The A2a/A2b receptor antagonist ZM-241385 blocks the cardioprotective effect of adenosine agonist pretreatment in in vivo rat myocardium

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Lasley RD, Kristo G, Keith BJ, Mentzer RM, Jr. The A2a/A2b receptor antagonist ZM-241385 blocks the cardioprotective effect of adenosine agonist pretreatment in vivo rat myocardium. Am J Physiol Heart Circ Physiol 292: H426–H431, 2007. First published September 15, 2006; doi:10.1152/ajpheart.00675.2006.—It is now well-recognized that the administration of adenosine before myocardial ischemia (i.e., pretreatment) protects against both reversible and irreversible injury, an effect that appears to increase for interactions among adenosine receptor subtypes in the brain and heart. The purpose of this study was to determine whether the adenosine A2a receptor modulates the infarct size-reducing effect of preischemic administration of adenosine receptor agonists in intact rat myocardium. Adult male rats were submitted to in vivo regional myocardial ischemia (25 min) and 2 h reperfusion. Vehicle-treated rats were compared with rats pretreated with the A1 agonist 2-chloro-N6-cyclopentyladenosine (CCPA, 10 μg/kg), the nonselective agonist 5'-N-ethylcarboxamidoadenosine (NECA, 10 μg/kg), or the A3 agonist 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-methylcarboxamidoadenosine (CGS-21680, 20 μg/kg). Additional CCPA- and NECA-treated rats were pretreated with the A1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 μg/kg), the A2a/A2b antagonist 4-[(2-[7-amino-2-[2-furyl]1,2,4]triazolo[2,3-a] [1,5,3]triazin-5-yl]-aminophenol (ZM-241385, 1.5 mg/kg) or the A1 antagonist 3-propyl-6-ethyl-5'-(ethylthio)carbonyl-2-phenyl-4-propyl-3-pyridine carboxylate (MRS-1523, 2 mg/kg). CCPA and NECA reduced myocardial infarct size by 50% and 35%, respectively, versus vehicle, but CGS-21680 had no effect. DPCPX blunted the bradycardia associated with CCPA and NECA, whereas ZM-241385 attenuated their hypotensive effects. Both DPCPX and ZM-241385 blocked the protective effects of CCPA and NECA. The A1 antagonist did not alter the hemodynamic effects of CCPA or NECA, nor did it alter adenosine agonist cardioprotection. None of the antagonists alone altered myocardial infarct size. These findings suggest that preischemic administration of an A2a receptor agonist does not induce cardioprotection, antagonism of the A2a and/or the A2b receptor blocks the cardioprotection associated with adenosine agonist pretreatment.

IT IS NOW WELL-RECOGNIZED that the administration of adenosine before myocardial ischemia (i.e., pretreatment) protects against both reversible and irreversible injury, an effect that appears to be mediated via the activation of cardiomyocyte A1 and/or A3 adenosine receptor subtypes (14, 21). The results of a very limited number of reports indicate that the administration of adenosine A2a agonists before ischemia is not cardioprotective (1, 13, 32, 33). However, there is now significant evidence that A2a receptor activation during reperfusion (i.e., postconditioning) reduces myocardial infarct size (7, 11, 12, 35–37). The results of in vivo studies suggest that A2a receptor postconditioning is mediated primarily via inhibition of inflammatory cell adhesion to vascular endothelial cells (7, 37). Additional reports that A2a receptor postconditioning can be induced in isolated perfused heart preparations (35, 36) suggest that direct myocardial effects may be involved. It has also been reported that rat, porcine, and human ventricular myocytes express A2a receptors (10, 20), raising the possibility that A2a receptor activation may modulate the cardiomyocyte response to ischemia-reperfusion.

Although the administration of A2a agonists before ischemia does not reduce infarct size, there are an increasing number of reports of interactions between A1 and A2a receptors. Interactions between adenosine A1 and A2a receptors have been reported in rat striatum and hippocampus (6, 8, 16–18, 23). Additional reports support the hypothesis that adenosine receptor subtype interactions may occur in the heart. Sympathetic nerve-mediated coronary constriction in stunned canine myocardium is induced by the infusion of a combination of adenosine A1 and A2a agonists but not by either agonist alone (3). It has been reported that cardiac adenosine A2a receptors modulate the adenosine A1 receptor anti-adrenergic effect (22, 34). There is also evidence implicating adenosine receptor subtype interactions in the beneficial effects of adenosine in ischemic-reperfused myocardium (11, 19, 26, 29, 30). Given the increasing evidence for adenosine receptor interactions in various tissues, including the heart, we tested the effects of A1, A2a, and A3 receptor antagonists on the cardioprotective effects of adenosine receptor agonist pretreatment in an in vivo rat model of regional myocardial ischemia.

MATERIALS AND METHODS

All animals in this study received humane care according to the guidelines set forth in The Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996). In addition, animal studies were approved by the University of Kentucky Institutional Animal Care and Use Protocol.

In vivo preparation. Adult Sprague-Dawley rats weighing 350–399 g were used. Rats were anesthetized with pentobarbital sodium (60 mg/kg ip) with supplemental doses as needed. The right jugular vein was cannulated for fluid and drug administration. The right femoral artery was cannulated for the measurement of blood pressure, heart rate, and blood gases. A tracheotomy was then performed, and the animals were connected to a small animal ventilator (model 683, Harvard Apparatus, South Natick, MA). Room air ventilation (with positive end-expiratory pressure) was supplemented with 100% O2. Respiration rate and tidal volume were adjusted to maintain normal arterial blood gases, which were determined every 30 min. Body temperature was monitored with a rectal temperature probe and maintained at 37.0–37.5°C with heating pads. A median sternotomy...
was performed and the pericardium removed. A 6-0 prolene suture was then passed below the left coronary artery in the area immediately below the left atrial appendage. The ends of the suture were then fed through a short length of propylene tubing to form a snare. After completion of the surgical procedures, the preparation was allowed to stabilize for 30 min before initiation of the experimental protocols. Regional myocardial ischemia was induced by pulling up on the snare and clamping it onto the epicardial surface using a small hemostat. Coronary artery occlusion was confirmed by epicardial cyanosis and a decrease in blood pressure.

**Determination of infarct size.** After 2 h reperfusion, the ligature at the coronary occlusion site was permanently tied off, and Evans blue solution (1%) was injected into the venous line to demarcate the left ventricular (LV) area at risk (AAR). The animal was then euthanized with a pentobarbital overdose, the heart was excised, and the atria and great vessels were removed. The heart was sliced from base to apex into three to four pieces (2 mm thickness), which were incubated in 1% triphenyltetrazolium chloride (TTC) solution in phosphate-buffered saline solution at 37°C for 15 min. After incubation each slice was compressed between two transparent acrylic plates separated by a distance of 2 mm to achieve uniform thickness. Images of each slice were taken with a CCD digital camera. The total slice area and the ischemic and infarcted areas of each slice were quantified using a graphic analysis software (Sigma Scan Pro Automated Image Analysis Software; Jandel Scientific, SPSS, San Rafael, CA). The AAR was devoid of Evans blue dye, whereas the infarcted tissue within the AAR was the TTC-negative-stained region. The percent AAR was calculated for each slice by dividing the AAR by the total slice area. The sum of the AAR for all slices was divided by the sum of the areas of all slices to obtain the percentage of the LV that was ischemic. Infarct size was expressed as the percentage of the AAR and AAR as the percentage of LV.

**Experimental protocol and study groups.** Rats were submitted to 25 min coronary artery occlusion and 2 h reperfusion. Rats were randomly assigned to receive either vehicle (10% DMSO in 1 ml normal saline iv; n = 6) or one of the following adenosine receptor agonists: 1) the adenosine A1 agonist 2-chloro-N6-cyclopentyladenosine (CCPA, 10 μg/kg, n = 6); 2) the adenosine agonist 5′-N-ethylcarboxamidoadenosine (NECA, 10 μg/kg, n = 5); or 3) the adenosine A2a agonist 2-[4-(2-carboxyethyl)phenethylamino]-5′-N-methylcarboxamidoadenosine (CGS-21680, 20 μg/kg, n = 5). Additional rats were treated with CCPA or NECA in the presence of one of the following adenosine receptor antagonists (n ≥ 5 per group): 1) the adenosine A1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 μg/kg); 2) the adenosine A2a/A2b antagonist 4′-2-[7-amino-2-(2-furyl)]1,2,4-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385, 1.5 mg/kg); or 3) the adenosine A3 antagonist 3-propyl-6-ethyl-5′-[ethylthio]carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS-1523, 2 mg/kg). A small subset of vehicle-treated rats was treated with the antagonists alone to verify their lack of effect on myocardial infarct size.

All agents were administered intravenously. The vehicle was given 30 min before ischemia. Adenosine agonists were administered 10 min before coronary occlusion, and the receptor antagonists were given 15 min before the agonists.

**Drugs.** CCPA, NECA, DPCPX, and MRS-1523 were obtained from Sigma-Aldrich (St. Louis, MO). ZM-241385 was acquired from Tocris (Ellisville, MI). All of these agents were made as concentrated stock solutions in DMSO and then diluted in saline to reduce the final DMSO concentration to ≤10%.

**Statistical analysis.** Results are expressed as means ± SE. Differences in infarct size were determined by one-way ANOVA, followed by Tukey’s post hoc test. Effects of adenosine agonists and antagonists on hemodynamics were analyzed by two-way ANOVA. A P value <0.05 was considered statistically significant.

**RESULTS**

The baseline hemodynamic data and the effects of the adenosine agonists and antagonists are shown in Figs. 1–3. There were no differences in baseline heart rates or mean arterial pressures (MAP) among the groups, with values ranging from 402 to 420 beats/min and 107–113 mmHg, respectively. Figure 1 illustrates the effects of the three adenosine agonists on hemodynamics before, during, and after regional myocardial ischemia. The adenosine A1 agonist CCPA decreased heart rate from 420 ± 11 to 150 ± 10 beats/min (P < 0.05 vs. vehicle control) immediately before ischemia, and the nonselective agonist NECA reduced heart rate from 415 ± 11 to 345 ± 31 beats/min (P < 0.05) (Fig. 1A). The A2a agonist CGS-21680 had no effect on heart rate. Heart rate in the CCPA group remained depressed throughout the remainder of the protocol, whereas heart rate in the NECA group recovered during ischemia. As shown in Fig. 1B, all three agonists induced significant decreases in MAP before and during ische-
mia, which remained depressed throughout ischemia and reperfusion in the CCPA and CGS-21680 groups.

Figure 2, A and B, illustrates the effects of three adenosine receptor antagonists on the hemodynamic effects induced by NECA (Fig. 3, A and B). The A1 receptor antagonist DPCPX blocked the immediate bradycardia associated with NECA, whereas ZM-241385 blocked the NECA-induced hypotension. The antagonists alone did not alter heart rate or MAP (data not shown).

The infarct size results are shown in Fig. 4, A and B. There were no differences in the AAR among the groups (range 37–44% of the left ventricle). Pretreatment with CCPA and NECA reduced infarct size from 51 ± 2% in vehicle-treated rats to 26 ± 3% and 33 ± 3%, respectively, of the ischemic zone (Fig. 4, A and B, respectively). The A2a agonist CGS-21680 had no effect on infarct size (47 ± 3% of the AAR). As shown in Fig. 4A, ZM-241385 blocked the infarct-reducing effect of CCPA to a similar extent as the A1 antagonist DPCPX. The A3 antagonist MRS-1523 did not alter the protective effect of CCPA. Similar effects were observed with NECA cardioprotection (Fig. 4B), as ZM-241385 blocked the infarct-reducing effect of this agonist (45 ± 2%) similar to DPCPX (50 ± 4%). The protective effect of NECA was not

Fig. 2. Effects of the three adenosine receptor antagonists on the hemodynamic effects of the adenosine A1 receptor agonist CCPA on HR (A) and MAP (B). The antagonists were administered 15 min before the agonists. Time points are as described in Fig. 1. CPX, A1 antagonist DPCPX; ZM, A2a antagonist ZM-241385; MRS, A3 antagonist MRS-1523. *P < 0.05 vs. vehicle-treated rats; +P < 0.05 vs. CCPA.

Fig. 3. Effects of the three adenosine receptor antagonists on the hemodynamic effects of the nonselective adenosine receptor agonist NECA on HR (A) and MAP (B). The antagonists were administered 15 min before the agonists. Time points are as described in Fig. 1. *P < 0.05 vs. vehicle-treated rats.
consistent with blockade of A2a receptors. The loss of cardioprotection with ZM-241385 was similar to that induced by the A1 receptor antagonist, modulates the cardioprotection induced by the adenosine receptor agonist DPCPX. In contrast, the adenosine A3 agonist MRS-1523 did not alter the effects of either agonist. These results suggest that the adenosine A2a and/or A2b receptor, but not the A3 receptor, modulates the cardioprotection induced by the adenosine A1 receptor.

Numerous studies over the years have examined the beneficial effects of administering adenosine or adenosine receptor agonists to the heart before the induction of ischemia. Initial studies with agonists and antagonists that exhibit some selectivity for the adenosine A1 receptor indicated that this protection was mediated via the A1 receptor (13, 14). The results of subsequent studies indicate that A1 receptor agonists also mimic this protective effect (21). It is thought that both of these receptors exert their protection via direct effects on the cardiac myocytes, although to date there remains no definitive evidence that A1 receptors are expressed in adult ventricular myocytes. Although there is evidence that adenosine A2a receptors are expressed in ventricular cardiomyocytes (10, 20), the results of a very limited number of studies indicate that the administration of A2a receptor agonists before ischemia does not induce cardioprotection (1, 13, 32, 33). The results of the present in vivo study confirm the inability of a selective A2a receptor agonist (CGS-21680) to reduce infarct size when administered before ischemia.

The novel finding of this study is that although an A2a agonist administered before ischemia did not induce myocardial protection, ZM-241385, an antagonist commonly used to block A2a receptors, blocked the infarct size-reducing effects of two distinct adenosine receptor agonists. ZM-241385 is a non-xanthine-based adenosine receptor antagonist that has a high affinity and selectivity for rat A2a receptors versus A1 and A3 receptors (9, 28). In both in vitro and in vivo studies, ZM-241385 exhibits high potency for A2a receptor-mediated effects versus A1 receptor-mediated effects (9, 24, 28). Our present results are consistent with these reports, because ZM-241385 significantly blunted the hypotensive effects of CCPA and NECA without altering the bradycardia induced by these agonists. Our findings that ZM-241385 blocked the cardioprotective effects of two adenosine receptor agonists, but an A2a agonist did not alter infarct size, may appear to be contradictory; however, these two results were obtained under different conditions. When both CGS-21680 and ZM-241395 were administered alone before ischemia, they exerted no effects on infarct size. In contrast, ZM-241385 exerted its blockade under conditions of simultaneous A1 receptor stimulation with the agonists CCPA and NECA.

We cannot exclude the possibility that ZM-241385, at the concentration used in this study, could have exerted its effects via blockade of the adenosine A2b receptor. Although this antagonist is selective for adenosine A2b receptors versus A1 and A3 receptors, it has been shown to exhibit some affinity for human A2b receptors (24), much as DPCPX does (5), and receptor selectivity is difficult to determine in vivo. There are some reports implicating the involvement of A2b receptors in myocardial protection; however, these effects have been observed during reperfusion in two different species with contrasting results (2, 27). Auchampach et al. (3), using three different antagonists, including DPCPX, concluded that blockade of A2b receptors following coronary reperfusion in dogs was cardioprotective. In contrast, Philipp et al. (27) reported that the A2b antagonist MRS-1754, but not DPCPX, blocked ischemic postconditioning in rabbit myocardium. Species differences may play a role in A2b receptors, since although ZM-241385 does bind to human A2b receptors (24), it has been reported that ZM-241385 exhibits no binding to rat A2b receptors (25). Definitive support for a role of adenosine A2b receptors in modulating myocardial ischemia-reperfusion injury will require more selective agonists and antagonists for the A2b receptor. Nonetheless, our observations are consistent with the hypothesis that stimulation of the A2a and/or A2b receptor modulates the cardioprotective effects of the A1 receptor.

DISCUSSION

The results of this study indicate that although adenosine A2a agonist CGS-21680 pretreatment was not protective, ZM-241835, an antagonist commonly used to block adenosine A2a receptors, blocked the infarct size-reducing effects of two distinct adenosine receptor agonists CCPA and NECA. ZM-241385 blocked the hypotension, but not bradycardia, associated with these agonists, consistent with blockade of A2a receptors. The loss of cardioprotection with ZM-241385 was similar to that induced by the A1 antagonist DPCPX. In contrast, the adenosine A1 agonist MRS-1523 did not alter the effects of either agonist. These results suggest that the adenosine A2a and/or A2b receptor, but not the A3 receptor, modulates the cardioprotection induced by the adenosine A1 receptor.

Numerous studies over the years have examined the beneficial effects of administering adenosine or adenosine receptor agonists to the heart before the induction of ischemia. Initial studies with agonists and antagonists that exhibit some selectivity for the adenosine A1 receptor indicated that this protection was mediated via the A1 receptor (13, 14). The results of myocardial protection; however, these effects have been observed during reperfusion in two different species with contrasting results (2, 27). Auchampach et al. (3), using three different antagonists, including DPCPX, concluded that blockade of A2b receptors following coronary reperfusion in dogs was cardioprotective.
Another non-xanthine adenosine receptor antagonist, MRS-1523, did not alter the cardioprotective effects of CCPA or NECA. This antagonist has been reported to have a very high affinity for rat A1 receptors, with 18-fold and 140-fold selectivities versus rat A2a and A1 receptors (15). Given the relative selectivity of this antagonist for the rat A1 receptor, these results suggest that the adenosine A3 receptor is not involved in the cardioprotective effect induced by the 10 μg/kg doses of either CCPA or NECA. Although the nonselective agonist NECA can stimulate adenosine A3 receptors, its affinity for the rat A1 receptor is 10- to 20-fold lower than for A1 and A2a receptors (5). In addition, the rat A1 receptor is very resistant to blockade by xanthine-based antagonists such as DPCPX (38). Thus our finding that MRS-1523 did not alter CCPA preconditioning is not surprising, and is consistent with Safran et al. (30), who reported that MRS-1523 did not block the cardioprotective effect of CCPA in neonatal rat myocytes. In contrast, Peart and Gross (26) reported that MRS-1523 blocked the preconditioning effect of CCPA in intact rat myocardium. We cannot explain the discrepancies between our present observations and those of Peart and Gross, since in both studies the same doses of MRS-1523 and CCPA were used.

The infarct size reduction in the present study could have been due to the prolonged A1- and A2a-mediated effects on heart rate and blood pressure, which persisted throughout reperfusion. However, several studies have demonstrated that the cardioprotective effects of A1 agonists can be dissociated from the prolonged bradycardia (26, 33). In addition, CCPA-induced cardioprotection was abolished with the A2a antagonist despite persistent bradycardia, and NECA exerted much less hemodynamic effects than CCPA. Although the antagonist ZM-241385 was administered 50 min before the onset of reperfusion, loss of protection with this antagonist could have been due to blockade of A2a receptors during reperfusion, since both CCPA and NECA were associated with some A2a receptor activation (based on decreases in arterial blood pressure). However, we do not think this is likely for several reasons. First, pretreatment with the A2a agonist CGS-21680 exerted the same extent and duration of hypotension (i.e., A2a stimulation) as did CCPA. If this persistent A2a receptor stimulation during reperfusion was contributing to the cardioprotection, then CGS-21680 pretreatment should have reduced infarct size. Second, blood pressures in the DPCPX + CCPA and DPCPX + NECA groups during reperfusion were similar to those with CCPA and NECA alone, suggesting that A2a receptors remained activated, yet cardioprotection was blocked. Finally, if reperfusion A2a activation associated with CCPA was contributing to protection, then A1 agonists should be able to induce protection when administered during reperfusion. However, there are several reports that reperfusion administration of A1 agonists is not cardioprotective (4, 11, 31, 33).

We think that the role of the A2a receptor in modulating the cardioprotection in the present study is due to the modulation of cardiac signal transduction. Stimulation of both adenosine A1 and A2a receptors is associated with the activation of multiple protein kinases that have been implicated in cardioprotection (11, 29, 36). In a previous study (29), we reported that ZM-241385 appeared to blunt AMP579-mediated increases in the phosphorylation of the myocardial mitogen-activated protein kinase, extracellular signal-regulated kinase, before ischemia. Our observations that the A2a agonist alone did not induce cardioprotection, but the antagonist ZM-241385 blocked protection induced by A1 activation, suggest that occupation of the A2a and/or A2b receptor, either by the A1 agonist or by increases in endogenous adenosine during ischemia or reperfusion, is necessary for adenosine A1 receptor-mediated cardioprotection. This hypothesis is consistent with the results of numerous reports indicating interactions or crosstalk between adenosine A1 and A2a receptors in various tissues, effects that have been described as both facilitating and inhibitory (6, 8, 16–18, 22, 23, 34). Lopes et al. (17) reported that ZM-241385 attenuated A1 receptor-mediated electrophysiological effects in rat hippocampal neurons at concentrations 10–40 times lower than those required to inhibit radioligand binding to A1 receptors. In a subsequent study (18), the same authors concluded that adenosine A3 receptor activation inhibited the tonic inhibitory effects of A1 receptors in the rat hippocampus. In normal ventricular myocardium there is evidence that the well-recognized adenosine A1 receptor-mediated anti-adrenergic effect is modulated by the A2a receptor (22, 34).

Adenosine receptor interactions have also been observed in ischemic-reperfused myocardium. Abe et al. (1) reported that in vivo neural stunning of sympathetic activation in canine coronary circulation was due to the combined effects of A1 and A2a receptor activation. We have previously reported (29) that the cardioprotective effect of the adenosine receptor agonist AMP579, which has a high affinity for both A1 and A2a receptors (31), was blocked by both the A1 antagonist DPCPX and the A2a antagonist ZM-241385. Reperfusion cardioprotection (i.e., postconditioning) with AMP579 in rabbit myocardium is mediated via A2a receptor activation, based on blockade by the A2a antagonist ZM-241385; however, the selective A2a agonist CGS-21680 was unable to mimic these effects (11, 35). Similarly, Maddock et al. (19) observed that reperfusion treatment with CGS-21680 alone was unable to protect the isolated perfused rat heart, but the A2a antagonist chlorostyryl-caffeine blocked A3 agonist-mediated protection. More directly related to our present observations are the results of Peart and Gross (26), who reported that CCPA-induced myocardial protection was blocked by opioid receptor blockade. These observations suggest that crosstalk between similar and distinct G protein-coupled receptors may play an important role in protection of ischemic myocardium.

In summary, the present study indicates that the adenosine A2a/A2b receptor antagonist ZM-241385 blocked the cardioprotective effects of the adenosine receptor agonists CCPA and NECA to the same extent as the A1 antagonist DPCPX. In contrast, the A3 antagonist MRS-1523 had no effect. These results suggest that occupation of the adenosine A2a and/or A2b receptor is necessary for the cardioprotective effects of adenosine A1 receptor activation in vivo rat myocardium.

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