Adenosine A₃ receptor activation protects the myocardium from reperfusion/reoxygenation injury

HELEN L. MADDOCK,* MIHAELA M. MOCANU,* AND DEREK M. YELLON
The Hatter Institute for Cardiovascular Studies, Division of Cardiology, University College London Hospitals and Medical School, London WC1E 6DB, United Kingdom

Received 1 October 2001; accepted in final form 29 May 2002

Maddock, Helen L, Mihaela M. Mocanu, and Derek M. Yellon. Adenosine A₃ receptor activation protects the myocardium from reperfusion/reoxygenation injury. Am J Physiol Heart Circ Physiol 283: H1307–H1313, 2002.—Ischemia–reperfusion induces both necrotic and apoptotic cell death. The ability of adenosine to attenuate reperfusion-induced injury (RI) and the role played by adenosine receptors are unclear. We therefore studied the role of the A₃ receptor (A₃R) in ameliorating RI using the specific A₃R agonist 1-[2-chloro-6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxi-N-methyl-b-D-ribofuranuronamide (2-Cl-IB-MECA). Isolated rat hearts and cardiomyocytes were subjected to ischemia or simulated ischemia, followed by reperfusion/reoxygenation. The end points were percent infarction/risk zone and annexin-V (apoptosis) and/or propidium iodide positivity (necrosis) and/or propidium iodide positivity (necrosis), respectively. In isolated hearts, 2-Cl-IB-MECA significantly limited infarct size (44.2 ± 2.7% in control vs. 21.9 ± 2.4% at 1 nM and 35.8 ± 3.3% at 0.1 nM, P < 0.05). In isolated myocytes, apoptosis and necrosis were significantly reduced compared with controls (5.7 ± 2.0% at 1 nM and 35.8 ± 2.7% in control vs. 21.9 ± 2.4% at 1 nM and 35.8 ± 3.3% at 0.1 nM, P < 0.0001). In both models, the beneficial effects were abrogated using the A₃R antagonist MRS-1191. The involvement of A₂a receptor activation was also examined. This is the first study to demonstrate that A₃R activation at reperfusion limits myocardial injury in the isolated rat heart and improves survival in isolated myocytes, possibly by antiapoptotic and antinecrotic mechanisms.

Reperfusion injury; necrosis; apoptosis

Adenosine is well recognized as a potent intermediary of myocardial protection, exerting its effects during both ischemia and reperfusion through receptor-mediated actions (19). There are four adenosine receptors, which have been cloned and designated A₁, A₂a, A₂b, and A₃ (40). Much attention has focused on the beneficial effects of activating adenosine A₁ and/or A₃ receptors in preconditioning the myocardium and hence reducing ischemic injury (36, 38). However, in terms of clinical relevance to acute myocardial infarction, it is important that a drug exerts its cardioprotective effects after the onset of ischemia and during reperfusion. Indeed, pretreatment with a drug before ischemia is seldom possible in the setting of acute myocardial infarction because it is not always possible to define that particular category of patients at risk until it is too late (48).

Reperfusion of ischemic myocardium is necessary for tissue salvage but can paradoxically cause lethal reperfusion injury and hence cell death (49). A large component of cell death during myocardial ischemia–reperfusion results from cellular necrosis. Furthermore, recent evidence suggests that programmed cell death (apoptosis) contributes in part to overall myocyte cell death during the reperfusion period (6, 10). It has been proposed that the apoptotic program is initiated shortly after the onset of apoptosis, but there is evidence that the process is amplified during reperfusion (6, 10, 29).

Therefore, there is a defined target in which pharmacological therapy can be designed to protect the myocardium against both the necrosis and apoptosis that occur as a consequence of reperfusion.

Studies investigating the effects of exogenous adenosine itself as well as adenosine A₁ and A₂a receptor agonists when administered at reperfusion have yielded conflicting results (9, 11, 23–25, 42). However, there are very little data investigating the effects of adenosine A₃ receptor activation on reperfusion injury, although Vinten-Johansen and colleagues (14) have shown that A₃ receptor activation at reperfusion attenuated neutrophil-mediated reperfusion injury in the dog. Notably, however, activation of adenosine A₃ receptors has been shown to reduce the degree of apoptosis in models of ischemia-reperfusion in the brain (1) and in HL-60 leukemia and U-937 lymphoma cell lines (47).

The objective of this study was therefore to determine whether activation of adenosine A₃ receptors using the specific adenosine A₃ agonist 1-[2-chloro-6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxi-N-methyl-b-D-ribofuranuronamide (2-Cl-IB-MECA) could protect the isolated perfused rat heart against cell death when given at the moment of reperfusion.

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*H. L. Maddock and M. M. Mocanu contributed equally to this work.

Address for reprint requests and other correspondence: D. M. Yellon, The Hatter Institute for Cardiovascular Studies, Div. of Cardiology, Univ. College London Hospitals and Medical School, Grafton Way, London WC1E 6DB, UK (E-mail: hatter-institute@ucl.ac.uk).

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Additional experiments were performed to rule out the possibility of a nonspecific activation of the adenosine A<sub>2a</sub> receptor that may contribute to the observed protection. For this purpose, we administered concomitantly the adenosine A<sub>3</sub> receptor antagonist 2-CI-IB-MECA and the A<sub>2a</sub> receptor antagonist 8-3-chlorostyryl caffeine (CSC) during reperfusion. Also, we investigated the role of a specific A<sub>2a</sub> agonist, CGS-21680, given at reperfusion.

Furthermore, we investigated the effects of adenosine A<sub>3</sub> receptor activation at reoxygenation on apoptotic and necrotic cell injury using an isolated rat myocyte model of simulated ischemia/reoxygenation.

MATERIALS AND METHODS

Drugs

A highly specific adenosine A<sub>3</sub> receptor agonist, 2-CI-IB-MECA, and an adenosine A<sub>2a</sub> receptor agonist, CGS-21680, were purchased from Tocris. 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4(±)-dihydropyridine-3,5 ditarboxylate (MRS-1191), a specific blocker of the adenosine A<sub>3</sub> receptor, and the adenosine A<sub>2a</sub> receptor antagonist CSC were purchased from Sigma. Drugs were selected for their high specificity for the rat tissue (4, 23). The drugs were dissolved in DMSO, and the aliquots were frozen at −20°C.

Ex Vivo Rat Hearts

Animals. Male Sprague-Dawley rats (350–400 g body wt) were used. All animals were obtained from the same source, fed a standard diet, housed under the same conditions, and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986. During the experiments, there were seven exclusions, these being due to technical problems (i.e., hearts not properly stabilized).

Heart preparation. The animals were anesthetized by an intraperitoneal administration of pentobarbital sodium (55 mg/kg) and anticoagulated with heparin (300 units). The hearts were then excised, placed in ice-cold buffer, and within 1 min mounted in a constant-pressure Langendorff system (80 mmHg). They were perfused retrogradely with a modified Krebs-Henseleit (KH) bicarbonate buffer containing (in mM) 118.5 NaCl, 25 NaHCO<sub>3</sub>, 4.8 KCl, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.7 CaCl<sub>2</sub>, and 12 glucose. All solutions were gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> balanced with argon (BOC gases). For control conditions and the controls, the percentage of infarcted tissue within the volume of the myocardium at risk was calculated (IR%).

Experimental groups. All hearts were allowed to stabilize for 15 min before being subjected to 35 min of regional ischemia followed by 120 min of reperfusion. One minute before reperfusion, they were randomly distributed into the following groups: 1) hearts perfused with normal buffer (controls, n = 11); 2) hearts perfused with different concentrations of the adenosine A<sub>3</sub> receptor agonist 2-CI-IB-MECA (100, 30, 10, 1 or 0.1 nM, n = 4, 4, 6, 8, and 4, respectively) added in the buffer throughout the reperfusion period; 3) hearts perfused for the same duration with a mixture of 1 nM 2-CI-IB-MECA and 1 µM MRS-1191, a specific A<sub>3</sub> antagonist (n = 6); or 4) perfused only with the antagonist added in the buffer (n = 4). There were also hearts that underwent the same protocol in the presence or absence of the adenosine A<sub>2a</sub> antagonist, 100 nM A<sub>2a</sub> agonist (CGS-21680, n = 5) or 100 nM A<sub>2a</sub> agonist (CGS-21680, n = 5) during reperfusion.

Rat Cardiomyocytes

Cardiomyocytes preparation. Rat ventricular myocytes were isolated by conventional enzymatic dissociation. In brief, male Sprague-Dawley rats (350–400 g body wt) were anesthetized with pentobarbital sodium (55 mg/kg) and anticoagulated with heparin (300 units) intraperitoneally. These hearts were then excised, mounted on a Langendorff apparatus, and perfused with modified ADS control buffer containing (in mM) 137 NaCl, 3.8 KCl, 0.49 MgCl<sub>2</sub>, 4 HEPES, 10 glucose, and 10 2,3-butanedione monoxime (pH 7.4). The perfusate was bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at 37°C. After 5 min, the hearts were switched to a modified Tyrode solution containing 1.0 mg/ml collagenase (Worthington type II) and 50 µM calcium for 10–15 min. They were then perfused for 5 min with ADS buffer containing 50 µM calcium alone. The hearts were then removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced and incubated in a shaking bath for 5 min in collagenase-containing solution. Cells were then filtered through nylon mesh and washed with restoration buffer (containing [in mM] 137 NaCl, 3.8 KCl, 0.49 MgCl<sub>2</sub>, 4 HEPES, 10 glucose, 10 2,3-butanedione monoxime, 2 carnitine, 5 creatine, 5 taurine, and 5 Na-pyruvate (pH 7.4)). The calcium concentration was gradually brought back to 1.25 mM.

Experimental protocol. The cardiac myocytes were exposed to lethal simulated ischemia as follows: the normal restoration medium was replaced with 2 ml Esumi ischemic buffer (5) containing (in mM) 137 NaCl, 12 KCl, 0.49 MgCl<sub>2</sub>, 0.9 CaCl<sub>2</sub>·H<sub>2</sub>O, 4 HEPES, 20 Na-lactate, 10 deoxyglucose, and 10 KCl (pH 6.5), and the cells were incubated at 37°C for 45 min in the hypoxic chamber in an atmosphere of 0% O<sub>2</sub>-5% CO<sub>2</sub> balanced with argon (BOC gases). For control conditions with or without drug treatments, myocytes were cultured with 2 ml modified Esumi control buffer for 2.75 h at 37°C in an atmosphere of 21% O<sub>2</sub>-5% CO<sub>2</sub> balanced with N<sub>2</sub> (normoxic environment). To investigate whether 2-CI-IB-MECA (adenosine A<sub>3</sub> agonist) protected adult cardiac myocytes from reoxygenation injury, the cells were incubated with different concentrations of 2-CI-IB-MECA (0.1, 1, 10, 30, and 100 nM) for 2 h at the point of reoxygenation and in the presence and absence of the selective adenosine A<sub>3</sub> receptor antagonist MRS-1191 (1 µM).
Assessment of apoptosis and necrosis. The cells were washed with 1 ml of cold PBS (pH 7.4; 140 mM NaCl, 5 mM KCl, and 1.8 mM CaCl₂). They were then incubated for 10 min in the dark at room temperature in annexin V-FITC solution (1:50 in annexin binding buffer). Propidium iodide (PI; 100 mg/ml) was added to the myocytes loaded with annexin V. Samples were analyzed immediately by flow cytometry using a Partec flow cytometer (Partec; Münster, Germany) equipped with a 488-nm argon laser, with settings optimized for detection of fluorescein and PI. Annexin V has been shown previously to detect the early stages of apoptosis by binding to the phosphatidyl serine (PS) residues, which are translocated on the external face of the cell membrane (2, 38, 40). Translocation of PS occurs early in apoptosis while the cell membrane is still intact. Cellular necrosis was determined using PI. The assay is based on the vital binding of PI to the nuclei of cells whose plasma membranes have become permeable due to cell damage (7). Results are expressed as the percent annexin V-positive/PI-negative (early apoptotic) and annexin-positive/PI-positive (necrotic) total numbers of cells.

Statistical Analysis

Data, expressed as means ± SE, were compared using one-way ANOVA and Fisher’s protected least-significant-difference test for multiple comparisons. The differences between groups were considered significant when \( P < 0.05 \).

RESULTS

Role of Adenosine A₃ Receptor Activation at Reperfusion Upon Infarction in Isolated Perfused Rat Heart

Homogeneity among experimental groups. The results of 45 successful experiments were included. The risk zone was not significantly different between groups (0.57–0.64 cm³). Heart rate, coronary flow, and left ventricular developed pressure were not significantly different among groups at the end of the stabilization period (data not presented).

Protective effect of adenosine A₃ agonist 2-Cl-IB-MECA is dose dependent. Our data showed that, in the isolated perfused rat hearts, there is a dose-dependent effect of the adenosine A₃ agonist 2-Cl-IB-MECA related to its ability to limit infarct size. Infarct developed in the risk zone (I/R%) was not different from control hearts when the agonist was present at concentrations of 30 nM or higher \( I/R% \) (44.2 ± 2.7 in control vs. 41.2 ± 5.6 at 100 nM and 40.2 ± 6 at 30 nM, \( P > 0.05 \)). However, lower concentrations of 2-Cl-IB-MECA significantly reduced the development of infarction \( I/R% \) (33.6 ± 4 at 10 nM and 21.9 ± 2.4 at 1 nM, \( P < 0.05 \) vs. control). The protective effect started to diminish when the agonist was further diluted \( I/R% \) (35.8 ± 3.3 at 0.1 nM) (Fig. 1).

Protective effect of 2-Cl-IB-MECA is due to a specific effect upon A₃ receptors. To elude the possibility that the protective effect we observed could have been caused by a nonspecific activation of other adenosine receptors, we used 2-Cl-IB-MECA, which has been previously shown to be a highly specific agonist of the adenosine A₃ receptor (4, 23). However, at high doses, this drug was observed to have marked vasodilator effects, increasing the coronary flow (Fig. 2), possibly due to some adenosine A₂ receptor activation. Furthermore, this effect was not accompanied by any protection (Fig. 1). There were no differences in heart rate among the groups, which can be an indicator that 2-Cl-IB-MECA has no major effect upon adenosine A₁ receptors at any concentration (data not shown).

In addition to that, the reduction in infarct size obtained with 1 nM 2-Cl-IB-MECA was completely abolished in the presence of 1 μM MRS-1191, a specific A₃ antagonist for the rat tissues \( I/R%: 44.2 ± 2.7 \) in controls vs. 21.9 ± 2.4 in 2-Cl-IB-MECA, \( P < 0.05 \), and vs. 40.7 ± 3.9 in 2-Cl-IB-MECA + MRS-1191, \( P > 0.05 \); Fig. 3). The antagonist alone did not modify the I/R% significantly compared with control hearts.
The A2a agonist failed to induce any protection in terms of infarct size reduction (I/R%: 44.4 ± 6; Fig. 4). Surprisingly, the coadministration, during reperfusion, of the A3 agonist and the A2a antagonist completely blocked the protection (I/R%: 53.0 ± 4). These results are considered in the DISCUSSION.

**Role of Adenosine A3 Receptor Activation at Reoxygenation Upon Rat Adult Ventricular Myocyte Survival**

Exposing the adult rat cardiac myocytes to 45 min of hypoxia followed by 120 min of reoxygenation induced significant injury, resulting in 17.1 ± 1.3% apoptotic cells and 23.1 ± 1.5% necrotic cells as measured using annexin-V-fluorescein and PI labeling, which detect early apoptotic and necrotic cells, respectively. To assess the role of adenosine A3 receptor activation in reducing myocyte injury during reoxygenation, we used the specific agonist 2-Cl-IB-MECA. The cardioprotective ability of the A3 agonist was observed at 1 nM, significantly decreasing the percentage of both apoptotic (5.7 ± 2.6% in 1 nM 2-Cl-IB-MECA vs. 17.1% ± 1.3 in control) and necrotic cells (13.7 ± 2.0% in 1 nM 2-Cl-IB-MECA vs. 23.1 ± 1.5% in control). At higher concentrations of 2-Cl-IB-MECA (>30 nM) and the lowest concentration (0.1 nM), there was no protection observed (Fig. 5A).

To determine whether the protection afforded by the adenosine A3 agonist is mediated by the A3 receptor, a selective antagonist of this receptor was used. The A3 antagonist MRS-1191 blocked the 2-Cl-IB-MECA-elicited cardioprotection in myocytes, increasing both necrosis (25.0 ± 1.7%) and apoptosis (17.1 ± 1.2%) at values not significantly different from the respective controls (23.1 ± 1.3% and 17.6 ± 2.2%, respectively; Fig. 5B).

### DISCUSSION

The major finding of the current study demonstrated that the activation of adenosine A3 receptors via administration of the specific adenosine A3 agonist 2-Cl-IB-MECA at reperfusion not only significantly reduced infarct size in the isolated perfused rat heart after ischemia but also exerted an antiapoptotic and antinecrotic effect in the isolated adult rat myocyte. Previous investigators have shown, in a variety of models, that preconditioning the heart via activation of adenosine receptors can protect against ischemia-reperfusion injury (19, 24, 36, 42). With regard to reperfusion injury, however, activation of adenosine A3 receptors has only been shown to attenuate neutrophil-mediated reperfusion injury (14), and no measures of infarct reduction or cell death have ever been reported. To our knowledge, this is the first study to identify a protective role for the adenosine A3 receptor in the heart when activated at reperfusion using infarct size as the end point of injury.

Our data are in agreement with those of Lubitz et al. (45), who demonstrated that treatment with the A3 agonist 2-Cl-IB-MECA after induced cerebral ischemia resulted in improved neuronal preservation, decreased intensity of reactive gliosis, and pronounced reduction of microglial infiltration. Furthermore, they also demonstrated a reduction of infarction in the brain after adenosine A3 receptor activation.

We observed a dose-dependent effect of the adenosine A3 receptor agonist on its ability to limit infarct size in the isolated perfused rat heart. At high doses of 100 and 30 nM, no protection was observed. However, we saw a significant reduction in myocardial infarction at 10 and 1 nM, respectively. At further dilution, this protection was not sustained. Therefore, it appears that the optimal dose for protection against reperfusion injury in this model using this A3 agonist is 1 nM. Our study using 2-Cl-IB-MECA showed similar profiles when apoptosis and necrosis were evaluated in rat cardiomyocytes as separate end points of injury.

**Fig. 3.** The effect of 1 μM MRS-1191 (adenosine A3 receptor antagonist) upon protection conferred by 2-Cl-IB-MECA at reperfusion. Results are presented as I/R %. *P < 0.01 vs. control.

**Fig. 4.** Infarct developed in the risk zone (I/R%) in control hearts and in hearts treated at reperfusion with 1 nM A3 agonist 2-Cl-IB-MECA, 1 μM A2a antagonist 8-3 chlorostyryl caffeine, 100 nM A2a agonist SGS-21680, or with both A3 agonist and A2a antagonist. *P < 0.01 vs. control.
Indeed, the adenosine A3 receptor has been reported to mediate both cell protection and cell death simply depending on the degree of receptor activation and/or specific pathophysiological conditions. A mild subthreshold activation of the adenosine A3 receptor has been shown to decrease apoptotic injury induced by either chemical toxicity or ischemia (1, 8, 32, 44, 47). In contrast, at high concentrations (>10 μM), 2-Cl-IB-MECA can trigger cell death by either necrosis or apoptosis (1, 31, 33).

Our results confirm those of Lasley et al. (20), who showed in isolated rat hearts that at ≥30 nM 2-Cl-IB-MECA caused an increase in vasodilatation that was attenuated by Sch-58261 (adenosine A2a receptor antagonist). In our study, protection was observed by 2-Cl-IB-MECA only at concentrations of 1 and 10 nM, which did not cause vasodilation, indicating no role for an A2a receptor-mediated vasodilation being involved in the 2-Cl-IB-MECA-induced protection.

However, some authors have previously demonstrated that the activation of the adenosine A2a receptor may be protective (15, 19) with respect to its inhibitory action upon the neutrophil-related processes (15).

To clarify the possible involvement of the A2a receptor in the protection observed in our model, we investigated the effect of a concomitant administration, at reperfusion, of an A3 agonist (2-Cl-IB-MECA) and an A2a antagonist (CSC) as well as the direct effect of A2a receptor stimulation at reperfusion using CGS-21680. The data we obtained showed that the A2a receptor antagonist appears to block the protective effect of the A3 agonist. However, the direct activation of the adenosine A2a receptor at reperfusion does not confer protection in terms of infarct reduction in our model.

Our explanation for these contradictory results is based on studies showing that an increased stimulation of the A3 receptor is in itself proapoptotic (Figs. 1 and 5A) (12, 45). We believe that this increased stimulation takes place when the A3 agonist, in a protective dose, is coadministered with the A2a antagonist. The blockage of the A2a adenosine receptor may allow for more adenosine, occurring naturally in ischemia, to interact with the A3 receptor. As a result, an indirect increased stimulation of the A3 receptor will take place, with its consequence being an increase in cell death. Of importance is the fact that the addition of an A2a agonist failed to demonstrate any protection, which highlights the potential problems with the A2a antagonist.

Furthermore, it has been shown that adenosine A3 receptors are expressed in resident inflammatory cells such as mast cells (28, 37), where they can stimulate the release of stored mediators including histamine, cytokines, and proteolytic enzymes as well as numerous other proinflammatory mediators. It could be argued, therefore, that at higher concentrations of 2-Cl-IB-MECA, adenosine A3 receptors may increase infarct size by potentiating the inflammatory response and hence reduce any beneficial effects observed through activation of A3 receptors via separate signaling path-
ways. We believe this can be discounted in our study as the same concentration-related protection via adenosine A3 receptors was observed in the mast cell-free system of the isolated rat myocyte.

Our data suggest that a significant component of the cell survival effect of 2-Cl-IB-MECA was in attenuating apoptosis. This was illustrated by the fact that at a concentration of 10 nM, a significant reduction in the apoptotic component of cell death was observed in the myocyte model, whereas, in the whole heart, although a significant reduction in infarct was seen at this dose, this did not produce maximum infarct size reduction.

The specificity of 2-Cl-IB-MECA to induce adenosine A3 receptor-mediated protection was confirmed using the specific antagonist MRS-1191, which is well documented to be highly specific for rat adenosine A3 receptors (4, 26). This antagonist has also been shown to have no effect on A2a receptor-mediated dilation of cerebral arterioles, again confirming its specificity for adenosine A3 receptors (22). We demonstrated that MRS-1191 completely abolished the protection conferred by 1 nM 2-Cl-IB-MECA when given at reperfusion in the isolated rat heart. Furthermore, this abrogation of protection was also observed in the isolated rat myocyte against both apoptosis and necrosis.

The mechanism involved in the A3 receptor-mediated effects on anti/proapoptotic pathways remains to be elucidated. Adenosine A3 receptors were demonstrated to specifically stimulate phosphorylation of the serine/threonine kinase Akt. The activation of Akt has been shown to inhibit programmed cell death (3, 16, 35). In cells exposed to UV light, adenosine A3 receptor activation has been shown to reduce apoptosis via the Akt pathway (8). It is therefore feasible that the protection observed in our study could be acting through this signaling pathway. It has also been proposed that stimulation of adenosine A3 receptors results in the phosphorylation of ERK1/2 (30). It is therefore conceivable that the protection induced by the adenosine A3 receptors maybe due to the triggering of specific reperfusion injury salvage kinases (48).

In summary, we demonstrated that activation of adenosine A3 receptors using the specific adenosine A3 receptor agonist 2-Cl-IB-MECA can protect the isolated perfused rat heart when given at the onset of reperfusion. Furthermore, the apoptotic component of cell death, which contributes to myocardial infarction, was also ameliorated by adenosine A3 receptor activation.

Further investigation into the role of adenosine A3 receptors in lethal reperfusion injury may lead to new therapeutic strategies in the treatment of ischemic heart disease. Compounds activating adenosine A3 receptors may have the added advantage, as potent therapeutic agents, by being free from the bradycardic and hypotensive side effects characteristic of the other adenosine receptor activation.

We are grateful to the British Heart Foundation for continued support.

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