Myocardial dysfunction is associated with activation of Na\(^+\)/H\(^+\) exchange immediately during reperfusion

THANE G. MADDAFORD AND GRANT N. PIERCE
Ion Transport Laboratory, Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, and Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada R2H 2A6

Maddaford, Thane G., and Grant N. Pierce. Myocardial dysfunction is associated with activation of Na\(^+\)/H\(^+\) exchange immediately during reperfusion. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2232–H2239, 1997.—Amiloride analogs block Na\(^+\)/H\(^+\) exchange and thereby protect the heart from myocardial ischemia-reperfusion injury. It is unclear whether drugs must be present before ischemia to be cardioprotective. After 60 min of global ischemia in the coronary-perfused right ventricular wall (RVW), as little as 1 min of exposure to dimethyl amiloride (DMA) immediately at the time of reperfusion protected the RVW. Delaying the drug attenuated the cardioprotection. If DMA was introduced in an ischemic solution near the end of ischemia, the cardioprotective effects were augmented. If the drug was washed out of the RVW vascular space before ischemia, cardioprotection was not observed. In contrast, in whole hearts, preischemic perfusion of the drug was necessary for cardioprotection and the cardioprotection remained even if the drug was washed out before ischemia. We conclude that Na\(^+\)/H\(^+\) exchange is active and contributes to contractile dysfunction during the first seconds of reperfusion. This is difficult to detect in the perfused whole heart, and the washout data suggest that this may be due to a limitation in drug delivery across the vascular wall. The data also suggest that the exchanger is not as active during ischemia itself as it is during reperfusion.

sodium; calcium; sodium/hydrogen exchange

There is clear evidence of a significant role for Na\(^+\)/H\(^+\) exchange in ischemia-reperfusion injury to the heart (for reviews, see Refs. 4, 8, 24, 29). Intracellular acidosis, which occurs during ischemia (3, 27, 32), is thought to induce a movement of extracellular Na\(^+\) into the myocardial cell in exchange for intracellular H\(^+\) through the Na\(^+\)/H\(^+\) exchanger. The intracellular Na\(^+\) then interacts with the Na\(^+\)/Ca\(^{2+}\) exchanger to induce an influx of extracellular Ca\(^{2+}\) into the cell. Ca\(^{2+}\) overload within the myocardial cell has been associated with contractile dysfunction and damage (6, 14, 30). Most of the evidence in favor of the existence of the above cascade of ionic events has been provided by studies that have employed drugs that block Na\(^+\)/H\(^+\) exchange and protect the heart from dysfunction and damage after ischemia-reperfusion (2, 7, 9, 10, 12, 14–20, 28, 30). However, although it now appears clear that activation of the exchanger is an important contributory factor in ischemia-reperfusion injury, it remains controversial whether the activation occurs during the ischemic period or during reperfusion. Several studies have reported that the drugs that block Na\(^+\)/H\(^+\) exchange must be present before and during the ischemic period to be cardioprotective (2, 7, 9, 10, 18, 20). Addition of the drugs at the time of reperfusion did not exert a beneficial effect. In contrast, other studies have shown that introduction of the Na\(^+\)/H\(^+\) exchange blocker during the reperfusion phase alone offers significant protection to the myocardium after ischemia (1, 5, 12, 17, 30). The controversy is not insignificant. A drug would be far more valuable in a clinical setting if it were cardioprotective during the reperfusion period and did not require a pretreatment period before the ischemic insult.

Because the effects of the Na\(^+\)/H\(^+\) exchange blockers during reperfusion are so controversial, further work defining the characteristics of action of the blockers of Na\(^+\)/H\(^+\) exchange during reperfusion is warranted to substantiate this effect. The purpose of the present investigation, therefore, was to determine in greater detail the temporal dependency of the cardioprotection observed during the reperfusion phase in the right ventricular wall (RVW). Second, we wanted to compare this effect in the RVW with the effects in whole hearts. Our results suggest that extravascular accumulation of the drug is time dependent and critical for the drug to exert cardioprotective effects in whole hearts. When the drug is introduced during reperfusion, this time may delay the drug's actions (and limit its efficacy).

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 300–350 g were anesthetized with an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) and killed by decapitation. The RVW was dissected free from the heart and mounted in a perfusion apparatus as described in detail previously (13, 14). The right ventricle was perfused by cannulating the right coronary artery and was maintained at a constant flow rate of 1.5 ml/min at 37°C with a peristaltic pump. For an extensive discussion of the advantages and characteristics of the coronary-perfused RVW compared with other cardiac muscle preparations, the reader is referred elsewhere (13). The whole heart was perfused in a retrograde Langendorff mode as described previously (7, 9, 18). This method requires a similar amount of time (~1–2 min) before perfusion is initiated in the two heart preparations following removal of the heart from the animal. To have our results relevant to previous reports, we attempted to simulate as closely as possible all aspects of whole heart perfusion used previously by others (7, 9, 18, 20). The flow rate for the whole hearts was 10 ml/min. The perfusion solution for the RVW and the whole heart was bubbled with 100% O\(_2\) and contained (in mM) 140 NaCl, 6 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 dextrose, and 6 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4.

We chose to use a HEPES-buffered solution instead of a bicarbonate-buffered solution for several reasons. First, the HEPES-buffered solution was used to isolate the Na\(^+\)/H\(^+\) exchange pathway and minimize the contribution of other
transport systems in the regulation of intracellular pH. Second, to resolve the controversy between our results and those of other labs, it was important to maintain consistency in solution composition with our previous work. A HEPES solution was used previously (14, 15, 17). Third, despite a recent study (28) that demonstrated a lack of effect of a blocker of Na\(^{+}/\)H\(^{+}\) exchange during reperfusion using bicarbonate-based solutions (1, 3, 5, 14, 30). Again, in an effort to reduce the number of variables potentially causing the controversy, it was thought to be reasonable to isolate the Na\(^{+}/\)H\(^{+}\) exchange pathway in a HEPES-perfused medium to ensure that data interpretation was clear. Cardiac tension was monitored as apicobasal displacement with a force transducer connected to the apex of the heart, as employed recently by many other investigators (7, 18, 21). Hearts were paced at 200 beats/min by electrodes at 200% of threshold voltage with 9-ms duration during the entire course of experiments.

Global ischemia was induced by turning off the peristaltic pump, and reperfusion was initiated by restarting the pump. Unless otherwise indicated, the duration of ischemia was 60 min in the RVW and 30 min in the whole heart. This duration of ischemia was chosen because it resulted in a similar percent recovery of active developed tension. The reperfusion period was 30 min. In a few selected experiments in the RVW, the ischemic period was interrupted for 5 min from the 54th to the 59th min of ischemia by initiating perfusion with a solution identical to that described above except it had been bubbled extensively with N\(_2\) to make it hypoxic and had a pH of 6.5. Drugs were introduced at various times as outlined in Fig. 1. After reperfusion, the heart was removed and trimmed of noncontractile tissue, blotted, and weighed.

Standard reagent-grade chemicals were obtained from Sigma Chemical (St. Louis, MO) and Mallinckrodt Specialty Chemicals (Mississauga, ON). Dimethyl amiloride (DMA) and methyl isobutyl-amiloride (MIA) were obtained from Research Biochemicals (Natick, MA). They were made at a 20 or 100 µM concentration and diluted immediately before use. DMA was carefully protected from light exposure. DMA was dissolved in water, whereas MIA was prepared in dimethyl sulfoxide (DMSO). DMSO, on its own, had no effect on any of the parameters measured in the present investigation.

Data were analyzed for significance by using a multivariate analysis of variance test followed by a Student-Newman-Keuls post hoc test. All values were represented as means ± SE with statistical significance set at a level of P < 0.05.

### RESULTS

#### Experiments With RVW

Will delaying the delivery of drug during reperfusion lessen cardioprotection? The effects of delaying the introduction of the drug during reperfusion were examined. DMA, if present for the first 3 min of reperfusion, protected against the rise in resting tension exhibited by drug-untreated RVW and significantly improved the recovery of developed tension (Fig. 2). If the drug was not administered until 3 min into the reperfusion period, there were reduced protective effects. If the delivery of DMA was delayed until the 6th min of reperfusion and the drug was continued to the 9th min, no significant cardioprotection was observed. Similar trends were observed with respect to the rates of relaxation and tension generation of the RVW in the various experimental groups during reperfusion (Fig. 3).

How long must the drug be present during the initial moments of reperfusion? The duration of drug delivery necessary to observe a cardioprotective effect was tested. If DMA was administered for the first 1, 3, or 6 min of reperfusion after 60 min of ischemia, improvements were observed in both resting tension and developed...
tension (Fig. 4). However, the longer periods of drug perfusion delayed the recovery of developed tension by 6–13 min. The critical time period for cardioprotection to be observed was clearly as early as the 1st min of reperfusion. The rates of both positive and negative tension generation (\(\frac{dT}{dt}\)) responded in a similar qualitative fashion as the maximal tension generation during the period of reperfusion (Fig. 5).

Could introduction of the drug late in ischemia afford cardioprotection? If introducing the drug during reperfusion is important, then introducing the drug as early into reperfusion or even before reperfusion may improve the cardioprotective efficacy of the drug. Others have shown that administration of blockers before ischemia protects the heart (2, 7, 9, 18, 20). However, we wanted to introduce the drug immediately before reperfusion but not before ischemia. The advantage of the latter protocol is that the effects of the drug during ischemia itself will be minimized. Therefore, the drug was introduced immediately before reperfusion without significantly disturbing the ischemic environment. An ischemic solution was administered for 5 min from min 54 to 59 of ischemia. The “prereperfusion” solution was hypoxic and acidic so that the tissue pH remained as undisturbed as possible (3, 27). One minute of global ischemia was then continued before the reperfusion period was initiated with the conventional reperfusion solution. This prereperfusion washout period from min 54 to 59 exerted no effects on recovery of developed tension or resting tension (Fig. 6). However, if DMA was included in this prereperfusion solution from min 54 to 59, significant cardioprotection was observed (Fig. 6). This cardioprotection was dependent on the concentration of DMA; A DMA concentration of 0.5 µM exerted no cardioprotection, whereas a DMA concentration of 1 µM exerted intermediate effects on resting tension and the recovery of developed tension.

Which drug administration protocol affords the best cardioprotection to RVW: preischemia, prereperfusion, or immediately during reperfusion? The effects of administering 20 µM DMA before ischemia were examined. DMA was cardioprotective when administered to the RVW for 3 min before the initiation of the ischemic insult (Fig. 7). These results were placed next to those obtained when DMA was administered at the end of ischemia but before reperfusion in the ischemia-mimetic solution (data from Fig. 6) or immediately on reperfusion (data presented in Fig. 2) for a direct comparison (Fig. 7). Introduction of the drug in the preischemic solution resulted in the best cardioprotection with a clear gradation of protective effects, depend-

Fig. 3. Effects on maximal rates of tension generation (+dT/dt) and relaxation (−dT/dt) of delaying delivery of 20 µM DMA during postischemic reperfusion of right ventricular wall. DMA was administered for first 3 min or from 3rd to 6th or 6th to 9th min of reperfusion. Absolute values for maximal +dT/dt (in g·s\(^{-1}\)·g wet tissue wt\(^{-1}\)) before ischemia were 1,606 ± 106, 1,882 ± 129, 1,647 ± 45, and 1,863 ± 224 in drug-untreated control group, and in groups receiving DMA for first 3 min or from 3rd to 6th or 6th to 9th min, respectively. Absolute values for maximal −dT/dt (in g·s\(^{-1}\)·g wet tissue wt\(^{-1}\)) before ischemia were 719 ± 45, 867 ± 41, 733 ± 28, and 802 ± 96 in control group and in groups receiving DMA for first 3 min or from 3rd to 6th or 6th to 9th min of reperfusion, respectively. *P < 0.05 vs. control group (n = 5–9).

Fig. 4. Effects of 20 µM DMA on developed and resting tension during reperfusion of right ventricular wall after 60 min of ischemia. DMA was present only for first minute or for first 3 or 6 min of reperfusion. Absolute values for maximal developed tension (in g/g wet tissue wt) before ischemia were 68.3 ± 3.1, 58.6 ± 4.6, 68.8 ± 3.9, and 71.7 ± 6.1 in drug-untreated control group and in groups receiving DMA for 1, 3, or 6 min, respectively. Absolute values for resting tension (in g/g wet tissue wt) before ischemia were 45.8 ± 2.8, 44.3 ± 3.5, 47.7 ± 2.7, and 52.7 ± 1.6 in control group and in groups receiving DMA for 1, 3, or 6 min, respectively. *P < 0.05 vs. control group (n = 6–9).
ing on the time that the drug was administered before reperfusion. Other than two early time points, there were no significant differences in the recovery of developed tension or in resting tension during reperfusion between the two groups that received DMA before reperfusion.

Does another type of blocker exert similar cardioprotection? Others have been unable to demonstrate cardioprotection when delivering MIA, another blocker of Na\(^+\)/H\(^+\) exchange, to whole hearts solely during the reperfusion period (9, 18, 20). The reason for the conflict in results could be due to a difference in the drug used or to the type of heart preparation used. To address the former explanation, we first examined the effects of MIA on ischemic injury in the RVW. As shown in Fig. 8, 15 min of preischemic drug perfusion resulted in a significant cardioprotection to the RVW. Fifteen minutes was chosen to be consistent with data obtained in other labs (9, 18, 20). Interestingly, the effects of 15 min of preischemic drug perfusion could be removed if the drug was washed out of the vascular space for 3 min before the start of ischemia.

Experiments With Langendorff-Perfused Whole Heart

Is there a difference between the RVW and the whole heart preparation with regard to how they respond to drug administration during preischemic period? We also examined the response of a Langendorff-perfused whole heart to MIA perfusion during ischemia. The perfusion conditions, the drug, its concentration, and the characteristics of its delivery were duplicated to match these works (9, 18, 20) as best as possible. We found that 1 \(\mu\)M MIA (and 20 \(\mu\)M DMA) did not exert a protective effect when administered to Langendorff-perfused hearts during reperfusion alone (data not shown). Consistent with previous reports (9, 18, 20), 1 \(\mu\)M MIA did exert a significant cardioprotective effect when introduced for 15 min before ischemia (Fig. 9). If the drug was introduced for 15 min and then washed out for 3 min immediately before ischemia, the cardioprotection remained. This was in striking contrast to the data in the RVW, which are depicted in Fig. 8. Three minutes of drug perfusion before ischemia were insufficient to induce a significant recovery of developed tension. All of the drug interventions attenuated the rise in resting tension.

Fig. 5. Effects on maximal \(\frac{dT}{dt}\) and \(-\frac{dT}{dt}\) of 20 \(\mu\)M DMA during postischemic reperfusion of right ventricular wall. DMA was present only for first minute or first 3 or 6 min of reperfusion. Absolute values for maximal \(\frac{dT}{dt}\) (in g·s\(^{-1}\)·g wet tissue wt\(^{-1}\)) before ischemia were 1,596 ± 106, 1,405 ± 115, 1,882 ± 129, and 1,694 ± 207 in drug-untreated control group and in groups receiving DMA for first minute or first 3 or 6 min of reperfusion, respectively. Absolute values for maximal \(-\frac{dT}{dt}\) (in g·s\(^{-1}\)·g wet tissue wt\(^{-1}\)) before ischemia were 742 ± 43, 599 ± 43, 867 ± 41, and 691 ± 70 in control group and in groups receiving DMA for first minute or for first 3 or 6 min of reperfusion, respectively. * \(P < 0.05\) vs. control group (\(n = 6–9\)).

Fig. 6. Effects of administering DMA in a hypoxic (pH 6.5) solution from min 54 to 59 of ischemia on recovery of developed tension and resting tension during reperfusion of right ventricular wall. DMA was not administered during reperfusion. Absolute values for maximal developed tension (in g/g wet tissue wt) before ischemia were 68 ± 3, 76 ± 6, 71 ± 4, 79 ± 8, and 92 ± 7 in control ischemic group (no washout), drug-untreated (pH 6.5) washout group, and in washout groups (pH 6.5) containing 20, 1, and 0.5 \(\mu\)M DMA, respectively. Absolute values for resting tension (in g/g wet tissue wt) before ischemia were 44 ± 3, 32 ± 3, 36 ± 3, 36 ± 2, and 35 ± 3 in control ischemic group (no washout), drug-untreated (pH 6.5) washout group, and in washout groups (pH 6.5) containing 20, 1, and 0.5 \(\mu\)M DMA, respectively. * \(P < 0.05\) vs. drug-untreated control group (pH 6.5); # \(P < 0.05\) vs. drug-untreated washout group (pH 6.5).
DISCUSSION

Many independent laboratories have demonstrated that amiloride and its family of analog drugs can protect the heart from ischemic dysfunction and damage (2, 3, 7, 9, 14–18, 20, 30). The mechanism of action of these drugs is clearly via inhibition of Na\(^+\)/H\(^+\) exchange and not due to nonspecific side effects at the drug concentrations used in the present study and elsewhere (11, 14–17, 23). The arguments in favor of this interpretation have been presented in detail previously (14–17, 23, 24) and are not repeated here. However, although these works have established the involvement of Na\(^+\)/H\(^+\) exchange in postischemic dysfunction, it is less clear precisely when the exchanger is activated. This is a critical issue from a clinical standpoint. If the drug must be administered before the ischemic insult to be effective, it will be less valuable, from a clinical perspective, than if it can be delivered at the time of reperfusion.

Na\(^+\)/H\(^+\) Exchange Is Active Immediately During Reperfusion

In the RVW, several observations in the present study support the contention that Na\(^+\)/H\(^+\) exchange is primarily active during the initial minute or seconds of reperfusion. First, cardioprotection was observed even when the drug was not present during ischemia. Second, a delay in administering the drug during reperfusion resulted in less cardioprotection. Third, the initial minute of reperfusion appears to be the critical time for cardioprotection. Even as little as 1 min of drug administration immediately at the time of reperfusion was sufficient to produce significant cardioprotective effects. Fourth, administering the drug in an ischemia-mimetic solution before reperfusion resulted in better protection than that observed even during the introduction of the drug immediately at the time of reperfusion. This protection was either due to the presence of the drug during late ischemia or during early reperfusion. One of the primary reasons we chose the RVW to use in these experiments was because of the long ischemic period required to induce contractile dysfunction. It then becomes very difficult to argue that the last 5 min of a 60-min ischemic insult were critical for activation of Na\(^+\)/H\(^+\) exchange. It is far more likely that the prereperfusion exposure to DMA augmented the cardioprotective effects, because the drug was now present immediately at the time of normal reperfusion. Preischemic perfusion of the drug did result in the best cardioprotection. However, the final recovery of developed tension at 30 min into reperfusion was similar (~55%) when the drug was introduced before ischemia or during the last 5 min of ischemia (Fig. 8). The effects on resting tension were also identical whether the drug...
was administered before ischemia or at the end of ischemia. Thus blocking the exchanger during the entire ischemic period did little to augment the cardioprotective effects.

These results strongly suggest that Na\(^+\)/H\(^+\) exchange is primarily active during reperfusion and not active or minimally active during ischemia. Biophysical data would support the interpretation that the exchanger is not active during ischemia. Nuclear magnetic resonance (NMR) studies have not demonstrated any change in pH during the latter stages of ischemia that could be used as evidence of Na\(^+\)/H\(^+\) exchange activation (3, 27). Furthermore, another NMR study (32) found the ischemic heart to be more acidic in the extracellular space than in the intracellular compartment. This would actually suppress the exchanger, not stimulate it (25). Finally, administration of a blocker of Na\(^+\)/H\(^+\) exchange during the ischemic period did not change intracellular or extracellular pH during ischemia (27), again suggesting that the exchanger is inactive during this period. One alternative possibility exists. Because we did not directly measure exchange activity, it is possible that the exchanger is active during ischemia, but this activity is not involved in causing the damage or dysfunction. This important distinction remains to be evaluated.

Comparison of Results using the RVW With Whole Heart

The above data provide convincing evidence for an activation of Na\(^+\)/H\(^+\) exchange immediately during the reperfusion period but do not explain why others do not observe a similar response (2, 7, 9, 10, 18, 20). Several factors may be postulated. A recent study (28) suggested that perfusate composition is an important determinant to observe the cardioprotective effects of a blocker of Na\(^+\)/H\(^+\) exchange during the reperfusion period alone. However, others have demonstrated that blockers of Na\(^+\)/H\(^+\) exchange can protect the heart in a bicarbonate-buffered medium when they are administered solely during the reperfusion period (1, 3, 5, 14, 30). Thus other factors may also be responsible.

The present results identify two other factors as potentially important mechanisms. First, the type of cardiac muscle preparation employed appears to be critical. Drug administration solely during reperfusion protected the RVW but did not protect the Langendorff-perfused whole heart. Furthermore, although 15 min of ischemic perfusion with blockers protected both the RVW and the whole heart, 3 min of washout were insufficient to remove these cardioprotective effects in the whole heart but not in the RVW. This observation is important because it suggests that the drug had accumulated in the interstitial space in whole hearts. With the Na\(^+\)/H\(^+\) exchanger site located on the cardiomyocyte, it is not surprising that extravascular accumulation of the drug is critical for cardioprotection. What is significant about the observation is that it would suggest that a second factor, the exchange time for drug movement across the vascular wall, may also be an important determinant for drug efficacy. The results with the drug washout suggest that MIA crossed the vascular wall much more easily in the RVW than in the Langendorff-perfused whole heart. The equilibration half-times for Ca\(^{2+}\) in the RVW are \(<5\) s and 1 min for the vascular space and the interstitial space, respectively (26). It is very likely that the drugs are equilibrating with these spaces at a significantly slower rate than Ca\(^{2+}\). Clearly, therefore, even when the drug is introduced immediately with the reperfusion solution, there will be a delay of \(~1\) min before the drug reaches its inhibitory site on the cardiomyocyte. If a 3-min exposure of the whole heart to the drug even before ischemia is insufficient to protect against contractile dysfunction, it is not surprising that introduction of the drug in the whole heart during reperfusion is ineffective. If the initial minutes of reperfusion are critical for observing the cardioprotective effects (as observed in the RVW), then this delay of \(>3\) min in the whole heart would represent a crucial factor for determining the effectiveness of the drug when it is administered solely during reperfusion. It is interesting that, with one exception (30), all of the studies that have reported a cardioprotective effect of a drug administered solely at the start of reperfusion used a preparation that did not contain a
vascular component [i.e., papillary muscles (12) and single cardiomyocytes (1, 12)], used the RVW (14–17), or used a blocker of Na+/H+ exchange that did not belong to the amiloride family (5).

It is relevant to note that amiloride transport is a carrier-mediated event that occurs in exchange for H+ (31). Thus it would be expected that, although drug movement across the vascular barrier would likely occur during ischemia, the fastest rates of transport would occur during reperfusion when the pH in the interstitial/cellular compartments would be far more acidic than in the vascular space (3, 27, 32).

Reasons for the apparently slower drug delivery in whole hearts as opposed to the RVW are not clear. Potential candidates include intrinsic differences between the right and left ventricles and no possibility in the RVW for drug equilibration with the dead space in the interventricular chamber. This requires direct determination, and unfortunately an extensive study in our laboratory using high-performance liquid chromatography to examine the MIA content of coronary effluents from the two preparations has not generated interpretable data.

In summary, activation of Na+/H+ exchange is an important pathological event during the initial seconds of reperfusion. This is difficult to detect in the perfused whole heart preparation using the amiloride analog drugs. Our washout data suggest that this may be due to a limitation in drug delivery across the vascular wall. The data also suggest that either the exchanger is not as active during ischemia itself as it is during reperfusion or, if the exchanger is active during ischemia, this activity is not critical to the processes of cardiac damage and dysfunction.

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G. N. Pierce is a Medical Research Council Scientist.

Address for reprint requests: G. N. Pierce, Institute of Cardiovascular Sciences, St. Boniface Hospital Research Centre, 351 Tache Ave., Winnipeg, MB, Canada R2H 2A6.

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