Timing of adenosine 2A receptor stimulation relative to reperfusion has differential effects on infarct size and cardiac function as assessed in mice by MRI

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Yang Z, Linden J, Berr SS, Kron IL, Beller GA, French BA. Timing of adenosine 2A receptor stimulation relative to reperfusion has differential effects on infarct size and cardiac function as assessed in mice by MRI. Am J Physiol Heart Circ Physiol 295: H2328–H2335, 2008.—The activation of adenosine 2A receptors before reperfusion following coronary artery occlusion reduces infarct size and improves ejection fraction (EF). In this study, we examined the effects of delaying treatment with the adenosine 2A receptor agonist ATL146e (ATL) until 1 h postreperfusion. The infarct size and EF were serially assessed by gadolinium-diethylene-triaminepentaacetic acid-enhanced MRI in C57BL/6 mice at 1 and 24 h postreperfusion. The infarct size was also assessed by 2,3,5-triphenyltetrazolium chloride staining at 24 h. Mice were treated with ATL (10 μg/kg ip) either 2 min before reperfusion (early ATL) or 1 h postreperfusion (late ATL) following the 45-min coronary occlusion. The two methods used to assess infarct size at 24 h postreperfusion (MRI and 2,3,5-triphenyltetrazolium chloride) showed an excellent correlation (R = 0.96). The risk region, determined at 24 h postreperfusion, was comparable between the control and ATL-treated groups. The infarct size by MRI at 1 versus 24 h postreperfusion was 25 ± 1% vs. 26 ± 1% of left ventricular mass (means ± SE) in control mice, 16 ± 2% versus 17 ± 2% in early-ATL mice, and 24 ± 2% versus 25 ± 2% in late-ATL mice (intragroup, P = not significant; and intergroup, early ATL vs. control or late ATL, P < 0.05). EF was reduced in control mice but was largely preserved between 1 and 24 h in both early-ATL and late-ATL mice (P < 0.05). In conclusion, after coronary occlusion in mice, the extent of myocardial death due to ischemia-reperfusion injury is 95% complete within 1 h of reperfusion. The infarct size was significantly reduced by ATL when given just before reperfusion, but not 1 h postreperfusion. Either treatment window helped preserve the EF between 1 and 24 h postreperfusion.

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METHODS

Animals and experimental protocol. This study conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1996) and was conducted under protocols approved by the Institutional Animal Care and Use Committee. Twenty-eight 10–12-wk-old male C57BL/6 mice, purchased from Jackson Laboratories (Bar Harbor, ME), were employed as summarized in Fig. 1. Baseline magnetic resonance (MR) images were acquired 3 to 5 days before surgery to ensure that all cardiovascular parameters were fully normalized before the invasive procedure. Animals underwent 45 min of left coronary artery occlusion followed by 24 h reperfusion. Mice were imaged again at 1 and 24 h after reperfusion. Immediately after the 24-h MRI follow-up, the mice were euthanized for MI size and risk region determination. Of these mice, the eight control mice were treated with vehicle (phosphate-buffered saline, 10 μl/g ip) 2 min before reperfusion. Another 20 mice were treated with ATL (10 μg/kg ip) either 2 min before reperfusion (early ATL, n = 10) or at 1 h after reperfusion (late ATL, n = 10). ATL (Adenosine Therapeutics, Charlottesville, VA) is a potent and selective A2AR agonist (5, 25, 33).

MR image acquisition. Cardiac MRI was performed on a Varian Inova 4.7T MR scanner with Magnex gradients using previously reported methods (32, 40, 41). In brief, pediatric ECG surface electrodes (Blue Sensor, Medicotest) were attached to the shaved forelimbs of mice to enable gated image acquisition with an MR-compatible small-animal physiological monitoring system (SA Instruments, Stony Brook, NY). During the imaging, the mice were anesthetized with inhaled isoflurane (1% in medical oxygen) within a birdcage respirator. Limbs of mice to enable gated image acquisition with an MR-compatible small-animal physiological monitoring system. Animals were anesthetized with pentobarbital sodium (100 mg/kg ip) and intubated. Artificial respiration was maintained with a rodent ventilator. A parasternal incision and thoracotomy were made by cutting the left third and fourth ribs and intercostal muscles with a cautery pen. Transient left anterior descending coronary artery (LAD) occlusion was accomplished by passing a 7-0 silk suture beneath the LAD at the lower margin of the left auricle, which was then tightened over a length of polyethylene-20 tubing. Reperfusion was achieved by removing the tubing 45 min later. Upon reperfusion, the chest was closed in layers. The endotracheal tube was removed once spontaneous breathing resumed. At the end of the experiments, 2,3,5-triphenyltetrazolium chloride (TTC) and Phthalocyanine blue staining were used to delineate the infarct and risk regions, respectively, according to standard protocols detailed previously (40, 41, 46). Mice were anesthetized and euthanized by aortic perfusion with 2 to 3 ml of 0.9% sodium chloride at 37°C and 3 to 4 ml of 1.0% TTC at 37°C in phosphate buffer (pH 7.4). The LAD was then reoccluded by retying the suture left in the myocardium after reperfusion so that the heart could be perfused with 2 to 3 ml of 10% Phthalocyanine blue dye (Heubach) to stain the fully perfused regions dark blue. The atria and right ventricle were then trimmed free of the LV, which was frozen and sectioned into 1-mm-thick short-axis slices before weighing and digital photography. The risk region, infarct zone, and LV areas were then determined by computer-assisted planimetry and converted to masses according to the weight of each tissue slice. The values for all slices comprising each heart were then summed to calculate the infarct size as a percentage of either risk region or LV mass.

Fig. 1. Study design. Time lines for the 3 groups of mice are illustrated, with the timing of drug treatments indicated above each time line and the methods of assessment indicated below. ATL, ATL146e; DE-MRI, delayed-enhancement magnetic resonance (MR) imaging; TTC, 2,3,5-triphenyltetrazolium chloride; d, day.
There was a significant decrease in body weight (to 92% of baseline weight) and a significant increase in heart rate (HR; to 15% above baseline) observed 24 h after surgery. There were no significant differences in body weight or HR among the three groups at each of the three time points. However, the LV weight in all three groups was significantly increased at 24 h postreperfusion compared with the same group at 1 h postreperfusion or baseline (Tables 1 and 2). The infarct size was significantly smaller in the early ATL-treated group (P < 0.05) compared with the control or late ATL-treated groups. As in the MRI analysis, the TTC analysis found no statistical difference in infarct size between the control and late ATL-treated groups (Tables 1 and 2).

**RESULTS**

All animals survived the surgical and MRI procedures. There was a significant decrease in body weight (to 92% ± 1% of baseline weight) and a significant increase in heart rate (HR; to 15% above baseline) observed 24 h after surgery. There were no significant differences in body weight or HR among the three groups at each of the three time points. However, the LV weight in all three groups was significantly increased at 24 h postreperfusion compared with the same group at 1 h postreperfusion or baseline (Tables 1 and 2).

**Measurement of MI size by in vivo MRI versus ex vivo TTC.** Figure 2A shows a graph in which the sizes of the contrast-enhanced areas in the three groups of mice from the 24-h MR images were plotted against the corresponding areas obtained from TTC staining. In all 28 mouse hearts, the areas of contrast enhancement ranged from 7 to 31% of LV mass with a mean of 23% ± 1% and the areas not stained by TTC ranged from 7 to 32% of LV mass with a mean of 22% ± 1%. A strong correlation was found between infarct size as assessed by MRI and TTC with R = 0.96, a slope close to unity (0.93) and a y-intercept close to zero (1.78). A Bland-Altman analysis revealed a mean difference of only 0.27% of LV mass between the two methods, with upper and lower 95% limits of agreement at 3.6% and −3.1%, respectively (Fig. 2B).

**Serial determination of MI size by in vivo MRI.** MI size was serially evaluated in vivo both in control and ATL-treated groups at 1 and 24 h after reperfusion. The risk region was determined by Phthalo blue staining after the 24-h MRI session. The infarct area was found among the three groups (Table 1). Infarct size, as assessed by MRI at 1 versus 24 h postreperfusion, was 25 ± 1% vs. 26 ± 1% of LV mass in control mice, 16 ± 2% vs. 17 ± 2% in early ATL-treated mice, and 24 ± 2% vs. 25 ± 2% in late ATL-treated mice (Table 2). No statistical differences in infarct size were found between 1 and 24 h postreperfusion in any of the groups by intragroup-paired analysis (P = not significant (NS)). The intergroup analysis demonstrated that the early-ATL group had a significantly smaller infarct size at both 1 and 24 h postreperfusion compared with either the control or late-ATL groups (P < 0.05). Furthermore, there was no statistical difference in infarct size between the control and late ATL-treated mice (Fig. 4).

**DISCUSSION**

This study is the first to use delayed-enhancement MRI to demonstrate that the process of myocardial death during reperfused myocardial infarction is essentially complete within 1 h postreperfusion in mice. This conclusion is further supported by evidence that the infarct-sparing effect of a potent A2A receptor agonist (ATL) is evident only when the drug is administered before reperfusion and disappears when administered 1 h postreperfusion. Interestingly, ATL also protects against the progressive loss in LV function that is normally observed between 1 and 24 h postreperfusion, regardless of whether it is

| Table 1. Results of terminal follow-up analysis and 2,3,5-triphenyltetrazolium chloride staining |
|----------------------------------|------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Baseline         | 24 h after 45 min of LAD Occlusion |
|                                | n    | Body wt, g     | Body wt, g | Heart wt, mg | RR/LV, % | If/R, % | If/LV, % |
| Control                        | 8    | 28.9 ± 0.4     | 26.4 ± 0.1† | 140 ± 3     | 41 ± 2   | 62 ± 1   | 26 ± 2   |
| Early ATL                      | 10   | 29.3 ± 0.2     | 27.1 ± 0.3‡ | 132 ± 4     | 39 ± 1   | 44 ± 4*  | 18 ± 2*  |
| Late ATL                       | 10   | 28.6 ± 0.4     | 26.4 ± 0.4‡ | 136 ± 4     | 41 ± 2   | 61 ± 4   | 24 ± 2   |

Values are means ± SE, n, number of mice. LAD: left anterior descending coronary artery; LV: left ventricle; RR: risk region; IF: myocardial infarct size; ATL: ATL146e. *P < 0.05 compared with control and late-ATL treatment groups by unpaired t-test; †P < 0.05 compared with corresponding group at baseline by paired t-test.
administered before or 1 h after reperfusion. ATL is a potent anti-inflammatory agent due to its selective activation of A₂A Rₐs present on leukocytes. The differential effects on myocardial infarction observed between early- and late-ATL treatment suggest that the initial inflammatory response (within the first hour after reperfusion) potentiates myocardial cell death and that the later inflammatory responses may not further increase infarct size but nevertheless may negatively impact cardiac function as measured 24 h postreperfusion.

Significance of defining appropriate treatment windows for myocardial salvage. The development of myocardial infarction after different periods of coronary occlusion followed by reperfusion has been studied for many decades, but reliable methods for the noninvasive serial assessment of myocardial infarction have only become available in recent years (17, 18, 26, 30, 40). The dynamic process of ischemic myocardial death begins with the transition from reversible to irreversible injury over time, which in large animals is characterized by a transmural wavefront proceeding from the subendocardium to the subepicardium. In dog models where the collateral circulation may be limited, the infarct size may be reduced only when ATL was administered 2 min before reperfusion (2). In experiments using a dog model with 90 min of regional ischemia and 48 h of reperfusion, N-(2-mercaptopropionyl)-glycine was found to provide a further reduction in infarct size (36). These same results (obtained from TTC-stained hearts (36)) have been confirmed using automated image analysis techniques (40) and others (1, 13, 31) have reported strong correlations (up to 0.97) between infarct size as determined by late Gd-enhanced cardiac MRI and ex vivo by TTC staining. A few early reports cautioned that extravascular Gd-based contrast agents may overestimate the infarct size (34, 35); however, more recent studies using automated image analysis techniques confirm strong correlations (up to R = 0.97) between infarct size as determined by late Gd-enhancement and TTC (13). In the current report, we performed a serial analysis of MI development and cardiac function in mice during reperfusion after 45 min of LAD occlusion. In agreement with our previous work (40), the current experiments confirm that the in vivo MRI measurement correlates well with ex vivo TTC staining (Figs. 2 and 3). Although the accuracy of TTC staining at early time points after reperfusion has been the subject of debate (10), careful comparisons indicate that TTC staining yields accurate results even when employed as early as 1 h postreperfusion (36). These same results (obtained from TTC-stained hearts from parallel groups of animals) can be taken as evidence that infarct size stabilizes within 1 h of reperfusion in rats. The current study employs a serial assessment of infarct size by delayed-enhancement MRI to confirm this finding in a single group of rodents, thus excluding the potential experimental confound of infarct size variability between groups. Because of the logistical constraints of recovering the mice from surgery, transferring them to the MRI facility, and preparing them for MRI; earlier time points following reperfusion were not investigated by MRI.

Timing of cardioprotective treatment with A₂A Rₐ agonist. In the current study, ATL, delivered at a dose free from hemodynamic effects (42), was administered either 2 min before or 1 h after reperfusion. The use of two independent means of infarct size determination (MRI and TTC) conducted at 24 h postreperfusion confirmed that infarct size was significantly reduced only when ATL was administered 2 min before reper-

### Table 2. Results of serial cardiac MRI

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<th>Parameter</th>
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<th>Late ATL</th>
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*Values are means ± SE, n, number of mice. DE-MRI, delayed-enhancement MRI; EDV, end-diastolic LV volume; ESV, end-systolic LV volume; EF, ejection fraction; SV, stroke volume; HR, heart rate; CO, cardiac output. *P < 0.05 compared with corresponding baseline results by paired t-test; †P < 0.05 compared with control (1 h postreperfusion results by paired t-test; P < 0.05 compared with early ATL by unpaired t-test.

CGS-21680 (but not ATL) is charged at physiological pH, which may prolong its retention in the vascular compartment. MI development during reperfusion in intact mice. Recently, the methods for the accurate and noninvasive measurement of myocardial infarction in humans (18, 20, 37) and intact animals (15, 17, 26, 30, 41) have been established, which allow investigators to serially monitor the development of myocardial infarction and assess its effect on cardiac function. Our group (40) and others (1, 13, 31) have reported strong correlations between MI size as determined in vivo by late Gd-enhanced cardiac MRI and ex vivo by TTC staining. A few early reports cautioned that extravascular Gd-based contrast agents may overestimate the infarct size (34, 35); however, more recent studies using automated image analysis techniques confirm strong correlations (up to R = 0.97) between infarct size as determined by late Gd-enhancement and TTC (13). In the current report, we performed a serial analysis of MI development and cardiac function in mice during reperfusion after 45 min of LAD occlusion. In agreement with our previous work (40), the current experiments confirm that the in vivo MRI measurement correlates well with ex vivo TTC staining (Figs. 2 and 3). Although the accuracy of TTC staining at early time points after reperfusion has been the subject of debate (10), careful comparisons indicate that TTC staining yields accurate results even when employed as early as 1 h postreperfusion (36). These same results (obtained from TTC-stained hearts from parallel groups of animals) can be taken as evidence that infarct size stabilizes within 1 h of reperfusion in rats. The current study employs a serial assessment of infarct size by delayed-enhancement MRI to confirm this finding in a single group of rodents, thus excluding the potential experimental confound of infarct size variability between groups. Because of the logistical constraints of recovering the mice from surgery, transferring them to the MRI facility, and preparing them for MRI; earlier time points following reperfusion were not investigated by MRI.
The infarct-sparing effect of ATL completely disappeared if the treatment was delayed to 1 h postreperfusion (Table 1). MRI showed that the infarct-sparing effect of early-ATL treatment was significant at 1 h postreperfusion and provided evidence that no further increase in infarct size occurred between 1 and 24 h postreperfusion. The results obtained with the A2AR agonist support the results of the serial MRI study, indicating that myocardial infarction in mice reaches its final size within 1 h postreperfusion.

Multiple lines of evidence have suggested that the A2AR is critical for adenosine-mediated protection against ischemia-reperfusion injury. A2AR-mediated inhibition of tissue ische-

![Fig. 2. Comparison of infarct size as determined using cardiac MRI vs. conventional postmortem histology. For the 3 groups that were imaged, areas of contrast enhancement in the MR images were compared with the areas free of TTC staining by histological analysis. Significant correlation was found between the 2 methods of assessing infarct size as percentage of left ventricular (LV) mass (A). The early ATL146e (ATL)-treated group (black circles) had significantly smaller infarct sizes compared with the untreated control (gray circles), or late-ATL group (white circles) (P < 0.05, see also Tables 1 and 2). Bland-Altman analysis revealed a mean difference of only 0.3% of LV mass between the 2 methods of measurement (MRI vs. TTC, B).

![Fig. 3. Contrast-enhanced MR images from a live mouse in the control group compared with corresponding tissue slices photographed post-TTC staining. The columns of MR images (left and middle) were acquired at 1 and 24 h postreperfusion, respectively. Red arrows (middle) demarcate the circumferential extent of delayed enhancement at each MR slice position. Right: corresponding slices from the same mouse heart after staining with Phthalo blue and TTC where blue-stained areas are nonischemic, red areas are ischemic but viable, and white areas are infarcted. Excellent agreement was found between the spatial location and extent of myocardial infarction as revealed by the contrast-enhanced (white) regions in the MR images and the necrotic (white) regions of tissue not stained red by TTC.]
activation is primarily due to its inhibitory effects on CD4+ T-cell-mediated inflammatory responses during early reperfusion (42, 43). Those findings, combined with the results of the current study, indicate that a single bolus administration of ATL just before reperfusion is adequate to suppress the proinflammatory, T-cell-dependent signaling cascade that contributes to the lethal myocellular injury initiated by reperfusion.

Although outside the scope of the current study, it is interesting to note the parallels between A2A receptor activation at reperfusion and the phenomenon of postconditioning (39). In particular, the timing of the ATL administration that proved cardioprotective in the current study (2 min before reperfusion) corresponds closely to the timing of endogenous adenosine release in the heart that results from a postconditioning protocol (i.e., applying brief pulses of reocclusion upon reperfusion). Furthermore, a number of recent studies have implicated adenosine receptors as mediators of postconditioning (19, 27, 39, 45), suggesting the possibility that selective adenosine agonists may serve as pharmacomimetics of postconditioning. Additional studies will be required to fully delineate the relationship between adenosine receptor stimulation and postconditioning.

Mechanisms of adenosine A2A receptor agonist in preserving LV function. Our previous work has shown that postinfarct LV dysfunction results not only from the akinetic infarct region but also from hypokinetic noninfarcted myocardium (38). In the current study, cardiac function not only declined precipitously by 1 h postreperfusion as a direct result of MI but also continued to progressively deteriorate between 1 and 24 h postreperfusion (P < 0.05) in untreated mice. Both early- and late-ATL treatment were effective in preventing this progressive decline in LV function (Table 2), suggesting that ATL may have improved LV function 24 h postreperfusion by acting on the adjacent and remote regions of the LV. We have previously shown that the administration of ATL significantly reduces the myocardial expression of TNF-α after reperfused MI (38), which suggests one potential mechanism of action given that TNF-α is known to induce cardiac dysfunction by multiple mechanisms (23). It is also interesting to note that the differential effects of early- and late-A2A receptor activation on infarct size and cardiac function in the current study suggest the possibility that the proinflammatory mechanisms governing myocellular death after reperfusion may differ from those responsible for progressive cardiac dysfunction.

Experimental limitations. Because of the time required to close the chest and revive the mice after surgery, it was not logistically feasible to assess earlier time points, such as 30 min postreperfusion, by MRI in the current study. To overcome this limitation, future studies could be performed with a closed-chest model, which would make it possible to achieve myocardial ischemia and reperfusion after the mouse is positioned inside the MRI scanner (44). Furthermore, the techniques used in this study to evaluate infarct size by MRI with Gd-DTPA enhancement or by TTC staining do not differentiate necrotic injury from apoptotic injury, although this would be of interest since A2A receptor agonists have been reported to inhibit apoptosis (3, 49). Finally, the 24 h follow-up period in the current study was too brief to establish that the short-term benefits of infarct size reduction with A2A receptor agonists can be translated into long-term benefits in terms of LV remodeling. Thus, although it is known that the degree of LV remodeling is proportional to initial infarct size (22), additional studies will be needed to establish this relationship in the setting of myocardial salvage.

Conclusions. By using both contrast-enhanced cardiac MRI for the noninvasive and serial determination of MI size and function and TTC staining for the ex vivo evaluation of MI size in mice, we found that, after a 45 min LAD occlusion, infarct size attains over 95% of its final (24 h) volume within 1 h postreperfusion. This conclusion is further substantiated by our finding that infarct size can be significantly reduced by an A2A receptor agonist administered just before reperfusion but not at 1 h postreperfusion. In untreated control animals subject to MI, LV function deteriorates significantly between 1 and 24 h postreperfusion. Either treatment window (early or late ATL)
suffices to improve LV function compared with untreated controls as assessed 24 h after reperfusion. Thus a single administration of a potent anti-inflammatory agent, ATL146e, shows potential for protecting patients against an acute pump failure early after large, anterior MI, even when administered too late to be effective in reducing infarct size.

**REFERENCES**


