Experimental Studies

Effect of Anoxic Preperfusion on Ischemic Myocardial Injury in Isolated Rat Hearts

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SUMMARY

Anoxic perfusion prior to sustained ischemia (anoxic preperfusion), reportedly improves postischemic functional recovery of the heart, but its mechanism has not been well understood. The present study aimed to characterize the cardioprotective effects of anoxic preperfusion and its relationship to extracellular Ca^{++} levels. Following 10 min of aerobic perfusion, isolated rat hearts were assigned to a 10 min aerobic perfusion or to a 10 min anoxic perfusion. The hearts were then subjected to 30 min of global ischemia and 30 min of aerobic reperfusion. When the perfusate-free Ca^{++} concentration was 2.0 mM, postischemic recovery of left ventricular developed pressure was significantly improved by anoxic preperfusion ($91.9 \pm 2.9\%$ of baseline value vs. $50.5 \pm 12.9\%$ after 30 min reperfusion in the controls). However, the improvement of postischemic ventricular function by anoxic preperfusion was abolished when perfusate Ca^{++} was reduced to 1.0 mM and the contractile function was rather suppressed during early reperfusion by anoxic preperfusion when the Ca^{++} level was 0.7 mM ($87.5 \pm 11.8\%$ vs. $115.6 \pm 13.9\%$ after 10 min of reperfusion). On the other hand, lactate accumulation during the global ischemia was significantly less in anoxic preperfused hearts compared with untreated hearts both when perfusate Ca^{++} was 0.7 mM (61.3 ± 5.1 vs. 85.9 ± 6.8 $\mu\text{mol/g}$ dry) and when it was 2.0 mM (43.8 ± 2.0 vs. 140.3 ± 14.1 $\mu\text{mol/g}$ dry). The amount of myoglobin released after global ischemia was not different between untreated and anoxic preperfused hearts regardless of the perfusate Ca^{++} level. The results suggest that anoxic preperfusion does not reduce ischemic myocardial necrosis, but it attenuates myocardial stunning. That effect of anoxic preperfusion on the stunning is dependent on the extracellular Ca^{++} level and is not totally explained by suppression of ischemia-induced lactate accumulation.

Key Words:

Anoxia Stunned myocardium Ischemia Preconditioning

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RECENT studies have disclosed a phenomenon whereby exposing the myocardium to brief transient ischemia potentiates myocardial resistance to subsequent ischemia.^{1)–6)} Insulting the myocardium with a brief period of ischemia is termed ischemic preconditioning and it has been shown to limit infarct size after long-lasting myocardial ischemia,^{1)–3)} reduce reperfusion arrhythmia,^{4),5)} and possibly attenuate myocardial stunning.⁶⁾ However, the mechanism of the preconditioning effect is not completely understood. One of the hypotheses is that the ischemic preconditioning depletes myocardial glycogen and suppresses the build-up of lactate, which could be a cytotoxic product,⁷⁾ during subsequent ischemia.^{2),4)} This hypothesis is based on a study by Neely and Grotyohann,⁸⁾ in which brief anoxic perfusion prior to global ischemia paradoxically improved postischemic ventricular function in the isolated rat heart. Although myocardial ATP was reduced by the anoxic preperfusion, accumulation of tissue lactate during ischemia was attenuated and the addition of exogenous lactate in the perfusate abolished the protective effect of anoxic preperfusion.⁸⁾ Based on these findings, they proposed that lactate and other catabolic products accumulating during ischemia play a key role in ischemic myocardial injury.⁸⁾ In contrast, Lagerstrom et al⁹⁾ reported that the postischemic ventricular developed pressure was significantly lower in rabbit hearts preperfused with anoxic perfusate or with substrate-free perfusate, both of which depleted myocardial glycogen and suppressed lactate accumulation during ischemia.

Lagerstrom et al⁹⁾ pointed out the possibility that the difference in the calcium level of the perfusate (1.25 mM vs. 2.5 mM) might be responsible for the different effects of anoxic preperfusion in the two studies.^{8),9)} While the relationship between the calcium level and the protective effect of anoxic preperfusion has not been examined, in a model of low flow ischemia, postischemic functional recovery was inversely correlated with calcium level in a study by Neely and Grotyohann.⁸⁾ Another important question remaining unanswered is whether the improvement of postischemic function by anoxic preperfusion is due to reduction of ischemic myocardial necrosis or to attenuation of myocardial stunning. To examine these questions, the present study analyzed the effect of anoxic preperfusion on myoglobin release and ventricular function after global ischemia in isolated hearts at different extracellular Ca^{++} levels (ranging from 0.7–2.0 mM).

MATERIALS AND METHODS

Preparation

Sprague-Dawley rats (body weight 200–300 g) were lightly anesthetized with diethyl ether and oxygen. After injection of heparin sodium (200 units) into the femoral vein, the heart was excised and immediately transferred to ice-cold perfusion medium before being mounted on a non-recirculating Langendorff apparatus. The heart was perfused at 100 cm H₂O pressure at 37°C. The perfusion fluid was modified Krebs-Henseleit bicarbonate buffer containing 11 mM glucose, and the concentration of CaCl was changed (0.75, 1.25, or 2.5 mM) according to the experimental protocol. The free Ca⁺⁺ level of the perfusate, which was determined with an ICAI Ionized Calcium Analyzer (Radiometer Copenhagen, Denmark), was 0.7, 1.0, or 2.0 mM. Aerobic perfusate was gassed with 95% O₂: 5% CO₂ and anoxic perfusate with 95% N₂: 5% CO₂. A catheter-tip manometer (model TCP-2, Nihon Kohden, Tokyo) was placed in the left ventricle via the mitral orifice to monitor left ventricular pressure throughout the experiment.

Experimental protocol

Six groups of hearts (n=6–13) were set up, as in Table I, to examine the effect of anoxic preperfusion under 3 different levels of extracellular calcium. Figure 1 illustrates the perfusion protocol. For each free Ca⁺⁺ level (i.e., 0.7, 1.0 or 2.0 mM Ca⁺⁺), the control group was subjected to 20 min of aerobic perfusion and then to 30 min of global ischemia and 30 min of reperfusion. The anoxic preperfusion group was initially perfused for 10 min with aerobic perfusate and then it was switched to anoxic perfusion for 10 min prior to the

Fig. 1. Perfusion protocols. For each free Ca⁺⁺ level (0.7, 1.0 or 2.0 mM) of the perfusing medium, a group of hearts received 30 min of global ischemia/30 min of reperfusion and another group was subjected to 10 min of anoxic perfusion prior to the 30 min of global ischemia.

global ischemia. Coronary effluent was collected for measuring coronary flow and for the assay of released myoglobin.

Using another group of rats, alterations of tissue metabolites by anoxic perfusion and by global ischemia following aerobic or anoxic perfusion were assessed. Ventricular myocardium with or without exposure to anoxic perfusion was quickly frozen immediately before and at the end of 30 min of global ischemia with a Woolenberger clamp cooled in liquid nitrogen ($n=5-8$ for each time period). The tissue samples were stored in liquid nitrogen until the assay.

Analytic methods

Coronary effluent was centrifuged at 3,000 rpm for 10 min and its absorbance at 417 nm was read in a spectrophotometer for determination of myoglobin concentration.¹⁰⁾ The standard curve for myoglobin assay was obtained using horse heart myoglobin (Sigma Co., MO, USA).

Frozen myocardium was freeze-dried and the metabolites were extracted by 0.6 N perchloric acid. ATP, creatine phosphate and lactate in the extracts were measured by standard enzymatic methods.¹¹⁾

Statistics

Results are presented as mean $\pm$ standard error. Multiple comparisons between the experimental groups were performed by analysis of variance (ANOVA) with the Bonferroni test. Differences in parameters in each group across time were tested by repeated measures ANOVA.

RESULTS

Effect of Anoxic Preperfusion on Postischemic Ventricular Function

The baseline hemodynamic parameters are summarized in Table I. Heart rate was similar in all groups regardless of differences in the perfusate Ca^{++} level. In contrast, ventricular developed pressure, rate-pressure products and coronary flow were larger in the groups perfused with the Krebs-Henseleit buffer containing higher Ca^{++} . There were no significant differences in the baseline parameters of the control group and the anoxic preperfused group receiving the buffer containing the same level of Ca^{++} .

Figure 2 presents the time course of developed pressure throughout the experiment. When the perfusate Ca^{++} was 0.7 mM (Fig. 2, upper panel), the developed pressure of control hearts recovered to the baseline level 5 min after the onset of reperfusion and was stable thereafter. In the anoxic preperfused

Table I. Baseline Hemodynamic Data Prior to Various Treatments of Oxygen Supply and Ischemia

Perfusate Free Ca ⁺⁺ (mM)	Subsequent Anoxic Tx	Heart Rate (/min)	Developed Pr (mmHg)	RPP (mmHg/min)	Coronary Flow (ml/min/g dry)
0.7	—	335±10	38±2	12895±855	63.3±5.4
0.7	+	336±5	35±5	11776±1365	76.7±8.9
1.0	—	372±11	60±13	22629±1498	100.1±9.1
1.0	+	380±10	54±4	20944±1658	103.9±6.3
2.0	—	346±12	91±5	31612±2260	105.1±5.8
2.0	+	357±8	80±3	28374±817	89.3±6.4

Mean±SEM. Perfusate Free Ca⁺⁺=free Ca⁺⁺ concentration in coronary perfusate; Anoxic Tx=anoxic preperfusion; Developed Pr=left ventricular developed pressure; RPP=rate pressure product.

group, developed pressure was reduced to $54.5\pm8.9\%$ of baseline during anoxic perfusion and recovery of the developed pressure was $33.4\pm16.8\%$ and $87.5\pm11.8\%$ after 5 and 10 min of reperfusion, respectively, which were significantly lower compared with those in the control group ($129.1\pm25.0\%$ and $115.6\pm13.9\%$).

When the perfusate-free Ca⁺⁺ was 1.0 mM (Fig. 2, middle panel), developed pressure after ischemia was approximately 50% of baseline in the control hearts in contrast to the complete recovery under 0.7 mM Ca⁺⁺. Anoxic perfusion itself reduced developed pressure to $34.0\pm6.6\%$ of baseline, but the recovery of developed pressure from global ischemia was not significantly different for control and anoxic preperfused hearts.

When the perfusate containing 2.0 mM Ca⁺⁺ was used (Fig. 2, lower panel), postischemic developed pressure in control hearts was slightly lower than in the hearts perfused with 1.0 mM Ca⁺⁺. However, under this level of free Ca⁺⁺, the developed pressure in the hearts which received anoxic preperfusion showed markedly better recovery after the global ischemia compared with untreated control hearts. At 30 min after reperfusion, developed pressure in the anoxic preperfused group was $91.9\pm2.9\%$ of baseline in contrast to $50.5\pm12.9\%$ in the control group ($p<0.05$).

Coronary Flow after Global Ischemia and Reperfusion

The time course of coronary flow during ischemia and reperfusion is presented for each perfusate Ca⁺⁺ level in Fig. 3. To assess coronary flow supply/demand status, coronary flow was expressed as the value per rate-pressure product (CF/RPP). Baseline CF/RPP was comparable between control and anoxic preperfused hearts for each of the perfusate Ca⁺⁺ concentrations (Fig.

Fig. 2. Left ventricular developed pressure after 30 min of global ischemia. Open circles are control hearts and closed circles are anoxic preperfused hearts. Upper panel: perfusate $\text{Ca}^{++}=0.7$ mM, middle panel: perfusate $\text{Ca}^{++}=1.0$ mM, lower panel: perfusate $\text{Ca}^{++}=2.0$ mM. Abbreviations: Pre=preperfusion; I=ischemia. Under 0.7 mM Ca^{++} , anoxic preperfusion suppressed developed pressure during 5–10 min reperfusion. In contrast, anoxic preperfusion significantly improved postischemic ventricular function under 2.0 mM Ca^{++} .

Fig. 3. Time course of coronary flow. Coronary flow (CF) was expressed as the ratio to rate-pressure products (RPP). Upper panel: perfusate $\text{Ca}^{++} = 0.7$ mM, middle panel: perfusate $\text{Ca}^{++} = 1.0$ mM, lower panel: perfusate $\text{Ca}^{++} = 2.0$ mM.

3). Anoxic perfusion caused an approximate 8-fold increase in coronary flow for each of the Ca^{++} levels. When perfusate Ca^{++} was 0.7 mM (Fig. 3, upper panel), CF/RPP was larger at 5–10 min of reperfusion in the anoxic-preperfused group than in the control group, for which high coronary flow

appears associated with suppressed functional recovery during that time period (shown in Fig. 2, upper panel). There was no significant difference in the normalized coronary flow during reperfusion between the control and anoxic preperfused hearts receiving perfusate with 1.0 or 2.0 mM Ca^{++} (Fig. 3, middle and lower panels).

Effect of Anoxic Preperfusion on Myoglobin Release after Ischemia

Figure 4 illustrates the time course of myoglobin release into the coronary effluent. Under perfusion with 0.7 mM Ca^{++} , the peak value and the total amount of myoglobin release after 30 min of ischemia and 30 min of reperfusion were not different ($434 \pm 61 \mu\text{g/g dry}$ vs. $509 \pm 152 \mu\text{g/g dry}$). The same was true for its release pattern between the hearts with and without anoxic preperfusion. When perfusion medium containing 1.0 or 2.0 mM Ca^{++} was used, peak myoglobin after reperfusion was higher in anoxic preperfused hearts than control hearts. However, total release of myoglobin was not different between the 2 groups for either Ca^{++} level ($772 \pm 131 \mu\text{g/g dry}$ vs. $613 \pm 105 \mu\text{g/g dry}$ in 1.0 mM Ca^{++} groups and $813 \pm 118 \mu\text{g/g dry}$ vs. $901 \pm 84 \mu\text{g/g dry}$ in 2.0 mM Ca^{++} groups).

Effect of Anoxic Preperfusion on Tissue ATP, CP and Lactate

Tissue metabolite data are summarized in Table II. When perfusate Ca^{++} was 0.7 mM, myocardial ATP and CP were significantly reduced after 30 min of global ischemia to 6.9 and 2.9 $\mu\text{mol/g dry}$, respectively, and lactate was increased to 85.9 $\mu\text{mol/g dry}$. Ten minutes of anoxic perfusion slightly reduced CP ($15.8 \pm 0.7 \mu\text{mol/g dry}$), but the ATP level was not changed. Lactate build-up during 30 min of ischemia was significantly suppressed ($61.3 \pm 5.1 \mu\text{mol/g dry}$) by anoxic preperfusion while depletion of ATP and CP was not modified.

When the perfusate Ca^{++} was 2.0 mM, baseline ATP, CP and lactate levels were similar to those of hearts perfused with 0.7 mM Ca^{++} . However, accumulation of lactate after 30 min of ischemia was more marked than in the hearts perfused with 0.7 mM Ca^{++} (140.3 ± 14.1 vs. $85.9 \pm 6.8 \mu\text{mol/g dry}$). In contrast to the experiment using 0.7 mM Ca^{++} , anoxic perfusion itself significantly reduced ATP and CP and slightly increased the lactate level of the hearts perfused with 2.0 mM Ca^{++} . Lactate accumulation during 30 min of ischemia was again significantly less ($43.8 \pm 2.0 \mu\text{mol/g dry}$) in the hearts preperfused with anoxic perfusate.

Fig. 4. Time course of myoglobin release after 30 min of global ischemia. Upper panel: perfusate $\text{Ca}^{++}=0.7$ mM, middle panel: perfusate $\text{Ca}^{++}=1.0$ mM, lower panel: perfusate $\text{Ca}^{++}=2.0$ mM. Peak myoglobin value was higher in anoxic preperfused hearts than in control hearts under 1.0 mM Ca^{++} (middle panel) and 2.0 mM Ca^{++} (lower panel), but the total amount of released myoglobin was not different between control and anoxic preperfused hearts under any perfusate Ca^{++} level.

Table II. Tissue ATP, CP and Lactate in Hearts Exposed to Various Conditions of Oxygen Supply Prior to 30 Min of Global Ischemia

Condition	N	Tissue Metabolites ($\mu\text{mol/g dry}$)		
		ATP	CP	Lactate
Perfusate $\text{Ca}^{++}=0.7 \text{ mM}$				
O_2 20 min	5	16.7 ± 1.4	20.0 ± 2.0	3.7 ± 0.2
+ Ischemia 30 min	8	$6.9 \pm 0.5^{*+}$	$2.9 \pm 1.2^{*+}$	$85.9 \pm 6.8^{*+}$
O_2 10 min + N_2 10 min	5	16.0 ± 0.3	15.8 ± 0.7	6.1 ± 0.7
+ Ischemia 30 min	8	$8.7 \pm 0.9^{*+}$	$5.8 \pm 0.8^{*+}$	$61.3 \pm 5.1^{*+ \#}$
Perfusate $\text{Ca}^{++}=2.0 \text{ mM}$				
O_2 20 min	5	18.2 ± 1.8	19.0 ± 2.6	10.3 ± 1.6
+ Ischemia 30 min	5	$3.4 \pm 0.6^{*+}$	$5.5 \pm 0.8^*$	$140.3 \pm 14.1^{*+}$
O_2 10 min + N_2 10 min	5	$8.7 \pm 1.8^*$	$5.8 \pm 0.5^*$	19.5 ± 1.0
+ Ischemia 30 min	5	$3.2 \pm 0.7^{*+}$	$1.9 \pm 0.6^*$	$43.8 \pm 2.0^{\#}$

Mean $\pm$ SEM. CP=creatine phosphate.

Comparison under the same Ca^{++} level: * $P < 0.05$ vs. O_2 20 min, + $p < 0.05$ vs. O_2 10 min + N_2 10 min, # $p < 0.05$ vs. O_2 20 min + Ischemia 30 min.

DISCUSSION

The present study showed that anoxic perfusion prior to global ischemia attenuated postischemic myocardial dysfunction in an isolated heart preparation. The beneficial effect of anoxic preperfusion was dependent on extracellular Ca^{++} and was not parallel to suppression of lactate build-up during ischemia. Anoxic preperfusion did not reduce myoglobin release after ischemia, suggesting that its effect on contractile function was due to attenuation of the myocardial stunning, not to reduction of ischemic myocardial necrosis.

The present observation that anoxic preperfusion improves postischemic contractile function corroborates an earlier observation by Neely and Grotyohann.⁸⁾ In addition, it was shown that the effect of anoxic preperfusion is dependent on the Ca^{++} level of the perfusate. Anoxic preperfusion suppressed lactate accumulation under both 0.7 mM and 2.0 mM Ca^{++} (Table II), but the postischemic functional recovery was not parallel to those lactate levels. Developed pressure during the early reperfusion period was rather suppressed by anoxic preperfusion under 0.7 mM Ca^{++} as shown in Fig. 2 although anoxic preperfused hearts recovered better than control hearts under 2.0 mM Ca^{++} . These findings suggest that suppression of lactate accumulation during ischemia does not necessarily accelerate postischemic functional recovery and that the beneficial effect of anoxic preperfusion may not be totally explained by the alteration of lactate level during ischemia.

The extent of ischemic necrosis and the degree of myocardial stunning are important determinants of postischemic contractile function¹²⁾ in addition to the factors that influence the contraction of normal myocardium (such as preload and afterload). Accordingly, improvement of postischemic ventricular function by anoxic preperfusion (Fig. 2, lower panel) could be due to limitation of ischemic necrosis, or attenuation of myocardial stunning, or both. However, no reduction in myoglobin release after ischemia by anoxic preperfusion was observed in the present study (Fig. 4). These findings suggest that anoxic preperfusion does not reduce ischemic myocardial necrosis and that probably its effect on contractile function reflects attenuation of myocardial stunning.

The present results argue against the hypothesis that the cardioprotective effect of ischemic preconditioning is mediated by glycogen depletion and suppression of lactate accumulation during subsequent ischemia,^{2),4)} which were observed in anoxic preperfused hearts. However, this does not exclude the possibility that different protocols of anoxic preperfusion delay ischemic myocardial necrosis via other mechanisms. A recent preliminary study by Shizukuda et al¹³⁾ suggests that myocardial infarct size in a dog model may be limited by anoxic preconditioning as well. However, in that study, they set up 10 min of normoxic perfusion between 5 min of hypoxic blood perfusion and 60 min of coronary occlusion, in contrast to the present protocol in which there was no normoxic perfusion period between anoxic perfusion and global ischemia. Nevertheless, the present findings do not support the hypothesis that ischemic preconditioning protects ischemic myocardium via attenuating the ischemia-induced lactate accumulation in the myocardium.

Although contractile function was better preserved in anoxic preperfused hearts during reperfusion compared with control hearts under 2.0 mM Ca^{++} (Fig. 2, lower panel), CF/RPP, the coronary flow normalized by rate-pressure products, was similar between control and anoxic preperfused hearts (Fig. 3, lower panel). Accordingly, modification of coronary flow supply-demand balance is unlikely to contribute to the improvement of postischemic contractile function by anoxic preperfusion.

The present study cannot specify the mechanism of the attenuation of stunning by anoxic preperfusion. However, one possibility is that proton production from ATP degradation during ischemia¹⁴⁾ might be suppressed by anoxic preperfusion. A possible difference in ATP consumption between anoxic preperfused and control hearts was suggested by the difference in ATP levels between preischemic and postischemic periods being smaller in the hearts receiving anoxic preperfusion than in the control hearts (Table II). If proton generation was attenuated, it would lead to less accumulation of Na^+

during ischemia through $\text{Na}^+\text{-H}^+$ exchange.^{15),16)} If that is the case, Ca^{++} influx via $\text{Na}^+\text{-Ca}^{++}$ exchange during reperfusion^{17),18)} would be reduced, which would modify the contractile function. This hypothesis also explains why the effect of anoxic preperfusion on the postischemic contractile function depended on the extracellular Ca^{++} level (Fig. 2). This explanation is, of course, speculative as the proton production or turnover of ATP during ischemia was not assessed in the present study.

Lagerstrom et al⁹⁾ also previously studied the effect of anoxic preperfusion on ventricular function after global ischemia and reperfusion in the rabbit heart. However, in contrast with the study by Neely and Grotyohann⁸⁾ and ours (Fig. 2), the postischemic ventricular developed pressure was significantly lower in hearts preperfused with anoxic perfusate or with substrate-free perfusate, both of which depleted myocardial glycogen and suppressed lactate accumulation during ischemia.⁹⁾ The reason for the negative results in their study in contrast with ours is unclear. If the Ca^{++} sensitivity of the rabbit heart were lower compared with that of the rat heart, the suppression of postischemic function in the anoxic preperfused rabbit heart might correspond to the delayed functional recovery by anoxic preperfusion in the rat heart under 0.7 mM Ca^{++} (Fig. 2, upper panel). However, there are also differences in the degree of suppression of lactate accumulation by anoxic preperfusion and in the duration of ischemia, which could possibly be responsible for the different outcomes between the negative study by Lagerstrom et al⁹⁾ and the positive studies, including ours.

In conclusion, anoxic perfusion prior to ischemia does not reduce ischemic myocardial necrosis, but significantly attenuates myocardial stunning. The effect of anoxic preperfusion on stunning is dependent on the extracellular Ca^{++} level, suggesting the important role of Ca^{++} flux alteration in this phenomenon.

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