Adenosine produces nitric oxide and prevents mitochondrial oxidant damage in rat cardiomyocytes

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Abstract

Objective: To examine if adenosine prevents oxidant-induced mitochondrial dysfunction by producing nitric oxide (NO) in cardiomyocytes.

Methods and results: Adenosine significantly enhanced the fluorescence of DAF-FM, a dye specific for NO, implying that adenosine induces synthesis of NO. Adenosine-induced NO production was blocked by both the nonspecific NOS inhibitor N\textsuperscript{G}-nitro-l-arginine methyl ester (\textit{l}-NAME) and N\textsuperscript{5}-(1-Iminoethyl)-l-ornithine dihydrochloride (\textit{l}-NIO), an inhibitor of endothelial NOS (eNOS), but not by N\textsuperscript{6}-(1-Iminoethyl)-l-lysine hydrochloride (\textit{l}-NIL), an inhibitor of inducible NOS (iNOS), indicating that adenosine activates eNOS. Adenosine also enhances eNOS phosphorylation and its activity. The adenosine A\textsubscript{2} receptor antagonist 8-(3-chlorostyryl)caffeine but not the A\textsubscript{1} antagonist 8-cyclopentyl-1,3-dipropylxanthine prevented the increase in NO production. CGS21680, an adenosine A\textsubscript{2} receptor agonist, markedly increased NO, further supporting the involvement of A\textsubscript{2} receptors. Adenosine-induced NO production was blocked by 4-Amino-5-(4-chlorophenyl)-7-(\textit{t}-butyl)pyrazolo(3,4-d)pyrimidine (PP2), a selective Src tyrosine kinase inhibitor, suggesting that Src tyrosine kinase is crucial for adenosine-induced NO production. Adenosine-induced NO production was partially reversed by both wortmannin and Akt inhibitor indicating an involvement of PI3-kinase/Akt. Pretreatment of cells with adenosine prevented H\textsubscript{2}O\textsubscript{2}-induced depolarization of mitochondrial membrane potential ($\Delta$\textit{V}\textsubscript{m}). The protective effect was blocked by \textit{l}-NAME and \textit{l}-NIO but not by \textit{l}-NIL, indicating that eNOS plays a role in the action of adenosine. The protective effect of adenosine was further suppressed by KT5823, a specific inhibitor of protein kinase G (PKG), indicating that PKG may serve as a downstream target of adenosine.

Conclusion: Adenosine protects mitochondria from oxidant damage through a pathway involving A\textsubscript{2} receptors, eNOS, NO, PI3-kinase/Akt, and Src tyrosine kinase.

Keywords: Adenosine; Nitric oxide; Mitochondria; Signal transduction

1. Introduction

It has been well documented that adenosine protects the myocardium from ischemia–reperfusion injury \[1,2\]. Although PKC and mitochondrial K\textsubscript{ATP} channels seem to be crucial, the mechanisms by which adenosine protects myocardium remain unclear \[3\]. It has been reported that eNOS plays a role in adenosine A\textsubscript{1} receptor-induced delayed preconditioning in mouse hearts \[4\]. Recently,
Yang et al. demonstrated that the protective effect of NECA, an agonist of adenosine, on reperfusion injury was abolished by L-NAME in intact rabbit hearts, suggesting a possible involvement of NO in the action of NECA [5]. Nonetheless, there has also been evidence that NO is not responsible for adenosine-induced early preconditioning [6] or adenosine-induced protective effect on reperfusion injury [7]. Although the reason for this discrepancy is not clear, the role of NO was determined by either suppressing NOS activity or detecting NOS expression, without direct evidence of whether adenosine verily altered intracellular NO concentration in cardiomyocytes or not. Accordingly, direct measurements of the change in cardiomyocyte NO concentration would greatly help ascertain the actual role of NO in the cardioprotective effect of adenosine. Moreover, given that adenosine enhances NO generation, it is tempting to determine the mechanisms underlying this phenomenon.

Reactive oxygen species (ROS) are involved in the pathogenesis of reperfusion injury [8], and have been reported to cause mitochondrial dysfunction in cardiomyocytes [9]. Recently mitochondrial dysfunctions such as \( \Delta \Psi_m \) dissipation, matrix Ca\(^{2+} \) overload and mitochondrial permeability transition (MPT) pore opening have been proposed to be associated with the mechanism for ischemia–reperfusion injury and modulation of the mitochondrial dysfunctions may lead to cardioprotection [10]. It has been addressed that exogenous NO exerts cardioprotection by modulating mitochondrial function [11]. NO was also shown to reduce cytochrome c release by inhibiting MPT pore opening [12]. Thus, it is plausible that adenosine prevents mitochondrial oxidant damage by elevating availability of NO within cardiomyocytes.

In the present study, we first tested whether adenosine can increase NO production in isolated rat cardiomyocytes. We then sought to investigate the detailed mechanisms whereby adenosine produces NO. Lastly, we determined whether adenosine could modulate mitochondrial oxidant damage, and whether NO mediates the effect of adenosine.

## 2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

### 2.1. Isolation of adult rat cardiomyocytes

Male Wistar rats weighing 250–350 g were anesthetized with thiobutabarbital sodium (100 mg/kg, i.P.). A midline thoracotomy was performed and the heart was removed and rapidly mounted on a Langendorff apparatus. The heart was perfused in a non-recirculating mode with Krebs–Henseleit buffer (37 °C) containing (in mM) NaCl 118, NaHCO\(_3\) 25, KCl 4.7, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 1.25, and glucose 10 for 5 min to wash out blood. The buffer was bubbled with 95% O\(_2\)/5% CO\(_2\). Then the heart was perfused with calcium-free buffer that contained all of the above components except CaCl\(_2\). After 5 min of perfusion, collagenase (type II) was added to the buffer (0.1%) and the heart was perfused in a recirculating mode for ~15 min. The heart was removed from the apparatus and the ventricles were placed into a beaker containing the calcium-free buffer. The ventricles were agitated in a shaking bath (37 °C) at a rate of 50 cycles/min until individual cells were released. The released cells were suspended in an incubation buffer containing all the components of the calcium-free buffer, 1% bovine serum albumin, 30 mM HEPES, 60 mM taurine, 20 mM creatine, and amino acid supplements at 37 °C. Calcium was gradually added to the buffer containing the cells to a final concentration of 1.2 mM. The cells were filtered through nylon mesh and centrifuged briefly. The cell viability was determined using trypan blue. Finally the cells were suspended in culture medium M199 for 4 h before experiments.

### 2.2. Confocal imaging of DAF-FM

To measure intracellular NO concentration, cardiomyocytes (1×10\(^5\) cells) cultured in a specific temperature-controlled culture dish were incubated with 2 \( \mu \)M DAF-FM diacetate in standard Tyrode solution containing (mM) NaCl 140, KCl 6, MgCl\(_2\) 1, CaCl\(_2\) 1, HEPES 5, and glucose 5.8 (pH 7.4) for 20 min. The cells were then mounted on the stage of an Olympus FV 500 laser scanning confocal microscope. The cells were randomly selected and imaged. The green fluorescence was excited at 488 nm and imaged through a 525 nm long-path filter. Temperature was maintained at 37 °C with Delta T Open Dish Systems (Biotopex, Butler, PA). The images recorded on a computer were quantified using Image J.

### 2.3. Confocal imaging of \( \Delta \Psi_m \)

\( \Delta \Psi_m \) was measured by loading cardiomyocytes with tetramethylrhodamine ethyl ester (TMRE, 100 nM). The red fluorescence was excited with a 543 nm line of argon-krypton laser line and imaged through a 560 nm long-path filter.

### 2.4. Western blotting analysis of phosphorylated eNOS activity

After exposure to adenosine (100 \( \mu \)M) for 10 min, cells were homogenized in ice-cold lysis buffer. Equal amounts of protein were loaded and electrophoresed on 4% SDS–polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with the primary antibody that recognizes phospho-eNOS (Ser 1177). The primary antibody binding was detected with a secondary anti-rabbit antibody and visualized by the ECL method.
2.5. NOS activity assay

NOS activity was determined by measuring the conversion of L-[3H]arginine to L-[3H]citrulline with the NOS activity assay kit from Calbiochem. Briefly, cardiomyocytes were homogenized in homogenization buffer (25 mM Tris–HCl, 1 mM EDTA, and 1 mM EGTA) and pelleted at 14,000 × g for 5 min at 4 °C. To determine eNOS activity, the supernatant (40 μg of protein per assay) was incubated for 30 min at 30 °C in 40 μl of reaction mix containing 25 μl of 2× reaction buffer (50 mM Tris–HCl, 6 μM tetrahydrobiopterin, 2 μM FAD, and 2 μM FMN), 5 μl of 10 mM NADPH, 1 μl of 3H-arginine (1 μCi/μl), 5 μl of 6 mM CaCl2, and 4 μl of H2O. To determine iNOS activity (Ca2+-independent), the assay was conducted in the presence of 2 mM EGTA without CaCl2. The reaction was stopped by adding 400 μl of stop buffer containing 50 mM HEPES and 5 mM EDTA, and the reaction mixture containing 100 μl of resin was transferred to a spin cup. The mixture was centrifuged for 30 s, and the eluate was added to a scintillation vial containing 5 ml of scintillation fluid. Finally, the radioactivity was quantified in a liquid scintillation counter.

2.6. Experimental protocol

In the experiments measuring NO generation, adenosine (or CGS21680) was given immediately after baseline (time 0') measurements, whereas the inhibitors were given 10 min prior to the application of adenosine (Fig. 1A). The measurements were terminated 10 min after the baseline recordings. In the study evaluating the effect of exogenous zinc on Δψm (Fig. 1B), cardiomyocytes were exposed to 100 μM H2O2 for 20 min to cause mitochondrial oxidant damage. Adenosine (or SNAP) was given 10 min before exposure to H2O2.

Fig. 2. (A) Confocal fluorescence images of cardiomyocytes loaded with DAF-FM. Adenosine (100 μM) dramatically increased fluorescence of DAF-FM. (B) Summarized data for DAF-FM fluorescence intensity 10 min after exposure to adenosine expressed as percentage of baseline. Six cells were imaged in each group. Adenosine enhanced NO production in a dose-dependent manner with the peak at 100 μM. *P<0.05 vs. control.

Fig. 3. Summarized data for DAF-FM fluorescence intensity 10 min after exposure to adenosine (100 μM). The effect of adenosine (n=9) on NO production was reversed by both l-NAME (200 μM, n=6) and l-NIO (10 μM, n=7) but not by l-NIL (100 μM, n=6). Ado, adenosine. *P<0.05 vs. control; #P<0.05 vs. adenosine.
2.7. Statistical analysis

Data are expressed as mean±S.E.M. and were obtained from 5 to 10 separate experiments. Statistical significance was determined using Student’s t-test or one way ANOVA followed by Tukey’s test. A value of P<0.05 was considered as statistically significant.

3. Results

To determine the exact role of NO in the cardioprotective effect of adenosine, we tested whether adenosine could induce NO production by loading cardiomyocytes with the NO-specific fluorescence dye DAF-FM diacetate. This membrane permeant probe is converted by intracellular esterases to DAF-FM, which reacts with NO to form a green-fluorescent product. DAF-FM has been reported to be useful for detection of NO in living cells [13,14]. Fig. 2A shows confocal images of cells loaded with DAF-FM. Compared to the control cells, cells treated with adenosine revealed a dramatic increase in DAF-FM fluorescence, indicating that adenosine can trigger NO production. Fig. 2B shows that adenosine increases NO production in a dose-dependent manner. The increase peaked with 100 μM adenosine. Fig. 3 shows that adenosine (100 μM)-induced increase in DAF-FM fluorescence was reversed by the NOS inhibitor L-NAME (200 μM), implying that adenosine increased NO production via NOS. To verify the specific NOS subtype that is responsible for the action of adenosine, we then treated cells with adenosine in the presence of either the selective iNOS inhibitor L-NIL (100 μM) or the potent eNOS inhibitor L-NIO (10 μM). As shown in Fig. 3, the increased DAF-FM signal was abolished by L-NIO but not by L-NIL, suggesting that eNOS but not iNOS accounts for adenosine-induced NO production. To confirm that eNOS is responsible for adenosine-induced NO production, we next determined if adenosine can activate eNOS. As shown in Fig. 4, cells treated with adenosine (100 μM) for 10 min indeed had a significant increase in phosphorylation of eNOS at Ser1177. We then determined if adenosine could alter NOS activity by measuring the conversion of L-[3H]arginine to L-[3H]citrulline. As shown in Fig. 5A, adenosine (100 μM) significantly increased eNOS activity, confirming the role of eNOS in NO generation. In contrast, adenosine had no effect on iNOS activity (Fig. 5B), supporting the above observation that eNOS but not iNOS is responsible for the action of adenosine on NO generation.
Adenosine receptors are divided into three major subtypes (A₁, A₂ and A₃), and adenosine primarily activates A₁ and A₂ receptors [15]. To clarify the specific receptor that is responsible for the action of adenosine, we tested if specific subtype adenosine receptor antagonists can alter the effect of adenosine on NO production. As shown in Fig. 6, the adenosine-induced increase in the fluorescence was abolished by the adenosine A₂ receptor antagonist CSC (10 μM) but not by the A₁ receptor antagonist DPCPX (1 μM). Furthermore, the adenosine A₂ receptor agonist CGS21680 (100 nM) mimicked the effect of adenosine to increase NO production, confirming that the activation of A₂ receptor accounts for the action of adenosine.

Akt/PKB mediates NO production in endothelial cells [16] and adenosine activates PI3-kinase in myocardium [17]. Therefore, it is possible that adenosine produces NO through activation of the PI3-kinase/Akt signal pathway. Fig. 7 indicates that adenosine-induced NO production was abolished partially but significantly by both the PI3-kinase inhibitors (wortmannin and LY294002) and the Akt inhibitor (10 μM). The involvement of Akt signal in the action of adenosine was also evidenced by the observation that adenosine-induced eNOS phosphorylation was blocked by the Akt inhibitor (Fig. 4). Since Src tyrosine kinase has been reported as a critical upstream signal of PI3-kinase/Akt activity, we tested if adenosine can modulate mitochondrial dysfunction caused by oxidant stress. Fig. 9A shows confocal images of cardiomyocytes treated with adenosine (100 μM) and the specific Src tyrosine kinase inhibitor PP2 (1 μM), indicating that Src tyrosine kinase may also play a role in the mechanism by which adenosine induces NO production. The importance of Src tyrosine kinase in NO production by adenosine can also be found in another experiment (Fig. 5) in which PP2 completely reversed the effect of adenosine on eNOS activity.

To determine the physiological significance of the effect of adenosine on NO production, we tested if adenosine can modulate mitochondrial dysfunction caused by oxidant stress. Fig. 9A shows confocal images of cardiomyocytes loaded with the ΔΨₘ indicator TMRE. The control cell exposed to 200 μM H₂O₂ for 20 min revealed a marked decrease in TMRE intensity, indicating that oxidant stress induces a loss of ΔΨₘ. Compared to the control, the cell treated with adenosine (100 μM) showed a much smaller change in TMRE fluorescence, implying that adenosine prevents the ΔΨₘ loss. To test if NO is involved in the

Fig. 6. DAF-FM fluorescence intensity 10 min after exposure to adenosine (100 μM). The effect of adenosine on NO production was abolished by the adenosine A₂ receptor antagonist CSC (10 μM, n=8) but not by A₁ receptor antagonist DPCPX (1 μM, n=7). The adenosine A₂ receptor agonist CGS21680 (100 nM, n=6) mimicked the effect of adenosine to increase NO generation. Ado, adenosine; CGS, CGS21680. *P<0.05 vs. control; #P<0.05 vs. adenosine.

Fig. 7. DAF-FM fluorescence intensity 10 min after exposure to adenosine (100 μM). The effect of adenosine on NO production was partially but significantly suppressed by the PI3-kinase inhibitors wortmannin (100 nM, n=10) and LY294002 (10 μM, n=6). The AKT inhibitor (10 μM, n=9) also prevented the increase in NO generation by adenosine. Ado, adenosine; Wort, wortmannin; LY, LY294002; AKTI, Akt inhibitor. *P<0.05 vs. control; #P<0.05 vs. adenosine.

Fig. 8. DAF-FM fluorescence intensity 10 min after exposure to adenosine (100 μM). The effect of adenosine on NO production was blocked by both the tyrosine kinase inhibitor genistein (100 μM, n=9) and the selective Src tyrosine kinase inhibitor PP2 (1 μM, n=7). Ado, adenosine; Gen, genistein. *P<0.05 vs. control; #P<0.05 vs. adenosine.
signaling mechanism by which adenosine prevents \( \Delta \psi_m \) loss, cells were pretreated with the NOS inhibitor L-NAME 10 min before the treatment with adenosine. As shown in Fig. 9A, L-NAME reversed the effect of adenosine on \( \Delta \psi_m \), suggesting that NO may serve as a downstream signal of adenosine receptor activation. The effect of adenosine was also blocked by the eNOS inhibitor L-NIO but not by the iNOS inhibitor L-NIL, confirming that eNOS is essential in the action of adenosine. To further investigate the downstream signal that mediates the protective effect of adenosine, we tested if KT5323, a specific inhibitor of PKG, could alter the action of adenosine. As shown in the bottom of Fig. 9A, the effect of adenosine was blocked by KT5823, indicating that PKG plays a role in the action of adenosine. TMRE intensity at the end of experiments (20 min after exposure to H\(_2\)O\(_2\)) was expressed as % of baseline and summarized in Fig. 9B.

Having demonstrated that NO may serve as a downstream signal of adenosine receptor activation. The effect of adenosine was also blocked by the eNOS inhibitor L-NIO but not by the iNOS inhibitor L-NIL, confirming that eNOS is essential in the action of adenosine. To further investigate the downstream signal that mediates the protective effect of adenosine, we tested if KT5323, a specific inhibitor of PKG, could alter the action of adenosine. As shown in Fig. 9A, the effect of adenosine was blocked by KT5823, indicating that PKG plays a role in the action of adenosine. TMRE intensity at the end of experiments (20 min after exposure to H\(_2\)O\(_2\)) was expressed as % of baseline and summarized in Fig. 9B.

Having demonstrated that NO may play a role in the protective effect of adenosine, we were interested to test if exogenous NO can mimic the effect of adenosine. As shown in Fig. 10A, compared to control, cells treated with SNAP revealed much less decrease in TMRE fluorescence,

Fig. 9. (A) Confocal fluorescence images of TMRE at baseline and 20 min after exposure to H\(_2\)O\(_2\) (200 \( \mu \)M) in rat cardiomyocytes. (B) Summarized data for TMRE fluorescence intensity 20 min after exposure to H\(_2\)O\(_2\) showing that SNAP (20 \( \mu \)M, \( n=6 \)) prevented dissipation of \( \Delta \psi_m \) caused by oxidant stress. \(*P<0.05\) vs. control.

Fig. 10. (A) Confocal fluorescence images of TMRE at baseline and 20 min after exposure to 200 \( \mu \)M H\(_2\)O\(_2\) in rat cardiomyocytes. (B) Summarized data for TMRE fluorescence intensity 20 min after exposure to H\(_2\)O\(_2\) showing that SNAP (20 \( \mu \)M, \( n=6 \)) prevented dissipation of \( \Delta \psi_m \) caused by oxidant stress. \(*P<0.05\) vs. control.
indicating that exogenous NO can mimic the effect of adenosine to prevent oxidant-induced dissipation of $\Delta \Psi_m$. This result further confirms the essential role of NO in the protective effect of adenosine.

4. Discussion

Adenosine can induce NO production in endothelial cells [20–23] and smooth muscle cells [24,25]. Although Ikeda et al. have reported an indirect effect of adenosine on NO production by chronically augmenting interleukin-1$\beta$-induced NO synthesis in neonatal rat cardiomyocytes [26], there has been no data describing a direct and acute effect of adenosine on NO production in cardiomyocytes. In the present study, we have demonstrated for the first time that adenosine is able to directly produce NO in cardiomyocytes. NO is synthesized from L-arginine by three isoenzymes: nNOS, iNOS, and eNOS. Among these enzymes, the existence of iNOS and eNOS in cardiomyocytes has been well documented in the literature [27]. In the present study, adenosine-induced NO production was blocked by the potent eNOS inhibitor L-NIO [28,29] but not by the selective iNOS inhibitor L-NIL [30], suggesting eNOS but not iNOS is responsible the effect of adenosine on NO production. The importance of eNOS in NO production by adenosine is further supported by the observation that adenosine enhances phosphorylation of eNOS at its activation site (Ser 1177). Moreover, adenosine significantly increased eNOS but not iNOS activity, corroborating the above observation. It has been suggested that a large amount of NO produced by iNOS is toxic, whereas eNOS is a protective enzyme [31]. Therefore, it is highly likely that adenosine-induced NO generation through eNOS can result in cardioprotection, which may explain the cardioprotective effect of adenosine.

It has been demonstrated that adenosine enhances interleukin-1$\beta$-induced NO production through activation of adenosine A2 receptor [24,26]. Activation of adenosine A2 receptors could increase NO production in porcine coronary endothelial cells [21] and in human umbilical vein endothelial cells [23]. In addition, 5’-(N-cyclopropyl)-carboxamidoadenosine (CPCA), a selective adenosine A2 receptor agonist, increases NO release in isolated perfused rat hearts [32]. The present results support these observations confirming that adenosine A2 receptors are responsible for adenosine-induced NO production. Since we have detected the changes in NO production by imaging rod-shaped single cardiomyocytes, the current data also suggest that adenosine directly increased NO production by stimulating A2 receptors on cardiomyocytes but not on endothelial cells.

Because adenosine A1 and A3 receptors but not A2 receptors are involved in ischemic preconditioning [33], it is unlikely that NO produced by adenosine (through activation of A2 receptors) plays a role in the mechanism underlying preconditioning. It has been proposed that activation of A2 receptors at reperfusion plays a role in protecting myocardium from reperfusion injury [34]. In canine hearts, CGS-23680 given at reperfusion significantly reduced infarct size [35]. In addition, Zhao et al. reported that adenosine at reperfusion can block ischemia–reperfusion-induced apoptosis by activating A2 receptors [36]. Recently, Xu et al. have demonstrated that AMP579, a novel adenosine A1/A2 receptor agonist, given at reperfusion significantly reduces infarct size in rabbit hearts [37,38], and that the activation of adenosine A2 receptors at reperfusion accounts for the protective effects of AMP579 [37]. Interestingly, Yang et al. have documented that the NECA-induced cardioprotection against infarction at reperfusion was blocked by both the adenosine A2 receptor antagonist CSC and the NOS inhibitor L-NAME [5]. Thus, it is tenable to propose that NO produced by the activation of adenosine A2 receptor may be involved in the mechanism by which adenosine or its analogues at reperfusion prevents reperfusion injury.

Akt is a downstream target of PI3-kinase [39], and the PI3-kinase/AKT signal pathway may mediate cardioprotection at reperfusion [40]. PI3-kinase/Akt is also important in phosphorylation of eNOS at Ser 1177 [16,41]. Therefore, it is possible that adenosine activates eNOS and thus generates NO through a pathway involving PI3-kinase and Akt. In the present study, adenosine-induced NO production was suppressed by the PI3-kinase inhibitors and Akt inhibitor, indicating an involvement of this pathway in NO generation. In addition, we have demonstrated that Src tyrosine kinase is also crucial for adenosine-induced NO production. It has been documented that Src tyrosine kinase serves as an essential upstream regulator of the estrogen-stimulated PI3-kinase/Akt/eNOS pathway [18]. Similarly, Src tyrosine kinase is an upstream stimulator of the PI3-kinase/Akt pathway that mediates high density lipoprotein (HDL)-induced eNOS activation [19]. It has been shown by others that acetylcholine and adenosine can activate PI3-kinase in an Src-tyrosine kinase dependent manner in rabbit hearts [17]. Therefore, it is conceivable that adenosine activates Src tyrosine kinase, which in turn activates PI3-kinase/Akt pathway leading to the activation of eNOS, although we could not determine the exact signaling sequence in the present study. In this study, we have also noted that adenosine-induced NO production was partially blocked by the inhibition of PI3-kinase/Akt pathway, making us speculate that there may be some other parallel signaling pathways contributing to Src tyrosine kinase-mediated eNOS activation by adenosine. Indeed, several different signal components such as HSP90 and PKA have been implicated in the activation of eNOS [42]. Interestingly, it was also proposed that MAP kinase serves as the downstream signal of Src tyrosine kinase leading to the activation of eNOS [19].

Mitochondria play an important role in both necrosis and apoptosis caused by ischemia–reperfusion. In isolated cardiomyocytes subjected to ischemia–reperfusion, the mitochondrial electron transport chain is the main source of ROS upon reperfusion [43]. The generated ROS not
only cause cellular membrane damage but also induce mitochondrial dysfunction by causing mitochondrial membrane protein oxidation and lipid peroxidation [44,45]. In neonatal rat cardiomyocytes, H$_2$O$_2$ causes dissipation of ΔΨ$_m$ and accumulation of matrix Ca$^{2+}$, which are crucial for cell death [9]. The dissipation of ΔΨ$_m$ causes uncoupling of oxidative phosphorylation resulting in a decrease in ATP synthesis. Therefore the loss of ΔΨ$_m$ may lead to necrosis, since cells die from necrosis when they cannot maintain adequate ATP [46]. It has been reported that Ca$^{2+}$ is accumulated in the mitochondrial matrix during ischemia–reperfusion and the accumulation of Ca$^{2+}$ can damage mitochondria either by inhibiting oxidative phosphorylation or by inducing MPT [10]. MPTP is characterized by a progressive permeabilization of the inner mitochondrial membrane, which gradually becomes permeable to protons, ions [47]. MPTP not only causes necrosis by accelerating cellular energy depletion but also induces apoptosis by releasing proapoptotic molecules [46]. Since mitochondrial dysfunctions (the loss of ΔΨ$_m$, Ca$^{2+}$ overload, and MPT) are so closely associated with the cell death pathways (both necrosis and apoptosis), interventions that prevent mitochondrial dysfunction could be cardioprotective. In the present study, adenosine attenuated oxidant-induced ΔΨ$_m$ dissipation. Since oxidant stress has been proposed to be an important mechanism of reperfusion injury [8], this finding may suggest that adenosine or its analogues is able to protect the heart from reperfusion injury by modulating mitochondrial functions. Our data have also shown that NO generated by eNOS but not by iNOS accounts for the protective effect of adenosine on mitochondrial oxidant damage. This finding is consistent with the observation that eNOS but not iNOS is responsible for adenosine-induced NO generation. We further found that the PKG inhibitor KT5823 can block the protective effect of adenosine, suggesting that PKG may play an important role in the action of adenosine on mitochondrial damage. PKG is an important downstream target of NO, and a recent study showed that cGMP attenuates oxidant-induced reduction of ΔΨ$_m$, cytochrome c release, MPTP opening through PKG [48]. Thus, it is likely that adenosine protects mitochondria by activating PKG via NO. Although PKC and mitochondrial K$_{ATP}$ channels are important in the protective effect of adenosine A$_1$ receptor activation, their roles in A$_2$ receptor activation remain unknown. In this study, the essential role of NO in the action of adenosine was further supported by the observation that the exogenous NO donor SNAP mimicked the effect of adenosine to prevent dissipation of ΔΨ$_m$ (Fig. 10). Thus, these findings together support the above-mentioned notion that NO produced by adenosine may be involved in the cardioprotection at reperfusion. It has been reported that the loss in NO release is responsible for the decreased adenosine protection against reperfusion injury of aging rat hearts [32]. In addition, NO has been implicated in the adenosine A$_1$/A$_2$ receptor agonist NECA-induced cardioprotection against reperfusion injury. Recently, Hausenloy and Yellon have proposed that NO produced from eNOS may play a role in the protection of reperfusion injury by blocking mitochondrial damage [40]. Therefore, it is reasonable to propose that NO mediates the protective effect of adenosine on mitochondrial oxidant damage, which may serve as a critical mechanism whereby reperfusion injury is reduced. Although NO is essential for the protective effect of adenosine, it should be mentioned that there may be some other signaling elements participating in the mechanism by which adenosine protects mitochondria, since the inhibitors of NOS could partially but not completely block the effect of adenosine. The observation that SNAP (75.3 ± 12.6 % of baseline) was less effective than adenosine (91.3 ± 4.9 % of baseline) in preventing dissipation of ΔΨ$_m$ further confirmed that some unknown signaling elements other than NO are also involved in the action of adenosine.

In summary (Fig. 11), we have demonstrated that adenosine increases NO production through activation of adenosine A$_2$ receptor, Src tyrosine kinase, PI3-kinase and eNOS, and that NO may play a crucial role in the protective effect of adenosine on mitochondrial oxidant damage in adult rat cardiomyocytes. These findings will provide new insights into the mechanisms by which adenosine (or its analogue) protects myocardium at reperfusion.

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