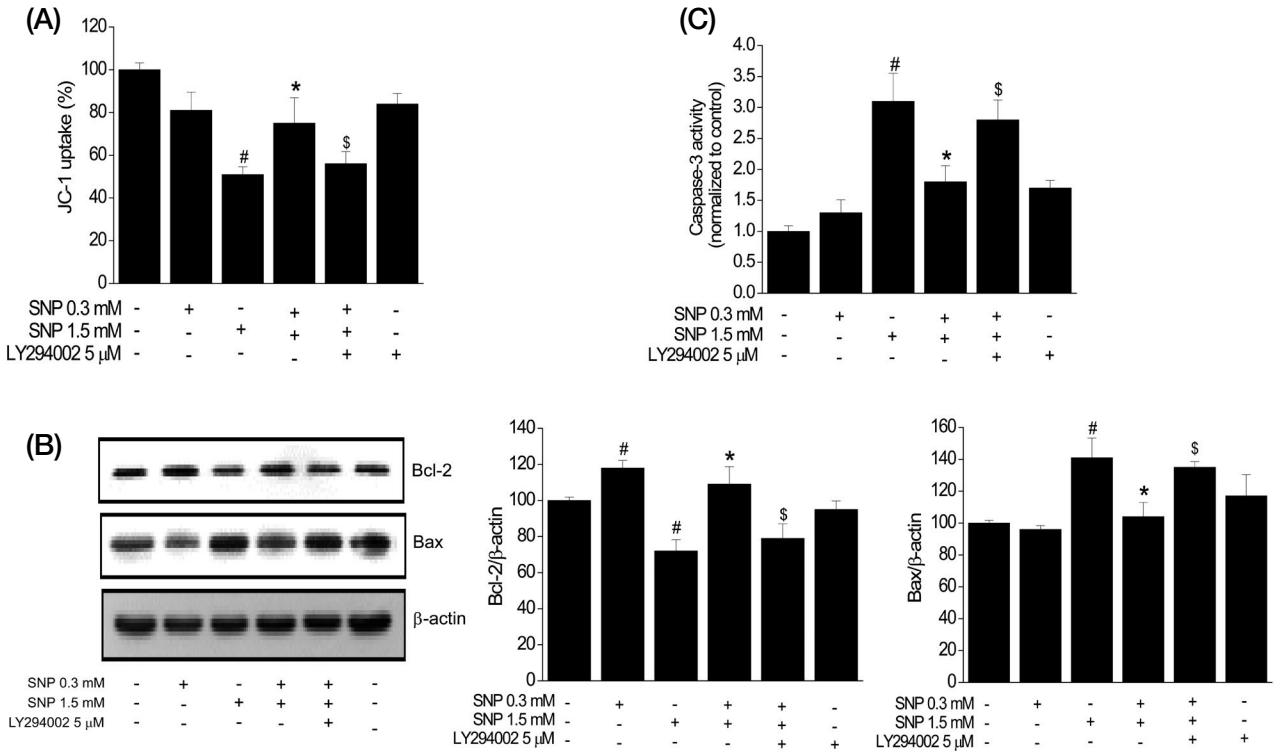

Figure 4. Effect of low NO preconditioning on the regulation of $\Delta\Psi_m$, Bcl-2, Bax, and caspase-3 activity. Cells were pretreated with low SNP for 4 h then incubated with high SNP for 24 h. After the incubation, cells were stained with JC-1 for 30 min and red/green fluorescence ratio was calculated as described in the Materials and methods section (A), protein extracts were assayed for Bcl-2 and Bax using western blot analysis (B), and caspase-3 activity was measured by colorimetric assay kit (C). Bcl-2 and Bax expressions were quantified by densitometry and normalized by their control. Data are represented as mean ± S.D. $^{\#}P<0.05$ versus untreated control, $^{*}P<0.05$ versus 1.5 mM SNP-treated cells and $^{\$}P<0.05$ versus 0.3 mM SNP and 1.5mM SNP-treated cells. Shown immunoblots are the representative of three independent experiments.

effects. Diverse effects have been shown between normal protective responses with two to six-fold increase in Akt activation vs pathological responses with high level (15-80 fold) of Akt activation[30]. Correspondingly, we observed that Akt activation occurs when there is a treatment of both low and high levels of NO. Furthermore, three-fold increase in Akt phosphorylation is seen in low NO preconditioning in the presence of high NO and this was abolished by LY294002, concurrent with the loss of cell viability induced by NO preconditioning. FoxO1 is an important transcription factor, which is associated with cell cycle regulation, oxidative stress and apoptotic gene expression[31]. Akt as an upstream regulator of FoxO1 inhibits FoxO1 activity by phosphorylating FoxO1 at three conserved phosphorylation sites[32,33]. According to our study, increased p-FoxO1 expression was observed during NO preconditioning, and its effect was blocked by LY294002, thus suggesting an important role of PI3/Akt/FoxO1 pathway in NO preconditioning-induced cardioprotection. We and others have previously established that cell death mechanisms mediated by NO have been attributed predominantly to the inactivation of mitochondrial respiratory chain, DNA damage and Bcl-2 downregulation/Bax upregulation[34,35]. This is consistent with our current studies, demonstrating that high NO-induced downregulation of Bcl-2 and upregulation of BAX were all reversed by low NO preconditioning. Additionally, antiapoptotic effects mediated by NO preconditioning were blocked by LY294002, downregulating of Bcl-2 and upregulating of Bax protein, implying that antiapoptotic effect were associated with Akt signaling.

Despite an important role of Akt in NO preconditioning, other effector signaling can also be involved in NO preconditioning-induced cardioprotection. NO exerts antiapoptotic effects via cGMP/PKG-mediated interruption of apoptotic signaling pathway[36], while in rodents, a NO-cGMP-PKA signal pathway has been described[37,38]. Since the involvement of PKA upon NO preconditioning is not fully elucidated in cardiomyocytes, we proposed possible mechanism whether NO

preconditioning activates PKA dependent pathway. Indeed, activation of PKA in the myocardium during IPC has been reported to limit infarct size of heart[22]. Prior work from our group suggest that PDE4 inhibitor increase cellular cAMP levels, leading to phosphorylation of PKA and CREB is essential in the cardioprotection against NO-induced cardiomyocytes apoptosis[21]. Similar to our previous reports, our observation confirmed that PKA-CREB signaling appears to mediate NO preconditioning-induced cardioprotection. Additionally, we observed inhibition of Akt pathway abolished NO preconditioning-induced CREB phosphorylation. Thus, it is evident that PKA-mediated cardioprotection is Akt-dependent mechanism.

Conclusion

In conclusion, the results presented here describe preconditioning mechanism by which low level of NO mediates cardioprotective effect during high levels of NO-induced apoptosis. These data demonstrate NO preconditioning protects cardiomyocytes from NO-induced apoptosis via activation of PI3K/Akt/PKA signaling pathway. Together, NO preconditioning may be an attractive potential therapeutic target for cardiac I/R injury.

Acknowledgements This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (NRF-2017R1A2B1011688 and 2018R1A2A3075684).

Conflicts of Interests Hyun-Jeong Kwak, Jae-Young Um & Sang-Seob Lee declare that they have no competing interests.

Human and animal rights The article does not contain any studies with human and animal and this study was performed following institutional and national guidelines.

References

1. Murry, C. E., Jennings, R. B. & Reimer, K. A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**, 1124-1136 (1986).
2. Bolli, R. *et al*. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res* **81**, 42-52 (1997).
3. Xuan, Y. T. *et al*. Endothelial nitric oxide synthase plays an obligatory role in the late phase of ischemic preconditioning by activating the protein kinase C epsilon p44/42 mitogen-activated protein kinase pSer-signal transducers and activators of transcription1/3 pathway. *Circulation* **116**, 535-544 (2007).
4. Beltran, B., Mathur, A., Duchen, M. R., Erusalimsky, J. D. & Moncada, S. The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A* **97**, 14602-14607 (2000).
5. Davidson, S. M. & Duchen, M. R. Effects of NO on mitochondrial function in cardiomyocytes: Pathophysiological relevance. *Cardiovasc Res* **71**, 10-21 (2006).
6. Razavi, H. M., Hamilton, J. A. & Feng, Q. Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. *Pharmacol Ther* **106**, 147-162 (2005).
7. Song, Y. H., Neumeister, M. W., Mowlavi, A. & Suchy, H. Tumor necrosis factor-alpha and lipopolysaccharides induce differentially interleukin 8 and growth related oncogene-alpha expression in human endothelial cell line EA.hy926. *Ann Plast Surg* **45**, 681-683 (2000).
8. Feng, Q. *et al*. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. *Circulation* **106**, 873-879 (2002).
9. Cohen, M. V., Yang, X. M. & Downey, J. M. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. *Cardiovasc Res* **70**, 231-239 (2006).
10. Bice, J. S., Jones, B. R., Chamberlain, G. R. & Baxter, G. F. Nitric oxide treatments as adjuncts to reperfusion in acute myocardial infarction: a systematic review of experimental and clinical studies. *Basic Res Cardiol* **111**, 23 (2016).
11. Kwak, H. J. *et al*. Preconditioning with low concentration NO attenuates subsequent NO-induced apoptosis in vascular smooth muscle cells via HO-1-dependent mitochondrial death pathway. *Toxicol Appl Pharmacol* **217**, 176-184 (2006).
12. Kwak, H. J., Park, K. M. Choi, H. E. & Park, H. Y. Protective mechanisms of NO preconditioning against NO-induced apoptosis in H9c2 cells: role of PKC and COX-2. *Free Radic Res* **43**, 744-752 (2009).
13. Cantley, L. C. The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657 (2002).
14. Terragni, J. *et al*. Phosphatidylinositol 3-kinase signaling in proliferating cells maintains an anti-apoptotic transcriptional program mediated by inhibition of FOXO and non-canonical activation of NFkappaB transcription factors. *BMC Cell Biol* **9**, 6 (2008).
15. Wu, H. *et al*. Nicorandil protects the heart from ischemia/reperfusion injury by attenuating endoplasmic reticulum response-induced apoptosis through PI3K/Akt signaling pathway. *Cell Physiol Biochem* **35**, 2320-2332 (2015).
16. Li, X. *et al*. Short-term hesperidin pretreatment attenuates rat myocardial ischemia/reperfusion injury by inhibiting high mobility group box 1 protein expression via the PI3K/Akt pathway. *Cell Physiol Biochem* **39**, 1850-1862 (2016).
17. Yu, H. *et al*. Gypenoside protects against myocardial

ischemia-reperfusion injury by inhibiting cardiomyocytes apoptosis via inhibition of CHOP pathway and activation of PI3K/Akt pathway in vivo and in vitro. *Cell Physiol Biochem* **39**, 123-136 (2016).
18. Matsui, T. *et al*. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* **104**, 330-335 (2001).
19. Engelhardt, S., Hein, L., Wiesmann, F. & Lohse, M. J. Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. *Proc Natl Acad Sci U S A* **96**, 7059-7064 (1999).
20. Makaula, S. *et al*. H-89, a non-specific inhibitor of protein kinase A, promotes post-ischemic cardiac contractile recovery and reduces infarct size. *J Cardiovasc Pharmacol* **45**, 341-347 (2005).
21. Kwak, H. J. *et al*. PDE4 inhibitor, roflumilast protects cardiomyocytes against NO-induced apoptosis via activation of PKA and Epac dual pathways. *Cell Signal* **20**, 803-814 (2008).
22. Yang, C., Talukder, M. A., aradharaj, S., Velayutham, M. V. & Zweier, J. L. Early ischaemic preconditioning requires Akt- and PKA-mediated activation of eNOS via serine1176 phosphorylation. *Cardiovasc Res* **97**, 33-43 (2013).
23. Duranski, M. R. *et al*. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest* **115**, 1232-1240 (2005).
24. Tripatara, P. *et al*. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J Am Soc Nephrol* **18**, 570-580 (2007).
25. Jung, K. H. *et al*. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke* **37**, 2744-2750 (2006).
26. Gonzalez, F. M. *et al*. Nitrite anion provides potent cytoprotective and antiapoptotic effects as adjunctive therapy to reperfusion for acute myocardial infarction. *Circulation* **117**, 2986-2994 (2008).
27. Bryan, N. S. *et al*. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injur. *Proc Natl Acad Sci U S A* **104**, 19144-19149 (2007).
28. Oerlemans, M. I. *et al*. Targeting cell death in the reperfused heart: pharmacological approaches for cardioprotection. *Int J Cardiol* **165**, 410-422 (2013).
29. Liu, H., Guo, X., Chu, Y. & Lu, S. Heart protective effects and mechanism of quercetin preconditioning on anti-myocardial ischemia reperfusion (IR) injuries in rats. *Gene* **545**, 149-155 (2014).
30. O'Neill, B. T. & Abel, E. D. Akt1 in the cardiovascular system: friend or foe? *J Clin Invest* **115**, 2059-2064 (2005).
31. Szydlowski, M., Jablonska, E. & Juszczynski, P. FOXO1 transcription factor: a critical effector of the PI3K-AKT axis in B-cell development. *Int Rev Immunol* **33**, 146-157 (2014).
32. Tzivion, G., Dobson, M. & Ramakrishnan, G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochim Biophys Acta* **1813**, 1938-1945 (2011).
33. Xin, Z. *et al*. FOXOs in the impaired heart: New therapeutic targets for cardiac diseases. *Biochim Biophys Acta* **1863**, 486-498 (2017).
34. Bolanos, J. P. *et al*. Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. *J Neurochem* **68**, 2227-2240 (1997).
35. Tamatani, M., Ogawa, S., Nunez, G. & Tohyama, M. Growth factors prevent changes in Bcl-2 and Bax expression and neuronal apoptosis induced by nitric oxide. *Cell Death Differ* **5**, 911-919 (1998).
36. Dimmeler, S., Haendeler, J., Nehls, M. & Zeiher, A. M. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J Exp Med* **185**, 601-607 (1997).
37. Garg, U. C. & Hassid, A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* **83**, 1774-1777 (1989).
38. Cornwell, T. L., Arnold, E., Boerth, N. J. & Lincoln, T. M. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* **267**, C1405-1413 (1994).