Reduction in myocardial infarct size at 48 hours after brief intravenous infusion of ATL-146e, a highly selective adenosine $A_{2A}$ receptor agonist

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Adenosine infusion during reperfusion has been shown to reduce infarct size compared with placebo in clinical trials of patients suffering from MI with large areas of ischemic anterior myocardium at risk (16, 21). Perhaps because of limited sample size, this reduction in ischemic injury was not associated with a significant mortality reduction. Adenosine administration may lead to unwanted effects such as heart block or hypotension. These effects result from nonselective activation of four adenosine receptor subtypes ($A_1$, $A_{2A}$, $A_{2B}$, and $A_3$). Selective activation of the adenosine $A_{2A}$ receptor may provide cardioprotection against reperfusion injury via an anti-inflammatory mechanism without nonselective activation of the other receptor subtypes caused by adenosine. In fact, a 2.5-h infusion of a nonvasodilating yet anti-inflammatory dose of the $A_{2A}$ receptor agonist ATL-146e beginning 30 min before reperfusion, decreased inflammation and infarct size in dogs examined 2 h after MI without increasing coronary blood flow. In the present study, adult dogs underwent 90 min of left anterior descending coronary artery occlusion. Thirty minutes before reperfusion, ATL-146e (0.01 $\mu$g kg$^{-1}$ min$^{-1}$; $n = 21$) or vehicle ($n = 12$) was intravenously infused and continued for 2.5 h (protocol 1) or 24 h (protocol 2). At 48 h after reperfusion hearts were excised and assessed for histological risk area and infarct size. Infarct size based on triphenyltetrazolium chloride (TTC) staining as a percentage of risk area was significantly smaller in ATL-146e-treated vs. control dogs (16.7 ± 3.7% vs. 33.3 ± 6.2%; $P < 0.05$; protocol 1). ATL-146e reduced neutrophil accumulation into infarcted myocardium of ATL-146e-treated vs. control dogs (30 ± 7 vs. 88 ± 16 cells/high-power field, $P < 0.002$). ATL-146e infusion for 24 h (protocol 2) conferred no significant additional infarct size reduction compared with 2.5 h of infusion. A 2.5-h ATL-146e infusion initiated 30 min before reperfusion results in marked, persistent (48 h) reduction in infarct size as a percentage of risk area in dogs with a reduction in infarct zone neutrophil infiltration. No significant further benefit was seen with a 24-h infusion.

reperfusion injury; myocardial infarction

MATERIALS AND METHODS

All animal experiments were performed with the approval of the University of Virginia Animal Care and Use Committee and were in compliance with the American Heart Association Position on Research Animal Use.

Experimental protocol. Protocol 1 randomized 32 adult mongrel dogs of both sexes (mean weight 22.4 ± 2.6 kg, range 19.5–29.5 kg) to vehicle ($n = 16$) or ATL-146e ($n = 16$). Protocol 2 utilized 10 adult mongrel dogs of both sexes, all of which received ATL-146e for 24 h. Induction of general anesthesia was achieved with pentobarbital sodium (30 mg/kg iv). After endotracheal intubation, dogs were mechanically ventilated on room air (Harvard Apparatus, Holliston, MA). Anesthesia was maintained with an intravenous pentobarbital sodium infusion. One gram of cefazolin and 80 mg of gentamicin were administered before surgery. A 5-Fr catheter was placed in the right femoral artery with the Seldinger technique and flushed with...
heparinized saline in order to monitor central arterial pressure and to serially sample arterial blood.

The dogs were then placed in the right lateral decubitus position on a heating pad set to regulate and maintain a constant normal body temperature. Heart rate, electrocardiogram, blood pressure, oxygen saturation, expired Pco₂, and body temperature were continuously monitored throughout the surgical procedure. In addition, arterial blood was periodically sampled and analyzed for pH, P O₂, and Pco₂ with a blood gas analyzer (Radiometer). After sterile skin preparation and draping, a thoracotomy was performed at the fifth intercostal space. The pericardium was divided, and the heart was suspended in a pericardial sling. All visible collaterals to the anterior wall of the left ventricle (LV) were ligated, followed by complete occlusion of the left anterior descending artery (LAD). We have previously demonstrated (6) that this technique produces a reproducible decrease in endocardial and transmural perfusion (i.e., risk area) during occlusion. After 60 min of occlusion (i.e., 30 min before reperfusion) intravenous infusion of ATL-146e (0.01 μg·kg⁻¹·min⁻¹) or vehicle (0.07% DMSO in saline) was randomly started in both the 2.5-h and 24-h infusion groups. The infusion was started 30 min before reflow in order to achieve a steady-state blood concentration before the ligatures were removed. The investigators performing the experiments were blinded to the identity of the solution being infused. Both groups of animals also received an intravenous 5-mg metoprolol bolus 30 min before reperfusion. If an animal was defibrillated or if an animal experienced >5 min of frequent nonsustained ventricular tachycardia, an additional 5 mg of intravenous metoprolol was administered at the time of the event. After 90 min of occlusion all coronary ligatures were removed to allow reperfusion. Arterial blood was sampled at the times depicted in Fig. 1. After 1 h of reperfusion, the chest was closed in layers. In the 24-h treatment group, the continuous infusion was maintained by subcutaneous implantation of an Alzet osmotic minipump (model 2001D; Durect, Cupertino, CA) that had been preloaded with the ATL-146e solution (4.4 μg/μL).

After the pentobarbital sodium infusion was discontinued, animals were given morphine analgesia (buprenorphine 0.15 mg iv and 0.15 mg sc). Animals were transferred to a heated, oxygenated recovery cage. Artemia were removed to allow reperfusion. Arterial blood was sampled at the time points shown in Fig. 1 was analyzed on an automated blood chemistry machine (Architect CI8200, Abbott Diagnostics, Abbott Park, IL). Complete blood cell counts with differentials were performed on samples collected at the time points shown in Fig. 1. Analysis was performed on an automated hematology analysis machine (Cell Line 4000, Abbott Diagnostics).

Hemodynamics. Forty-eight hours after MI, animals were placed under pentobarbital sodium anesthesia as described above. Risk area and infarct size were delineated with monosodium blue dye and triphenyltetrazolium chloride (TTC), respectively, as previously described by our laboratory (6). The hearts were then harvested, and the LV was sectioned from base to apex into four short-axis slices, each ~1 cm thick. These slices were stained with TTC (4). Sections were then digitally scanned. The infarct area, risk area, and normal myocardial area of each section were quantified with Sigmascan software (Systat, San Jose, CA) by a blinded operator. The average of the infarct area from each of the four sections was divided by the average of the risk area to arrive at the infarct area as a percentage of risk area as previously described by our laboratory (6). A priori, animals with a risk area <15% of the total myocardial area were excluded.

Histology. Forty-eight hours after MI, animals were placed under pentobarbital sodium anesthesia as described above. Risk area and infarct size were delineated with monosodium blue dye and triphenyltetrazolium chloride (TTC), respectively, as previously described by our laboratory (6). A priori, animals with a risk area <15% of the total myocardial area were excluded.

Myocardial neutrophil quantification. Transmural heart fragments from the grossly infarcted zone were randomly sampled, excised, and fixed in 3.7% paraformaldehyde solution. After dehydration and clearing with xylene, tissue samples were embedded in molten paraffin at 60°C. The paraffin-embedded specimens were then sectioned at 5 μm and stained for neutrophils with a commercially available mouse anti-human antibody (MCA8T4GT, Serotec, Raleigh, NC). Sixteen photomicrographs (×20) were taken serially every 2 mm across each section. The number of neutrophils per field was quantified with ImagePro software (MediaCybernetics, Bethesda, MD). The investigator quantifying neutrophil counts was blinded to whether the specimens were from dogs given ATL-146e or vehicle.

Statistics. One-way ANOVA with Tukey post hoc correction was used to compare infarct sizes between the groups. A two-tailed Student’s t-test was used for comparisons of neutrophil counts between control and ATL-146e-treated animals. Two-way repeated-measures ANOVA was used to analyze the mean arterial pressure and heart rate responses between the groups over time. Fisher’s exact test was used when comparing the number of dogs in each group that died from ventricular fibrillation (VF) and the number of dogs in each group that survived VF arrest. Statistical analysis was performed with SigmaStat v2.03 (SPSS, Chicago, IL).

RESULTS

Sample size. Thirty-two dogs were originally randomized to protocol 1, control (n = 16) or ATL-146e (n = 16). The final study group included 25 dogs (control n = 12; ATL-146e n = 13). Four dogs in the control group and two dogs in the ATL-146e group died secondary to lethal arrhythmias. One dog in the ATL-146e group was excluded from the study because of a small risk area in accordance with predetermined criteria.

Protocol 2 (24-h infusion) included 10 adult mongrel dogs, with a final study group of 8. One dog in protocol 2 died from lethal arrhythmia. Another dog was excluded because of a small risk area.

Hemodynamics. There was no difference in heart rate or mean arterial blood pressure between ATL-146e-treated or control animals at any monitored time point (Fig. 2). Importantly, no fall in systemic blood pressure was observed consequent to ATL-146e infusion.

Ventricular fibrillation and metoprolol use. In protocol 1, there was a trend toward a higher death rate from refractory VF in the control dogs (ATL-146e: 2/16 vs. control: 4/16); however, this trend did not reach statistical significance. Among the surviving animals there was no difference between groups in
infarct size than that achieved by just a 2.5-h infusion (24 h: ATL-146e for 24 h did not achieve a greater reduction in infarct size compared with control animals (ATL-146e: 16.7% vs. control: 33.3%)).

Histological infarct size. Risk areas were similar between treatment groups (Fig. 3). However, infarct size by TTC staining as a percentage of risk area at 48 h after reperfusion was reduced by 49% in dogs receiving the 2.5-h ATL-146e infusion compared with control animals (ATL-146e: 16.7% vs. control: 33.3%; P < 0.05). Infusion of ATL-146e for 24 h did not achieve a greater reduction in infarct size than that achieved by just a 2.5-h infusion (24 h: 17.1 ± 4.3% vs. 2.5 h: 16.7 ± 3.7%; P = 0.942).

Myocardial neutrophil infiltration. Infarct zone neutrophil infiltration (Figs. 4 and 5) differed markedly between the two groups at 48 h after MI (ATL-146e: 30.5 ± 6.8 vs. control: 87.6 ± 15.7 cells per field; P = 0.002). As shown, the ATL-146e-treated dogs had significantly fewer neutrophils in the reperfused infarct zone.

DISCUSSION

The present study demonstrates that a brief, 2.5-h infusion of a nonvasodilating, anti-inflammatory dose of the adenosine A2A receptor agonist ATL-146e started 30 min before reperfusion confers a sustained reduction in infarct size as a percentage of risk area at 48 h after reflow. Histological infarct size in treated dogs was significantly smaller than observed after reperfusion with vehicle alone (16.7% vs. 37.3%) as assessed by blinded observers. This was associated with a substantial reduction in inflammation as measured by infarct zone neutrophil infiltration. The present findings are in accordance with previously reported data at 2 h after MI in which the radiolabeled leukotriene B4 antagonist RP517, an imaging agent that targets circulating neutrophils, showed reduced infarct zone neutrophil uptake in dogs treated with ATL-146e during reperfusion (6). There was no additional benefit from an extended 24-h infusion of ATL-146e over the short 2.5-h infusion with respect to infarct size reduction. Other potential benefits of a more prolonged infusion were not explored in this study. Our findings also support the hypothesized mechanism of benefit of ATL-146e, which is a reduction in the acute inflammatory response consequent to reperfusion, leading to a reduction in infarct size (25, 28, 29). The low ATL-146e dose used does not increase myocardial blood flow (7).

Because dogs have variable coronary anatomy, each animal may have a slightly different coronary anatomic distribution and collateralization. It is speculated that infarct size estimated by serial troponin I concentrations did not achieve statistical significance because these indexes were not controlled for variations in risk area as opposed to histological infarct size measurements, which were normalized to risk area. The importance of controlling for risk area is critical in human studies.
assessing reperfusion injury (17). Nevertheless, the serial troponin assessments were concordant with the histological data and the myocardial neutrophil assessments.

The present experimental model involved producing MI at the time of thoracotomy. As expected, surgical trauma to the chest wall caused a significant leukocytosis. Nonetheless, neutrophils counts from the infarct zone of ATL-146e-treated animals were significantly lower than those of control animals.

**Mechanism of action of ATL-146e.** The adenosine receptors are G protein-coupled seven-transmembrane receptors. The A₁ receptor produces the bradycardia and heart block associated with adenosine. Additionally, the A₁ receptor has a proinflammatory effect on neutrophil function. The A₂B receptor contributes to the vasodilatory and hypotensive response of adenosine. A₂B (dogs and primates) and A₃ (rodents) receptor activation can be proinflammatory in bronchial smooth muscle tissue and may facilitate allergic reactions in susceptible subjects. On the other hand, there have also been reports showing both anti-inflammatory (8, 26) and anti-infarct (1) effects of A₃ adenosine receptor activation. Nevertheless, the cardioprotective effects of adenosine during reperfusion injury appear to be mediated at least in part by A₁ receptor activation (2, 13, 15, 18). Activation of A₂A receptors results in increased intracellular adenosine 3',5'-cyclic monophosphate (cAMP) concentration, resulting in activation of protein kinase A and inhibition of multiple steps in the inflammatory cascade. A₂A receptors are present on a variety of cells including various types of leukocytes, cardiac myocytes, and endothelial cells. ATL-146e is a highly selective A₂A receptor agonist that has been shown to have more potent binding activity to recombinant human and canine A₂A receptors than the commercially available A₂A receptor agonist CGS-21680 (23). Data from Yang et al. (28) in A₂A receptor-knockout mice demonstrated that the cardioprotective mechanism of ATL-146e is via interaction with the A₂A receptor.

The leading hypothesis regarding the potent anti-inflammatory action of A₂A receptor activation via ATL-146e in reducing reperfusion injury involves A₂A receptor-mediated reduction of CD4⁺ lymphocyte activation. In the liver and kidney, NKT cells are the principal targets of A₂A receptor activation. A₂A receptor agonism on NKT cells has been shown to reduce neutrophil-mediated reperfusion injury in these two organs (11, 14). It is not yet known whether this is also the case in the heart; however, the data presented here demonstrate that ATL-146e treatment reduced inflammation in the reperfused zone, as evidenced by a reduction in neutrophil infiltration, compared with vehicle. In a recent study using an isolated, blood-perfused heart model, Rork et al. (20) showed that, even in the absence of neutrophils, ATL-146e reduced tissue-resident mast cell degranulation and protected against postischemic myocardial necrosis. Thus current evidence demonstrates that the anti-inflammatory and infarct-sparing effects of A₂A adenosine receptor activation result from inhibition of multiple inflammatory cell types including NKT cells, mast cells, and neutrophils. An alternative or complimentary, yet nonexclusive, mechanism may be the concept of postconditioning described by Kin et al. (10) as well as others that involves delayed washout of endogenous adenosine upon reperfusion, which exerts a cardioprotective effect.

Other investigators have also studied the role of the A₂A receptor in reducing infarct size in acute models of reperfused MI in large animals. CGS-21680, a commercially available A₂A receptor agonist, is ~50 times less potent and less selective than ATL-146e. Schlack et al. (22) reported a 60-min LAD occlusion-reperfusion study in which intracoronary CGS-21680 was started 5 min before reperfusion. After 6 h of reperfusion, infarct size as a percentage of risk area was significantly smaller with CGS-21680. Jordan et al. (9) subsequently reported similar results in a canine model of LAD occlusion-reperfusion after 3 h of reperfusion. More recently, Lasley et al. (12) reported data from a porcine model of infarction and reperfusion with intracoronary CGS-21680. Infarct size as a percentage of risk area assessed after 3 h of reperfusion was reduced with intracoronary CGS-21680. The porcine model is interesting in that pigs are generally regarded as having less collateral myocardial perfusion than dogs.

Unlike adenosine, the dose of ATL-146e utilized in the present study conferred a beneficial effect on infarct size as a percentage of risk area without requiring any increase in myocardial blood flow (7). Furthermore, unlike the present study, none of the aforementioned studies evaluated the impact of adenosine receptor agonism in the presence of contemporary pharmacological treatment with metoprolol at the time of reperfusion.

**Study limitations.** Coronary flow was not assessed during ATL-146e infusion in each of the experiments reported here. However, coronary flow during various infusions of ATL-146e, including the dose used here in the same experimental animal preparation, has been published previously by our group (7). In addition, our conclusions are based on experiments in which collateral flow was not measured. Therefore, we cannot completely discount the possibility that differences in infarct size between control and ATL-146e-treated groups...
can be attributed to differences in collateral flow and not the effect of the drug.

**Clinical implications.** The present experimental study demonstrated a sustained reduction in infarct size after administration of a 2.5-h intravenous ATL-146e infusion beginning 30 min before reperfusion. It is hypothesized that ATL-146e administration to acute ST-elevation MI (STEMI) patients will be superior to the effect of adenosine since ATL-146e does not activate the A2A receptor causing hypotension and ATL-146e does not bind to the A1 receptor, which can be associated with bradycardia and atioventricular block and with proinflammatory effects. In recent clinical trials (AMISTAD I and II) conducted to assess whether adenosine would limit myocardial reperfusion injury, patients in both studies assigned to adenosine treatment had an increased incidence of hypotension and heart block (16, 21). Additionally, patients assigned to adenosine in AMISTAD I had an increased incidence of bradycardia (16). Adverse hemodynamic effects such as hypotension consequent to the A2A agonist infusino were not observed in this canine experimental study (see Figs. 2 and 3). Furthermore, the benefit of ATL-146e occurred in the presence of beta-blocker administration. Thus the infarct-reducing effect of ATL-146e is complementary to any infarct-sparing effect of early intravenous metoprolol administration. This is important because early beta-blocker therapy is part of the standard of care in treating patients with MI. If ongoing chronic studies demonstrate no adverse effect of ATL-146e on infarct healing, as was seen with steroid administration after MI, then a randomized clinical trial assessing the efficacy of ATL-146e in reducing MI is warranted.

In summary, the highly selective adenosine A2A receptor agonist ATL-146e limits reperfusion injury when given as a brief 2.5-h infusion starting 30 min before reperfusion. Despite the short time course of this infusion there is no rebound reperfusion injury when infarct size is assessed at 48 h after MI. No added benefit with regard to infarct size was observed with the more prolonged 24-h infusion of the drug. The beneficial effect of ATL-146e appears to be via its anti-inflammatory properties and persists in the presence of intravenous metoprolol given at the time of reperfusion. These data will be useful for the design of a randomized clinical trial assessing this novel treatment for attenuating reperfusion injury.

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**DISCLOSURES**

D. K. Glover and J. Linden have equity interest/stock options in Adenosine Therapeutics; G. A. Beller has Founders Stock in and is a member of the advisory board of Adenosine Therapeutics.

**REFERENCES**


19. Rork TH, Wallace KL, Kennedy DP, Marshall MA, Lankford AR, Linden J. Adenosine A2A receptor activation reduces infarct size in the isolated, perfused mouse heart by inhibiting resident cardiac mast cell...


