

ISCHEMIC AND PHARMACOLOGICAL INDUCTION OF DELAYED CELLULAR PROTECTION IN iNOS GENE-DISRUPTED MICE MYOCYTES

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Inducible nitric oxide synthase (iNOS) has been implicated as a mediator in myocardial protection, but this property of iNOS is still conflicting. Therefore, the present study was designed to assess whether iNOS really contributes to the ischemically and pharmacologically induced delayed cellular protection (DCP) in mice myocytes. The following groups of cultured iNOS gene-knockout (iNOS^{-/-}), and its respective wild-type (wt) mice myocytes subjected to simulated ischemia (SI) at 20 h were studied: (a) wt + SI: with ischemia alone; (b) iNOS^{-/-} + SI: with ischemia alone; (c) iNOS^{-/-} + heat shock (HS): iNOS^{-/-} and HS; (d) iNOS^{-/-} + sub-lethal simulated ischemia (SSI): iNOS^{-/-} and SSI; (e) iNOS^{-/-} + A₁AR agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA): iNOS^{-/-} and 1 μM CCPA; (f) iNOS^{-/-} + A₁AR agonist (2S)-N⁶-[2-endo-norbornyl]adenosine (S-ENBA): iNOS^{-/-} and 1 nM S-ENBA; (g) iNOS^{-/-} + K_{ATP} channel opener pinacidil (Pin): iNOS^{-/-} and 0.05 μM Pin, and (h) iNOS^{-/-} + mitochondrial K_{ATP} channel opener diazoxide (Diazo): iNOS^{-/-} and 100 μM Diazo. The release of LDH into the medium as well as the amount of LDH remaining in the cells was used as a marker of cellular injury and cell viability. The cellular resistance was acquired by iNOS^{-/-} mice myocytes due to HS, SSI, CCPA, S-ENBA, pinacidil and diazoxide treatment, which was evidenced by reduction of LDH (U/L) release from 51.14 ± 1.35 (iNOS^{-/-}) to 42.20 ± 1.01 (iNOS^{-/-} + HS); 45.57 ± 0.75 (iNOS^{-/-} + SSI); 42.87 ± 0.87 (iNOS^{-/-} + CCPA); 43.21 ± 0.70 (iNOS^{-/-} + S-ENBA); 37.81 ± 0.99 (iNOS^{-/-} + Pin) and 36.79 ± 0.68 (iNOS^{-/-} + Diazo), p < 0.01. Our data suggest that heat shock (HS), sub-lethal simulated ischemia (SSI), A₁ adenosine agonists CCPA, S-ENBA and K_{ATP} channel openers pinacidil (membrane K_{ATP} channel), diazoxide (mitochondrial K_{ATP} channel) induce delayed cellular protection in mice myocytes against subsequent sustained simulated ischemia without the involvement of iNOS. Further, our data also suggest that pinacidil and diazoxide are more potent inducers of delayed cellular protection among others in iNOS^{-/-} mice myocytes against sustained simulated ischemia.

Key words: *simulated ischemia, K_{ATP} channel, iNOS, late protection, heat shock*

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Abbreviations: A₁AR – A₁ adenosine receptor, CCPA – chloro-N⁶-cyclopentyl adenosine (A₁AR agonist), DCP – delayed cellular protection, Diazo – diazoxide (mitochondrial K_{ATP} channel opener), iNOS – inducible nitric oxide synthase, iNOS-/- – inducible nitric oxide synthase knockout, Pin – pinacidil (K_{ATP} channel opener), SI – simulated ischemia, wt – wild type

INTRODUCTION

Brief episodes of ischemia increase the tolerance of myocardium to injury by a second ischemic insult 24 to 72 h later [4]. This phenomenon is known as the delayed phase or second window of protection (SWOP). The delayed phase of preconditioning is preceded by an early phase, which is known to last 2 to 3 h after the initial preconditioning insult [5, 6, 14, 17, 18, 24]. Adenosine A₁ receptor (A₁AR) activation has also been shown to trigger delayed preconditioning in a number of animal species [5, 11]. Further, pharmacological studies have provided evidence that opening of K_{ATP} channels is necessary for the infarct-sparing effects of late preconditioning with ischemia [8] and A₁AR agonist treatment [12]. While delayed myocardial protection has been shown to be efficacious in reducing infarct size resulting from ischemia, the mechanisms regarding the role of inducible nitric oxide synthase (iNOS) that mediate this protection remain unclear. Bolli's group [13, 25] has shown the implication of the synthesis of nitric oxide (NO) as being an important mediator of delayed ischemic preconditioning. Where NO production has been abrogated, the resistance to ischemia is lost. NO can be synthesized by any of the three nitric oxide synthase (NOS) isoforms, nNOS or eNOS or iNOS found in myocardium [9]. Endogenous NO formation is catalyzed by a family of NOS enzymes that directly produce NO from L-arginine and NADPH in a calmodulin-dependent reaction that stoichiometrically produces citrulline as a co-product [10]. The corresponding protein products have been named according to their original sites of identification. Endothelial and neuronal nitric oxide synthases (eNOS and nNOS, respectively) are constitutively expressed NOS isoforms whose activity is regulated by cytosolic concentration of calcium and by the presence of cofactors such as tetrahydrobiopterin (BH₄), magnesium, and NADPH [2]. The iNOS is a calcium-independent synthase whose ac-

tivity appears to be depended upon protein transcription [1], while the iNOS protein is negligible in unstressed myocardium [3, 13]. However, iNOS is markedly induced following ischemic stress by signaling cascades resulting in the up-regulation of transcription factors [16, 27]. This protein transcription leads to upregulation of iNOS and thus NO synthesis has led many investigators to suggest that iNOS is the source of NO in delayed preconditioning. Recently, Robert et al. [23] demonstrated the role of eNOS in delayed A₁AR-mediated preconditioning for protecting the ischemic heart. Also in our preliminary studies, pharmacological induction of delayed cellular protection (DCP) has been observed in iNOS gene-disrupted mice myocytes [21]. The potential importance of this observation in the context of heat shock or ischemic as well as pharmacological preconditioning has not been explored.

To investigate, whether the iNOS isoform is involved in the mediation of delayed ischemic or heat shock or pharmacological preconditioning, we used sub-lethal simulated ischemia for ischemic preconditioning, sub-lethal heat shock for heat shock preconditioning, and A₁AR agonists, 2-chloro-N⁶-cyclopentyl adenosine (CCPA) or (2S)-N⁶-[2-endo-norbornyl]adenosine (S-ENBA) or K_{ATP} channel opener, pinacidil (Pin) or mitochondrial K_{ATP} channel opener, diazoxide (Diazo) for pharmacological preconditioning as a trigger for delayed preconditioning in both, mice with targeted disruption of the iNOS gene and their wild-type counterparts.

MATERIALS and METHODS

Isolation and maintenance of mice myocytes

Myocytes were isolated as previously reported [19, 20] with the following modifications: the hearts from adult male mice (iNOS-/- or wt, 29–35 g or 12–14 weeks) were dissected free of major blood vessels and minced into smaller fragments with a surgical blade. These fragments were incubated for 15 min at 37°C in Hank's Balanced Salt Solution (HBSS, calcium free) containing 0.05% trypsin and 0.05% collagenase type II (GIBCO/BRL) and 0.002% deoxyribonuclease (Sigma) with continuous shaking in a water bath. At 15 min intervals, the supernatants were collected and the residual tissue re-incubated in fresh enzyme solution. Myocytes were obtained from the supernatants by pass-

ing through 200 μM mesh nylon gauze [19, 20] followed by centrifugation at 2,500 rpm. Each batch of cells was re-suspended in 5 ml of medium containing 8% FBS, DMEM; Ham's F12 (1:1) in uncoated Falcon Petri dishes (Becton Dickinson, Oxford, CA) for 1–1.5 h at 37°C with 5% CO_2 . A total of nine supernatants were collected and each processed separately. The supernatants (4–9) containing more than 90% of cylindrical, striated myocytes were plated in serum-containing medium. Approximately 2×10^5 cells/ml were plated into 6-well Falcon tissue culture plates, previously coated with collagen type IV (GIBCO/BRL). The medium was changed regularly at 48–72 h interval.

Experimental protocol

The detailed experimental protocol is shown in Figure 1. Five days old cultured mice (iNOS^{-/-} or wt) myocytes were subjected to sustained simulated ischemia (SI) by treating with buffer containing 0.75 mM sodium dithionite, 12 mM KCl, 20 mM dl-lactic acid and 10 mM 2-deoxy-D-glucose (pH 6.5) for 1 h at 37°C [19, 20]. The cells were returned to normal medium for further incubation for 2.5 h.

In the first set of experiments, iNOS^{-/-} mice myocytes were treated with heat shock at 42°C for 30 min or sub-lethal simulated ischemic (SSI) buffer (20 mM dl-lactic acid, 10 mM 2-deoxy-D-

glucose, 4 mM Hepes, 137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl_2 and 0.9 mM CaCl_2 , pH 6.5) for 30 min at 37°C and washed out with normal medium, then exposed to SI next day.

In the second set, iNOS^{-/-} mice myocytes were treated with A_1AR agonists 1 μM CCPA or 1nM S-ENBA and exposed to SI next day.

In the final set, iNOS^{-/-} mice myocytes were treated with K_{ATP} channel opener 0.05 μM Pin or mitochondrial K_{ATP} channel opener 100 μM Diazo and exposed to SI next day.

Determination of cellular injury

Cellular injury caused by SI was assessed by measuring LDH release into the medium as well as LDH remaining in the cells using commercially available kit (Sigma St. Louis, MO). LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). The formation of reduced nucleotide (NADH) results in an increase in absorbance at 340 nm, which is directly proportional to LDH activity in the sample. One unit of LDH activity is defined as the amount of enzyme that will catalyze the formation of 1 μ mole of NADH/minute.

Determination of cell viability

The remaining cells were treated with 1% Triton X-100 in 6-well plates after aspiration of culture medium. The cell lysates from each well was used for estimation of LDH activity (% of intact cells), 2.5 h following SI.

Statistical analysis

Group data are expressed as means \pm SEM. Inter-group comparison was done by using analysis of variance (ANOVA). A Bonferroni multiple comparison test was used to compare between different groups. Statistical differences were considered significant if p value was less than 0.05.

RESULTS

Effect of heat shock (HS) or sub-lethal simulated ischemia (SSI) on protection of iNOS^{-/-} mice myocytes against subsequent sustained simulated ischemia (SI)

In the first set of experiments, wt myocytes subjected to SI demonstrated insignificant ($p > 0.05$)

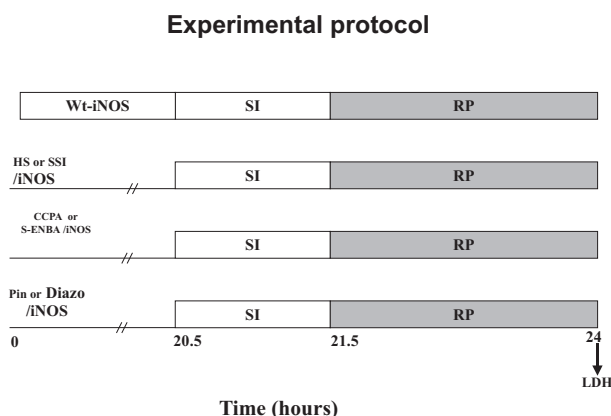


Fig. 1. Experimental protocol: Cultured mice myocytes (iNOS^{-/-} or wt) were treated with sub-lethal simulated ischemia (SSI) or heat shock (HS) or pharmacological preconditioning with A_1 adenosine receptor agonists CCPA or S-ENBA or K_{ATP} channel opener pinacidil or mitochondrial K_{ATP} channel opener diazoxide as described in Materials and Methods. Twenty hours after SSI or HS or pharmacological preconditioning with A_1AR agonists CCPA or S-ENBA or K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo treatment, the mice myocytes (iNOS^{-/-}) were exposed to SI for 1 h followed by 2.5 h of recovery period (RP)

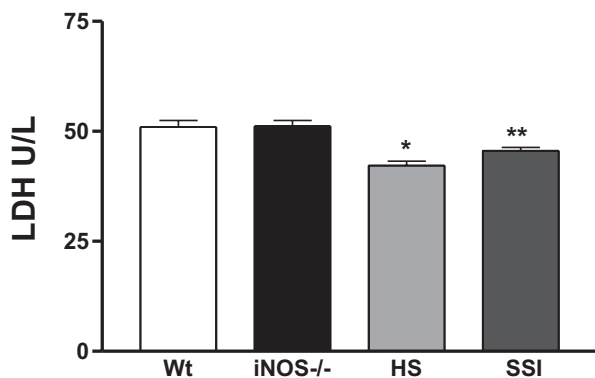


Fig. 2. The delayed cellular protective effect of SSI and HS on iNOS^{-/-} mice myocytes compared to non-treated iNOS^{-/-} mice myocytes as well as wild-type (wt) mice myocytes subjected to SI. Cellular injury was assessed as release of LDH (U/l) into the medium and retained in the cells. Results represent means ± SE of 12 measurements (* $p < 0.01$ wt or iNOS^{-/-} vs. iNOS^{-/-} + HS and wt or iNOS^{-/-} vs. iNOS^{-/-} + SSI groups)

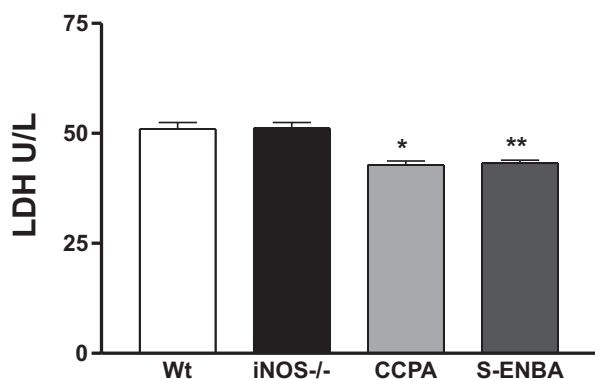


Fig. 3. The pharmacological induction of DCP with CCPA or S-ENBA (A₁AR agonists) in iNOS^{-/-} mice myocytes compared to non-treated iNOS^{-/-} mice myocytes as well as wild-type (wt) mice myocytes subjected to SI. Cellular injury was assessed as release of LDH (U/l) into the medium and retained in the cells. Results represent means ± SE of 12 measurements (* $p < 0.01$ wt or iNOS^{-/-} vs. iNOS^{-/-} + CCPA and wt or iNOS^{-/-} vs. iNOS^{-/-} + S-ENBA groups)

difference in LDH release as compared to the iNOS^{-/-} mice myocytes (Fig. 2). HS treatment-induced DCP in iNOS^{-/-} mice myocytes resulted in a significant attenuation in the cellular injury as indicated by reduction in the release of LDH from 51.14 ± 1.35 U/l (iNOS^{-/-}) to 42.20 ± 1.01 U/l (iNOS^{-/-} + HS), $p < 0.01$ (Fig. 2). Similarly, a significant attenuation in the cellular injury in sub-lethal simulated ischemia-treated group of iNOS^{-/-} mice myocytes was indicated by reduction in the release of LDH from 51.14 ± 1.35 U/l (iNOS^{-/-}) to 45.57 ± 0.75 U/l (iNOS^{-/-} + SSI), $p < 0.01$ (Fig. 2).

Effect of pharmacological preconditioning with A₁ adenosine receptor agonists CCPA or S-ENBA on protection of iNOS^{-/-} mice myocytes against subsequent sustained simulated ischemia (SI)

In the second set of experiments, pharmacological preconditioning with A₁AR agonist CCPA induced DCP in iNOS^{-/-} mice myocytes resulting in a significant attenuation in the cellular injury as indicated by reduction in the release of LDH from 51.14 ± 1.35 U/l (iNOS^{-/-}) to 42.87 ± 0.87 U/l (iNOS^{-/-} + CCPA), $p < 0.01$ (Fig. 3). Similarly, a significant attenuation in the cellular injury in the group of iNOS^{-/-} mice myocytes treated with another A₁AR agonist S-ENBA was indicated by reduction in the release of LDH from 51.14 ± 1.35 U/l (iNOS^{-/-}) to 43.21 ± 0.70 U/l (iNOS^{-/-} + S-ENBA), $p < 0.01$ (Fig. 3).

Effect of pharmacological preconditioning with K_{ATP} channel opener pinacidil or mitochondrial K_{ATP} channel opener diazoxide on protection of iNOS^{-/-} mice myocytes against subsequent sustained simulated ischemia (SI)

In the third set of experiments, pharmacological preconditioning with K_{ATP} channel opener Pin induced DCP in iNOS^{-/-} mice myocytes resulting in a significant attenuation in the cellular injury as indicated by reduction in the release of LDH from 51.14 ± 1.35 U/l (iNOS^{-/-}) to 37.81 ± 0.99 U/l (iNOS^{-/-} + Pin), $p < 0.01$ (Fig. 4). Similarly, a significant attenuation in the cellular injury in the group of iNOS^{-/-} mice myocytes treated with mitochondrial K_{ATP} channel opener Diazo was indicated by reduction in the release of LDH from 51.14 ± 1.35 U/l (iNOS^{-/-}) to 36.79 ± 0.68 U/l (iNOS^{-/-} + Diazo), $p < 0.01$ (Fig. 4).

Comparative effects of preconditioning with HS, SSI, CCPA, S-ENBA, Pin and Diazo on protection of iNOS^{-/-} mice myocytes against subsequent sustained simulated ischemia (SI)

In the last set of experiments a comparison has been done between different preconditioning procedures. Cellular injury was assessed as release of

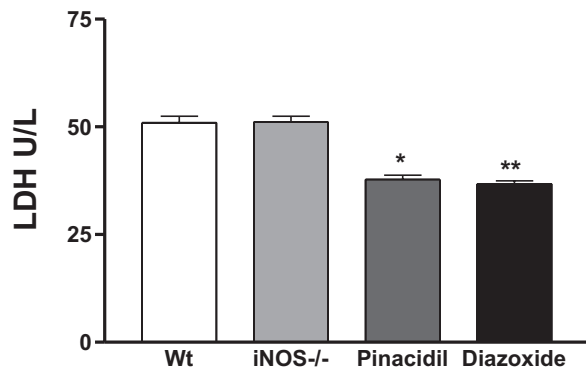


Fig. 4. The pharmacological induction of DCP with K_{ATP} channel opener Pin and mitochondrial K_{ATP} channel opener Diazo in iNOS^{-/-} mice myocytes compared to non-treated iNOS^{-/-} mice myocytes as well as wild-type (wt) mice myocytes subjected to SI. Cellular injury was assessed as release of LDH (U/l) into the medium and retained in the cells. Results represent means \pm SE of 12 measurements (* $p < 0.01$; ** $p < 0.01$ wt or iNOS^{-/-} vs. iNOS^{-/-} + Pin and wt or iNOS^{-/-} vs. iNOS^{-/-} + Diazo groups)

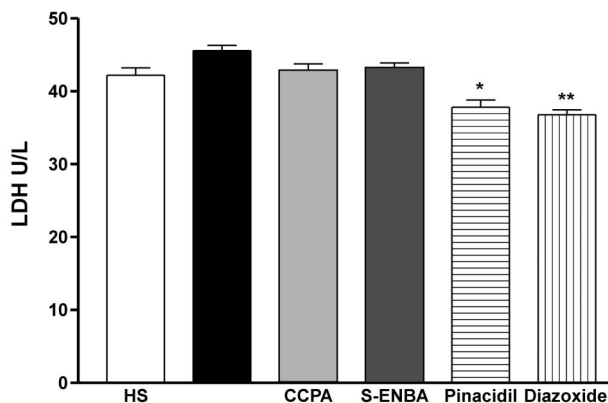


Fig 5. The comparative effects of delayed preconditioning (SSI; HS; CCPA; S-ENBA; Pin and Diazo) on iNOS^{-/-} mice myocytes. Cellular injury was assessed as release of LDH (U/l) into the medium and retained in the cells. Results represent means \pm SE of 12 measurements (* $p < 0.01$; ** $p < 0.01$ iNOS^{-/-} + HS; iNOS^{-/-} + SSI; iNOS^{-/-} + CCPA or iNOS^{-/-} + S-ENBA vs. iNOS^{-/-} + Pin or iNOS^{-/-} + Diazo groups)

LDH U/l into the medium and retained in the cells. Results represent means \pm SE of 12 measurements (* $p < 0.01$; ** $p < 0.01$ iNOS^{-/-} + HS; iNOS^{-/-} + SSI; iNOS^{-/-} + CCPA or iNOS^{-/-} + S-ENBA vs. iNOS^{-/-} + Pin or iNOS^{-/-} + Diazo groups) (Fig. 5).

DISCUSSION

In this study, we found sub-lethal simulated ischemia (SSI)- or HS-induced DCP in iNOS^{-/-} mice myocytes against subsequent sustained SI.

Secondly, we also found that pharmacological preconditioning with A_1AR agonists CCPA or S-ENBA induced DCP in iNOS^{-/-} mice myocytes against subsequent sustained SI. Finally, pharmacological preconditioning with K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo also induced DCP in iNOS^{-/-} mice myocytes against subsequent sustained SI.

Bolli et al. [7] proposed the NO hypothesis for the late phase of myocardial protection. A number of recent studies, using pharmacological inhibitors of iNOS [15, 25] or iNOS^{-/-} mice with targeted deletion of iNOS [13, 26], have implicated NO in late phase of myocardial protection. Moreover, they have also demonstrated that transient ischemia results in protein kinase C (PKC) activation and translocation [22], activation of the transcription factor NF- κ B and ultimately iNOS induction and expression [27], providing further support for the hypothesis. With this evidence in mind, we anticipated that delayed preconditioning triggered by SSI or HS or by pharmacological preconditioning, such as A_1AR agonists CCPA or S-ENBA or K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo would also be mediated *via* a similar iNOS-dependent mechanism. Evidence supports a delayed protection in which the beneficial effects of preconditioning are manifest as late as 72 h after a prolonged ischemic event [5, 6, 14, 17, 18]. A_1AR activation has been shown to trigger delayed preconditioning in a number of animal species [5, 11]. Further, pharmacological studies have provided evidence that opening of K_{ATP} channels is necessary for the infarct-sparing effects of late preconditioning with ischemia [8] and adenosine A_1AR agonist treatment [12]. In this study, we demonstrate the induction of DCP with SSI or HS or by pharmacological preconditioning with A_1AR agonists CCPA or S-ENBA or K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo. These are entirely consistent with the previous reports [5, 6, 8, 11, 12, 14, 17, 18]. Remarkably however, iNOS^{-/-} mice myocytes (with no iNOS gene) display a similar degree of DCP following SSI or HS or by pharmacological preconditioning with A_1AR agonists CCPA or S-ENBA or K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo administered 24 h earlier against subsequent sustained SI.

One potential explanation for this observation is that there is an iNOS-independent signaling medi-

ating DCP following SSI or HS or by pharmacological preconditioning with A₁AR agonists CCPA or S-ENBA or K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo administration 24 h earlier against subsequent sustained SI. The present study appears to contradict some of the recent investigations for late phase of myocardial protection in mice with targeted disruption of the iNOS gene. In an *in vivo* model using ischemia to precondition the myocardium, Bolli et al. [8] demonstrated that delayed protection was absent in the iNOS^{-/-} mice. Similar results have been found using both monophosphoryl lipid A (MLA) and CCPA as a trigger for late phase of pharmacological preconditioning [26, 28], in which ischemia/reperfusion protocol was performed *in vitro* 24 h after the *ip* drug administration. There are two potential explanations for the observed difference between these studies and the data presented here. One would be the difference in the models of the mice used in the studies where we have used cellular model of iNOS^{-/-} mice myocytes. However, from our observations, there is no difference between the *in vivo*, *in vitro* isolated mouse heart and the cellular model of adult mice myocytes in their infarct-limiting cardioprotective response to ischemic as well as pharmacological delayed preconditioning (data not shown). The other explanation is that the other authors [26, 28] used *ip* route for drug administration in iNOS^{-/-} mice 24 h before the subsequent ischemic insult, whereas we added drugs directly to the iNOS^{-/-} mice myocytes by dissolving them in the medium, 24 h before the subsequent sustained SI [21].

In summary, the present data provide evidence for the first time that SSI or HS induced DCP in iNOS^{-/-} mice myocytes against subsequent sustained SI. Secondly, we also found that pharmacological preconditioning with A₁AR agonists CCPA or S-ENBA induced DCP in iNOS^{-/-} mice myocytes against subsequent sustained SI. Finally, pharmacological preconditioning with K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo also induced DCP in iNOS^{-/-} mice myocytes against subsequent sustained SI. Further, our data also suggest that Pin and Diazo are more potent inducers of DCP among others in iNOS^{-/-} mice myocytes against sustained SI. Further studies using eNOS^{-/-} and nNOS^{-/-} mice myocytes will be necessary to unravel the mystery of NOS role in

mediation of delayed cellular tolerance to SI injury in cultured mice myocytes.

CONCLUSION

In conclusion, it appears that the HS or ischemic as well as pharmacological preconditioning with A₁AR agonists CCPA or S-ENBA and K_{ATP} channel opener Pin or mitochondrial K_{ATP} channel opener Diazo induced DCP in iNOS^{-/-} mice cultured myocytes against subsequent sustained SI. Induction of DCP through HS or ischemic or pharmacological preconditioning does not depend on iNOS as evidenced by genetically disrupted iNOS^{-/-} mice myocytes.

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