A Food and Drug Administration–Approved Antiviral Agent that Inhibits Adenylyl Cyclase Type 5 Protects the Ischemic Heart Even When Administered after Reperfusion

Claudio A. Bravo, Dorothy E. Vatner, Ronald Pachon, Jie Zhang, and Stephen F. Vatner
Department of Cell Biology and Molecular Medicine, Rutgers, New Jersey Medical School, Newark, New Jersey

ABSTRACT

A Food and Drug Administration–approved antiviral agent, known as vidarabine or adenine 9-β-D-arabinofuranoside (AraA), has features of inhibiting adenylyl cyclase type 5 (AC5) and protects against chronic coronary artery occlusion (CAO). The goal of this investigation was to determine whether AraA protects against myocardial ischemia, even when delivered after coronary artery reperfusion (CAR). AraA, delivered after CAR in wild-type controls, reduced infarct size by 55% compared with vehicle-treated controls, whereas an equal dose of adenosine reduced infarct size only when administered before CAR. A 5-fold greater dose of adenosine was required to reduce infarct size when delivered after CAR, which also reduced arterial pressure by 15%, whereas AraA did not affect pressure. The reduction in infarct size with AraA was prevented by a MEK/extracellular signal–regulated kinase blocker, a pathway also involved in the mechanism of protection of the AC5 knockout (KO) model. Infarct size was also reduced in cardiac-specific AC5 KO mice similarly in the presence and absence of AraA, further suggesting that AraA protection involves the AC5 pathway. AraA reduced infarct size in chronically instrumented conscious pigs when delivered after CAR, and in this model, it also reduced post-CAR coronary hyperemia, which could be another mechanism for cardioprotection (i.e., by reducing oxidative stress during CAR). Thus, AraA inhibits AC5 and exhibits unique cardioprotection when delivered after CAR, which is critical for clinical translation.

Introduction

Adenylyl cyclase (AC) is an enzyme that converts ATP into the second-messenger cAMP, which is involved universally in physiologic regulation. There are nine major AC isoforms; the two major isoforms are in the heart: AC5 and AC6. AC5 is expressed in most other organs; for example, its central nervous system role affects behavior (Kim et al., 2006, 2007, 2008), and its role in skeletal muscle regulates exercise performance (Vatner et al., 2015). Our laboratory developed a mouse with disruption of adenylyl cyclase type 5 (AC5 knockout, KO), which lives a third longer and eats more but weighs less than their wild-type littermates (Yan et al., 2007). The AC5 KO mice are also protected against diabetes (Ho et al., 2015) and chronic heart disease (Okumura et al., 2007) through its protection against oxidative stress (Yan et al., 2007; Lai et al., 2013). Since it is not feasible to alter a gene in patients, development of a pharmacologic analog of AC5 knockdown or inhibition is required. One prototype, a Food and Drug Administration–approved antiviral drug known as vidarabine or AraA (Iwatsubo et al., 2004), does have features of AC5 inhibition and also protects against chronic ischemic heart disease (Iwatsubo et al., 2012).

The goal of this investigation was to determine whether AraA also protects against acute myocardial ischemia. One of the major limitations to discovering novel agents to reduce infarct size in patients is that most drugs studied experimentally must be administered before coronary artery occlusion (CAO) or at least before coronary artery reperfusion (CAR) occurs; however, that limits their clinical utility, since patients coming to the hospital with myocardial infarction must be treated with angioplasty immediately, not allowing time for drug delivery. Therefore, finding a drug that is equally effective when administered after CAR would be of major significance. This important point became a critical part of the experimental design of this investigation, that is, to compare effects of AraA before and after CAR and also compare it with one of the most well recognized agents for protecting ischemic myocardium, adenosine (Mahaffey et al., 1999; Ross et al., 2001). At least four adenosine receptor isoforms are expressed in the heart: A1, A2a, A2b, and A3 (Mubagwa and Flameng, 2001). Mostly, the A1 and A3 receptors, which are expressed in cardiomyocytes, have been studied extensively for their crucial role in protection against cardiac ischemia (Auchampach and Gross, 1993; Liang and Jacobson, 1998); however, although A2a and A2b, which are expressed in the blood vessels, may also

ABBREVIATIONS: AC, adenylyl cyclase; AraA, adenine 9-β-D-arabinofuranoside; cAC5 KO, cardiac specific AC5 knockout; CAO, coronary artery occlusion; CAR, coronary artery reperfusion; ERK, extracellular signal–regulated kinase; KO, knockout; LAD, left anterior descending; LV, left ventricle; pERK, phosphorylated ERK; TTC, triphenyl tetrazolium chloride.
play role in cardioprotection (Norton et al., 1992; Eckle et al., 2007; Morrison et al., 2007; Cohen and Downey, 2008; Rork et al., 2008; McIntosh and Lasley, 2012; Headrick et al., 2013). The mechanism of adenosine induced cardioprotection involves the activation of phospholipase C and subsequently protein kinase C, which eventually lead to preventing opening of mitochondrial permeability transition pore formation (Xiang et al., 2010) and also the MEK-extracellular signal–regulated RK pathway (Germack and Dickenson, 2005). A second goal was to determine whether the mechanism of AraA’s cardioprotection potentially involved AC5, which was done by examining cardiac ischemic protection in a cardiac-specific AC5 KO in the presence and absence of AraA. In addition, we examined the MEK-extracellular signal–regulated kinase (ERK) pathway for AraA since that pathway mediates many of the effects in the AC5 KO (Yan et al., 2007). Since another limitation to translating therapy from a mouse genetic model to humans is species difference, the FDA requires effectiveness in a large mammalian model. This requirement was addressed in the current investigation in a chronically instrumented pig model (Shen et al., 2008; Kudej et al., 2011). Another advantage of that model is that it permits instantaneous and continuous measurements of coronary blood flow before and during CAO and after CAR. Since CAR damage has been linked to the magnitude of reactive hyperemia (Braunwald and Kloner, 1985; Peng et al., 1989; Yellon and Hausenloy, 2007; Hausenloy and Yellon, 2013), we also examined this mechanism in the pig model.

Materials and Methods

All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at the Rutgers New Jersey Medical School. All the investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Mice Ischemia and Reperfusion Model. Either 3- to 4-month-old male C57BL/6 mice from the Jackson Laboratory (Bar Harbor, ME) or mixed-background SVJ129/C57BL/6 bred inhouse were anesthetized by an i.p. injection of pentobarbital (60 mg/kg), followed by tracheal intubation and mechanical ventilation.

The heart was accessed via a thoracotomy at the fourth intercostal space and a 7-0 silk suture passed under the left anterior descending coronary artery (LAD) at the point where it emerged from under the left atrial flap. Myocardial ischemia was achieved by occluding the LAD against a 22-gauge J-shaped stainless steel probe and verified by visually noting the regional akinesis and blanching of the left ventricle. The chest was closed in layers, with the long end of the probe remaining outside the chest wall, allowing the animal to be removed from the ventilator. To induce an infarct size between 30% and 35%, 30 minutes of ischemia was induced in the pure C57BL/6 and 60 minutes in the mixed background SVJ129/C57BL/6. The reperfusion was initiated by carefully pulling the probe out from under the ligature and then removing it from the chest cavity. After the surgical procedure, the mouse was allowed to recover on a warmed surface, with supplemental oxygen delivered through a nose cone. Ischemia and reperfusion were verified by continuous three-lead electrocardiogram, which was obtained during the entire experiment. Mice fully recovered from the surgical procedure were returned to standard animal housing conditions. The mice were kept alive for 24 hours after reperfusion, when the mice were sacrificed and dual staining of the heart with triphenyl tetrazolium chloride (TTC) and Alcian Blue was used to measure infarct size.

Swine Ischemia and Reperfusion Model and Reactive Hyperemia Measurement. Yorkshire pigs were placed under anesthesia and instrumented through a left thoracotomy; an LAD coronary artery flow probe was placed proximal to a hydraulic occluder to ensure zero flow during the complete LAD occlusion. Tygon catheters were inserted in the ascending aorta and left atrium, and a solid-state pressure gauge was implanted in the left ventricle (LV) to measure their respective pressures. Finally, two pairs of ultrasonic dimension crystals were implanted in the posterior and in the anterior wall, for measurements of LV diameter and ischemic versus non-ischemic zone wall thickness (Shen et al., 2008; Kudej et al., 2011). After 4 days of recovery, the pigs were subjected to 1-hour LAD occlusion, followed by reperfusion as previously described (Shen et al., 2008). At 3 hours of CAR, the animals were sacrificed, and the infarct size was measured with TTC staining as previously described (Shen et al., 2008).

The reactive hyperemia was tested by inflating the previously implanted hydraulic occlude on the coronary artery for 15 seconds and then releasing it to examine the reactive hyperemia. Coronary blood flow was continuously recorded with a chronically implanted ultrasonic probe on the artery undergoing CAO and CAR until it returned to preocclusion levels. The hyperemic response was evaluated by computing blood flow debt and the blood flow debt repayment by digital measurement of the area under the curve; the data were presented as debt-repayment ratio as previously described (Coffman and Gregg, 1969). AraA or the vehicle was administered at a rate of 0.012 mg/kg per minute continuously for 5 minutes, starting within the first 5 minutes after release of the occlusion.

Cardiac-Specific AC5 Generation. Cardiac-specific AC5 KO (AC5floxflox transgenic core) was generated by using the flox-cre recombinant technique (Sauer, 1998) in the Transgenic Core at New Jersey Medical School.

Drugs. Adenine 9-β-D-arabinofuranoside (AraA) was purchased from Sigma-Aldrich (St. Louis, MO). AraA was administered at a dose of 0.06 mg/kg (0.012 mg/kg per minute) via an i.v. infusion for 5 minutes that was started within the first 5 minutes into CAR in both the mouse and pig experiments. The time point for drug administration was based on clinical relevance (i.e., the earliest time the drug might be administered to patients with myocardial infarction and angioplasty). The dose selected was the lowest that was able to consistently reduce infarct size. The MEK blocker, U0126, purchased from Promega (Madison, WI), was administered i.v. at a dose of 0.2 mg/kg at the beginning of CAO (Soeki et al., 2010), and AraA was administered after reperfusion. Adenosine was purchased from Sigma-Aldrich, and it was administered via a 5-minute i.v. infusion either at 0.06 or 0.3 mg/kg per dose.

Western Blotting. Western blotting of tissue lysate from the viable region of the LV was conducted with commercially available antibodies against the phosphorylated form and total MEK/ERK.

Data Analysis and Statistics. Comparison of the two groups was performed using the Student’s t test. For comparison among multiple groups, we used one-way analysis of variance followed by Bonferroni post hoc analysis. P < 0.05 was taken as a minimal level of significance.

Results

AraA Reduced Infarct Size in Mice After Ichiaemia and Reperfusion Injury. The i.v. infusion of AraA immediately before CAO reduced infarct size to 22% ± 0.6% (n = 6) compared with the vehicle group that had an infarct size of 31% ± 2.4% (n = 5) (P = 0.002). The i.v. injection of AraA right after CAR reduced the infarct size to 15% ± 1.9% (n = 11) versus vehicle 33 ± 1.1% (n = 32) (P < 0.001). The size reduction when AraA was infused after CAR versus before CAO (P = 0.026) is shown in Fig. 1.
To compare the potency for reduction of infarct size of AraA and adenosine, adenosine was given at the same dosage before CAR and after CAR. Adenosine given before CAR reduced infarct size to 14% ± 1.9% (n = 6) compared with the vehicle group that had an infarct size of 31% ± 2.4% (n = 5) (P < 0.001). The administration of adenosine at the same dose after CAR did not reduce infarct size, 38% ± 1.0%, values not lower than the infarct size obtained in the vehicle group (33 ± 1.1%); however, a five times greater dose of adenosine administered after CAR did reduce infarct size to 21% ± 1.5% (P < 0.001). Adenosine and AraA, at doses that reduced infarct size after CAR, were compared on measurements of LV systolic pressure and mean arterial pressure. Adenosine reduced LV systolic pressure by 7%, which decreased from 103 to 96, and reduced mean arterial pressure by 15%, which decreased from 90 to 77, whereas AraA had negligible effects on hemodynamics.

**Infarct Size Reduction by AraA Is Mediated by the MEK/ERK Pathway.** Mice were injected via jugular vein catheterization with either vehicle or AraA, and ERK and phosphorylated ERK (pERK) were measured by Western blot. pERK, which is the active form of this enzyme, was increased in the AraA-treated group compared with the vehicle treated (P = 0.02) (Fig. 2A).

To evaluate its role in the mechanism of AraA infarct size protection, the MEK inhibitor U0126 was administered before CAO/CAR. U0126 abolished the cardioprotection obtained with AraA (Fig. 2B).

**Infarct Size Reduction Obtained with AraA Is Similar to Cardiac-Specific AC5 KO.** Since AraA has been previously described as an AC5 inhibitor, the next step was to evaluate whether the infarct size reduction obtained with this compound is AC5 mediated. For this aim, we used the cardiac specific AC5 KO (cAC5 KO). To induce a similar infarct size in this mixed background strain to the C57BL/6, it was necessary to induce a 60-minute CAO, which resulted in a 37% infarct size in the wild-type mice. Infarct size was reduced in the cAC5KO mice treated with vehicle compared with that in vehicle-treated wild-type mice (28%, P < 0.005). In cAC5 KO mice treated with AraA, infarct size was not reduced further than in cAC5 KO treated with vehicle, indicating that whereas cardiac AC5 deletion protects the heart from CAO/CAR, no further protection was induced by AraA, suggesting that similar mechanisms mediated cardioprotection in both cAC5 KO and AraA (Fig. 3).

**AraA Reduces Infarct Size in a Swine Model of CAO/CAR, and Reactive Hyperemia Reduction Might Play a Role.** In chronically instrumented conscious pigs with CAO/CAR, i.v. administration of AraA at the beginning of CAR reduced infarct size by 25% compared with vehicle treatment (P < 0.05) (Fig. 4A). The reactive hyperemia seen after 15 seconds of CAO, measured by the area under the curve, was diminished by 37% with i.v. administration of AraA (vs. before AraA P < 0.05) (Fig. 4C).

**Discussion**

The major finding of this investigation is that AraA affords marked protection against myocardial ischemic injury, even when administered after CAR. That its protection was as effective after CAR as before CAR is critical clinically since any drug to be used to reduce ischemic injury will be administered to patients after CAR. When they arrive at the hospital, CAR is induced immediately, leaving no time for adjunctive drug therapy, until after CAR. Many agents that protect the ischemic heart are only effective when administered either before CAO or at least before CAR. For example, in the present study, adenosine, one of the most effective anti-ischemic agents (Mahaffey et al., 1999; Ross et al., 2005), was studied both before and after CAR. Whereas AraA exhibited similar efficacy before and after CAR at the same dose, adenosine required 5 times the dose after CAR to be as effective as AraA or to be as effective as adenosine was when it was administered before CAR. Thus, even though AraA and adenosine share some of the same pathways to mediating cardioprotection (Germack and Dickenson, 2005; Vatner et al., 2015), some of the pathways must be different to explain why AraA exerts more potent protection after CAR.

It is also important for cardioprotective agents not to reduce arterial pressure, since reduced driving pressure would reduce coronary flow and compromise the ischemic heart even further. Therefore, it is important to note that AraA did not reduce pressure, whereas adenosine, administered after CAR, induced a marked reduction in arterial pressure. AraA’s trivial hemodynamic effects were also observed recently by Wada et al. (2016).

Since it is known that AraA has features of AC5 inhibition (Iwatsubo et al., 2012), it was important to determine whether the mechanism of its cardioprotection was mediated by AC5. We reasoned that if AraA and AC5 inhibition induced cardioprotection through different mechanisms, then the cardioprotection would be augmented when AraA was superimposed on the AC5 KO mouse, whereas if the mechanisms were overlapping, no additional protection would be afforded by superimposition of AraA on the AC5 KO mouse. To address this concept, we first had to demonstrate that the AC5 KO mouse induced cardioprotection, which had not been done previously, even though the mouse was shown to be protected against chronic cardiac stress (Okumura et al., 2007). To
examine the effects of AC5 KO on acute ischemic protection, we developed and studied a cardiac-specific AC5 KO. The cAC5 KO mouse did demonstrate protection against CAO/CAR with reduced infarct size, and since infarct size was not reduced further in the cAC5 KO mouse with superimposition of AraA, we concluded that the mechanisms are likely overlapping.

To further support the involvement of AC5 inhibition in AraA’s cardioprotective action, we examined the role of the MEK/ERK pathway. We previously demonstrated that the MEK/ERK pathway is involved in the longevity model of the AC5 KO (Yan et al., 2007) and also in the protection induced by chronic administration of AraA in ameliorating heart failure progression after permanent CAO (Iwatsubo et al., 2012). In the present investigation, we first demonstrated that AraA increased ERK phosphorylation (pERK) in the heart of wild-type mice, and blocking this pathway with U0126, which is a known MEK inhibitor (Soeki et al., 2010; Iwatsubo et al., 2012), also blocked the cardioprotection induced by AraA. Therefore, a MEK/ERK mechanism, which is important in the AC5 KO mouse (Yan et al., 2007), mediates the cardioprotection with AraA, as it does with chronic CAO (Iwatsubo et al., 2012).

Given that remarkable infarct size reduction obtained with AraA was observed, when delivered after CAR, it was important to examine the effects of AraA on the reactive hyperemia observed after CAR. Infarct size expansion and the concept of reperfusion damage have been linked to the hyperemia after CAR (Braunwald and Kloner, 1985; Peng et al., 1989; Yellon and Hausenloy, 2007; Hausenloy and Yellon, 2013). To examine this question, we used a chronically instrumented pig model, in which coronary artery blood flow was measured instantaneously and continuously with an implanted ultrasonic flow probe. The amount of reactive hyperemia after CAR was reduced significantly in the pigs, which received AraA, versus those with vehicle treatment. Thus, reduced CAR hyperemia, which reduces oxidative stress, could be another mechanism mediating the reduced infarct size with AraA administered after CAR. In support of this, cardioprotection in the AC5 KO mouse is mediated to a great extent by reducing oxidative stress (Yan et al., 2007; Lai et al., 2013).

It is also conceivable that AraA reduces myocardial stunning, originally described after brief episodes of myocardial ischemia not leading to infarction (Heyndrickx et al., 1975) and later found to be involved in the mechanism of hibernating myocardium (Wijns et al., 1998). Although these protocols have not been examined with AraA, it is known that AraA improves the recovery of myocardial function after complete
coronary occlusion, where myocardial stunning may be superimposed on the positive effects induced by reduced infarct scar development (Iwatsubo et al., 2012).

It has been argued that AraA is not a pure AC5 inhibitor (Braeunig et al., 2013; Brand et al., 2013). That might be so, but it does not mean that the salutary mechanism of AraA on myocardial ischemic protection does not have inhibition of AC5 in its mechanism. It is difficult to explain why superimposition of AraA on the AC5 KO mouse does not induce enhanced infarct size protection if AraA and the AC5 KO mouse have different cardioprotective mechanisms. Moreover, the similarity in MEK/ERK pathway mechanisms in the AC5 KO mouse and AraA supports a common mechanism of action. Finally, AraA reduced AC activity significantly in AC5 transgenic mice, but not at all in AC5 KO mice, and exerted little effect in either wild-type or AC6 transgenic mice (Iwatsubo et al., 2012). Regardless of whether AraA is a 100% pure AC5 inhibitor, this does not detract from its remarkable ability to reduce infarct size and coronary hyperemia when administered after CAR.

It is tempting to speculate that a pharmacologic analog of the AC5 KO mouse (e.g., AraA or one of its analogs) could develop into a clinically useful adjunct to reperfusion therapy in view of its potent ischemic protection when delivered after CAR. Furthermore, one limitation of drug development from genetically altered mouse models to the clinical setting is species differences; however, the similarity of salutary effects of AraA in a large mammalian model, the pig, lends further support to its potential utility in translation to the clinics.

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Authorship Contributions

Participated in research design: D. E. Vatner, S. F. Vatner.
Conducted experiments: Bravo, Pachon.
Performed data analysis: Bravo, Pachon, Zhang.
Wrote or contributed to the writing of the manuscript: D. E. Vatner, S. F. Vatner, Zhang, Bravo.
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Address correspondence to: Dr. Stephen F. Valner, University Professor, Director, Cardiovascular Research Institute, Department of Cell Biology and Molecular Medicine. Rutgers, New Jersey Medical School, Newark, New Jersey. E-mail: valners@njms.rutgers.edu