Attenuation of oxidant stress during reoxygenation by AMP 579 in cardiomyocytes

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Attenuation of oxidant stress during reoxygenation by AMP 579 in cardiomyocytes. Am J Physiol Heart Circ Physiol 281: H2585–H2589, 2001.—AMP 579, an adenosine A1/A2 receptor agonist, has a strong anti-infarct effect when administered just before reperfusion. Because oxidative stress has been proposed to contribute to myocardial reperfusion injury, we tested whether AMP 579 can reduce the production of reactive oxidant species (ROS) during reoxygenation in cultured chick embryonic cardiomyocytes. The intracellular fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH) was used to detect ROS. The cells were subjected to 60 min of simulated ischemia, followed by either 15 min or 3 h of reoxygenation. AMP 579 (0.5 and 1 μM), when started 10 min before reoxygenation, significantly reduced ROS generation from 4.86 ± 0.30 (arbitrary units) in untreated cells to 2.72 ± 0.31 and 1.85 ± 0.14, respectively (P < 0.05). Cell death that was assessed by propidium iodide uptake was markedly reduced by AMP 579 (49.6 ± 4.7% of control cells vs. 25.4 ± 2.4%, P < 0.05). In contrast, adenosine did not alter ROS generation or cell death. Attenuation of ROS production by AMP 579 was completely prevented by simultaneous exposure of cells to the selective adenosine A2 antagonist 8-(13-chlorostyryl) caffeine. These results indicate that AMP 579 directly protects cardiomyocytes from reperfusion injury by a mechanism that attenuates intracellular oxidant stress. Furthermore, adenosine could not duplicate these effects.

Materials and Methods

Cardiac cell culture. Ten-day-old embryonic chick ventricular myocytes were prepared using a method first described by Barry et al. (2) and modified by Vanden Hoek et al. (37). Briefly, hearts were harvested and placed in Hanks’ balanced salt solution lacking magnesium and calcium. Ventricles were minced, and myocytes were dissociated by treatment with trypsin (0.025%, Life Technologies; Grand Island, NY) applied four to six times at 37°C with gentle agitation. Isolated cells were then transferred to a solution with trypsin inhibitor for 8 min, filtered through a 100-mesh, centrifuged for 5 min at 1,200 rpm at 4°C, and finally resuspended in a nutritive medium described previously by Chandel et al. (7) and Duranteau et al. (11). Resuspended cells were placed in a petri dish in a humidified incubator (5% CO2-95% air, 37°C) for 45 min to promote early adherence of fibroblasts. Nonadherent cells were counted with a hemocytometer, and viability was measured using trypan blue (0.4%).

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Intensity values are reported as the percentages of initial values after the background value was subtracted.

Experimental protocol. After an equilibration period of 60 min, control cells were subjected to 60 min of simulated ischemia, as detailed above, followed by either 15 min (ROS generation studies) or 3 h (cell viability studies) of reoxygenation. In treatment groups, the cells were exposed to either AMP 579 or adenosine starting 10 min before reoxygenation. To determine which adenosine receptor subtype was responsible for the effect of AMP 579 on ROS production, cells were exposed simultaneously to AMP 579 and 8-(13-chlorostyryl) caffeine (CSC) (1 μM), a selective A2 antagonist.

Chemicals. AMP 579 was a gift from Aventis Pharmaceuticals. Adenosine, CSC, and all other chemicals were purchased from Sigma.

Statistical analysis. Data are expressed as means ± SE. Two-way analysis of variance with repeated measures and Fisher’s test were used to test for differences among the groups. A P value <0.05 was considered to be significant.

RESULTS

ROS generation. Figure 1 shows the time course of changes in mean DCF fluorescence intensity for the four study groups. On reoxygenation, the DCF fluorescence intensity in the control group (n = 6) increased dramatically, indicating an increased ROS production after reintroduction of oxygen. AMP 579 at 0.5 μM (n = 6) greatly attenuated the burst of ROS at reoxygenation and production was further attenuated by 1 μM AMP 579 (n = 7). In contrast, adenosine (100 μM) had no effect on the increase in DCF fluorescence. Figure 2 summarizes the peak values of DCF fluorescence during reoxygenation. AMP 579 significantly reduced the peak value from 4.86 ± 0.30 (arbitrary units) in control cells to 2.72 ± 0.31 and 1.85 ± 0.14 in cardiomyocytes exposed to 0.5 and 1 μM AMP 579, respectively (P < 0.05). Adenosine at a concentration of 100 μM (n = 4) did not affect the peak value (5.28 ± 0.22).

To probe further this effect of AMP 579 on production of ROS by reperfused cardiomyocytes, cells (n = 3) were exposed simultaneously to AMP 579 (10 μM) and


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CSC. In these experiments, peak DCF fluorescence was significantly attenuated by AMP 579 from 4.27 ± 0.69 (arbitrary units) to 2.17 ± 0.64 (P < 0.025). This attenuation was completely prevented by CSC (5.03 ± 1.43) (Fig. 2).

Cell viability. Percent cell death (PI uptake) was monitored continuously throughout the experiment and the data are shown in Fig. 3. Reoxygenation was accompanied by the onset of cell death. After 3 h of reoxygenation, cell death was 49.6 ± 4.7% in the control group (n = 8) and was significantly reduced by 1 µM AMP 579 (n = 6) (25.4 ± 2.4%, P < 0.05). In contrast, cell death was not significantly altered by 100 µM adenosine (n = 6) (46.4 ± 2.2%).

DISCUSSION

The present study reveals that AMP 579 can significantly attenuate ROS generation by hypoxic cardiac cells when they are reoxygenated. This attenuation of oxidant stress may well be related to the mechanism by which AMP 579 protects the reperfused heart against infarction. Although an increasing body of data has demonstrated the cardioprotective effects of AMP 579 in various animal models (6, 20, 33, 40), the mechanism underlying the protection of the agent is still unclear. Most baffling is the observation that activation of the adenosine A2 receptor seems to be a requisite for the protection, yet adenosine or A2 receptor agonists will not duplicate the protective effect (6, 23).

Nakamura et al. (23) reported that AMP 579 inhibits neutrophil activation and neutrophil-mediated coronary dysfunction, suggesting neutrophil suppression in the action of AMP 579. However, we found that AMP 579 protects against infarction, even in neutrophil-free isolated hearts (40). The present study extends that observation. Cardiomyocyte viability was preserved by the use of AMP 579 in a cell model, which contained no neutrophils or any other cell type, indicating that the cardiomyocyte is the direct target for this drug.

Myocardial reperfusion injury refers to a toxic event caused by the act of reperfusion. If a reperfusion injury is present, it should be possible to attenuate it by an intervention given at the time of reperfusion (27). There are several hypotheses regarding the mechanism by which reperfusion injury might occur in the heart. These include calcium overload (24), apoptosis (1), osmotic swelling (15), and the generation of ROS. Whereas it is clear that reperfusion of the ischemic heart is accompanied by a burst of ROS, the consequence of their presence is controversial. In a previous study (36) of chick cardiomyocytes, both the free radical scavenger N-2-mercaptopropionyl glycyne and the ferrous ion chelator 1,10-phenanthrolone attenuated ROS production while increasing cell viability and improving return of cell contraction. There are compelling data to indicate that ROS generated at reperfusion contribute to stunning of myocardium (3). Stunned myocardium is a temporary lesion in which the contractility of the heart is greatly reduced. However, unlike infarction, these effects are fully reversible. It has been proposed that ROS contribute to infarction of the heart as well. The evidence supporting a contribution to the infarction process was gathered from reports that treatment with antioxidants given only at reperfusion could reduce infarct size in animal models (14, 16, 22). Unfortunately, many laboratories have been unable to reproduce those findings (21, 26, 31, 34). As a result, many believe that ROS stun the reperfused heart, but that insufficient ROS are produced to significantly contribute to cell killing (10, 17, 28).

In the present study, AMP 579 greatly attenuated the burst of ROS accompanying reoxygenation. Because the effective concentration of AMP 579 was as low as 0.5 µM, it is unlikely that AMP 579 acted as a direct scavenger, nor could AMP 579 have caused expression of endogenous antioxidants so quickly. The most probable explanation is that AMP 579 exerted a direct effect on myocardial cells, which caused them to generate fewer ROS. The important question then becomes whether suppression of ROS is the mechanism whereby AMP 579 limits infarct size in the whole heart. That could be the case only if ROS actually contribute to infarction. It is also equally possible that

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Fig. 2. Peak values of DCF fluorescence representing generation of ROS in isolated chick cardiomyocytes exposed to AMP 579, adenosine, and 8-(13-chlorostyryl) caffeine (CSC). *P < 0.05 vs control.

Fig. 3. Effects of AMP 579 and adenosine on cell death. Propidium iodide (PI) was used to provide an index of cell death. *P < 0.05 vs control.
AMP 579 acted to preserve the integrity of the cell through some ROS-independent process, and, as a result, the less-injured cells simply produced fewer ROS at reperfusion.

Reperfused hearts are thought to produce ROS at reperfusion as a result of defects in their metabolic pathways. These include defects in electron transport or perhaps activation of Ca\textsuperscript{2+}-dependent oxidases as Ca\textsuperscript{2+} enters the injured cell. In a previous study (39), AMP 579 markedly reduced the degree of myocardial contracture on reperfusion in the isolated rabbit heart model, suggesting that the drug acted to keep calcium out of the cells. Oxidant stress could cause a depression in the membrane Ca\textsuperscript{2+} exchangers or the Na\textsuperscript{+}-K\textsuperscript{+} ATPase that maintains the sodium gradient for the exchanger (8, 9). Oxidant stress also depresses the sarcoplasmic reticulum Ca\textsuperscript{2+} pump ATPase (25, 32). These changes would result in intracellular Ca\textsuperscript{2+} overload and myocardial contracture. On the other hand, it is just as plausible that AMP kept Ca\textsuperscript{2+} out of the cell by some mechanism unrelated to ROS and, in the absence of elevated Ca\textsuperscript{2+}, fewer ROS were produced. In the latter scenario, the reduced ROS would have been the result of the protection rather than its cause. Furthermore, these studies cannot completely exclude the possibility that the effect of AMP 579 on ROS is unrelated to its necrosis-sparing action.

In previous studies, we (40) and others (33) found that the effect of AMP 579 required adenosine receptor activation. The present investigation confirms and extends these earlier observations. A selective adenosine A\textsubscript{2} antagonist completely prevented the ability of AMP 579 to attenuate the burst of ROS upon reperfusion. Thus, somehow, A\textsubscript{2} activity is necessary for AMP 579 to exert its salutary effect. We also evaluated the effect of 100 μM adenosine given with the same timing as AMP 579. That level of adenosine should have nearly saturated both A\textsubscript{1} and A\textsubscript{2} receptors. Yet surprisingly, adenosine was unable to reduce either ROS generation or, and cell death responded in parallel fashion to both AMP 579 and adenosine alone. The fact that both ROS production and cell death responded in parallel fashion to both AMP 579 and adenosine would suggest that the two processes are related to one another.

These studies were performed in cultured embryonic ventricular myocytes. This model has proved to be very effective for the study of ROS production during and after ischemia (35, 37) and for investigation of mechanisms of ischemic preconditioning (35, 41). And it is now helping to uncover the mode of action of a drug known to be quite protective in intact hearts. These cultured cells have surface receptors, can be preconditioned, and contract, and, therefore, are differentiated. One cannot exclude the possibility that some genes are active that are subsequently downregulated in adult cardiomyocytes. But the ability to grow cells into a monolayer to have a fixed, adherent cell population to observe greatly facilitates studies such as those described here.

In summary, the novel adenosine A\textsubscript{1}/A\textsubscript{2} receptor agonist AMP 579 given just before simulated reperfusion strongly attenuates the rate at which isolated chick cardiomyocytes die following a period of simulated ischemia. AMP 579 also decreases the burst of ROS seen at reoxygenation suggesting a cause-and-effect relationship between cell death and ROS generation. This effect on ROS production is dependent on adenosine A\textsubscript{2} receptor binding. Yet adenosine could not duplicate either of these effects, indicating that AMP 579 has protective actions apart from its effect on adenosine receptors.

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