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N^6-(3-Iodobenzyl)-adenosine-5′-*N*-methylcarboxamide Confers Cardioprotection at Reperfusion by Inhibiting Mitochondrial Permeability Transition Pore Opening via Glycogen Synthase Kinase 3β

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ABSTRACT

Although the adenosine A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (IB-MECA) has been reported to be cardioprotective at reperfusion, little is known about the mechanisms underlying the protection. We hypothesized that IB-MECA may protect the heart at reperfusion by preventing the opening of mitochondrial permeability transition pore (mPTP) through inactivation of glycogen synthase kinase (GSK) 3β. IB-MECA (1 μM) applied during reperfusion reduced infarct size in isolated rat hearts, an effect that was abrogated by the selective A_3 receptor antagonist 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)-methyl]ester (MRS1334) (100 nM). The effect of IB-MECA was abrogated by the mPTP opener atractyloside (20 μM), implying that the action of IB-MECA may be mediated by inhibition of the mPTP opening. In cardiomyocytes, IB-MECA attenuated oxidant-induced loss of mitochondrial membrane potential ($\Delta\Psi_m$), which was reversed by MRS1334. IB-MECA also reduced Ca^{2+}-induced mitochondrial swelling. IB-MECA enhanced phosphorylation of GSK-3β (Ser^9) upon reperfusion, and the GSK-3 inhibitor 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763) (3 μM) mimicked the protective effect of IB-MECA by attenuating both infarction and the loss of $\Delta\Psi_m$. In addition, the effect of IB-MECA on GSK-3β was reversed by wortmannin (100 nM), and IB-MECA was shown to enhance Akt phosphorylation upon reperfusion. In contrast, rapamycin (2 nM) failed to affect GSK-3β phosphorylation by IB-MECA, and IB-MECA did not alter phosphorylation of either mTOR (Ser^{2448}) or 70s6K (Thr^{389}). Taken together, these data suggest that IB-MECA prevents myocardial reperfusion injury by inhibiting the mPTP opening through the inactivation of GSK-3β at reperfusion. IB-MECA-induced GSK-3β inhibition is mediated by the PI3-kinase/Akt signal pathway but not by the mTOR/p70s6K pathway.

It is well known that activation of adenosine A_3 receptors by selective agonists applied before ischemia can trigger pharmacological preconditioning to protect the heart against ischemia/reperfusion injury (Tracey et al., 1997; Takano et al., 2001; Zhao and Kukreja, 2002). However, because pretreatments are seldom possible in the clinical setting of acute myocardial infarction, it is important to determine whether A_3 receptor activation after the onset of ischemia or during reperfusion can also confer cardioprotection. In this regard, Vinten-Johansen's group has reported that the selective A_3 receptor agonist 2-CI-IB-MECA given at reperfusion protects isolated rabbit hearts by decreasing polymorphonuclear neutrophil-endothelial cell interactions (Jordan et al., 1997). Likewise, several recent studies have also shown a cardioprotective effect of A_3 receptor activation with IB-MECA or 2-CI-IB-MECA upon reperfusion in rat (Maddock et al., 2002), guinea pig (Maddock et al., 2003), and dog (Auchampach et al., 2003) hearts. Nevertheless, curiously little is known about the cellular and molecular mechanisms that mediate the cardioprotection induced by A_3 receptor activation at reperfusion.

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ABBREVIATIONS: 2-CI-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide; mPTP, mitochondrial permeability transition pore; GSK, glycogen synthase kinase; PI3-kinase, phosphatidylinositol 3-kinase; mTOR, molecular target of rapamycin; p70s6K, 70-kDa ribosomal protein S6 kinase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MRS1334, 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)-methyl]ester; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione; $\Delta\Psi_m$, mitochondrial membrane potential; TMRE, tetramethylrhodamine ethyl ester; MOPS, 4-morpholinepropanesulfonic acid; MRS1191, 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; NIM811, *N*-methyl-4-isoleucine-cyclosporin.

Suleiman et al. (2001) and Weiss et al. (2003) have proposed that the opening of the mitochondrial transition pore (mPTP) plays an essential role in myocardial ischemia/reperfusion injury and that blockade of the pore opening is cardioprotective. Interestingly, it has been demonstrated that the mPTP remains closed during ischemia but opens at the onset of reperfusion (Griffiths and Halestrap, 1995) and that inhibition of mPTP opening at early reperfusion can protect the heart from reperfusion injury (Hausenloy et al., 2003; Halestrap et al., 2004). Because adenosine A_3 receptor activation at reperfusion can exert cardioprotection against reperfusion injury, it is possible that IB-MECA applied at reperfusion may induce cardioprotection by modulating the opening of the mPTP. If this is the case, it is necessary to investigate the signaling mechanisms underlying the inhibition of the mPTP opening by IB-MECA. Inhibition of GSK-3β has been shown to contribute to opioid-induced cardioprotection at reperfusion (Gross et al., 2004) and serves as one important mechanism by which pharmacological preconditioning prevents the mPTP opening in cardiomyocytes (Juhaszova et al., 2004). GSK-3β has high basal activity and is activated by phosphorylation of its Tyr^{216} residue, whereas phosphorylation at Ser^{9} decreases its activity (Cohen and Frame, 2001). Some intracellular signals such as PI3-kinase/Akt, mTOR/p70s6K, and mitogen-activated protein kinases have been proposed to decrease GSK-3β activity by phosphorylating it (Cohen and Frame, 2001; Murphy, 2004). Because these signals may play an important role in cardioprotection against reperfusion injury (Hausenloy and Yellon, 2004) and stimulation of adenosine A_3 receptors can activate Akt (Gao et al., 2001) and ERK (Schulte and Fredholm, 2003), it is possible that IB-MECA-induced cardioprotection at reperfusion may also be due to suppression of GSK-3β activity.

In this study, we examined whether IB-MECA given at reperfusion protects the heart by modulating mPTP opening via GSK-3β and to dissect the signaling mechanisms that mediate the protective effect of IB-MECA.

Materials and Methods

All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Chemicals and Antibodies. IB-MECA, MRS1334, SB216763, wortmannin, and rapamycin were purchased from Tocris Cookson Inc. (Ellisville, MO) and were dissolved in a final concentration of 0.01% dimethyl sulfoxide. Atractyloside was obtained from Sigma Chemical (St. Louis, MO) and was directly dissolved in Krebs-Henseleit bicarbonate solution. All antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA).

Perfusion of Isolated Rat Heart. Rat hearts were isolated and perfused as described previously (Xu et al., 2001). Male Wistar rats (300–350 g) were anesthetized with thiobutabarbital sodium (100 mg/kg i.p.). The hearts were removed rapidly and mounted on a Langendorff apparatus. The hearts were perfused with Krebs-Henseleit buffer containing 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose, which was heated to 37°C and gassed with 95% O_2-5% CO_2. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. The left ventricular pressure and heart rate were continuously recorded with a PowerLab system (ADInstruments, Mountain View, CA). A 5-0 silk suture was placed around the left coronary artery, and the ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. All hearts were allowed to stabilize for at least 20 min. Ischemia was induced by pulling the snare and then fixing it by clamping the tubing with a small hemostat. Total coronary artery flow was measured by timed collection of the perfusate dripping from the heart into a graduated cylinder.

Measurement of Infarct Size. At the end of the experiments, the coronary artery was reoccluded, and fluorescent polymer microspheres (2–9 μM diameter; Duke Scientific Corporation, Palo Alto, CA) were infused to demarcate the risk zone as the tissue without fluorescence. The hearts were weighed, frozen, and cut into 1-mm slices. The slices were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer at 37°C for 20 min. The slices were immersed in 10% formalin to enhance the contrast between stained (viable) and unstained (necrotic) tissue and then squeezed between glass plates spaced exactly 1 mm apart. The myocardium at risk was identified by illuminating the slices with UV light. The infarcted and risk zone regions were traced on a clear acetate sheet and quantified with ImageTool. The areas were converted into volumes by multiplying the areas by slice thickness. Infarct size is expressed as a percentage of the risk zone.

Isolation of Adult Rat Cardiomyocytes. Rat cardiomyocytes were isolated enzymatically (Xu et al., 2005). Male Wistar rats weighing 250 to 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 nonrecirculating mode with Krebs-Henseleit buffer (37°C) containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, and 10 mM glucose for 5 min to wash out blood. The buffer was bubbled with 95% O_2-5% CO_2. Then the heart was perfused with a 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 of 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. Finally the cells were suspended in culture medium M199 for 4 h before experiments.

Confocal Imaging of $\Delta\Psi_m$. $\Delta\Psi_m$ was measured using confocal microscopy as reported previously (Xu et al., 2005). In brief, cardiomyocytes cultured in a specific temperature-controlled culture dish were incubated with tetramethylrhodamine ethyl ester (TMRE, 100 nM) in standard Tyrode's solution containing 140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 5.8 mM glucose, pH 7.4, for 20 min. Cells were then mounted on the stage of an Olympus FV 500 laser scanning confocal microscope. The red fluorescence was excited with a 543-nm line of an He-Ne laser line and imaged through a 560-nm long path filter. Temperature was maintained at 37°C with Delta T Open Dish Systems (Bioptechs, Butler, PA). The images recorded on a computer were quantified using Image J.

Measurement of Mitochondrial Swelling. The mPTP opening was determined by measuring Ca^{2+}-induced mitochondrial swelling (Wang et al., 2004). Mitochondria were isolated from rat cardiomyocytes as described previously (Baines et al., 2003). Cardiomyocytes were exposed to either IB-MECA or dimethyl sulfoxide (control) for 10 min before mitochondrial isolation. Mitochondrial swelling was assessed spectrophotometrically as a decrease in absorbance at 520 nm (A_{520}). Mitochondria (0.3 mg/ml) were suspended in a buffer containing 120 mM KCl, 10 mM Tris·HCl, 5 mM KH_2PO_4, and 20 mM MOPS. The mPTP opening was induced by 200 μM $CaCl_2$.

Mitochondrial swelling was estimated by calculating percentage decrease in A_{520} 10 min after addition of Ca^{2+}.

Western Blotting Analysis. Myocardial samples taken from risk zones were homogenized in ice-cold lysis buffer. Equal amounts of protein were loaded and eletrophoresed on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with nonfat milk and then incubated with the primary antibodies (1:1000) that recognize phosphorylation of GSK-3β (Ser^9), Akt, mTOR, or p70s6K at 4°C overnight. The primary antibody bindings were detected with a secondary anti-rabbit antibody (1:2000) and visualized by the enhanced chemiluminescence method. Equal loading of samples was confirmed by reprobing membranes with antibodies that recognize total proteins (phospho and nonphospho).

Experimental Protocols. In the perfused heart study, all hearts were subjected to a 30-min regional ischemia followed by 2 h of reperfusion. Infusion of IB-MECA or SB216763 was started 5 min before the onset of reperfusion and continued for 70 min. Inhibitors were administered for 75 min, starting 5 min before the infusion of IB-MECA. Biopsies were collected from risk zones at 10 min before reperfusion, and 5 and 10 min after the onset of reperfusion. Infarct size was measured 2 h after reperfusion. In the $\Delta\Psi_m$ measurement study, cardiomyocytes were exposed to 100 μM H_2O_2 for 20 min. IB-MECA or SB216763 was given 5 min before exposure to H_2O_2 for 25 min. MRS1334 was given 5 min before exposure to IB-MECA.

Statistical Analysis. Data are expressed as means ± 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.

Results

There were no significant differences in baseline hemodynamic indexes, heart weight, and body weight among the experimental groups. There was no significant difference in the risk zone among the groups. As shown in Fig. 1, infarct size in the control hearts was 37.9 ± 3.1% of the risk zone. Treatment with 1 μM IB-MECA at reperfusion markedly reduced infarct size to 21.5 ± 3.0% of the risk zone, suggesting that IB-MECA can prevent reperfusion injury. The infarct-sparing effect of IB-MECA was abrogated by 20 μM atractyloside (38.9 ± 2.7% of the risk zone), an opener of the mPTP, indicating that IB-MECA prevents reperfusion injury by modulating the mPTP opening. Atractyloside itself did not alter infarct size (35.7 ± 3.8 of the risk zone). SB216763 (3 μM), a potent and selective inhibitor of GSK-3, reduced infarct size (25.1 ± 1.3% of the risk zone) when given at reperfusion by mimicking the effect of IB-MECA. To confirm that the inactivation of GSK-3β is an upstream event of the mPTP closing, we examined whether the protective effect of SB216763 is altered by the mPTP opener atractyloside. Figure 1 shows that the infarct-sparing effect of SB216763 was reversed by atractyloside, indicating that the inactivation of GSK-3β is crucial for the inhibition of the mPTP opening. Figure 1 also shows that the anti-infarct effect of IB-MECA is abolished by the selective A_3 antagonist MRS1334 (100 nM), suggesting that IB-MECA protected the heart via activation of A_3 receptors. To determine the role of mTOR in the action of IB-MECA, we tested whether the mTOR inhibitor rapamycin could alter the effect of IB-MECA. As shown in Fig. 1, the protection of IB-MECA was not altered by rapamycin (2 nM), implying that mTOR may not contribute to the action of IB-MECA.

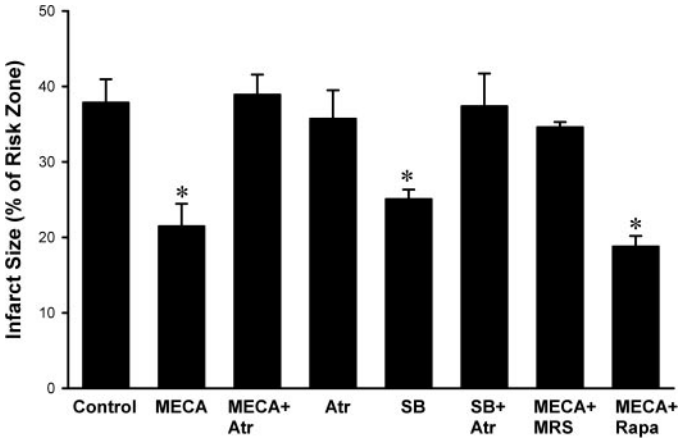


Fig. 1. Effect of IB-MECA (MECA) on myocardial infarct size in isolated rat hearts. IB-MECA (n = 6) given at reperfusion significantly reduced infarct size compared with control (n = 8), an effect that was reversed by the mPTP opener atractyloside (Atr, n = 6). The GSK-3β inhibitor SB216763 (SB, n = 6) mimicked the effect of IB-MECA by reducing infarct size, an effect that was reversed by atractyloside (n = 6). The anti-infarct effect of IB-MECA was blocked by the specific A_3 receptor antagonist MRS1334 (MRS, n = 6) but not by rapamycin (Rapa, n = 6). Each bar represents the mean ± S.E.M. of six to eight experimental observations. *, $P < 0.05$ versus control.

To further determine whether IB-MECA can modulate the mPTP opening caused by mechanisms other than ischemia, we tested the effect of IB-MECA on $\Delta\Psi_m$ in isolated rat cardiomyocytes exposed to oxidant stress. Figure 2A shows representative confocal images of cardiomyocytes loaded with the $\Delta\Psi_m$ indicator TMRE. Exposure of cells to 100 μM H_2O_2 for 20 min showed a marked decrease in TMRE fluorescence, implying that this oxidant stress paradigm causes loss of $\Delta\Psi_m$. Because the loss of $\Delta\Psi_m$ is caused by the mPTP opening (Crompton, 1999), this result indicates the mPTP opening by oxidant stress. In contrast, the cells treated with 1 μM IB-MECA revealed a much smaller change in TMRE fluorescence, indicating that IB-MECA prevents the mPTP opening caused by oxidant stress. To examine the role of GSK-3β in the action of IB-MECA, we examined whether the GSK-3 inhibitor SB216763 can attenuate the mPTP opening. SB216763 prevents the loss of $\Delta\Psi_m$ by oxidant stress, as indicated by a much smaller decrease in TMRE fluorescence. This result suggests that inhibition of GSK-3β may contribute to the action of IB-MECA. To confirm that the action of IB-MECA is mediated by activation of adenosine A_3 receptors, we tested whether the specific A_3 antagonist MRS1334 can block the effect of IB-MECA. As shown in the figure, IB-MECA failed to preserve TMRE fluorescence in the cell treated with MRS1334, indicating that adenosine A_3 receptors are involved in the action of IB-MECA. Figure 2B represents the summarized data for TMRE fluorescence intensity 20 min after exposure to H_2O_2. To corroborate the protective effect of IB-MECA on the mPTP opening, we examined whether IB-MECA could prevent Ca^{2+}-induced mitochondrial swelling. As shown in Fig. 2C, mitochondria isolated from the cardiomyocytes treated with 1 μM IB-MECA revealed a much smaller decrease in A_{520} 10 min after addition of Ca^{2+}, implying that IB-MECA may protect the heart by targeting mPTP.

To determine whether phosphorylation of GSK-3β plays a role in the protective effect of IB-MECA, we measured GSK-3β phosphorylation (Ser^9) at reperfusion in perfused rat hearts. As shown in Fig. 3, myocardial samples taken from

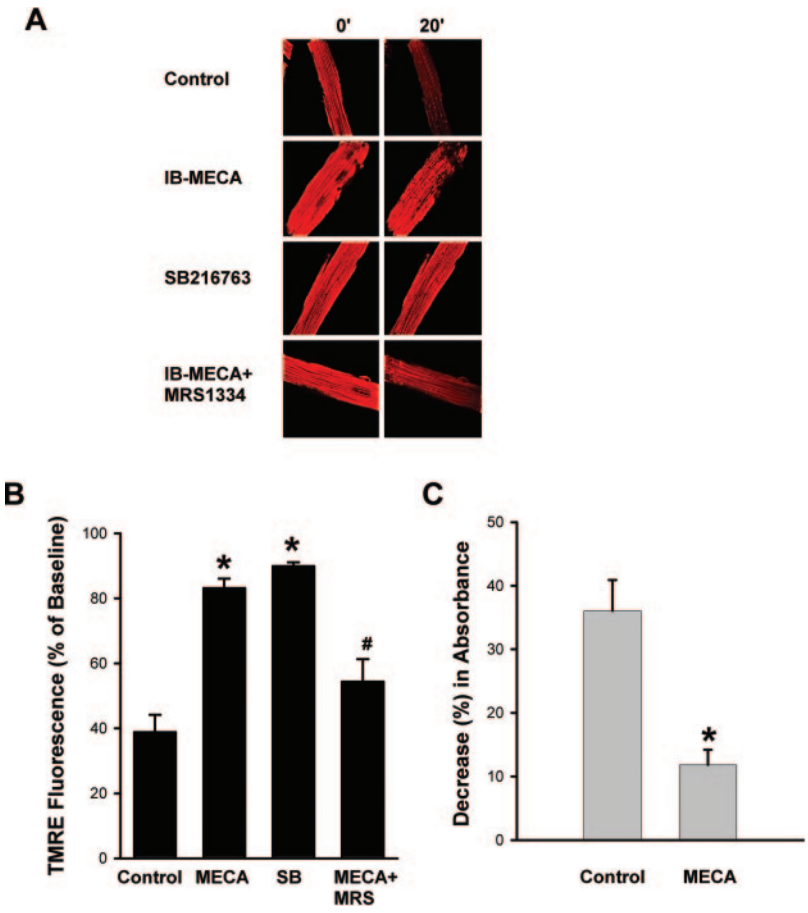


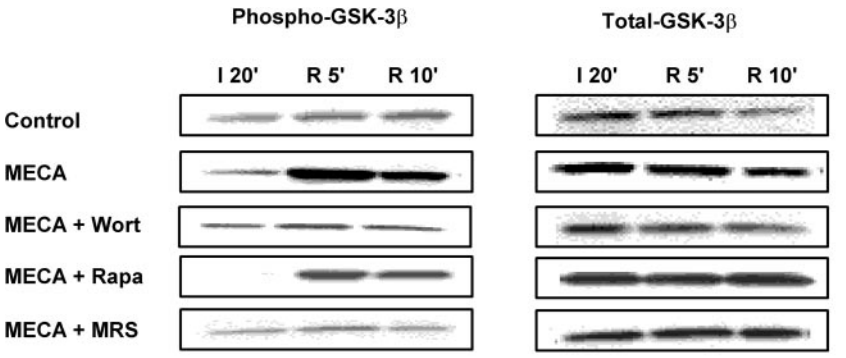
Fig. 2. A, confocal fluorescence images of adult rat cardiomyocytes loaded with the $\Delta\Psi_m$ indicator TMRE. B, summarized data for TMRE fluorescence intensity 20 min after exposure to H_2O_2. Both IB-MECA (MECA, $n = 7$) and SB216736 (SB, $n = 6$) prevented the loss of $\Delta\Psi_m$ caused by oxidant stress (control, $n = 10$). The protective effect of IB-MECA was blocked by the selective adenosine A_3 receptor antagonist MRS1334 (MRS, $n = 6$). Each bar is the mean ± S.E.M. *, $P < 0.05$ versus control; #, $P < 0.05$ versus IB-MECA. C, IB-MECA (MECA, $n = 8$) prevents Ca^{2+}-induced mitochondrial swelling (control, $n = 8$). Mitochondrial swelling was measured as a decrease in absorbance at 520 nm (A_{520}). Each bar indicates a decrease (percentage) in A_{520} by Ca^{2+} (200 μM) over 10 min. Data are presented as the mean ± S.E.M. of eight experimental observations *, $P < 0.05$ versus control.

the risk zone at 5 and 10 min of reperfusion in hearts treated with IB-MECA revealed a significant increase in GSK-3β phosphorylation compared with that of the control (405.6 ± 116.2% in IB-MECA and 116.1 ± 18.9% in control at 5 min of reperfusion; compared with that at 10 min of ischemia; $P < 0.05$) (Fig. 3, bottom), indicating that IB-MECA can suppress GSK-3β activity upon reperfusion. IB-MECA failed to enhance GSK-3β phosphorylation in the presence of the PI3-kinase inhibitor wortmannin (171.0 ± 48.8%), implying that the effect of IB-MECA on GSK-3β phosphorylation is mediated by the PI3-kinase/Akt pathway. In contrast, the mTOR inhibitor rapamycin did not alter IB-MECA-induced GSK-3β phosphorylation (399.9 ± 60.8%). In addition, MRS1334 completely reversed the effect of IB-MECA on GSK-3β phosphorylation, confirming the involvement of adenosine A_3 receptors. Figure 4 shows changes in phosphorylation of Akt upon reperfusion. IB-MECA markedly enhanced Akt phosphorylation at reperfusion compared with control (296.6 ± 53.4% in IB-MECA and 116.9 ± 5.8% in control at 5 min of reperfusion compared with that at 10 min of ischemia; $P < 0.05$), corroborating the crucial role of the PI3-kinase/Akt pathway in the action of IB-MECA. Figure 5 shows that the values for phosphorylation of mTOR and p70s6K in control and IB-MECA perfused rat hearts. Phosphorylation of either mTOR (125.2 ± 19.7% in control versus 130.6 ± 35.6% in IB-MECA) or p70s6K (90.0 ± 8.9% in control versus 103.2 ± 18.9% in IB-MECA) was not altered by IB-MECA, supporting the fact that the mTOR/p70s6K pathway is not implicated in the action of IB-MECA.

Discussion

Our data demonstrate for the first time that IB-MECA administered at reperfusion protects the hearts by inhibiting the mPTP opening in rat hearts. The preventive effect of IB-MECA on the mPTP opening is mediated by inactivation of GSK-3β at reperfusion. The PI3-kinase/Akt pathway but not the mTOR/p70s6K pathway contributes to IB-MECA-induced GSK-3β inhibition. Activation of adenosine A_3 receptors is responsible for the action of IB-MECA.

In this study, the selective adenosine A_3 receptor agonist IB-MECA protected isolated rat hearts from ischemia/reperfusion injury when applied at reperfusion, suggesting that IB-MECA is capable of attenuating reperfusion injury. This finding is consistent with several previous reports. Auchampach et al. (2003) has shown that IB-MECA given immediately before reperfusion significantly reduced infarct size in dog hearts. Likewise, Maddock et al. (2003) also demonstrated that IB-MECA infused at reperfusion markedly reduced myocardial stunning in guinea pig hearts. IB-MECA has been shown to be ~50-fold more selective for A_3 receptors than for A_1 and A_{2A} receptors in rats (Klotz, 2000). Thus, it is reasonable to propose that the cardioprotective effect of IB-MECA at reperfusion is mediated mainly by adenosine A_3 receptors. This finding is supported by the present observation that the effects of IB-MECA on the mPTP opening and GSK-3β activity were blocked by the selective adenosine A_3 receptor antagonist MRS1334. In support of our findings, Maddock et al. (2003) also reported that the anti-stunning



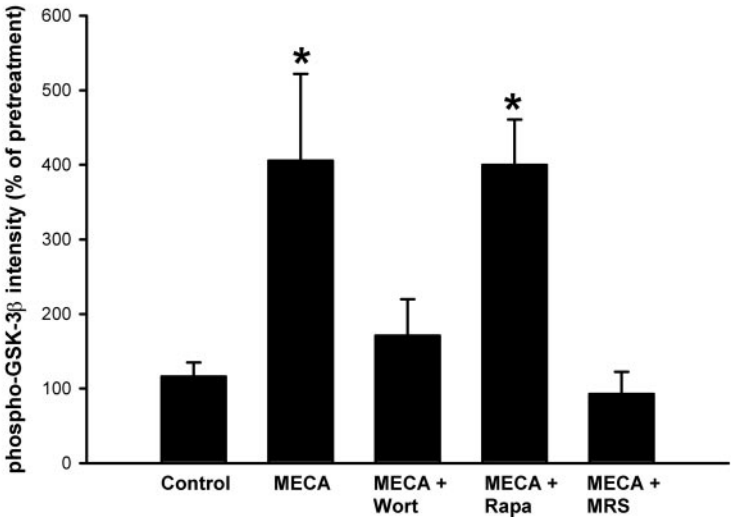
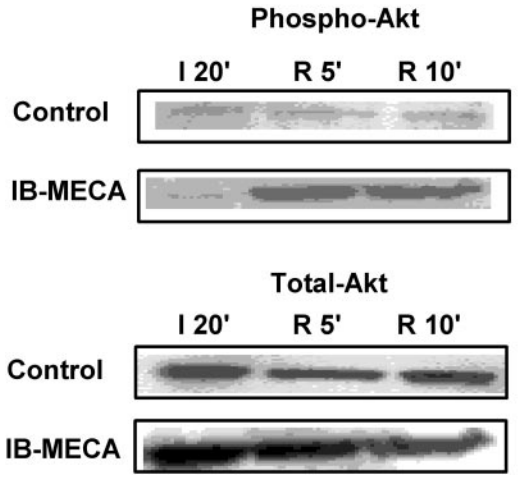


Fig. 3. Western blotting analysis of phospho-GSK-3β at Ser^{9} and total GSK-3β protein in isolated rat hearts. Top, representative Western blot of myocardial samples acquired from the risk zone at 20 min of ischemia (I 20′), 5 (R 5′) and 10 (R10′) min after onset of reperfusion. Bottom, percentage change in GSK-3β phosphorylation at 5 min of reperfusion relative to the pretreatment value (at 20 min of ischemia). IB-MECA (MECA, $n = 6$) significantly increased GSK-3β phosphorylation (control, $n = 5$), and this was prevented by the PI3-kinase inhibitor wortmannin (Wort, $n = 7$) and the adenosine A_3 receptor antagonist MRS1334 (MRS, $n = 5$). The p70s6k inhibitor rapamycin (Rapa, $n = 5$) did not alter IB-MECA-induced GSK-3β phosphorylation. Each bar is the mean ± S.E.M. of five to seven experimental observations *, $P < 0.05$ versus control.



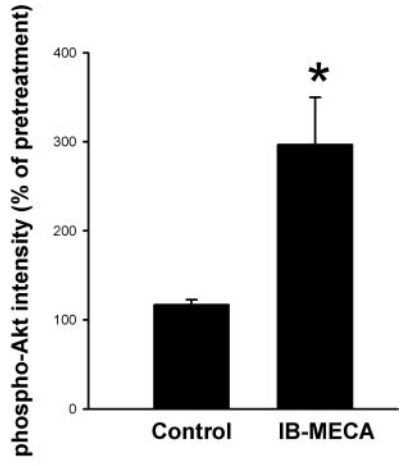


Fig. 4. Western blotting analysis of phospho-Akt and total Akt (Ser^{473}) in isolated rat hearts. Top, representative Western blot of myocardial samples acquired from the risk zone at 20 min of ischemia (I 20′), 5 (R 5′) and 10 (R10′) min after onset of reperfusion. Bottom, percentage changes in Akt phosphorylation at 5 min of reperfusion relative to the pretreatment value (at 20 min of ischemia). IB-MECA ($n = 6$) markedly enhanced Akt phosphorylation. Each bar is the mean ± S.E.M. of six experimental observations *, $P < 0.05$ versus control.

effect of IB-MECA at reoxygenation was prevented by the selective adenosine A_3 receptor antagonist 1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine. In addition, the cardioprotective effect of 2-CI-IB-MECA on reperfusion/reoxygenation injury was abrogated by the specific A_3 receptor antagonist MRS1191 in rat myocardium (Maddock et al., 2002). Moreover, in a previous study Linden's group showed that IB-MECA can protect rat mast cells from apoptosis by activating adenosine A_3 receptors (Gao et al., 2001). Therefore, it is tenable to propose that adenosine A_3 receptor activation at reperfusion is capable of protecting the heart from reperfusion injury and thus has the clinical potential to treat patients with acute myocardial infarction. This might be further supported by a recent study in which Kin et al. (2005) demonstrated that endogenous activation of adenosine A_3 receptors is involved in the cardioprotective effect of postconditioning.

Despite of the importance of A_3 receptor activation in cardioprotection at reperfusion, the cellular and molecular mechanisms underlying the action of A_3 receptors remain

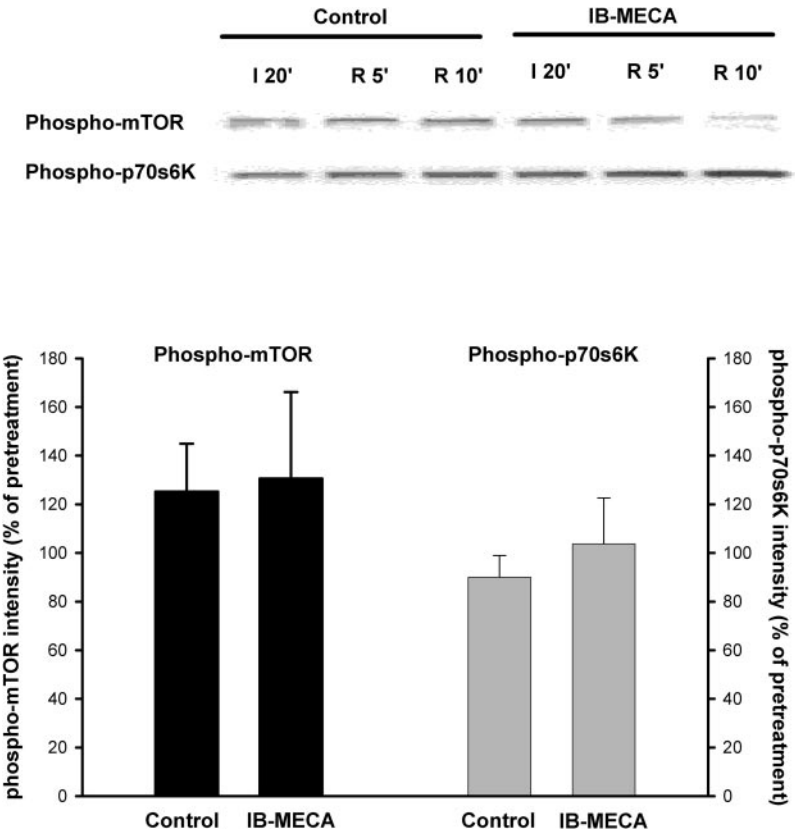


Fig. 5. Western blotting analysis of phospho-mTOR (Ser^{2448}) and phospho-p70s6K (Thr^{389}) in isolated rat hearts. Top, representative Western blot of myocardial samples acquired from the risk zone at 20 min of ischemia (I 20′), 5 (R 5′) and 10 (R 10′) min after onset of reperfusion. Bottom, percentage changes in mTOR and p70s6K phosphorylation at 5 min of reperfusion relative to the pretreatment value (at 20 min of ischemia). IB-MECA did not alter the phosphorylation of mTOR ($n = 5$, control $n = 4$) or p70s6K ($n = 7$, control, $n = 5$). Each bar is the mean ± S.E.M. of four to seven experimental observations.

unknown. Some recent studies have proposed that the mPTP opening plays a critical role in reperfusion injury. It has been demonstrated that the mPTP opens in the first few minutes of reperfusion but not during ischemia (Griffiths and Halestrap, 1995). Interestingly, Hausenloy et al. (2003) have demonstrated that inhibition of mPTP opening by sanglifehrin-A during the first few minutes of reperfusion leads to cardioprotection against infarction. Recently, the same group also reported that suppression of mPTP opening at the onset of reoxygenation with cyclosporin and sanglifehrin-A protects human myocardium against lethal hypoxia/reoxygenation injury (Shanmuganathan et al., 2005). Moreover, specific inhibition of the mPTP opening by NIM811 or cyclosporin at reperfusion was proven to provide a powerful antinecrotic and antiapoptotic protection against ischemia/reperfusion injury (Argaud et al., 2005a). A recent study by Argaud et al. (2005b) showing that postconditioning protects the heart from ischemia/reperfusion injury by inhibiting the mPTP opening further supports the essential role of inhibition of the mPTP opening in prevention of reperfusion injury. Therefore, it is possible that IB-MECA may modulate the mPTP opening, leading to cardioprotection at reperfusion. In the present study, we have shown that IB-MECA-induced cardioprotection against infarction was abrogated by the opening of the mPTP with atractyloside and that oxidant-induced mPTP opening was prevented by IB-MECA. However, the observation that the anti-infarct effect of IB-MECA was abolished by atractyloside may not exactly imply an inhibition of the mPTP opening in the action of IB-MECA, since atractyloside itself may induce cardiac damage regardless of the presence of IB-MECA, if mPTP opening serves as a critical mechanism for ischemia/reperfusion injury. Thus, we further tested whether IB-MECA could modulate Ca^{2+}-induced mitochondrial swelling, a direct index of the mPTP opening. Our data showed that IB-MECA indeed prevents mitochondrial swelling, indicating that IB-MECA is able to prevent mPTP opening. These observations indicate that inhibition of mPTP opening is an important mechanism by which adenosine A_3 receptor activation protects the heart at reperfusion. Therefore, it is likely that the mPTP may serve as a common target for various cardioprotectants that modulate reperfusion injury.

Inhibition of the mPTP opening by pharmacological preconditioning has been proposed to be attributable to suppression of GSK-3β activity (Juhaszova et al., 2004). In addition, a recent study by Gross et al. (2004) has shown that inhibition of GSK-3β is critical for the protective effect of opioids at reperfusion. Thus, it is rational to hypothesize that GSK-3β may play a role in the protective action of IB-MECA by modulating mPTP opening. In agreement with this hypothesis, we have demonstrated that IB-MECA significantly increased GSK-3β phosphorylation, thus inhibiting its activity at reperfusion, and that the selective GSK-3 inhibitor SB216763 mimicked the effects of IB-MECA on infarct size and the mPTP opening. These observations suggest that GSK-3β may play an essential role in the protective effect of IB-MECA, presumably by modulating the mPTP opening. However, it should be mentioned that in the present study we determined the potential role of GSK-3β in the action of IB-MECA on the mPTP opening by the fact that IB-MECA increases GSK-3β phosphorylation at reperfusion and that SB216763 mimics the effect of IB-MECA on the mPTP opening. For a clearer definition of the exact role of GSK-3β in the action of IB-MECA, it would be desirable to examine whether

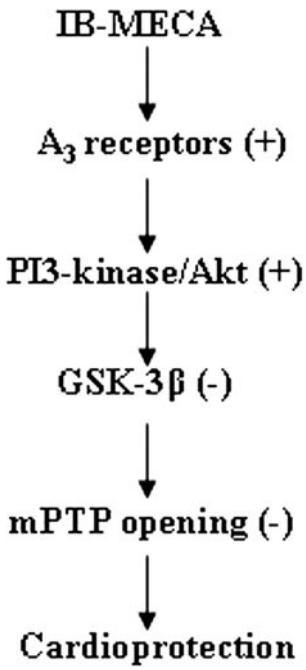


Fig. 6. Signal transduction pathway leading to the cardioprotection of IB-MECA at reperfusion.

transfection of cardiomyocytes with a constitutively active GSK-3β gene can reduce or abolish the effect of IB-MECA on mPTP opening. In addition, although SB216763 can mimic the protective effects of IB-MECA, we do not completely rule out the role of the α isoform, since SB216763 can inhibit both α and β isoforms of GSK-3.

Some intracellular kinases such as PI3-kinase/Akt, mTOR/p70d6K, and mitogen-activated protein kinase can inactivate GSK-3β (Cohen and Frame, 2001) and have been proposed to be critical for prevention of myocardial reperfusion injury (Hausenloy and Yellon, 2004). Our data have shown that GSK-3β phosphorylation by IB-MECA was reversed by the PI3-kinase inhibitor wortmannin and that IB-MECA markedly increased Akt phosphorylation at reperfusion. This is consistent with the finding of a previous study in which the antiapoptotic effect of IB-MECA was reversed by 10 and 50 nM wortmannin (Gao et al., 2001). These findings indicate that the PI3-kinase/Akt signal pathway may be involved IB-MECA-induced GSK-3β inactivation in the heart, presumably as a negative upstream regulator of GSK-3β activity. In addition, Schulte and Fredholm (2002) have also reported that activation of adenosine A_3 receptor activation in Chinese hamster ovary cells leads to strong stimulation of ERK via PI3-kinase.

The mTOR/p70s6K signal pathway has also been proposed to be an important upstream regulator of GSK-3β (Frame and Cohen, 2001). A recent study has reported that opioid-induced GSK-3β phosphorylation at reperfusion was abrogated by blockade of the mTOR/p70s6K pathway with rapamycin, suggesting that this pathway plays a role in the action of opioid on GSK-3β phosphorylation at reperfusion (Gross et al., 2004). Sollott et al. have also proposed that the mTOR/p70s6K pathway plays a role in pharmacological preconditioning-induced GSK-3β inhibition that leads to suppression of the mPTP opening (Juhaszova et al., 2004). In contrast, our data have shown that the mTOR/p70s6K inhibitor rapamycin did not alter IB-MECA-induced GSK-3β phosphorylation and that IB-MECA failed to enhance phosphorylation of either mTOR or p70s6K upon reperfusion. Although we do not know the reason for the discrepancy, it should be mentioned that the mTOR/p70s6K pathway may not always serve as the upstream regulator of GSK-3β activity. Obviously, further studies to determine the exact roles of the mTOR/p70s6K pathway in GSK-3β phosphorylation induced by various cardioprotectants at reperfusion will provide useful information on this matter.

In summary (Fig. 6), this study demonstrates that adenosine A_3 receptor activation with IB-MECA at reperfusion protects the heart by inhibiting the mPTP opening via inactivation of GSK-3β. The PI3-kinase pathway but not the mTOR/p70s6K pathway contributes to IB-MECA-induced GSK-3β inactivation.

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