ARRHYTHMIA AFTER THE RELEASE OF INHIBITED OXIDATIVE PHOSPHORYLATION

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Automaticities due to delayed afterdepolarizations, elicited upon reoxygenation, are thought to be caused by Ca overload. Since tissue Ca uptake upon reoxygenation has been reported to be closely related to metabolic inhibition during hypoxic perfusion, the relationship between the degree of metabolic inhibition during hypoxia and reoxygenation-induced arrhythmias was investigated in guinea pig papillary muscles. (1) Arrhythmias occurred after 60 min substrate-free hypoxia, but not after 30 min hypoxia. The chance of automaticities was closely related with the increase in resting tension achieved during hypoxic period. The incidence of arrhythmias was, however, lower after 90 or 120 min hypoxia. (2) There were arrhythmias after 20–30 min hypoxia with glycolytic inhibition (20 mM 2-deoxyglucose + 5 mM acetate). On the other hand, 60-min hypoxia in the presence of 5 mM glucose did not elicit arrhythmias on reoxygenation. (3) Stimulation of glycolysis (50 mM glucose) during substrate-free hypoxia prolonged the action potential duration, but did not cause arrhythmias. Washout of cyanide (1 mM) after 60 min perfusion in the presence of oxygen, caused arrhythmias and aftercontractions. These results suggest that the degree of metabolic inhibition during hypoxia is closely related to Ca overload and the resultant arrhythmias upon reoxygenation. The release of inhibited oxidative phosphorylation, rather than the reintroduction of oxygen per se, was thought to be the key mechanism of reoxygenation-induced arrhythmias.

THE electrophysiologic basis for reperfusion arrhythmias¹ has been proposed to be the heterogeneity of electrical recovery resulting in reentry or enhanced automaticity. Kaplinsky et al² proposed two distinct types of arrhythmias. One type, which occurred during the first 1 min of reperfusion, was due to reentry. The

second type, which occurred between 2 and 7 min after the onset of reperfusion, was thought to be due to enhanced automaticity.

We have shown that triggered activities due to delayed afterdepolarizations were induced upon reoxygenation following severe hypoxic condition³. These abnormal activities resembled those described for cardiac preparations with Ca overload^{4,5}. Since ryanodine, which has been shown to inhibit Ca release from the sarcoplasmic reticulum (SR)^{6,7} abolished automaticities, delayed afterdepolarizations, and aftercontractions, these activities were likely to be caused by Ca overload and intracellular Ca oscillatory

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Fig.1. Arrhythmia, delayed afterdepolarizations, and aftercontractions in guinea pig papillary muscle after 60 min substrate-free hypoxia. The muscle was reoxygenated for 5 min with Krebs solution which contained 5 mM glucose. The upper trace is membrane potential, and the lower one is tension. Stimulation BCL was 500 msec marked by arrowheads. Delayed afterdepolarizations and aftercontractions grew up in each stimulation, and triggered activities were elicited near the peak of delayed afterdepolarization following the fourth stimulation.

release from the SR.

The chance of inducing automaticities was related to the increase in resting tension achieved during hypoxic conditioning³. The increase in resting tension has been shown to be due to either a fall in ATP⁸ or a rise in intracellular Ca⁹ but is likely to be due to a low ATP concentration and rigor formation⁸. On the other hand, the intracellular Ca homeostasis¹⁰ has been shown to be closely related to the metabolic energy in heart cells. Furthermore, Ca influx or tissue Ca uptake upon reoxygenation has been shown to be related to ATP depletion¹¹ or an increase in resting tension¹². These findings suggest that there is a connection between the degree of metabolic inhibition during hypoxia and Ca overload on reoxygenation.

The first purpose of this study was to correlate reoxygenation arrhythmias with variable degrees of metabolic inhibition during hypoxia, using the increase in resting tension as an indirect index of ATP depletion. We changed the duration of substrate-free hypoxia, and the glycolytic activity during hypoxia by using glucose or glycolytic inhibitor, 2-deoxyglucose^{8,13,14}

The second purpose was to investigate the role of metabolic inhibition in reoxygenation arrhythmias, and it was shown that the release of inhibited oxidative phosphorylation, and not the reintroduction of oxygen, is the causal mechanism of reoxygenation-induced arrhythmias.

METHODS

Papillary muscles were obtained from the right ventricles of guinea pig hearts. Animals weighing 250–300g were sacrificed by cervical dislocation and the hearts removed and placed in Krebs solution (mM): NaCl 113.1, KCl 4.6, CaCl₂ 2.45, MgCl₂ 1.2, NaH₂PO₄ 3.5, NaHCO₃ 21.9 and glucose 5, equilibrated with 95% O₂ – 5% CO₂ (pH 7.4). Papillary muscles (about 3 mm long, 0.5 mm wide) were excised and mounted in a Perspex bath (1 ml volume) perfused with solution at 37±0.2 °C.

Mural ends of muscles were secured in a clamp and tendinous ends were tied by a silk by a thread to a stainless steel rod extending from the head of a force transducer. Stimulating pulses (2 msec duration, 2 x threshold intensity) were applied to

Fig. 2. Automaticities upon reoxygenation after variable duration of hypoxia with different glycolytic state. The incidence of automaticities was shown after variable duration of hypoxia with different glycolytic state. Circles show muscles with substrate-free hypoxia, triangles show the muscles made hypoxic during glycolytic inhibition (2-DG and acetate), and the squares show muscles made hypoxic in the presence of 5 mM glucose. Muscles that displayed arrhythmias on reoxygenation are scored by filled symbols, and those did not by open symbols. Data were presented on plots of the increase in resting tension at the end of hypoxia versus duration of hypoxia.

muscles through a bipolar Ag—AgCl electrode. Muscle length was adjusted until resting tension was 50–100 mg (sufficient for about 75% maximum twitch tension in control experiments). A 60 min equilibration period in oxygenated Krebs solution preceded all experimental procedures.

Action potentials were recorded with 3 M KCl-filled microelectrode (8–10 M Ω) connected to a high input impedance amplifier (M-707, WPI) via an Ag—AgCl pellet.

Action potentials and tension were displayed on a Tektronix oscilloscope and a Gould pen recorder.

Hypoxic conditions were achieved by adding 95% N₂ — 5% CO₂ (pH 7.4) to the solutions. Stimuli were applied at a basic

cycle length (BCL) of 1000 msec during equilibration and hypoxic periods. During reoxygenation or after the washout of CN, stimulation at BCL 1000 msec was interrupted 30 sec for each minute by trains of 10 stimuli (BCL 200–800 msec) separated by 5-sec rests. The increase in resting tension above control value was measured at the end of hypoxia or at the end of CN perfusion.

Sodium cyanide, sodium acetate, and 2-deoxy-D-glucose (2-DG) were purchased from Sigma. CN and acetate were kept as stock solutions and added in small volumes to Krebs solution. The pH of CN-Krebs solutions was adjusted with HCl, and solutions were freshly prepared every 10 min during CN experiments. 2-DG solution was prepared by the addition of 2-DG powder to glucose-free Krebs solution.

RESULTS

Arrhythmia on reoxygenation after variable duration of substrate-free hypoxia

The muscles were reoxygenated with Krebs solution containing 5 mM glucose after variable (30–120 min) duration of substrate-free hypoxia. There were arrhythmias from 1 to 10–15 min after reoxygenation following 60 min substrate-free hypoxia, and the peak was at 3–7 min. Fig. 1 shows arrhythmias at 6 min after reoxygenation elicited at the BCL of 500 msec (stimuli marked by arrowheads). Delayed afterdepolarizations and aftercontractions were elicited following stimulated action potentials and twitches. The amplitude of these activities increased gradually, and elicited triggered activities near the peak of delayed afterdepolarization after 4 trains of stimuli. These arrhythmias were shown to be triggered activities caused by delayed afterdepolarizations in myocardial cells as reported in the previous paper³

Figure 2 (circles) shows the incidence of arrhythmic activities which is presented on a plot of duration of substrate-free hypoxia versus increase in resting tension at the end of hypoxia. Muscles that exhibited reoxygenation-induced arrhythmias are scored by the filled circles and those without arrhythmias are indicated by the open circles. As we have already reported³ there

Fig.3. Stimulation of glycolysis during hypoxia. After the muscle was submitted to 60 min substrate-free hypoxia (panel A), the perfusate was switched to 50 mM glucose for 15 min in the presence of continued hypoxia. Panel B shows the activities at 10 min after the switch, and stimulation BCL was 1000 msec (left) or 200 msec (right). The action potential duration prolonged, but there were no automaticities nor aftercontractions. The perfusate was then switched to the oxygenated solution which contained 5 mM glucose. Aftercontractions appeared shortly after the switch. Panel C shows the aftercontractions at BCL 1000 msec (left) and automaticity after a short train with BCL 200 msec (right) at 6 min after the switch.

were no arrhythmias when reoxygenation was imposed after 30 min hypoxia ($n=5$) but there was a high incidence after 60 min hypoxia (24 of 30 muscles). The chances of automaticities after 60 min substratefree hypoxia were closely related to the increase in resting tension achieved at the end of hypoxia. All muscles with an increase in resting tension of over 150 mg showed automaticities upon reoxygenation. The incidence of arrhythmias was, however, lower after 90 min (6 out of 10 muscles), or 120 min (5 of 10 muscles) in spite of a similar

increase in resting tension.

Reoxygenation after hypoxia with a different state of glycolysis

Since the increase in resting tension during hypoxia has been suggested to reflect a deficiency of ATP causing rigor, and has been shown to be changed by the metabolic condition during hypoxia, we changed the glycolytic state during hypoxic period.

We have already reported¹⁴ that the perfusion with 2-deoxyglucose (2-DG) in the presence of acetate, as an exogenous substrate for oxidative phosphorylation, inhibited glycolysis, but did not change the action potential duration. The papillary muscles were perfused with 20 mM 2-DG and 5 mM sodium acetate for 90 min, and were made hypoxic for 10–30 min. During hypoxia, the action potential duration shortened to 15% of the control in 5 min, and developed tension became hardly visible within 6 min, and resting tension began to increase quickly after 5–6 min reaching over 150 mg within 10 min.

There were no arrhythmias in 6 muscles reoxygenated after 10–15 min of hypoxia (Fig. 2 : triangles). Reoxygenation after 20–30 min of hypoxia in this condition caused delayed afterdepolarizations and aftercontractions, and elicited arrhythmias in 9 of 10 muscles. Fig. 2 shows that the increase in resting tension after 20–30 min hypoxia in the presence of 2-DG and acetate is also in the same range with those after 10–15 min hypoxia.

Sixty min hypoxia ($n=5$) or 120 min hypoxia ($n=3$) in the presence of 5 mM glucose did not cause a large increase in resting tension, and did not elicit arrhythmias upon reoxygenation (Fig. 2 : squares).

It was shown that the duration of hypoxic perfusion which could induce automaticities upon reoxygenation, changed according to the metabolic state (glycolytic condition) during hypoxia. Another noteworthy finding is that the increase in resting tension is not the only factor to determine the incidence of automaticity. The duration of hypoxia is also a determining factor. The short duration of hypoxia (10–15 min hypoxia during glycolytic inhibition) did not elicit automaticities upon reoxygenation even if there was a sufficiently large increase in

Fig. 4. Arrhythmia, delayed afterdepolarization and aftercontraction after the washout of sodium cyanide (CN) perfusion. (A) One mM CN was added in the substrate-free oxygenated solution for 60 min, and CN was washed out with the oxygenated solution which contained 5 mM glucose. The records were taken at 2 min after the washout. The upper trace is membrane potential, and the lower is tension, and the stimulation BCL (shown by the arrowheads) are 1000, 500, and 200 msec from the top to the bottom panels. There were delayed afterdepolarizations and aftercontractions at each BCL. The automaticity was elicited after a short train of BCL 200 msec. (B) Plots of the increase in resting tension at the end of perfusion versus duration of CN exposure. Circles show the muscles that were perfused with 1 mM CN in oxygenated substrate-free perfusate for 60 min, and triangles show the muscles that were perfused with 1 mM CN in substrate-free hypoxic solution for 30 min. All the muscles showed automaticities after the washout of CN or the washout and the simultaneous reoxygenation.

resting tension.

Stimulated glycolysis during hypoxia

After 60 min substrate-free hypoxia, the muscles were perfused with 50 mM glucose for 15 min in the presence of continued hypoxia (Fig. 3). The action potential duration prolonged and developed tension increased, but there were no automaticities nor aftercontractions. Then, the perfusate was switched to the oxygenated solution containing 5 mM glucose. Automaticities and aftercontractions developed shortly after the switch, and these activities subsided within 12 min after the switch. Automaticities were, however, elicited only following stimuli at 200 msec, suggesting that stimulated glycolysis might have alleviated

the metabolic inhibition during hypoxia. Events of similar time course were obtained in other 2 muscles. There were no abnormal activities when muscles were perfused with 50 mM glucose and continued hypoxia for 30 min following 60 min substrate-free hypoxia in 3 muscles.

Though stimulation of glycolysis with 50 mM glucose during hypoxia was shown to recover the action potential duration and contraction incompletely, reoxygenation with 5 mM glucose was necessary to elicit automaticity and aftercontraction. The reintroduction of oxygen and/or the restoration of oxidative phosphorylation could have caused reoxygenation arrhythmias.

Inhibition of oxidative phosphorylation with

Fig. 5. Critical role the release of the inhibited oxidative phosphorylation. After the muscle was perfused with 1 mM CN in substrate-free hypoxic solution for 30 min (panel A), the perfusate was switched to the oxygenated solution which contained 1 mM CN and 50 mM glucose for 15 min. Panel B shows the activities at 10 min after the switch stimulated at BCL 1000 or 500 msec. The perfusate was then switched to the oxygenated solution containing 5 mM glucose (i.e., the washout of CN). Panel C shows the records at 5 min after the switch at BCL 1000 or 500 msec. There were aftercontractions and the arrhythmias during the stimulation at BCL 500 msec.

cyanide

Cyanide inhibits oxidative phosphorylation by blocking the mitochondrial electron transport. In order to investigate the role of the release of inhibited oxidative phosphorylation as the mechanism of reoxygenation automaticities, muscles were perfused with oxygenated substrate-free solution containing 1 mM sodium cyanide (CN) for 60 min, after which the CN solution was washed out.

Though the time course and the degree of the action potential duration shortening during CN perfusion was similar with changes during substrate-free hypoxia, resting tension began to increase later than substrate-free hypoxia (from 40–50 min after the CN perfusion vs 25–35 min after substrate-free hypoxia). Automaticities were elicited in all 5 muscles after the washout of CN, and Fig. 4 (A) is an example. Delayed afterdepolarizations and aftercontractions developed at 2 min after the washout, and an extrasystole was elicited following a short train of BCL 200 msec. There was automaticity during BCL 1000 msec at 3 min after the washout. In 5 muscles treated with 1 mM CN and hypoxia for 30 min, automaticity occurred in all muscles following the washout of CN and the simultaneous

reoxygenation, suggesting additive effects of these two means of the inhibition of oxidative phosphorylation. The increase in resting tension after 60 min perfusion with 1 mM CN (circles) and that of 30 min CN plus hypoxia (triangles), are shown in Fig. 4, and are similar to or larger than those after 60 min substrate-free hypoxia (Fig. 2). The reintroduction of oxygen per se is unlikely to be the causal mechanism of reoxygenation automaticities, and the release of the inhibited oxidative phosphorylation (i.e., the washout of CN or reoxygenation) could be the underlying mechanism of reoxygenation automaticities.

Figure 5 shows more clearly the critical effect of the CN washout, and not of reoxygenation. Muscles were perfused for 30 min with the hypoxic solution which contained 1 mM CN (Fig. 5A). Then the perfusate was switched to the solution which contained 50 mM glucose and 1 mM CN, and reoxygenated for 15 min (Fig. 5B). The action potential duration and developed tension were incompletely recovered but there were no automaticities nor aftercontractions.

The next switch to the oxygenated solution which contained 5 mM glucose and the washout of CN, caused large aftercontractions and automaticities after the switch

(Fig. 5C). Similar results were obtained in other 2 preparations.

DISCUSSION

In a previous paper³ it was suggested that a rapid Ca influx¹⁵ which occurred upon reoxygenation after severe hypoxic conditioning caused oscillatory Ca release from the SR¹⁶ and resulted in aftercontractions and triggered activity due to delayed afterdepolarization. The degree of Ca influx and the Ca uptake on reoxygenation have been correlated with the degree of ATP depletion¹¹ and the increase in resting tension¹² during hypoxia. To investigate the relationship between reoxygenation-induced automaticities and the degree of metabolic inhibition during hypoxia, we changed the duration of hypoxic period and the state of glycolysis during hypoxia. The degree of metabolic inhibition was estimated indirectly by the increase in resting tension.¹⁷

Reoxygenation automaticity and the metabolic state during hypoxia

Thirty min substrate-free hypoxia caused only a small increase in resting tension and did not elicit automaticity upon reoxygenation. There was a high incidence of automaticity after 60 min hypoxia, when there was a large increase in resting tension. The chance of automaticities declined after 90–120 min of hypoxia, even though the increase in resting tension was similar to those of 60 min hypoxia. These findings coincide with the report on release enzyme upon reoxygenation in isolated heart preparation,¹⁸ though there is no evidence of a correlation between arrhythmias and enzyme release as an index of reoxygenation injury. They have reported that reoxygenation after 30–35 min of hypoxia caused only a small increase in enzyme release, and that there was a large increase in enzyme release between 35–45 min of hypoxia. There was, however, a progressive reduction in the amount of enzyme released after approximately 60 min.

McDonald and MacLeod¹⁹ have reported that exposure of the muscles to medium containing 5 mM glucose and nitrogen for a period of longer than 5 min, resulted in a

decreased ability to develop contractility and to synthesize ATP. The damage to the ATP generating system occurred between 5–15 min after the onset of hypoxia and would seem to imply that mitochondrial function was affected. The more severe, possible irreversible, damage of myocardial cells after prolonged hypoxia could be the reason of the lower incidence of automaticity comparing with the muscles after 60 min substrate-free hypoxia.

It was also shown that 60–120 min hypoxia in the presence of 5 mM glucose as well as 30 min substrate-free hypoxia did not cause a large increase in resting tension and did not elicit automaticities upon reoxygenation. It was suggested that reoxygenation after 30 min hypoxia without exogenous substrate, or after 60–120 min hypoxia with 5 mM glucose did not cause automaticities, because reoxygenation damage after these conditions was not severe enough to elicit massive Ca influx on reoxygenation.

On the other hand, 20–30 min hypoxia in the presence of 2-DG and acetate caused a large increase in resting tension, and elicited automaticities upon reoxygenation. Since CN in the presence of 2-DG has been shown to increase intracellular Ca concentration after 20 min in isolated rat heart cells,²⁰ it is likely that intracellular Ca was increased more⁹ and/or intracellular ATP was depleted more⁸ during hypoxia with glycolytic inhibition. The relationship between reoxygenation automaticities and the increase in resting tension achieved during hypoxia in these different conditions of glycolysis, suggests that the close relationship exists between reoxygenation-induced automaticities and the degree of metabolic inhibition during hypoxia.

There were, no automaticities after 10–15 min hypoxia in the presence of 2-DG and acetate, though there was a similar increase in resting tension. It appears that critical period of time after severe metabolic inhibition is necessary until intracellular Ca homeostasis is disturbed. This assumption is supported by the reoxygenation injury in isolated rat heart cells reported by Stern et al.²¹ They have reported that the isolated cells which were reoxygenated within 10 min in the rigor state all recovered, and that those which spent more than 20 min in the

rigor state invariably rounded up when reoxygenated.

Mechanism of reoxygenation automaticity

The causative mechanism of reoxygenation automaticities was shown not to be the prolongation of the action potential duration²² since the stimulation of glycolysis during hypoxia with 50 mM glucose prolonged the action potential duration without inducing automaticities nor aftercontractions. The reintroduction of oxygen and/or the restoration of oxidative phosphorylation could be the key mechanism of reoxygenation automaticities.

However, the findings that the washout of CN after 60 min CN perfusion in the continued presence of oxygen caused automaticities and aftercontractions, suggests that reoxygenation automaticities are likely to be caused by the release of inhibited oxidative phosphorylation, and that the reintroduction of oxygen per se might not be the causal mechanism. This assumption was further strengthened by the result in Fig. 5, and the reports that the addition of CN before reoxygenation decreased Ca uptake²³ and enzyme release²⁴ upon reoxygenation. The other effects of CN, for example, the inhibition of Ca release from mitochondria²⁵ have been ascribed to these protective effects of CN on reoxygenation injury. Similar effects of CN on the action potential duration and the increase in resting tension comparing with those of substrate-free hypoxia in this study, as well as on contraction and ATP content²⁶ suggest that the inhibition of oxidative phosphorylation is the main effect of CN.

The possible linking mechanism of the release of the inhibited oxidative phosphorylation and the reoxygenation-induced automaticities could be : (1) mitochondrial Ca uptake occurring on reoxygenation²³ or on the washout of CN²⁷ might be the causal mechanism of these two events. Hearse et al.¹⁸ suggested that the primary event in the oxygen paradox is likely to be the oxygenation-induced re-energization of the electron transport chain which could trigger uncontrolled mitochondrial Ca uptake from the cytoplasm; and/or (2) the involvement of free radical system in these conditions, since mitochondria have been shown to

produce oxygen radicals as an energy-dependent interaction²⁸. The damage to cell membrane on reoxygenation might result from the process of lipid peroxidation, which could be caused by the action of oxygen radicals produced in the cell membrane, mitochondria, or white blood cells in vivo²⁹.

In conclusion, though the ultimate mechanism of reoxygenation automaticities remains to be solved, the conditioning during hypoxia which elicited reoxygenation automaticities, was shown to be closely related to the degree of metabolic inhibition. Secondly, the restoration of the inhibited oxidative phosphorylation, and not the reintroduction of oxygen, was thought to be the causative mechanism of reoxygenation automaticities.

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