Facilitating Effect of Cold Shock on Recovery From Anoxia-Induced Contractile Depression in Isolated Rat Heart and Heart Muscle

Takao Okada

The effect of rapid cooling on the recovery process from anoxia-induced hypodynamic state was studied in isolated rat ventricular muscle and ventricle. Following 10–15 minutes’ perfusion of N₂-saturated Krebs solution, the muscle was reoxygenated. When the muscle was rapidly cooled for 10–30 seconds during the early phase of reoxygenation, the rate of recovery from contracture significantly increased ($p<0.01$). Rapid cooling was also effective on the recovery from contracture induced by superfusion of Krebs solution with lowered sodium chloride concentration, but it did not affect the recovery from rigor induced by CN⁻. In the recovery from sustained anoxia (60 minutes), cooling facilitated reattainment of tension development and reduction in contracture tension. Similarly, in whole heart, 2–3 episodes of rapid cooling for about 60 seconds significantly accelerated the recovery of pressure development after 30 minutes of anoxia. At 60 minutes after reoxygenation, pressure development in hearts that were reoxygenated without rapid cooling was 29.8 ± 26.9% (mean ± SD) of pressure developed before anoxia. This value increased to 93.8 ± 27.5% following recurrent rapid cooling ($p<0.001$). At the same time, rapid cooling prevented any significant elevation in resting tension (development of oxygen paradox). These results indicate that excess intracellular calcium ions were removed by rapid cooling. This relief of the myocardium from calcium overload is believed to improve mitochondrial function and result in facilitated recovery of contractile activity. (Circulation Research 1988;62:338–346)

Recent advances in myocardial protection during global ischemia have preserved, to some extent, the contractile and metabolic activities of cardiac muscle. However, it is also imperative to facilitate the recovery of contractile performance of cardiac muscle that was ischemic. It has been reported that the excess entry of calcium ion during ischemia or reperfusion leads to mitochondrial calcium overload. This calcium overload is thought to prevent the recovery of ATP synthesis and, therefore, also the recovery of myocardial contractile activity.

In the 1960s, Sakai and his coworkers found that contracture could be induced in skeletal muscle by rapidly cooling the perfusate to 0–4°C in the presence of low concentration of caffeine. This phenomenon, known as rapid cooling contracture, could be immediately and completely reversed by rapidly raising the temperature. They also found that similar contracture could be induced in cardiac and smooth muscles. In cardiac muscle, it was possible to induce contracture just by lowering the temperature rapidly without raising the Ca²⁺ concentration, by reducing the Na⁺ concentration, or by adding caffeine. Since the membrane was depolarized only slightly by the cold shock, it was believed that the contracture developed as a result of rapid release of activator calcium from internal stores, probably from the sarcoplasmic reticulum, without the generation of action potential. However, in smooth muscle, Magaribuchi et al found that calcium is also released from other store sites, such as mitochondria.

Assuming that cardiac mitochondria are similar to those of smooth muscle, in view of their response to physical shock, they would also release calcium if they were overloaded with calcium. To clarify this hypothesis and to find a way of accelerating the recovery process from anoxic impairment of cardiac contractile performance, rapid cooling contracture was induced in rat ventricular muscle and ventricle during the early period of reoxygenation after anoxia. It was found from a series of experiments that rapid cooling accelerates the recovery from anoxic contracture while simultaneously facilitating the recovery of tension or pressure development.

Materials and Methods

Preparation Procedures

Male Sprague-Dawley rats, weighing 350–450 g, were killed under light ether anesthesia. Hearts were quickly excised, and after retrograde coronary perfusion, thin and uniformly shaped trabeculae or papillary muscles (width 0.1–0.3 mm and length 2–4 mm) were dissected from the right ventricle. The muscle was placed horizontally in an experimental chamber (3 x 1 x 1 cm³) and stimulated electrically via a pair of platinum plates with a voltage about 20% above the threshold. One end of the muscle was connected to a force transducer (compliance 2.5 μm/g) on a micrometer, and the other end was tied to a fixed hook with a 3-0 silk thread. The muscle was stretched until the resting force reached about 10–20% of the developed
Okada Cold Shock Facilitates Recovery From Anoxia

95% O\textsubscript{2}-5% CO\textsubscript{2} or 95% N\textsubscript{2}-5% CO\textsubscript{2}

FIGURE 1. Schematic diagram of experiment carried out on whole heart. Solutions in each reservoir for afterload (R1 or R2) and preload (R3) were warmed and bubbled with the same gas mixture. Left ventricular pressure (PC-460, Millar Instruments, Houston, Texas), perfusion pressure (TP-200T, Nihon Kohden, Tokyo), and temperature of perfusate were monitored. For induction of anoxia, afterload was switched from R1 to R2, and the solution in R3 was quickly changed to sucrose solution. C1, aortic cannula; C2, atrial cannula; PT1, pressure transducer for perfusion pressure; PT2, pressure transducer for left ventricular pressure; S, stopcocks; T, thermometer.

tension and was then allowed to stabilize for about 1 hour. The muscle length was kept constant throughout the procedure, and the dimensions of the muscle were measured at the end of the experiment. All experiments were performed at 30–32°C with a stimulation frequency of 0.5 Hz, except when the muscle was rapidly cooled.

Solutions

The content of solutions were (mM) control solution: NaCl 116.0, NaHCO\textsubscript{3} 25.0, CaCl\textsubscript{2} 1.5, KCl 4.7, MgSO\textsubscript{4} 0.6, KH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 5.5, Na pyruvate 2.0 with 4.0 g/1 dextran-40; 0.5-Ca solution: concentration of CaCl\textsubscript{2} was lowered to 0.5 mM without any other change from the control solution; sucrose solution: glucose and sodium pyruvate were replaced by dose-matched sucrose and sodium chloride, respectively.

The control and 0.5-Ca solutions were oxygenated with a 95% O\textsubscript{2}-5% CO\textsubscript{2} gas mixture, while the sucrose solution was equilibrated with a 95% N\textsubscript{2}-5% CO\textsubscript{2} gas mixture. Under control conditions, Po\textsubscript{2} of solution was 400–500 mm Hg, while under anoxic conditions, it was less than 20 mm Hg. In some cases, sodium pyruvate and some of the sodium chloride in the control solution were replaced by 118 mM choline chloride with a resultant Na\textsuperscript{+} concentration of 25 mM, or 17% that of the control solution (25-Na solution). In other experiments, 2 mM NaCN buffered with 5 mM N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES) was added to the control solution (CN\textsuperscript{-} solution); the solution was prepared according to the method of Allen and Orchard. All solutions were adjusted to pH 7.4.

Perfusion and Experimental Procedures

The experimental chamber was continuously perfused at a rate of 8 ml/min without recirculation. For the change of perfusate, the chamber was emptied at a rate of 4 ml/sec, and an inflow tube was switched to the new solution. At the beginning of reperfusion, the chamber was quickly (1.4 ml/sec) filled with the new solution to minimize the time that the muscle was exposed to air (usually 2–3 seconds).

Rapid cooling contracture (duration of 10–30 sec-

FIGURE 2. Development of rapid cooling contracture. Upon rapid cooling (arrow), rat right ventricular muscle developed contracture. Tonic contracture was subsequently sustained for more than 20 seconds with gradual decrease. Noisy parts of tension record are artifacts induced by changes of solution. Muscle quickly recovered from contracture upon rewarming and developed twitch tension in response to electrical stimulation.
ond) was induced by the 0.5-Ca solution, which had been cooled on ice. After the warm solution had been pumped out, cooled 0.5-Ca solution was injected manually into the chamber using a syringe (temperature of solution in the chamber was about 4°C). Judging from the complete recovery of contractile performance that occurred with rewarming, neither the rapid cooling contracture itself nor exposure of the muscle to air affected the contractile activity, even though the cold solution was not oxygenated.

The experimental procedure for the standard experiment was as follows. At the beginning of the experiment, the muscle was induced to contract isometrically in the oxygenated control solution. After the stability of isometric tension was confirmed, the perfusing solution was changed to sucrose solution that had been equilibrated with nitrogen. After the development of anoxic contracture was observed, the solution was switched to oxygenated 0.5-Ca solution, and the muscle was allowed to recover from the contracture. When the resting tension had returned to the control level, the solution was switched to the control solution. With these procedures, resting and developed tension recovered to the control level, and it was possible to repeat anoxic contracture 3-4 times in 1 muscle. Rapid cooling contracture was applied 10-20 seconds after reoxygenation.

Since the rate of development and recovery from anoxic contracture differed between muscle specimens, the effects of rapid cooling contracture and other interventions were evaluated by comparing them with the corresponding control experiments in the same muscle using paired t test.

Experiments on Whole Heart

Rat hearts were excised using the same procedure as that described above. Inserting catheters into aorta and left atrium, the heart was connected to the experimental system (Figure 1). In this system, the heart contracted against 80 mm Hg afterload with a preload of 10 cm H2O that was applied by hydrostatic pressure. Left ventricular pressure was measured via a needle inserted into the left ventricular cavity from the apex. Two stimulus needle electrodes were attached to the connective tissues around the pulmonary artery and on the right ventricular free wall. Each heart was stimulated at a constant rate (rate varied from heart to heart in the range of 1-4 Hz because of differences in spontaneous beat rates).

While the left ventricular pressure, perfusion pressure, and temperature of the perfusate were monitored, the heart was stabilized under perfusion with oxygenated control solution (34-37°C). Anoxia was induced by switching the perfusate to sucrose solution that had been equilibrated with nitrogen. Under control conditions, the coronary circulation was maintained by solution ejected from the ventricle in an anterograde direction, while during the anoxic period, retrograde coronary perfusion occurred due to the afterload because the heart stopped beating. After a 30-minute period of anoxia, the heart was reoxygenated with the control solution, and the recovery of pressure development was monitored for 60 minutes. In 6 hearts, rapid cooling was applied 3-4 times during the early period of reoxygenation by injection of cooled 0.5-Ca solution. The duration of each cooling period was about 1 minute, and the perfusion pressure was kept at less than 110 mm Hg during the injection.

Results

Rapid Cooling Contracture and Its Effect on Recovery From Contracture

Upon rapid cooling of rat ventricular muscle, contracture developed (Figure 2). The level of contracture

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Table 1. Changes in Time Constant and Time to Complete Relaxation From Contracture Induced by 25-Na Solution

<table>
<thead>
<tr>
<th>Muscle</th>
<th>RCC (-)</th>
<th>RCC (+)</th>
<th>Complete relaxation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.92</td>
<td>0.99</td>
<td>4.43</td>
</tr>
<tr>
<td>2</td>
<td>2.03</td>
<td>0.86</td>
<td>5.84</td>
</tr>
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<td>3</td>
<td>2.57</td>
<td>1.22</td>
<td>6.27</td>
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<tr>
<td>4</td>
<td>1.88</td>
<td>0.87</td>
<td>5.36</td>
</tr>
<tr>
<td>5</td>
<td>2.45</td>
<td>1.18</td>
<td>5.79</td>
</tr>
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</table>

Relaxation from contracture induced by 25-Na solution was fitted to exponential, and time constant and time to complete relaxation were calculated.

RCC (-), muscle was recovered without induction of rapid cooling contracture; RCC (+), rapid cooling contracture was induced during early phase of relaxation.
Cold Shock Facilitates Recovery From Anoxia

Okada

FIGURE 4. Effect of rapid cooling contracture (RCC) on recovery from contracture induced by CN⁻ solution. Upon addition of 2 mM NaCN to control solution, contracture developed (Panel A). When perfusate returned to normal control solution, muscle recovered from contracture (Panel B). Rapid cooling contracture did not affect time course of recovery from CN⁻ solution--induced contracture (Panel C).

was not dependent on the Ca²⁺ concentration of the cooling solution but on the Ca²⁺ concentration of the solution in which the muscle had been immersed before cooling (not shown). Together with the slight (10–20 mV) depolarization of the cell membrane observed during contracture, this indicates that Ca⁺⁺ influx through the cell membrane participates little, if at all, in the development of contracture. Upon rewarming with control solution, the muscle quickly recovered from contracture, and in response to electrical stimulation, twitch tension development recovered within 10–20 seconds to a level equivalent to the tension before contracture. Thus, the interposed rapid cooling contracture did not affect the contractile activity of the muscle in control solution.

In 5 muscles, contracture was induced by 25-Na solution. The muscle could be made to completely recover from contracture by superfusing a solution containing a normal concentration of sodium chloride (0.5-Ca solution). Induction of rapid cooling contracture during the early period of recovery accelerated the rate of decrease in contracture tension in all five experiments (Figure 3 and Table 1). In three other muscles, another contracture was elicited by superfusion of CN⁻ solution. In this form of contracture, the depression of developed tension was more marked than the elevation of resting tension (Figure 4A). Upon reperfusion of control solution, the muscle completely recovered. Rapid cooling contracture, however, neither accelerated nor delayed the recovery process from the contracture (Figures 4B and 4C); time constants of recovery process with or without rapid cooling contracture changed only slightly (3.25, 3.58, and 4.21 and 2.96, 3.68, and 4.23 minutes, respectively).

FIGURE 5. Induction of anoxia. To induce anoxia, sucrose solution was equilibrated with nitrogen-carbon dioxide gas mixture. Resulting oxygen partial pressure of perfusate was less than 20 mm Hg (top panel). With induction of anoxia, tension development was decreased and resting tension elevated. Muscle was reoxygenated with 0.5-Ca solution after short period of anoxia (bottom panel).

Effect of Rapid Cooling Contracture on Recovery From Anoxic Contracture

Although rat ventricular muscle was resistant to anoxia, total replacement of glucose by sucrose and the absence of sodium pyruvate significantly accelerated the development of anoxic contracture. With the induction of anoxia in sucrose solution (n = 13), resting tension rose to 40–100% of total tension (development of anoxic contracture) within 6–15 minutes (Figure 5). Upon superfusion with oxygenated 0.5-Ca solution, the muscle recovered from anoxic contracture in an exponential manner (Figure 6, top panel). After switching to control solution, the developed tension also recovered to the control level within 5–20 minutes in a somewhat stepwise manner.

Rapid cooling contracture was induced in the muscle during the early period of reoxygenation with 0.5-Ca solution. As shown in the bottom panel of Figure 6, contracture tension dropped by a significant degree immediately after the rapid cooling contracture. The subsequent rate of recovery from anoxic contracture was also significantly accelerated in comparison with the control experiment (Figure 6, top panel). With only one exception, the time constant of recovery process
from anoxic contracture was reduced from 5.10 ± 3.18 (mean ± SD) to 3.50 ± 2.64 minutes by rapid cooling contracture. Similarly, the time to complete relaxation, i.e., the time needed to return to the original resting tension level, was reduced from 7.21 ± 4.20 to 4.83 ± 3.58 minutes (Table 2). These differences were proved to be statistically significant (p < 0.01) by paired difference t test.

**Effect of Rapid Cooling Contracture on Recovery From Sustained Anoxia**

When the duration of anoxia was sustained for 60 minutes and the muscle was reoxygenated with control solution, the muscle did not show complete recovery from anoxic contracture even after 60 minutes of reoxygenation (n = 6). At this point, the active tension development was less than 30% of the control level. However, when several (2–5) episodes of rapid cooling contracture were induced during the early period of reoxygenation (n = 3), contracture tension decreased and active tension increased with each intermittent cold shock. Although the limited amount of data made statistical analysis inappropriate, the resultant amount of active tension development at 60 minutes after reoxygenation increased to 40–70% of the control level (Figure 7).

**Effect of Rapid Cooling on Recovery From Anoxia in Whole Heart**

Isolated rat heart (left ventricle) developed stable pressure for more than 4 hours under coronary perfusion with oxygenated control solution (n = 14). Anoxia was induced by sucrose solution (Figure 8). Upon perfusion with sucrose solution, pressure development decreased to zero within several minutes and diastolic pressure was usually elevated by 2–20 mm Hg. Anoxic conditions were maintained for 30 minutes, and the heart was then reoxygenated with control solution. In the early period of reoxygenation, 2–4 episodes of rapid cooling were applied to the ventricle to assess the resultant effect on the recovery of left ventricular pressure development. In whole heart, rapid cooling elicited only a slight elevation of diastolic pressure, even though the duration of cooling was increased to about 1 minute.

Upon reperfusion with oxygenated control solution, the right atrium started to contract within 5–15 minutes;
Table 2. Time Course of Recovery From Anoxia With or Without Rapid Cooling Contracture

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Rapid cooling contracture</th>
<th>Duration (min)</th>
<th>Contracture (g/mm²)</th>
<th>r</th>
<th>Time constant (min)</th>
<th>Recovery (min)</th>
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<td>1.08</td>
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Duration of anoxia and contracture tension are values measured from actual experimental records, while time constant and time to complete recovery are values calculated from exponential fitting. Although there were no statistical differences in duration of anoxia and contracture tension at onset of reoxygenation, time constant (5.10 + 3.18-3.50 + 2.64 minutes) and time to complete recovery (7.21 + 4.20-4.83 + 3.58 minutes) significantly (p<0.01 by paired difference t test) decreased with induction of rapid cooling contracture.

Query: the contraction then spread to other parts. Figure 9 shows the effect of rapid cooling on the recovery of pressure development (n = 6). As shown in the upper panel, recovery of pressure development was facilitated by 3 episodes of rapid cooling with only a slight elevation of diastolic pressure at 60 minutes after reoxygenation. When the heart was reoxygenated without rapid cooling (n = 6), as shown in the lower panel, recovery of pressure development was slow, and the amount of pressure development was very small at 70 minutes after reoxygenation. Furthermore, 3 of 6 hearts showed gradual but significant elevation of diastolic pressure. However, rapid cooling usually accelerated the decay of diastolic pressure; with a delay of 5-10 minutes, diastolic pressure decreased to its original level (Figure 10). The effect of rapid cooling on the recovery of left ventricular pressure development is summarized in Figure 11. At 60 minutes after reoxygenation, the pressure development in hearts, which were reoxygenated without rapid cooling, was 29.8±26.9% of the pressure before anoxia. This value was increased to 93.8±27.5% by recurrent rapid cooling (p<0.001).

Discussion

Rapid cooling contracture was shown to facilitate the process of recovery from contracture induced by lowered Na⁺ concentration (Figure 3). Similarly, it accelerated the recovery from a short period of anoxia (Figure 5). However, rapid cooling contracture was found not to affect recovery from a CN⁻-induced rigor state (Figure 4); thus, it was suggested that cardiac muscles were relieved from calcium overload by rapid cooling contracture.

It is widely believed that ischemia- or anoxia-induced cellular calcium overload generates mitochondrial calcium overload.10-12 Such calcium overload would induce uncoupling of mitochondrial oxidative
phosphorylation, resulting in irreversible cell injury. Consequently, facilitation of recovery of mitochondrial ATP synthesis would in turn facilitate recovery of anoxia-induced myocardial contractile depression. Although cardiac mitochondria, under normal conditions, are not expected to play an important role in calcium regulation, under condition of calcium overload, mitochondria might release Ca$^{2+}$ by cold shock. Thus, rapid cooling contracture may relieve mitochondrial calcium overload and facilitate the recovery of ATP synthesis, resulting in facilitated recovery of contractile activity. This hypothesis was supported by experimental results (Figure 9) that, although incomplete, show a decrease in rigor tension and an increase in developed tension were attained by rapid cooling contracture. It may also be possible that generation of oxygen free radicals was suppressed by the cold shock itself or, alternatively, by relief from myocardial calcium overload.

The released Ca$^{2+}$ has to be excluded to extracellular space. The mechanism of calcium removal is not yet clear, but a possible explanation would be as follows: During cold shock, the released Ca$^{2+}$ is excluded through a Na–Ca exchange mechanism, with a reported low temperature dependence. This hypothesis is supported by the fact that cold shock with 0.5-Ca solution was more effective than cold shock with control solution containing 1.5 mM CaCl$_2$ (not shown).

**Figure 8.** Induction of anoxia in whole heart. Experimental record of, from top to bottom, perfusion pressure (P.P.), temperature of perfusate (T), and left ventricular pressure ($P_{LV}$). With anoxic perfusion, left ventricular pressure development decreased and diastolic pressure was elevated. This heart was exceptional in that diastolic pressure was quickly elevated to level of afterload (80 mm Hg). The heart was stimulated at 3 Hz. Interposed calibration (bottom right).

**Figure 9.** Effect of rapid cooling (RC) on recovery from anoxia. Recovery of pressure development was observed for 60 minutes after 30-minute anoxia period. Top panel: Three episodes of RC were applied during early period of reoxygenation with stimulus rate of 2.1 Hz (10 min, start of reoxygenation). Bottom panel: Conventional reoxygenation without RC. Note significant elevation of diastolic pressure after reoxygenation (stimulus rate, 3.3 Hz).
Furthermore, it was often observed that with induction of rapid cooling contracture, a significant drop in resting tension was followed by a transient small elevation (Figure 7). This result may indicate that intracellular Na⁺ increased by the cold shock is exchanged for Ca²⁺ when the perfusate is rewarmed. Calcium efflux through Na-Ca exchange is estimated to be approximately 1.2 μmol/kg wet wt/sec at 6 μM [Ca²⁺].\(^{13}\) If this value, or a value close to it, is applicable, it would be sufficient to remove excess intracellular Ca²⁺ during a cold period of 10–30 seconds.

Rapid cooling of the heart did not produce significant elevation of diastolic pressure, probably because not all heart cells were cooled and developed contracture. After 30 minutes of anoxia, left ventricular pressure development completely disappeared, but with rapid cooling, pressure development dramatically increased to almost complete recovery. Reoxygenation without rapid cooling often resulted in significant elevation of diastolic pressure. This elevation was greater than that in diastolic pressure during anoxia, suggesting that cardiac cells had been severely damaged. This injury induced by reoxygenation (oxygen paradox)\(^{19,20}\) did not occur in hearts that underwent rapid cooling, indicating a protective effect of rapid cooling on extreme calcium overload.

There have been many studies aimed at finding a way to protect cardiac muscle and to preserve the contractile activity during and after ischemia. These reports have suggested the advantage of hypothermia during ischemia (for a review, see Rosenkranz and Buckberg\(^{21}\)). However, the aim of the present study was to find a way to facilitate the recovery of cardiac muscle already in an anoxic condition. Some researchers have reported a detrimental effect of hypothermic reperfusion, indicating that it inhibits regeneration of high energy phosphate compounds.\(^{22,23}\) The present results do not contradict these findings. Although sustained (10–30 minutes) hypothermia may depress ATP synthesis to a greater extent than utilization, a short period of cooling (10–60 seconds) would not significantly affect the content of ATP but, rather, would facilitate ATP synthesis under normothermic conditions after cooling.

Thus, it seems that many concerns remain to be solved; i.e., the difference between anoxia and ischemia\(^{24,25}\) or species differences in the reaction of muscle tissue to anoxia. Furthermore, it seems certain that calcium overload is not the only cause of the series of events that occur after anoxia or reoxygenation. However, the present findings suggest that rapid cooling may prove to be an effective means of facilitating the recovery of cardiac muscle from an anoxic and calcium-overload state in clinical cases.

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