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Sublethal Simulated Ischemia Promotes Delayed Resistance Against Ischemia via ATP-Sensitive (K^+) Channels in Murine Myocytes: Role of PKC and iNOS

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ABSTRACT

In this study, we examined whether sublethal simulated ischemia (SSI) induces delayed cellular protection in mouse cardiac myocytes, and whether the delayed cellular protection depends on the activation of protein kinase C-ε (PKC-ε), inducible nitric oxide synthase (iNOS), and ATP-sensitive K^+ (K_{ATP}) channels against subsequent sustained simulated ischemia (SI). The following groups of mouse cardiac myocytes were studied: (a) SI: incubation with SI buffer for 1 h; (b) SSI: incubation with SSI buffer for 30 min; (c) SSI + PKC inhibitor, chelerythrine chloride (CCl): SSI and 1 μ*M* CCl; (d) SSI + iNOS inhibitor, S-methylthiourea (SMT): SSI and 100 n*M* SMT; (e) SSI + K_{ATP} channel blocker, glibenclamide (Glb): SSI and 50 μ*M* Glb; (f) SSI + mitochondrial K_{ATP} channel blocker, 5-hydroxydecanoate (5-HD): SSI and 50 μ*M* 5-HD. The release of lactate dehydrogenase into the medium and the amount remaining in the cells was measured, and A_1 adenosine receptor, PKC-ε, and iNOS were detected through western blot analysis. The delayed cellular protection acquired due to SSI showed a decreased release of lactate dehydrogenase (%) from 46.51 ± 1.60 to 37.00 ± 1.34 ($p < 0.001$) and was blocked by CCl (47.08 ± 0.95), SMT (48.08 ± 1.18), Glb (45.88 ± 1.31), and 5-HD (47.20 ± 1.56). Simultaneously, SSI-induced up-regulation of A_1 adenosine receptor, PKC-ε, iNOS, and opening of both membrane and mitochondrial K_{ATP} channels also was observed compared with controls. *Antioxid. Redox Signal.* 6, 375–383.

INTRODUCTION

ISCHEMIC PRECONDITIONING with brief periods of ischemia/reperfusion protects against subsequent sustained ischemia. Preconditioning is the most potent cardioprotective adaptation known to reduce the adverse effects of sustained ischemia (10, 17, 21–23, 26, 32, 37). There is abundant evidence that earlier repetitive short episodes of coronary occlusion and reperfusion reduce the size of myocardial infarction in nearly all species (22, 23, 26) and that preconditioning improves postischemic functional recovery (10, 25). The concept that adenosine released during the preconditioning stimulus triggers the development of delayed protection was first proposed by Baxter *et al.* (4) and subsequently expanded by others (8). There is also a well established notion that protein kinase C (PKC) is essential for the mechanism of late preconditioning as proposed by Baxter *et al.* (5), who found that the delayed infarct-sparing effects of preconditioning in rabbits were abrogated by pretreatment with the PKC inhibitor chelerythrine chloride (CCl). Conversely, administration of PKC activator dioctanoyl-*sn*-glycerol induced cardioprotection 24 h later (6). Furthermore, inhibition of PKC-ε translocation (activation) by CCl blocked the beneficial effects of late preconditioning. Thus, activation of PKC after preconditioning was isoform-

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selective, and PKC-ε appears to be the specific PKC isotype responsible for the development of delayed protection (30).

The role of inducible nitric oxide synthase (iNOS) in delayed preconditioning was first reported by Bolli's group (16), who demonstrated that the late phase of preconditioning was associated with up-regulation of myocardial iNOS, and that targeted disruption of the iNOS gene completely abrogated the delayed infarct-sparing effect, providing molecular genetic evidence for an obligatory role of iNOS in the cardioprotection afforded by the late phase of ischemic preconditioning.

Furthermore, pharmacological studies have provided evidence that opening of ATP-sensitive K^+ channels (K_{ATP}) is necessary for the infarct-sparing effects of late preconditioning with ischemia (8) and A_1 adenosine receptor (A_1AR) agonist treatment (3). Previous studies have shown that ischemic protection induced by pharmacological agents such as monophosphoryl lipid A or ischemic preconditioning is mediated by a nitric oxide (NO)-sensitive mechanism (9). In addition, it has been shown that NO may modulate K_{ATP} channels. In short, all these mediators have been proposed to be involved in the cardioprotective phenomenon *in vivo,* including adenosine receptors, PKC-ε, iNOS, and K_{ATP} channels. With the description of clinical correlates of ischemic preconditioning (11, 35, 36) and the possibility of harnessing this powerful adaptive response in the management of ischemic heart disease, earnest investigation continues in pursuit of its physiological mechanisms. Potential clinical interventions directed at inducing and delaying the protective effects of ischemic preconditioning will require a thorough understanding of the cellular and molecular events mediating its effects at the cellular level. Further, it is unclear whether cultured ICR strain mouse cardiac myocytes share the same beneficial effects of ischemic preconditioning with the involvement of cellular and molecular mediators *in vitro.* We hypothesized that the delayed cytoprotective effect caused by the opening of K_{ATP} channels may be mediated by iNOS via A_1AR and PKC activation in sublethal simulated ischemia (SSI)-treated cardiac myocytes against subsequent sustained simulated ischemia (SI). Therefore, the purpose of this study was to demonstrate the delayed cellular protection with SSI in cardiac myocytes, and to elucidate the cellular and molecular mechanisms involved in SSI-induced delayed cellular protection in mouse cardiac myocytes.

MATERIALS AND METHODS

Isolation and maintenance of mouse cardiac myocytes

Cardiac myocytes were isolated as previously reported (28) with the following modifications: the hearts from adult male mice (ICR strain, 29–35 g or 12–14 weeks each) 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 Hanks' balanced salt solution (calcium-free) containing 0.05% trypsin and 0.05% collagenase type II (GibcoBRL, Grand Island, NY, U.S.A.) and 0.002% deoxyribonuclease (Sigma, St. Louis, MO, U.S.A.) with continuous shaking in a water bath. At 15-min intervals, the supernatants were collected and the residual tissue reincubated in fresh enzyme solution. Cardiac myocytes were obtained from the supernatants by passing through 200 μ*M* mesh nylon gauze (28) followed by centrifugation at 2,500 rpm. Each batch of cell pellet was suspended in 5 ml of medium containing 8% fetal bovine serum with Dulbecco's modified Eagle medium/Ham's F12 (1:1) in uncoated Falcon Petri dishes (Becton–Dickinson, Oxnard, CA, U.S.A.) 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 >90% cylindrical, striated myocytes (confirmed by immunohistochemistry with the adult cardiac myosin heavy chain) were plated in serum-containing medium. Approximately 2×10^5 cells/ml were plated into six-well Falcon tissue culture plates, previously coated with collagen type IV (GibcoBRL). The medium was changed regularly at 48–72 h intervals.

Experimental protocol

The detailed experimental protocol is shown in Fig. 1. Five-day-old cultured mouse (ICR strain) cardiac myocytes were treated with SSI buffer (20 m*M* DL-lactic acid, 10 m*M* 2-deoxy-D-glucose, 4 m*M* HEPES, 137 m*M* NaCl, 3.8 m*M* KCl, 0.49 m*M* $MgCl_2$, and 0.9 m*M* $CaCl_2$, pH 6.5) for 0.5 h at 37°C, washed out with normal medium, and then exposed to SI the next day. In the second group, cardiac myocytes were treated with 1 μ*M* CCl (PKC blocker) 0.5 h before SSI buffer treatment and exposed to SI the next day. Appropriate controls for cardiac myocytes were run under identical conditions without the drug. Twenty hours following the preconditioning (SSI) protocol, mouse myocytes were exposed to SI by treating with buffer containing, in addition to SSI, the following ingredients: 0.75 m*M* sodium dithionite, 12 m*M* KCl, 20 m*M* DL-lactic acid, and 10 m*M* 2-deoxy-D-glucose, pH 6.5, for 1 h at 37°C (27, 28). The cells were returned to normal medium for further incubation for 2.5 h. For determining the role of K_{ATP} channels in SSI-mediated cellular protection, the SSI-preconditioned cardiac myocytes were treated with 50 μ*M* glibenclamide (Glb) or 100 μ*M* 5-hydroxydecanoate (5-HD) during SI and during the postischemic period as well. One more group of SSI-treated cardiac myocytes also received 100 n*M* *S*-methylthiourea (SMT), an iNOS inhibitor, during SI and postischemic recovery period of 2.5 h.

Gel electrophoresis and Western blotting

Cells were rinsed with phosphate-buffered saline and lysed with boiling lysis solution, containing 1% sodium dodecyl sulfate, 1 m*M* sodium vanadate, 10 m*M* Tris, pH 7.4, and the scraped cells were transferred to a microcentrifuge tube and boiled for additional 5 min. The samples were then sonicated briefly and centrifuged (12,000 *g*, 15°C) for 5 min. Protein was measured using the Bio-Rad protein assay based on the Bradford dye binding procedure with bovine serum albumin as a standard. The protein mixture was divided into aliquots and stored at −80°C. At the time of analysis, samples were thawed and ~20 μg of total protein per lane was loaded on a slab gel. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10% acrylamide gels (1-mm thick). After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell, Keene, NH, U.S.A.) by electroelution. Protein

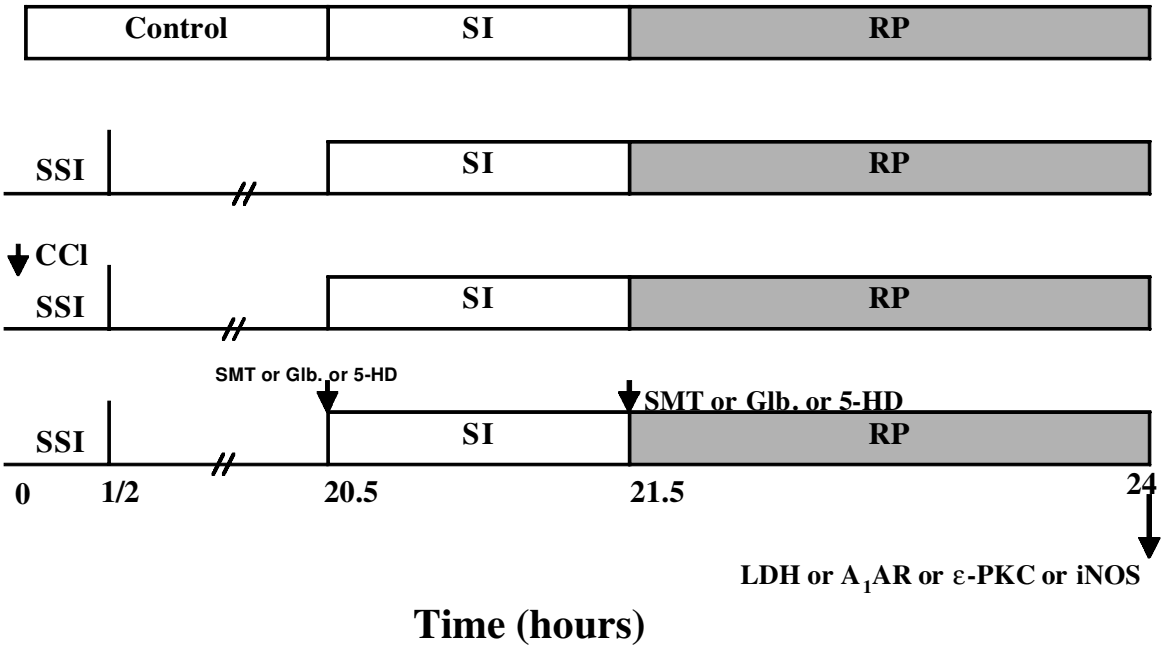

FIG. 1. Experimental protocol. Cultured mouse cardiac myocytes were treated with SSI buffer as described in Materials and Methods. Twenty hours after SSI buffer treatment, the cardiac myocytes were exposed to SI for 1 h followed by a 2.5-h period (RP).

transfer was confirmed by using prestained molecular-weight markers (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Complete transfer of the protein was ascertained by staining the gel in Coomassie Blue and transfering prestained molecular-weight markers on the PVDF membrane. Following blocking with nonfat dry milk, the PVDF membranes were incubated with monoclonal and polyclonal antibodies cross-reacting with A_1AR protein (Alpha Diagnostic, San Antonio, TX, U.S.A., or RBI, Sigma), PKC-ε (GibcoBRL), and iNOS (GibcoBRL). The second antibody was a horseradish peroxidase-conjugatedanti-rabbit IgG. The membranes were developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) and exposed to x-ray film for the appropriate time.

Determination of cellular injury

Cellular injury caused by SI was assessed by measuring lactate dehydrogenase (LDH) release into the medium, as well as LDH remaining in the cells, using a commercially available kit (Sigma). LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of 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 μmol of NADH/min.

Determination of cell viability

After the aspiration of whole overlay (culture medium), the remaining cells or attached cells were treated with 1% Triton X-100 in six-well plates. The cell lysates from each well were used for estimation of LDH activity to assess percentage of intact or attached or viable cells, 2.5 h following SI.

Statistical analysis

Group data are expressed as means ± SEM. Intergroup comparison was done by using ANOVA. A Bonferroni multiple comparison test was used to compare between different groups. Statistical differences were considered significant if p value was <0.05.

RESULTS

Up-regulation of PKC-ε and A_1AR

Figure 2A shows expression of PKC-ε (92 kDa) in the cultured mouse cardiac myocytes subjected to preconditioning with SSI, the sublethal simulated ischemic preconditioning also called metabolic preconditioning (MP). Western blot analysis shows an up-regulation of PKC-ε with MP compared with the control group. There was insufficient amount of protein to analyze by western blot when we separated protein from particulate and cytosolic fractions. Because of this, we decided to perform the experiment with whole-cell homogenates. Figure 2B shows expression of A_1AR (36 kDa) in the cultured cardiac myocytes subjected to preconditioning with MP. Western blot data show an up-regulation of A_1AR protein with MP compared with the control group. In a separate experiment, we confirmed that the antibody we used in this report is solely recognizing the A_1AR. Experiments were performed with A_1AR antibody displacement (data not shown.) using corresponding

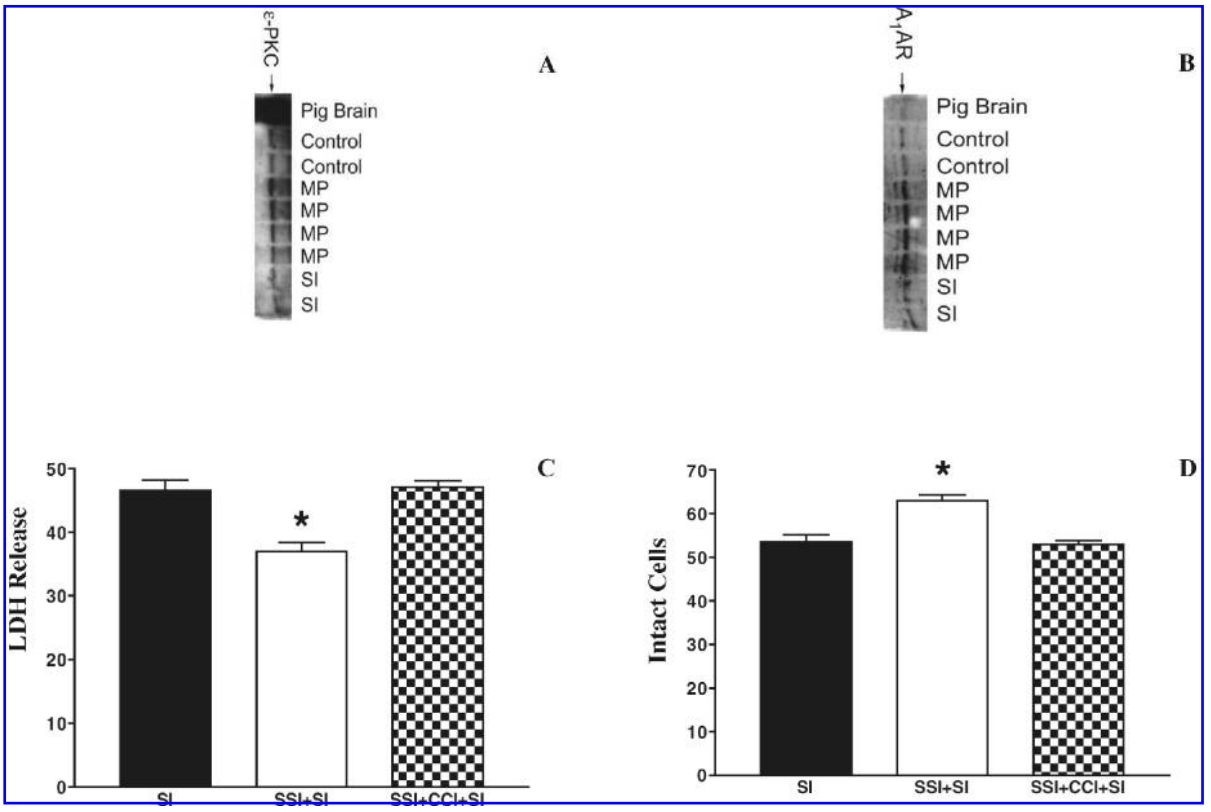

FIG. 2. **(A)** Western blot for PKC-ε (90 kDa). Samples were taken from mouse cardiac myocytes after SSI [also called metabolic preconditioning (MP) treatment] ($n = 4$), control ($n = 2$), and SI ($n = 2$); pig brain was used as a positive control. **(B)** Western blot for A_1AR (36 kDa). Samples were taken from cardiac myocytes after MP treatment ($n = 4$); control ($n = 2$), and SI ($n = 2$); pig brain was used as a positive control. **(C)** The % of LDH release. Percentage release of LDH from cardiac myocytes into the medium is significantly different from nonpreconditioned as well as preconditioned group treated with CCl. All these groups were subjected to SI. **(D)** The % of LDH remaining in cardiac myocytes as an index of cell viability (% of intact cells). Results represent means ± SE of 12 measurements (*$p < 0.01$ SI versus SSI and versus SSI + CCl group).

peptide to rat A_1AR gene, provided by Alpha Diagnostic International.

Cellular resistance against SI

Mouse cardiac myocytes subjected to SI demonstrated a significant increase in LDH release as compared with the SSI-preconditioned cells (Fig. 2C). SSI-induced delayed cellular protection resulted in a significant attenuation in the cellular injury as indicated by reduction in the release of LDH (from 46.51 ± 1.60% to 37.00 ± 1.34%, $p < 0.01$; Fig. 2C). Similarly, the percentage of viable cells in the SSI preconditioned group also increased by 10% (from 53.49 ± 1.60% to 63.00 ± 1.34%, $p < 0.01$; Fig. 2D) compared with the SI group.

Effect of CCl on preconditioned cardiac myocytes

SSI-induced delayed cellular resistance was totally abolished by CCl (PKC inhibitor), and the treatment resulted in a significant increase in the release of LDH (from 37.00 ± 1.34% to 47.08 ± 0.95%, $p < 0.01$; Fig. 2C). In a separate experiment, the CCl-treated cells did not show any significant release of LDH into the medium compared with controls (data not shown). This shows that 1 μ*M* CCl has no cytotoxic effect of its own. Furthermore, the percentage of cellular viability (percentage of intact cells) was also significantly reduced after PKC inhibition (from 63.00 ± 1.34% to 52.92 ± 0.95%, $p < 0.01$; Fig. 2D).

Expression of iNOS

Figure 3A shows expression of iNOS (130 kDa) in the cultured mouse cardiac myocytes subjected to preconditioning with SSI. Western blot shows an up-regulation of iNOS with SSI-treated cardiac myocytes compared with nontreated control cardiac myocytes.

Effect of SMT on preconditioned cardiac myocytes

SMT (iNOS inhibitor) totally abolished the acquired cellular resistance due to SSI pretreatment, which resulted in an increase in release of LDH from 37.00 ± 1.34% to 48.08 ± 1.18% ($p < 0.01$; Fig. 3B). Viability index also went down from 63.00 ± 1.34% to 51.92 ± 1.18% after SMT treatment in SSI-preconditioned mouse myocytes ($p < 0.01$; Fig. 3C). In a separate experiment, the cells were exposed to 100 n*M* SMT alone, and there was no significant release of LDH into the

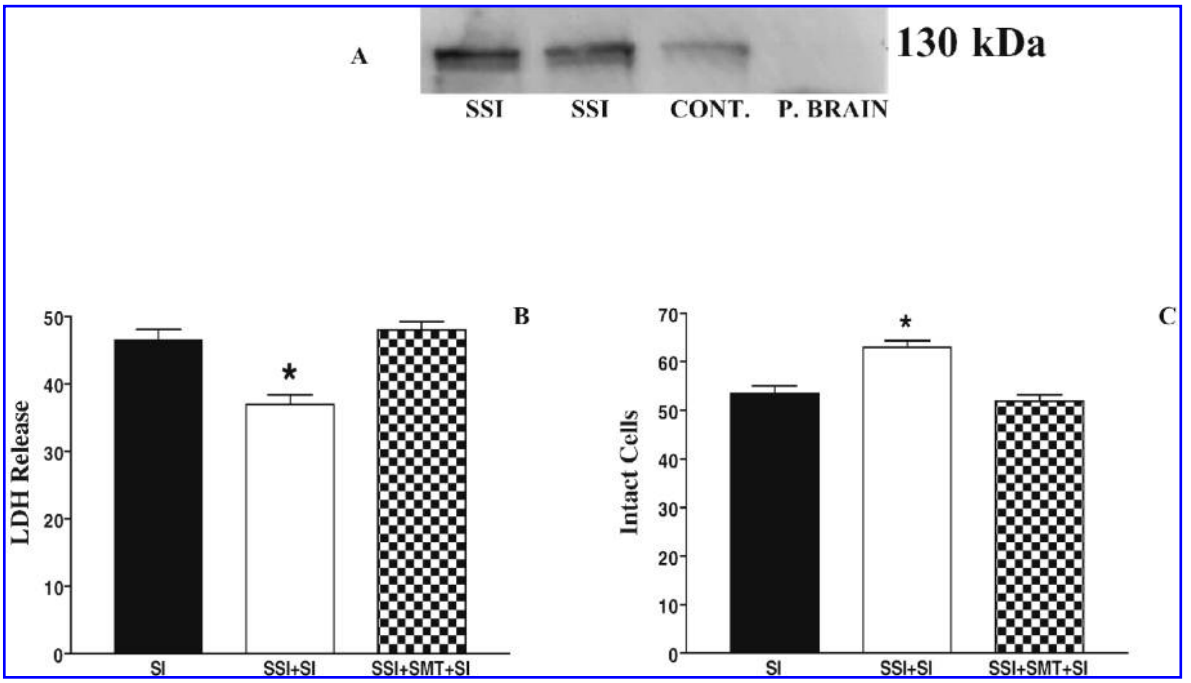

FIG. 3. **(A)** Western blot showing expression of iNOS (130 kDa) in SSI-treated cardiac myocytes ($n = 2$); pig brain was used as a positive control. **(B)** Effect of SMT (100 n*M*) on SSI-induced delayed preconditioning. Reduced cellular injury acquired with SSI treatment was abrogated by SMT. **(C)** The % of LDH remaining in cardiac myocytes as an index of cell viability (% of intact cells). Results represent means ± SE of 12 measurements (*$p < 0.01$ SI versus SSI and versus SSI + SMT group).

medium compared with controls (data not shown). This shows that SMT has no cytotoxic effect of its own.

Effect of Glb and 5-HD on preconditioned cardiac myocytes

Treatment of SSI-preconditioned cardiac myocytes with Glb (K_{ATP} channel blocker) during SI and the postischemic period resulted in significantly increased release of LDH from 37.00 ± 1.34% to 45.88 ± 1.31% ($p < 0.01$; Fig. 4A). The viability of SSI-treated mouse myocytes was decreased from 63.00 ± 1.34% to 54.12 ± 1.31% after Glb treatment ($p < 0.01$; Fig. 4B). 5-HD (mitochondrial K_{ATP} channel blocker) caused a significant increase in the release of LDH following SI in SSI-preconditioned mouse myocytes (from 37.00 ± 1.34% to 47.20 ± 1.56%, $p < 0.01$; Fig. 5A). The viability of the cells decreased when SSI-treated mouse myocytes were exposed to 5-HD (63.00 ± 1.34% to 52.80 ± 1.56%, $p < 0.01$; Fig. 5B). In

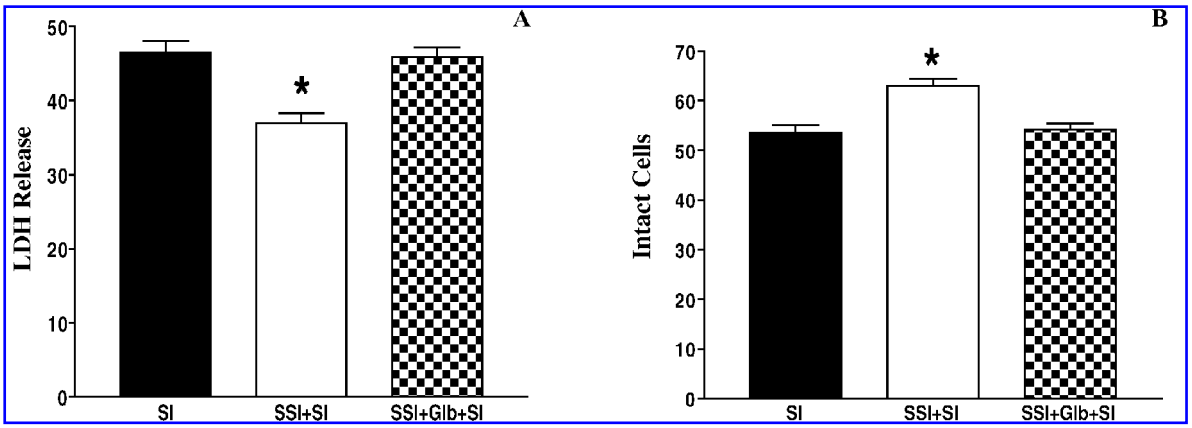

FIG. 4. **(A)** Effect of Glb on SSI-treated cardiac myocytes. Percentage release of LDH from cardiac myocytes into the medium is significantly different when preconditioned group is compared with the group treated with Glb. All these groups were subjected to SI. **(B)** The % of LDH remaining in cardiac myocytes as an index of cell viability (% of intact cells). Results represent means ± SE of 12 measurements (*$p < 0.01$ SI versus SSI and versus SSI + Glb group).

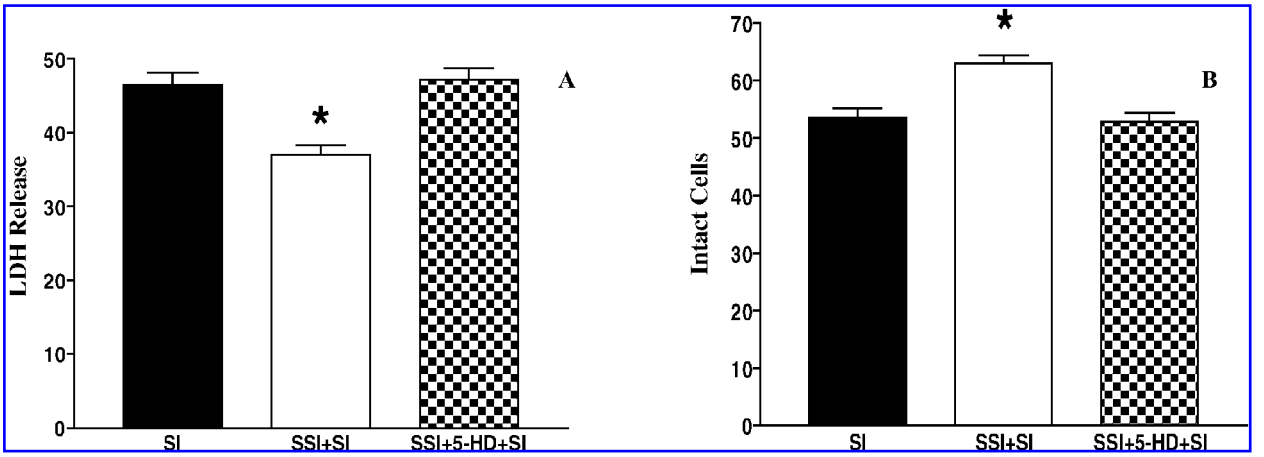

FIG. 5. **(A)** Effect of 5-HD on SSI-treated mouse cardiac myocytes. Percentage release of LDH from cardiac myocytes into the medium is significantly different when preconditioned group is compared with the group treated with 5-HD. All these groups were subjected to SI. **(B)** The % of LDH remaining in cardiac myocytes as an index of cell viability (% of intact cells). Results represent means ± SE of 12 measurements (*$p < 0.01$ SI versus SSI and versus SSI + 5HD group).

a separate experiment, the 50 μ*M* Glb- or 100 μ*M* 5-HD-treated cells did not show any significant release of LDH into the medium compared with controls (data not shown). This shows that 50 μ*M* Glb or 100 μ*M* 5-HD does not have any cytotoxic effect of its own.

DISCUSSION

The main goal of this investigation was to demonstrate whether SSI induces a delayed cytoprotective effect in the cultured ICR strain mouse cardiac myocytes against sustained SI. In addition, we wanted to understand whether A_1AR, PKC-ε, and iNOS, which are implicated in the delayed phase of pharmacological and ischemic preconditioning in the heart (28, 30) with modulation of K_{ATP} channels (9), also play a role in SSI-induced delayed cellular protection in cultured cardiac myocytes. This study demonstrated for the first time that cultured ICR strain mouse cardiac myocytes could be preconditioned with SSI. This preconditioning phenomenon has delayed effects on cardiac myocytes with associated expression of A_1AR, PKC-ε, iNOS, and opening of K_{ATP} channels due to phosphorylation. Expression of these mediators is believed to be involved in protection of preconditioned myocardium (2, 9, 13, 19, 28).

SSI and A_1AR

Ischemic preconditioning of the heart is a phenomenon by which a brief period of ischemia followed by reperfusion protects the myocardium from irreversible injury or infarction that results from a subsequent more prolonged or severe ischemic insult. An exact mechanism to explain the protective actions of myocardial preconditioning has not been elucidated, although some evidence strongly implicates a role for adenosine (12), and adenosine is known to be produced in cardiac myocytes (34). Komamura *et al.* (20) demonstrated that intracoronary infusion of ATP, which is degraded to adenosine, reduces the extent of no-reflow and infarct size in dog. Adenosine production increases during hypoxia in the heart (7, 33). A_1AR in the myocardium has been most extensively implicated in the protective effects of ischemic preconditioning (12, 22). It is possible that this receptor may have a role in delayed cellular protection during SSI preconditioning in cardiac myocytes similar to myocardial A_1AR. Overexpression of myocardial A_1AR protects the heart from ischemic damage (24). Bernardo *et al.* (8) showed the transient activation of A_1AR by delayed preconditioning with 2-chloro-N^6-cyclopentyladenosine in the rabbit heart. Such a transient activation of A_1AR may be involved in the mechanism of delayed cellular protection induced by specific A_1AR through SSI in mouse cardiac myocytes.

SSI and PKC

Considering that the half-life of adenosine is short, A_1AR activated through SSI preconditioning would not be cytoprotective but, rather, its "activation" may have initiated a cascade of signaling events leading to cytoprotection. A_1AR stimulation has been reported to activate phospholipase C and phospholipase D (13). Interestingly, A_1AR stimulation tends to cause a more prolonged accumulation of diacylglycerol (13). These findings could indicate that A_1AR activation leads to delayed activation and expression of PKC in cells. The present study demonstrates the overexpression of PKC-ε in MP-treated cardiac myocytes compared with nontreated cells. Furthermore, the cellular protection in cardiac myocytes was blocked with CCl (PKC inhibitor). These data suggest that PKC is involved in SSI-mediated delayed cellular protection. Ping *et al.* (29) further support this notion by showing the involvement of PKC-ε in ischemic preconditioning in the hearts of conscious rabbit. Qiu *et al.* (30) have suggested that PKC-ε has more implication than any other isoform in myocardial protection. However, we have tested both PKC-ε and PKC-δ

with N^6-(2-endo-norbornyl)adenosine (ENBA; A_1AR agonist), because some of the reports show the involvement of PKC-δ also in preconditioning. We found no change in PKC-δ expression in the ENBA-treated group compared with control. However, a remarkable change was observed for PKC-ε expression in the ENBA-treated group compared with control (data not shown). Therefore, we did not pursue PKC-δ and other PKC isoforms for this study. The present study is the first study to report the SSI-induced preconditioning phenomenon in cardiac myocytes with the elevation of PKC-ε. Our findings are similar to the results of Qiu *et al.* (30), who were able to inhibit PKC-ε activation by CCl and block the development of late preconditioning. Thus, activation of PKC after preconditioning is isoform-selective and ε appears to be the specific PKC isotype responsible for the development of delayed protection in cardiac myocytes.

SSI and iNOS

In the present study, we have also demonstrated the upregulation of iNOS in SSI-treated cardiac myocytes compared with nontreated cells. This expression was associated with delayed cellular protection in cardiac myocytes, and this cellular protection was totally blocked by SMT (iNOS inhibitor). These data suggest that iNOS is involved in SSI-mediated delayed cellular protection in cardiac myocytes. Recently, Imagawa *et al.* (18) demonstrated the involvement of iNOS in delayed protection against myocardial infarction. Our results show that cellular protection in the SSI-treated cardiac myocytes was totally blocked by SMT during SI. This is in agreement with recent work of Bolli *et al.* (9) showing that aminoguanidine, an inhibitor of iNOS, given prior to infarction also blocked the delayed protective effects of ischemic preconditioning. Thus, it is likely that the induction and activation of iNOS are important steps in the development of delayed cellular protection in SSI-treated cardiac myocytes, and synthesis of NO might be involved as a possible mediator of delayed cellular preconditioning.

SSI and K_{ATP} channel

Gross and Auchampach (14) suggested the involvement of the K_{ATP} channel in preconditioning. In this study using a canine model of infarction, the infarct size limiting the effect of preconditioning was abolished by Glb, a K_{ATP} channel blocker. K_{ATP} channels are inhibited by intracellular ATP, opened by pinacidil and cromakalim, and closed by sulfonylureas such as Glb and tolbutamide (1, 31). Many K^+ channel openers act as exogenous ischemic preconditioners that enable the heart to survive during limited periods of ischemia by opening coronary K_{ATP} channels (15). In the present study, we observed significant delayed cellular resistance during SI in SSI-treated cardiac myocytes, and this acquired cellular tolerance was completely blocked by Glb and 5-HD. These data provide conclusive evidence that both mitochondrial and sarcolemmal K_{ATP} channels can block the SSI-stimulated activation of A_1AR-induced delayed cellular protection in mouse cardiac myocytes against SI.

In summary, the present data provide evidence for the first time that mouse cardiac myocytes can be induced to have a delayed preconditioning effect with SSI. Furthermore, these data elucidate the signaling relationship and demonstrate the enhanced expression of A_1AR, PKC-ε, iNOS, and the opening of K_{ATP} channels during delayed cellular tolerance of cardiac myocytes. This is based on the following evidence. First, the cellular resistance against SI was acquired due to SSI treatment against SI with the expressions of A_1AR, PKC-ε, and iNOS. Further, cellular tolerance acquired in cardiac myocytes with SSI treatment was blocked by CCl (PKC blocker), SMT (iNOS inhibitor), Glb (K_{ATP} channel blocker), and 5-HD (mitochondrial K_{ATP} channel blocker). However, the role of sarcolemmal or mitochondrial K_{ATP} channels in delayed cellular resistance against SI injury in cardiac myocytes is not clear. Further studies will be necessary to unravel the mechanism(s) by which the activation of A_1AR, PKC-ε, and iNOS play a role in the opening of sarcolemmal or mitochondrial channels or both, which ultimately leads to the delayed cellular tolerance against SI injury in cultured mouse cardiac myocytes.

Conclusion

In conclusion, it appears that the delayed preconditioning phenomenon in mouse cardiac myocytes is a polygenic process that requires the synthesis of multiple proteins. A number of signaling molecules possibly trigger by the activation of A_1AR through the SSI. These signaling elements include PKC-ε and other effector molecules, which may possibly be involved directly or indirectly in the transcription and translation of iNOS. This may lead to phosphorylation of both sarcolemmal and mitochondrial K_{ATP} channels or only mitochondrial K_{ATP} channels, resulting in the delayed cellular protection in mouse cardiac myocytes.

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ABBREVIATIONS

A_1AR, A_1 adenosine receptor; CCl, chelerythrine chloride (PKC blocker); ENBA, N^6-(2-endo-norbornyl)adenosine; Glb, glibenclamide (K_{ATP} channel blocker); 5-HD, 5-hydroxydecanoate (mitochondrial K_{ATP} channel blocker); iNOS, inducible nitric oxide synthase; K_{ATP}, ATP-sensitive K^+ channel; LDH, lactate dehydrogenase; MP, metabolic preconditioning; NO, nitric oxide; PKC, protein kinase C; PVDF, polyvinylidene difluoride; SI, simulated ischemia; SMT, *S*-methylthiourea (iNOS inhibitor); SSI, sublethal simulated ischemia.

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