Free Radical Research, August 2009; 43(8): 744–752

informa
healthcare

Protective mechanisms of NO preconditioning against NO-induced apoptosis in H9c2 cells: role of PKC and COX-2

HYUN-JEONG KWAK*, KYOUNG MI PARK*, HYE-EUN CHOI, & HYUN-YOUNG PARK

Division of Cardiovascular Diseases, Center for Biomedical Sciences, National Institutes of Health, Eunpyung-gu, Seoul 122-701, Korea

(Received 18 March 2009; revised 8 May 2009)

Abstract

Cardiomyocyte apoptosis is involved in several cardiovascular diseases, including ischemia, hypertrophy and heart failure. Nitric oxide (NO) signalling is crucial in the regulation of cardiomyocyte apoptosis, capable of both inducing and preventing apoptosis depending upon the level of NO production. Growing evidence suggests that NO preconditioning has cardioprotective effects, but the mechanism remains unclear. The purpose of this study was to elucidate how NO preconditioning inhibits subsequent NO-induced apoptosis in H9c2 cells. According to the data, preconditioning with a low concentration of sodium nitroprusside (SNP, 0.3 mM) inhibited subsequent high-concentration-SNP (1.5 mM)-induced apoptosis and this effect was reversed by the protein kinase C (PKC) inhibitor chelerythrine and the cyclooxygenase-2 (COX-2) inhibitor rofecoxib. Low-concentration-SNP-mediated protection involved extracellular signal regulated kinase 1/2 (ERK1/2), a signal transducers and activators of transcription 1/3 (STAT1/3) activation and increased COX-2 expression. Activation of ERK1/2 and STAT1/3 was abolished by chelerythrine. However, COX-2 expression was not inhibited, implying that the COX-2-mediated protective effect occurred via a PKC-independent pathway. The results showed that low-concentration-SNP preconditioning suppresses subsequent high-concentration-SNP-induced apoptosis by ERK1/2-STAT 1/3 activation via PKC-dependent mechanisms in H9c2 cells. COX-2 also plays a role in NO-induced preconditioning, but is independent of PKC.

Keywords: *Cardiomyocyte, ischemia, preconditioning, nitric oxide, protein kinase C, COX-2*

Introduction

Programmed cell death or apoptosis is a conserved process that plays a critical role in embryonic development and adult homeostasis. In humans and mice, deregulated apoptosis has been implicated in the pathophysiology of major illnesses, including cancer and cardiovascular diseases [1]. In particular, apoptosis of cardiomyocytes is involved in several cardiovascular diseases and has been recognized as a cellular mechanism of ischemic/reperfusion (I/R) injury in the heart [2,3]. Apoptosis can be induced in cardiomyocytes by several stimuli. Among them, nitric oxide (NO), which plays a key role in cardiovascular regulation, has been reported to be a bidirectional regulator of apoptosis, as it can either promote or inhibit apoptosis in cardiomyocytes [4–8]. In this regard, high levels of NO produced by inducible nitric oxide synthase (iNOS) [9,10] and exogenous NO promote apoptosis, whereas basal levels of NO production from endothelial nitric oxide synthase (eNOS) protect cardiomyocytes from apoptosis [11]. Therefore, the amount of NO and its origin appear to be important factors in the survival outcome of cardiomyocytes. Given its pro- and anti-apoptotic capacity within the heart, NO may serve as a valuable therapeutic target in myocardial ischemia and heart failure.

Ischemic preconditioning is a cardioprotective phenomenon [12] that is thought to be a universal protective mechanism in the body. Recently, this

*These authors contributed equally to this work.

Correspondence: Hyun-Young Park, MD, PhD, Division of Cardiovascular Diseases, Center for Biomedical Sciences, National Institutes of Health, Nokbun-dong, Eunpyung-gu, Seoul 122-701, Korea. Tel: +82-2-380-1534. Email: hypark65@nih.go.kr

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760903040602

concept has been extended to preconditioning induced by non-ischemic stress, including NO, hydrogen peroxide and reactive oxygen species (ROS). Preconditioning with NO was reported to provide cardioprotection similar to ischemic preconditioning against I/R [13–16]. The effect of preconditioning was blocked by treatment with L-nitro-arginine, a non-selective NOS inhibitor [14]. Recently, we found that low-concentration NO triggered preconditioning and provided protection from high-concentration-NO-induced apoptosis through the induction of heme oxygenase-1 (HO-1) in vascular smooth muscle cells (VSMC) [17]. Nevertheless, to our knowledge, the intracellular signalling events that are activated by NO and the molecular mechanisms underlying this radical trigger of preconditioning in H9c2 cells remain unknown. In the present study, we examined the effect of NO preconditioning on H9c2 cells, focusing on protein kinase C (PKC) and cyclooxygenase-2 (COX-2), which are well-characterized intracellular signalling mediators that act during preconditioning.

The PKC family of enzymes plays a role in several cellular signal transduction pathways and is implicated in numerous physiological and pathological processes [18]. In the heart, PKC plays a crucial role in preconditioning [19]. It was observed that the protection following ischemic preconditioning is abolished by PKC inhibitors *in vivo* and *in vitro* [20–22]. Specifically, considerable evidence supports the idea that NO triggers preconditioning through the activation of PKC; and NO preconditioning is abrogated by PKC inhibitor treatment of the heart [23], indicating that this NO-mediated preconditioning involves a PKC-mediated signalling pathway. A plausible target for PKC-mediated signalling events is the family of p44/42 mitogen-activated protein kinases (MAPKs) that have been isolated from heart tissue [24,25]. Previous studies have shown that ischemic preconditioning induces a rapid activation of extracellular signal regulated kinase 1/2 (ERK1/2) via the PKC-dependent pathway and can be abolished by the ERK inhibitor PD98059 in isolated cardiomyocytes [26]. It was also reported that NO induced by eNOS is required for the development of the cardioprotective effect of preconditioning by activating the PKCε-ERK1/2 pathway [13]. Furthermore, ischemic preconditioning was found to upregulate ERK1/2- signal transducers and activators of transcription 1/3 (STAT1/3)-COX-2 sequential induction via the PKC (especially ε)-dependent pathway [27].

COX is an enzyme involved in the metabolism of arachidonic acid (AA). Two important forms of COX have been identified. COX-1 is a form that is constitutively expressed in most cells. COX-2 is rapidly induced by cytokines and various stress stimuli and is present in the brain, heart and kidney [28]. COX-2 is the rate-limiting enzyme for prostaglandin (PG) synthesis, catalysing the conversion of AA to prostaglandin H_2 (PGH_2) [29]. Recently, COX-2 induction has also been implicated as a mediator of cell protection [30]. For example, COX-2 activation protects cardiomyocytes against oxidative stress. There is convincing evidence that the expression and activity of COX-2 are upregulated by ischemic preconditioning and this increase in COX-2 mediates protective effects against myocardial infarction (MI) [31], suggesting that the COX-2 pathway is also essential in conferring the cardioprotection afforded by preconditioning. Although there have been some contradicting reports that the inhibition of COX-2 activity reduces cardiac damage after MI [32], little is known about how COX-2 induction by low-concentration-NO preconditioning regulates cardioprotective effects.

Therefore, we investigated whether preconditioning with low-concentration NO could protect against high-concentration-NO-induced apoptosis in H9c2 cells and also investigated the underlying mechanism of this protective effect, with emphasis on the roles of PKC, ERK1/2, STAT1/3 and COX-2. Here, we report that low-NO preconditioning protected H9c2 cells from subsequent NO-induced apoptosis. Furthermore, this anti-apoptotic effect of NO preconditioning was modulated by dual signalling mechanisms, involving both PKC-dependent-ERK-STAT1/3 phosphorylation and the PKC-independent COX-2 pathway.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and other cell culture reagents were purchased from Gibco-BRL (Grand Island, NY). Sodium nitroprusside (SNP), FITC-annexin V, phorbol myrystate acetate (PMA) and chelerythrine were purchased from Sigma (St Louis, MO). PD98059 were obtained from Calbiochem (San Diego, CA). Specific antibody against ERK1/2, p-ERK1/2 and COX-2 were the products of Santa Cruz (Santa Cruz, CA) and STAT1/3 was from Upstate biotechnology (Charlottesville, VA). STAT1/3^{Tyr705} and p-STAT1/3^{ser727} were from Cell Signaling (Beverly, MA). PGE_2 EIA kit was purchased from Cayman chemical (Ann Arbor, MI). All other reagents were the products of Sigma chemical (St. Louis, MO) unless indicated otherwise.

Cell culture

The H9c2 embryonal rat heart-derived cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in DMEM with 10% FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells from passages 20–30 were used for all studies.

To achieve quiescence, cells were starved for 24 h in 0.1% FBS.

Cell viability assay

Cell viability was determined by a 3-[4,5-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Subconfluent H9c2 cells in 12 well plates were incubated with various inhibitors for 1 h before being exposed to low SNP (0.3 mM) for 4 h and then high SNP (1.5 mM) was treated for 18 h. After SNP stimulation, 100 μl/well of MTT solution (5 mg/ml in PBS) was added and the cells were incubated for 3 h more. The medium was replaced with dimethylsulfoxide (DMSO). The plates were shaken for 20 min, then optical density (OD) was measured at 570 nm using the microplate reader (Boehringer Mannheim, IN).

FITC-annexin V and propidium iodide double staining by flow cytometry

Double staining with FITC-annexin V and propidium iodide (PI) was performed for flow cytometry analysis. Cells were washed with PBS and resuspended in a binding buffer. FITC- annexin V (1 μg/ml) and PI (20 μg/ml) were added. The mixture was incubated for 10 min in the dark and FACS analysis was done using FACSVantage system (Becton Dickinson, San Jose, CA).

Analysis of PGE_2 concentration

H9c2 cells (4×10^5 cells/well) were pretreated with inhibitors for 1 h and then incubated with 0.3 mM SNP for 4 h. After the incubation, high SNP (1.5 mM) was treated for 18 h. The levels of PGE_2 concentration was measured in cell culture media using Cayman chemical EIA kit and measured according to the manufacture's instruction.

Western blot analysis

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 ERK1/2, p-ERK1/2, COX-2, STAT1/3, STAT1/3^{Tyr705} and p-STAT1/3^{ser727} in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4°C. Primary antibody was removed by washing the membranes three times in TBS-T and the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2000). Following three washes in TBS-T, immuno-positive bands were visualized by ECL kits (Santacruze, CA) and exposed to X-ray film (Amersham, Piscataway, NJ).

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. A p-value < 0.05 was regarded as a significant difference.

Results

Preconditioning with low-concentration NO protects H9c2 cells from high-concentration-NO-induced apoptosis

To examine the role of NO, we treated H9c2 cells with SNP. SNP did not affect cell viability up to 0.3 mM, but it significantly decreased cell viability at concentrations ≥ 0.5 mM (data not shown). In order to determine whether the protective effect of NO preconditioning reported in other cell types also occurs in H9c2 cells, we treated cells with SNP. Preconditioning of H9c2 cells with various concentrations of SNP for 4 h protected cells from 1.5 mM SNP-induced cell death at non-toxic concentrations of SNP (0.1 ~ 0.3 mM). In contrast, preconditioning with 0.5 mM SNP resulted in no cytoprotective effects (Figure 1A). Preconditioning of the cells with low SNP also attenuated high-SNP-induced apoptosis, as determined by an annexin-V assay (Figure 1B).

PKC is a mediator of NO preconditioning against high-concentration-NO-induced H9c2 cell apoptosis

As a first step in investigating the role of PKC in NO preconditioning, we used the PKC inhibitor chelerythrine. Pretreatment with chelerythrine (1 μM) 1 h before preconditioning with 0.3 mM SNP abolished the protective effect of preconditioning against high-SNP-induced apoptosis (Figure 2A). We then evaluated whether direct PKC activation leads to protection against NO-induced apoptosis using PKC activators. Phorbol myristate acetate (PMA) treatment partially reversed high-SNP-induced apoptosis (Figure 2B). These results suggest that the protective effect of low-NO preconditioning is mediated by a PKC-dependent pathway.

ERK1/2 signalling is involved in NO preconditioning via a PKC-dependent pathway in H9c2 cells

To investigate the molecular mechanism underlying NO preconditioning, we then investigated the role of ERK1/2 signalling in NO-induced preconditioning. As shown in Figure 3A, treatment with the mitogen-activated protein kinase kinase (MAPKK) inhibitor PD98059 (10 μM) prior to NO preconditioning abrogated the protective effect of NO preconditioning against high SNP-induced cell death. Our data showed that ERK1/2 was down-regulated by high-SNP

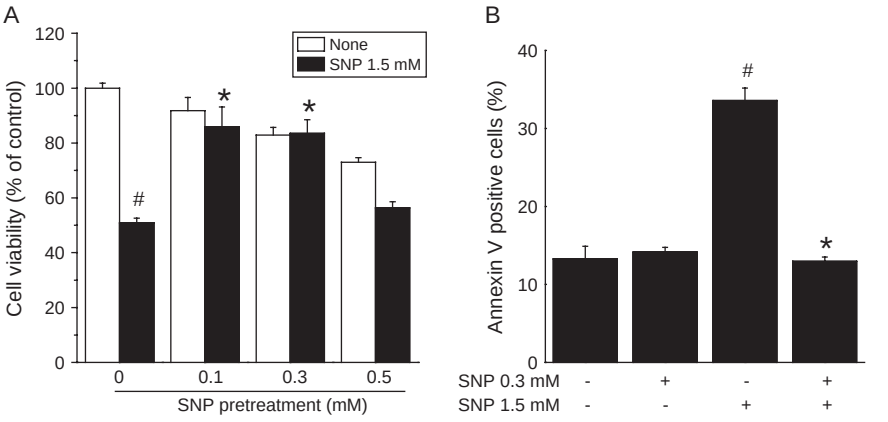

Figure 1. Preconditioning using low SNP protects H9c2 cells from subsequent high-SNP-induced apoptosis. (A) Cells were treated with SNP at the indicated concentrations for 4 h. Then, they were incubated with 1.5 mM SNP for 24 h and their viability was analysed. Cell viability was also measured after SNP treatment using a MTT assay. Relative cellular viability was calculated by dividing the optical density of SNP-treated cells by the optical density of untreated control cells. The viability of the control cells was set at 100. (B) Cells were pretreated with 0.3 mM SNP for 4 h, followed by incubation with high SNP for 24 h as indicated. After the SNP treatment, the cells were washed and tested for apoptosis using an FITC-annexin V assay. Data shown are the mean ± SD of four experiments (each performed in duplicate). $^{\#}p < 0.05$ vs untreated control, $^{\star}p < 0.05$ vs 1.5 mM SNP-treated control.

treatment, whereas low-NO preconditioning against high-SNP treatment markedly increased ERK1/2 phosphorylation. Furthermore, ERK1/2 phosphorylation was completely blocked by chelerythrine treatment, suggesting that ERK signalling by NO preconditioning is mediated by a PKC-dependent pathway (Figure 3B).

STAT1/3 signalling is involved in NO preconditioning via a PKC-ERK1/2-dependent pathway in H9c2 cells

It was previously reported that ischemic preconditioning activates STAT1/3 via a PKC-ERK1/2 dependent pathway *in vivo* [27]. Therefore, we next examined whether PKC-ERK1/2 activation by NO preconditioning can activate STAT1/3^{ser727} in H9c2 cells. According to our data, low-SNP preconditioning significantly increased STAT1/3 phosphorylation of the Ser-727 residue against high-SNP treatment, whereas STAT1/3 phosphorylation was not detectable in cells exposed to high-SNP treatment without preconditioning. We then examined the involvement of the PKC-ERK1/2 pathway in STAT1/3 activation. When cells were pretreated with chelerythrine or PD98059 prior to low-SNP exposure, phosphorylation of STAT1/3 was inhibited (Figure 4). No significant Tyr705 phosphorylation of STAT1/3 was observed (data not shown). Taken together, these

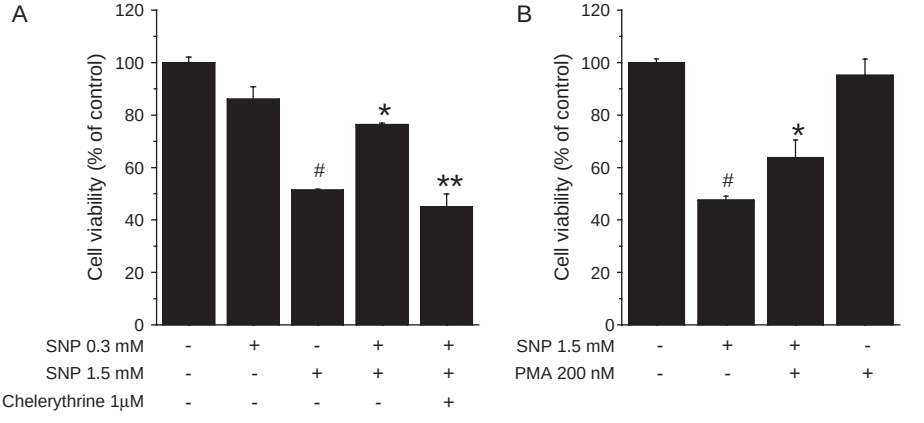

Figure 2. Low-SNP-mediated preconditioning protects against subsequent high-SNP-induced apoptosis via a PKC-dependent pathway. (A) Chelerythrine was added 30 min before the low-SNP pretreatment (for 4 h). After 4 h of preconditioning with low SNP, cellular apoptosis was induced by high-SNP treatment (1.5 mM for 24 h). (B) Cells were pretreated with 200 nM PMA for 1 h and then apoptosis was induced by 1.5 mM SNP for 24 h. Following incubation, the cells were washed and the cellular viability was measured using the MTT assay. Data shown are the mean ± SD of four experiments (each performed in duplicate). $^{\#}p < 0.05$ vs untreated control, $^{\star}p < 0.05$ vs 1.5 mM SNP-treated group, $^{\star\star}p < 0.05$ vs 0.3 mM plus 1.5 mM SNP-treated group.

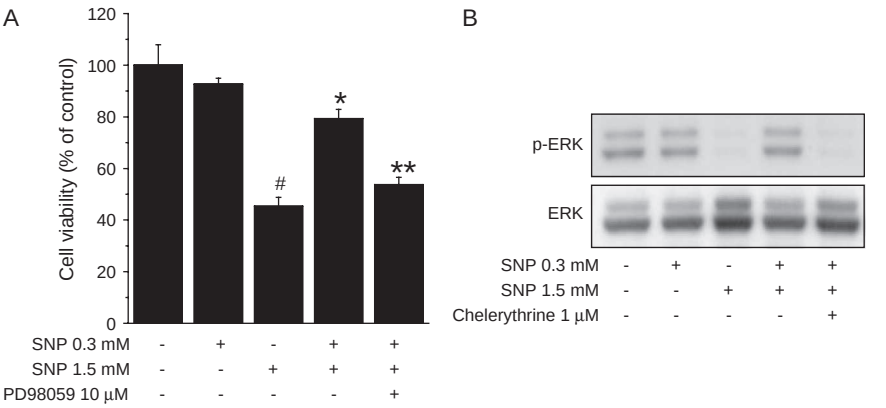

Figure 3. Effects of chelerythrine on ERK1/2 phosphorylation caused by low-SNP-induced preconditioning. (A) Cells were pretreated with 10 μM PD98059 for 30 min before low-SNP pretreatment for 4 h, followed by incubation with high SNP for 24 h as indicated. Cellular viability was determined using the MTT assay. (B) Cells were pretreated with 1 μM chelerythrine for 30 min and then incubated with 0.3 mM SNP for 4 h. Following incubation, the cells were treated with high SNP (1.5 mM) for 20 min. The cells were harvested for ERK1/2 activation and detected using immunoblot analysis. $^{\#}p < 0.05$ vs untreated control, $^{*}p < 0.05$ vs 1.5 mM SNP-treated group, $^{**}p < 0.05$ vs 0.3 mM plus 1.5 mM SNP-treated group.

data indicate an obligatory role for PKC in the activation of ERK1/2 and the Ser phosphorylation of STAT1/3 for the induction of NO preconditioning.

COX-2 induction by low-concentration-NO preconditioning protects H9c2 cells from high-concentration-NO-induced apoptosis

Since COX-2 is known to play a crucial role in the protective effect of ischemic preconditioning [31], we explored whether COX-2 is also involved in the NO preconditioning mechanism. According to our data, COX-2 induction was dramatically increased by low-SNP treatment, whereas high-SNP treatment slightly increased COX-2 expression. Moreover, COX-2 induction by low-SNP preconditioning was maintained after high-SNP treatment (Figure 5A). Treatment with the COX-2-specific inhibitor rofecoxib (30 μM) abrogated the protective effect of low-SNP-induced preconditioning, implying that COX-2 is involved in NO preconditioning (Figure 5B).

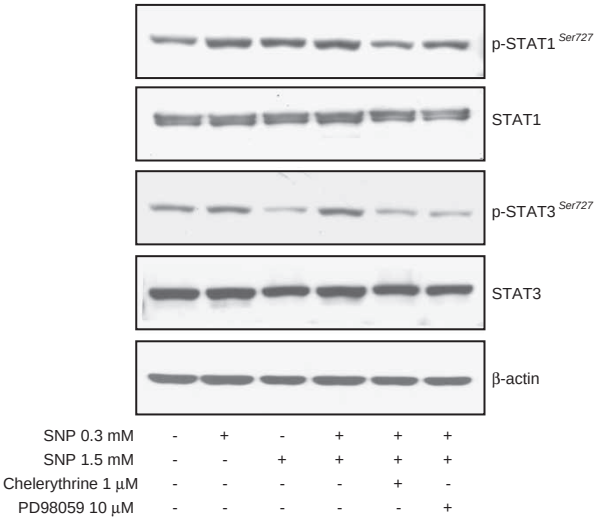

Figure 4. Effects of PD98059 and chelerythrine on STAT1/3^{Ser727} phosphorylation caused by low-SNP-induced preconditioning. Cells were pretreated with PD98059 (10 μM) or chelerythrine (1 μM) for 1 h and then incubated with 0.3 mM SNP for 4 h. After incubation, the cells were treated with high SNP (1.5 mM) for 20 min. Total protein was isolated and subjected to immunoblot analysis for STAT1/3^{Ser727} activation. The results are representative of at least three similar experiments performed on different experimental sets.

NO preconditioning upregulates COX-2 protein levels in a PKC-independent manner

To explore the involvement of the PKC pathway in COX-2 induction, PKC or ERK1/2 inhibitors were administered 1 h before low-SNP preconditioning treatment, followed by exposure to high levels of SNP. Interestingly, neither treatment affected the COX-2 levels, indicating that the COX-2 pathway is regulated by a PKC-independent signalling pathway (Figure 6A). In accordance with COX-2 expression, the level of PGE_2 increased significantly following low-SNP preconditioning. PGE_2 production was not blocked by chelerythrine or PD98059 treatment, supporting the concept that COX-2 induction by low-NO preconditioning is independent of PKC (Figure 6B).

Discussion

Recent studies have shown that preconditioning using NO helps to ameliorate ischemia/reperfusion in a variety of animal models, including heart [14,33]. However, precisely how NO preconditioning confers cellular protection on H9c2 cells remains unclear. In the present study, we showed that NO preconditioning using a low concentration of SNP exerts protective effects against high-concentration-SNP-induced apoptosis in H9c2 cells. Furthermore, this protective

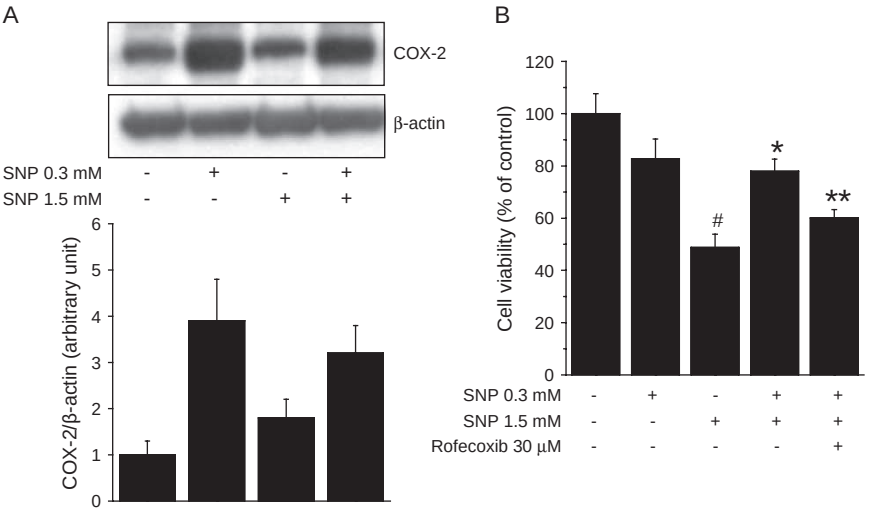

Figure 5. COX-2 induction by low SNP-mediated preconditioning protects against subsequent high-SNP-induced apoptosis. (A) Cells were exposed to low SNP for 4 h followed by exposure to high SNP for 5 h. After 5 h, total protein was isolated and subjected to immunoblot analysis for COX-2. COX-2 levels were quantified by densitometry and normalized to β-actin. (B) Rofecoxib (30 μM) was added for 30 min before low-SNP pretreatment for 4 h. After 4 h, cellular apoptosis was induced by high-SNP treatment (1.5 mM for 24 h). Cell viability was analysed using the MTT assay. Data shown are the mean ± SD of four experiments (each performed in duplicate). $^{\#}p < 0.05$ vs untreated control, $^{\star}p < 0.05$ vs 1.5 mM SNP-treated group, $^{\star\star}p < 0.05$ vs 0.3 mM and 1.5 mM SNP-treated group.

effect is primarily mediated by a PKC-dependent ERK1/2-STAT1/3 pathway and a PKC-independent COX-2 pathway.

NO has been reported to induce apoptosis in a variety of cell types, including macrophages, chondrocytes, smooth muscle cells and cardiac myocytes [34–37]. However, NO can produce dual effects depending upon the concentrations used. It has been reported that in H9c2 cells, a small amount of NO had an anti-apoptotic effect, whereas higher concentrations of NO promoted apoptosis [4–8]. Furthermore, studies have shown that NO exerts protective effects against ischemia/reperfusion injury *in vivo* in a range of low concentrations [38], but high concentrations of NO are cytotoxic [39]. In agreement with such reports, our data also indicated that preconditioning H9c2 cells with low concentrations of NO protected the cells from subsequent high-concentration-NO-induced apoptosis. To further understand the mechanisms underlying NO preconditioning, we examined the roles of PKC and COX-2 as possible modulators

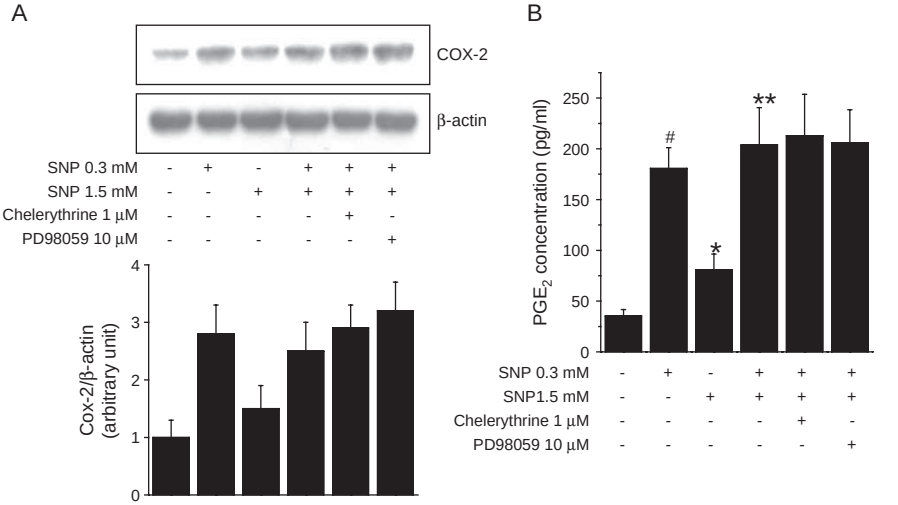

Figure 6. COX-2 induction by low-SNP-induced preconditioning is independent of PKC. Cells were pretreated with PD98059 (10 μM) or chelerythrine (1 μM) for 1 h and then incubated with 0.3 mM SNP for 4 h. Following incubation, cells were treated with high SNP (1.5 mM) for 5 or 18 h. (A) After 5 h, total protein was isolated and subjected to immunoblot analysis for COX-2. COX-2 levels were quantified by densitometry and normalized to β-actin. (B) After 18 h, the supernatant was collected and the PGE_2 concentration measured using a PGE_2 ELISA kit. Data shown are the mean ± SD of four experiments (each performed in duplicate). $^{\#}p < 0.05$ vs untreated control, $^{\star}p < 0.05$ vs 0.3 mM SNP-treated group, $^{\star\star}p < 0.05$ vs 1.5 mM SNP-treated group.

of the protective effect observed in the present study.

One of the best characterized intracellular signalling events during ischemic preconditioning is the activation of PKC [19]. The potential role of PKC enzymes in cardioprotection has been the subject of many investigations. Recent studies have shown that PKC activation contributes to ischemic tolerance in a variety of experimental animal models [19–22]. Also, PKC activation by NO preconditioning protected rabbit heart from I/R injuries [23]. Thus, we specifically investigated the role of PKC and the underlying mechanisms that mediate the cytoprotective process induced by NO preconditioning in H9c2 cells.

Pretreatment with low concentrations of SNP (up to 0.3 mM) protected cell viability after high-SNP treatment. This protective effect of low-SNP pretreatment was abrogated when the PKC inhibitor chelerythrine was co-administered with low SNP, suggesting that PKC plays an important role in low-SNP-induced cellular protection mechanisms. Furthermore, the known PKC activator phorbol myristate acetate (PMA) alone could mimic the protective effect of low-SNP pretreatment. These results strongly substantiated our assumption that PKC is one of the major molecules mediating the protective effects of NO preconditioning. Several studies have reported the importance of PKCε in cardioprotection induced by preconditioning [13,27]. In the heart, NO produced a cardiac protective effect through the activation of PKCε [23]. However, in the present study, we did not identify which PKC isoforms are involved in the NO preconditioning pathway. Thus, assessment of the PKC isoform relevant to the protection induced by low-NO preconditioning is of interest. Our preliminary study showed that PKCδ and PKCε are involved in protective mechanisms induced by NO preconditioning. We are currently working on this issue.

It is generally accepted that ERK1/2 is activated by I/R injuries and that it plays an important role in the ventricular recovery and protection of the ischemic heart [40,41]. However, little is known about how NO preconditioning directly regulates the ERK1/2 pathway in H9c2 cells. According to our data, preconditioning with low-SNP-induced ERK1/2 phosphorylation protected against H9c2 cell apoptosis caused by high-SNP treatment. Regarding the ERK1/2 pathway in PKC-mediated preconditioning, studies have demonstrated the importance of PKC in the activation of ERK1/2 by ischemic preconditioning [26]. Overall, such findings agree with our results that chelerythrine inhibits the ERK1/2 phosphorylation induced by low-SNP preconditioning.

It has been suggested that the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway is essential for the development of preconditioning [42]. Ser phosphorylation of STATs was induced by the ERK1/2 pathway [43,44]. Indeed, it is well documented that activation of ERK1/2 by ischemic preconditioning induces Ser phosphorylation of STAT1/3 via a PKC-dependent mechanism in the heart [27]. Therefore, we postulated that Ser phosphorylation of STAT1/3 is mediated by a PKC-ERK1/2 signalling cascade in NO preconditioning. We found that low-SNP preconditioning led to an increase in pSer(727)-STAT1/3, which is inhibited by chelerythrine and PD98059, demonstrating that Ser phosphorylation of STAT1/3 by low-NO preconditioning is mediated primarily via a PKC-dependent pathway. Nevertheless, we found no evidence of Tyr phosphorylation of STAT1/3 by immunoblot analysis, indicating that Tyr phosphorylation is not involved in our low-SNP preconditioning mechanisms (data not shown). These data suggest that a NO-mediated PKC pathway plays a critical role in the activation of downstream ERK1/2-Ser phosphorylation of STAT1/3.

COX-2 plays a necessary role in mediating the cardioprotection induced by ischemic preconditioning. Inhibition of COX-2 activity during preconditioning results in complete loss of protection against infarction [31,32]. Thus, we hypothesized that NO preconditioning upregulates COX-2 expression to mediate cytoprotection in H9c2 cells. Our data showed that low-NO preconditioning induced the production of COX-2 protein and its inhibitor, rofecoxib, reversed the protective effect against high-NO-induced cell death, demonstrating that COX-2 induction by low-NO preconditioning is involved in cardioprotection in H9c2 cells. However, the signalling mechanism by which NO preconditioning induces the synthesis of COX-2 protein is incompletely understood. There is evidence that COX-2 transcription after ischemic preconditioning is modulated by PKCε-mediated ERK1/2 and STAT1/3 Ser phosphorylation [27]. This was also demonstrated in our study, since PKC-mediated ERK1/2-Ser phosphorylation of STAT1/3 activation occurred following NO preconditioning. Interestingly, treatment with chelerythrine and PD98059 has not been shown to inhibit COX-2 induction by NO preconditioning, indicating that this event is mediated by a distinct PKC-independent signalling pathway. Thus, which upstream signalling pathway leads to the induction of COX-2 should be further investigated. We are currently working on this issue and it will be addressed in a future report.

Myocardial I/R has been implicated in the induction of inducible nitric oxide synthase (iNOS) that lead to increased production of NO, however the role of NO in the heart has yielded conflicting reports regarding the severity of I/R injury. It seems that high, non-physiological levels of NO produced by iNOS actually promote cellular necrosis and apoptosis through formation of peroxynitrite, while basal NO

production from endothelial nitric oxide synthase (eNOS) is essential for cardiomyocyte survival and can provide cardioprotective effects [45]. According to this knowledge, NO appears to be an important regulator of apoptosis in the heart during normal physiological conditions as well as in myocardial I/R. Since, as shown in our *in vitro* study, preconditioning with low-concentration NO inhibited high-concentration NO-induced apoptosis in cardiomyocyte, further studies will be needed to examine whether NO preconditioning also protects myocardial infarction *in vivo* via PKC- and COX-2-dependent pathways.

In conclusion, our results suggest that PKC activation by NO preconditioning using low concentrations of SNP suppress H9c2 cell apoptosis induced by high SNP. Furthermore, we demonstrated that the mechanism of NO preconditioning was modulated by ERK1/2-Ser phosphorylation of STAT1/3 via a PKC-dependent pathway and COX-2 induction by a PKC-independent pathway. Therefore, our findings provide new insights into the mechanisms of NO preconditioning and its downstream molecule may serve as a valuable therapeutic target in myocardial I/R injury.

Acknowledgements

This work was supported by the Korea National Institute of Health intramural research grant 4800-4845-300-210.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Rudin CM, Thompson CB. Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu Rev Med 1997;48:267–281.

[2] van Empel VP, Bertrand AT, Hofstra L, Crijns HJ, Doevendans PA, De Windt LJ. Myocyte apoptosis in heart failure. Cardiovasc Res 2005;67:21–29.

[3] Kitsis RN, Mann DL. Apoptosis and the heart: a decade of progress. J Mol Cell Cardiol 2005;38:1–2.

[4] Beltran B, Mathur A, Duchen MR, Erusalimsky JD, 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 USA 2000;97:14602–14607.

[5] Davidson SM, Duchen MR. Effects of NO on mitochondrial function in cardiomyocytes: pathophysiological relevance. Cardiovasc Res 2006;71:10–21.

[6] Pinsky DJ, Aji W, Szabolcs M, Athan ES, Liu Y, Yang YM, Kline RP, Olson KE, Cannon PJ. Nitric oxide triggers programmed cell death (apoptosis) of adult rat ventricular myocytes in culture. Am J Physiol 1999;277:H1189–H1199.

[7] Razavi HM, Hamilton JA, Feng Q. Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. Pharmacol Ther 2005;106:147–162.

[8] Iwase H, Robin E, Guzy RD, Mungai PT, Vanden Hoek TL, Chandel NS, Levrau J, Schumacke PT. Nitric oxide during ischemia attenuates oxidant stress and cell death during ischemia and reperfusion in cardiomyocytes. Free Radic Biol Med 2007;43:590–599.

[9] Ing DJ, Zang J, Dzau VJ, Webster KA, Bishopric NH. Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ Res 1999;84:21–33.

[10] Song W, Lu X, Feng Q. Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. Cardiovasc Res 2000;45:595–602.

[11] Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. Circulation 2002;106:873–879.

[12] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–1136.

[13] Xuan YT, Guo Y, Zhu Y, Wang OL, Rokosh G, Bolli R. 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 2007;116:535–544.

[14] Bolli R, Bhatti ZA, Tang XL, Qiu Y, Zhang Q, Guo Y, Jadoon AK. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. Circ Res 1997;81:42–52.

[15] Qiu Y, Rizvi A, Tang XL, Manchikalapudi S, Takano H, Jadoon AK, Wu WJ, Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. Am J Physiol 1997;273:H2931–H2936.

[16] Takano H, Tang XL, Qiu Y, Guo Y, French BA, Bolli R. Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant-sensitive mechanism. Circ Res 1998;83:73–84.

[17] Kwak HJ, Par KM, Lee S, Lim HJ, Go SH, Eom SM, Park HY. 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 2006;217:176–184.

[18] Newton AC. Protein kinase C: structure, function, and regulation. J Biol Chem 1995;270:28495–28498.

[19] Ytrehus K, Liu Y, Downey JM. Preconditioning protects ischemic rabbit heart by protein kinase C activation. Am J Physiol 1994;266:H1145–H1152.

[20] Beguin PC, Belaidi E, Godin-Ribuot D, Levy P, Ribuot C. Intermittent hypoxia-induced delayed cardioprotection is mediated by PKC and triggered by p38 MAP kinase and Erk1/2. J Mol Cell Cardiol 2007;42:343–351.

[21] Baxter GF, Goma FM, Yellon DM. Involvement of protein kinase C in the delayed cytoprotection following sublethal ischaemia in rabbit myocardium. Br J Pharmacol 1995;115:222–224.

[22] Baxter GF, Mocanu MM, Yellon DM. Attenuation of myocardial ischaemic injury 24 h after diacylglycerol treatment in vivo. J Mol Cell Cardiol 1997;29:1967–1975.

[23] Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. Circ Res 1999;84:587–604.

[24] Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben Levy R, Ashworth A, Marshall CJ, Sugden PH. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. Circ Res 1996;79:162–173.

[25] Bogoyevitch MA, Ketterman AJ, Sugden PH. Cellular stresses differentially activate c-Jun N-terminal protein

kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. J Biol Chem 1995;270:29710–29717.

[26] Ping P, Zhang J, Cao X, Li RC, Kong D, Tang XL, Qiu Y, Manchikalapudi S, Auchampach JA, Black RG, Bolli R. PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. Am J Physiol 1999;276:H1468–H1481.

[27] Xuan YT, Guo Y, Zhu Y, Wang OL, Rokosh G, Messing RO, Bolli R. Role of the protein kinase C-epsilon-Raf-1-MEK-1/2-p44/42 MAPK signaling cascade in the activation of signal transducers and activators of transcription 1 and 3 and induction of cyclooxygenase-2 after ischemic preconditioning. Circulation 2005;112:1971–1978.

[28] Yasojima K, Schwab C, McGeer EG, McGeer PL. Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. Brain Res 1999;830:226–236.

[29] Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 1996;271:33157–33160.

[30] McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci USA 1999;96:272–277.

[31] Shinmura K, Tang XL, Wan Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. Pro. Natl Acad Sci USA 2000;97:10197–10202.

[32] LaPointe MC, Mendez M, Leung A, Tao Z, Yang XP. Inhibition of cyclooxygenase-2 improves cardiac function after myocardial infarction in the mouse. Am J Physiol Heart Circ Physiol 2004;286:H1416–H1424.

[33] Koti RS, Seifalian AM, Davidson BR. Protection of the liver by ischemic preconditioning: a review of mechanisms and clinical applications. Dig Surg 2003;20:383–396.

[34] Messmer UK, Brune B. Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. Biochem J 1996;319:299–305.

[35] Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146:75–85.

[36] Nishio E, Fukushima K, Shiozaki M, Watanabe Y. Nitric oxide donor SNAP induces apoptosis in smooth muscle cells through cGMP-independent mechanism. Biochem Biophys Res Commun 1996;221:163–168.

[37] Arstall MA, Sawyer DB, Fukazawa R, Kelly RA. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ Res 1999;85:829–840.

[38] Gourine AV, Bulhak AA, Gonon AT, Pernow J, Sjoquist PO. Cardioprotective effect induced by brief exposure to nitric oxide before myocardial ischemia-reperfusion in vivo. Nitric Oxide 2002;7:210–216.

[39] Rakhit RD, Mojet MH, Marber MS, Duchen MR. Mitochondria as targets for nitric oxide-induced protection during simulated ischemia and reoxygenation in isolated neonatal cardiomyocytes. Circulation 2001;103:2617–2623.

[40] Omura T, Yoshiyama M, Shimada T, Shimizu N, Kim S, Iwao H, Takeuchi K, Yoshikawa J. Activation of mitogen-activated protein kinases in in vivo ischemia/reperfused myocardium in rats. J Mol Cell Cardiol 1999;31:1269–1279.

[41] Punn A, Mockridge JW, Farooqui S, Marber MS, Head RJ. Sustained activation of p42/p44 mitogen-activated protein kinase during recovery from simulated ischaemia mediates adaptive cytoprotection in cardiomyocytes. Biochem J 2000;350:891–899.

[42] Xuan YT, Guo Y, Han H, Zhu Y, Bolli R. An essential role of the JAK-STAT pathway in ischemic preconditioning. Proc Natl Acad Sci USA 2001;98:9050–9055.

[43] Decker T, Kovarik P. Serine phosphorylation of STATs. Oncogene 2000;19:2628–2637.

[44] Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol 1997;17:6508–6516.

[45] Liu P, Hock CE, Nagele R, Wong PY. Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. Am J Physiol 1997;272:H2327–H2336.

This paper was first published online on iFirst on 25 June 2009.