Induction of Kinetic Cell Death and Its Underlying Mechanisms

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Using a modified Langendorff system, we established an experimental model of kinetic cell death in the rat heart. After calcium (5.5 mM) was loaded for 20 min and 10^{-7} mol isoproterenol was then delivered to the perfusion medium, the hearts developed tonic contracture. Histological examination of the myocardium revealed widespread kinetic cell death. Stimulation of α -adrenoceptors with phenylephrine did not induce kinetic cell death, even after calcium loading.

In our kinetic cell death model in the rat, the outflow of creatine phosphokinase into the perfusion medium was increased after isoproterenol application, with a peak occurring at 45 min. The peak transient increase in the intracellular calcium ion concentration (the Ca-transient) was measured using Fura2/AM, and was found to be augmented by increasing the calcium concentration of the perfusion medium.

These results suggest that in the presence of an increased intracellular calcium concentration, even slight stimulation of β -adrenoceptors can easily induce myocardial injury.

CALCIUM plays an important role in the excitation of muscles and in excitation-contraction coupling. On the other hand, an excessive influx of calcium into myocytes is thought to cause myocardial damage, which has been demonstrated in such phenomena as the calcium paradox¹ and the ischemia-reperfusion injury².

We have demonstrated in mongrel dogs that after calcium loading epinephrine instantly destroys myocytes at a dose that is harmless without calcium loading, and we have named this phenomenon "kinetic cell death"³.

Epinephrine has two actions, the α -stimulation and β -stimulation of adrenoceptors. To clarify the mechanism of kinetic cell death, we investigated the relationships between types of cell death and α - and β -stimulation, creatine phosphoki-

nase, and changes in the peak transient increase in the intracellular calcium ion concentration after calcium loading in rats.

MATERIALS AND METHODS

Wistar rats aged 17 weeks were used. The rats were killed and their hearts were excised rapidly.

Catheters were inserted in the cut end of the ascending aorta of each heart and retrograde perfusion was performed using a modified Langendorff perfusion system. The perfusion medium (the Krebs-Henseleit buffer) was aerated continuously with a mixture of 95% O₂ and 5% CO₂. The composition of the medium (in millimoles) was as follows: NaCl, 118.1; KCl, 4.7; MgSO₄, 1.3; KH₂PO₄, 1.2; NaHCO₃, 25.0; and glucose 16.7. A concentration of CaCl₂ of either 2.0 or 5.5 mM was added to this medium. The heart rate was set at 330 beats/min. The

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Fig.1. Induction of kinetic cell death in the rat hearts. Calcium loading and application of the triggers. (Group 3)

temperature was maintained at 37°C. Isoproterenol and phenylephrine were used as triggers to stimulate α - and β -adrenoceptors, respectively. Experiments were performed in two stages, as described below. Stage one: Induction of kinetic cell death

The heart preparations were divided into three groups depending on the method of treatment:

- Group 1: No calcium loading (control group) (n=5);
- Group 2: Application of the triggers without calcium loading (n=24);
- Group 3: Calcium loading and application of the triggers (n=28).

In the case of no calcium loading, 2.0 mM calcium was added to the perfusion medium, while during calcium loading 5.5 mM calcium was added. For Groups 2 and 3, phenylephrine or isoproterenol (both at 10^{-9} , 10^{-8} , or 10^{-7} mol) was delivered rapidly by means of a one-shot pressure injection in the medium. The same procedural sequence was repeated in 4~8 rats. Ten min after application of the trigger, the preparations were removed from the apparatus for examination (Fig. 1).

The preparations were fixed in 10% neutral buffered formalin, and sections about 3 mm in thickness were cut from the central part of each ventricle. These sections were dehydrated in alcohol, following which fatty tissue was removed, and the sections defatted, embedded in paraffin. They were then cut into 4- μ m thin sections and stained with Heidenhain-iron-hematoxylin. For electron microscopy, the myocardial tissue from each preparation was fixed in 6.5% glutaraldehyde and double-fixed in 1% osmium. The tissue was then dehydrated in alcohol, embedded in Quetol 653, sectioned using an ultramicrotome, and observed under an electron

microscope (HV-12A).

Stage two:

1) Measurement of the left ventricular filling pressure

The left ventricular filling pressure was measured continuously in 4 preparations while kinetic cell death was induced. Distilled water was injected into a balloon-tipped catheter, which was inserted into the left ventricle via the left auricle, and the left ventricular filling pressure was then continuously recorded using a pressure manometer.

2) Measurement of creatine phosphokinase (CPK)

The CPK level in the perfusion medium was measured several times over a 120-min period after trigger application (10^{-7} mol isoproterenol) in 4 preparations from each of Groups 2 and 3. The CPK concentration in the medium was measured every 2 min for the first 16 min after isoproterenol application and then every 15 min. Group 3, the calcium concentration in the medium was changed from 5.5 to 2.0 mM 5 min after isoproterenol application.

3) Measurement of the Ca-transient

The peak transient increase in the intracellular calcium ion concentration (the Ca-transient), resulting from changing the calcium concentration in the perfusion medium from 2.0 to 5.5 mM, was measured using the calcium-sensitive fluorescent dye, Fura2/AM. The preparations were treated with the medium containing 5 μ M Fura2/AM and 0.2% Cremophor. The anterior wall of each preparation was then exposed to the exciting light at two wavelengths (340 and 380 nm). The fluorescence emitted at 500 nm was measured using a spectrofluorometer (CAF100) (Fig. 2).

RESULTS

1. Pathological changes

No morphological changes were noted in the myocardial cells in Groups 1 or 2. In contrast, all 8 preparations in Group 3 developed tonic contraction within 5 min after the application of 10^{-7} mol isoproterenol, and then stopped beating (Fig 3). The left ventricles in Group 3 showed the histological changes which typically result from such hypercontraction along their entire cir-

Fig. 2. Method of measuring calcium transients.

Trigger		Ca-load (-) n=24		Ca-load (+) n=28	
		No. of KD/n	Incidence (%)	No. of KD/n	Incidence (%)
α -Stimulation (Phenylephrine)	1×10^{-9} mol	0/4	0	0/4	0
	1×10^{-6} mol	0/4	0	0/4	0
	1×10^{-7} mol	0/4	0	0/4	0
β -Stimulation (Isoproterenol)	1×10^{-9} mol	0/4	0	0/4	0
	1×10^{-6} mol	0/4	0	1/4	25
	1×10^{-7} mol	0/4	0	8/8	100

Fig. 3. Difference in effects of α and β stimulation.

cumferences, while electron micrographs also showed evidence of hypercontraction of the myofibrils. The glycogen granule count was normal, and the mitochondria maintained their structural integrity (Figs. 4a and 4b).

2. Changes in left ventricular filling pressure at the same time as kinetic cell death

Isoproterenol applied after calcium loading increased first the systolic pressure and then the diastolic pressure, and produced tonic contraction of the myocardium in all cases (Fig. 5).

3. Changes in CPK

Isoproterenol applied without a preceding calcium load did not increase the outflow of CPK. However, isoproterenol applied after calcium loading increased the CPK outflow after application, with a peak occurring at 45

min (Fig. 6).

4. Correlation between the Ca-transient and the calcium concentration in the perfusion medium

Following Fura2/AM loading, the fluorescence signal at 340 nm increased during systole and decreased during diastole. In contrast, at a wavelength of 380 nm, the signal decreased during systole and increased during diastole. Thus, opposing changes in the fluorescence intensity were obtained at these two wavelengths.

As previously shown in Fig. 5, the left ventricular filling pressure rose after a change in the calcium concentration of the perfusion medium from 2.0 mM to 5.0 mM. After an increase in the calcium concentration of the medium, the Ca-transient was also augmented (Fig. 7).

DISCUSSION

On the basis of our previous histopathological studies regarding sudden death and acute myocardial infarction, we have reported that the death of myocytes, which plays a key role in myocardial infarction, can be interpreted as kinetic cell death³. We have also reported that after calcium loading in dogs, low doses of caffeine and epinephrine caused the tonic hypercontraction of myocytes³. In the present experiment, we also found that kinetic cell death can be

Fig.4-a. After 10^{-7} mol isoproterenol injection under 5.5 mM calcium perfusion. (Heidenhain-iron-Hematoxylin stain; cross section $\times 10$, contraction band lesions $\times 400$)

Fig.4-b. Electron microscopical findings. ($\times 5000$)

induced by β -stimulation after calcium loading.

Rona⁴ demonstrated experimentally that a high dose of isoproterenol could cause an infarct-like form of myocardial necrosis. In the present experiment, we showed that a small dose of isoproterenol, which itself

could not cause any damage to the myocardium, could produce myocyte injury after calcium loading.

Several theories have been presented to explain how a high dose of isoproterenol induces myocardial necrosis. They include relative ischemia⁴; changes in the coronary

Fig. 5. Changes of left ventricular pressure.

Fig. 6. Changes of CPK release after isoproterenol injection.

microcirculation⁵; an alteration of membrane permeability⁶; and the cellular influx of calcium ions⁷.

Fleckenstein et al found that a high subcutaneous dose (30 mg/kg) of isoproterenol greatly increased the uptake of Ca^{45} into the myocardium, and suggested that this large influx of calcium into myocardial cells could lead to myocardial necrosis⁷.

We have previously reported that the intra-coronary arterial injection of calcium causes myocardial necrosis which is typically accompanied by hypercontraction⁸. This finding also supports the possibility that calcium can cause myocardial injury.

Isoproterenol at low doses (in the microgram per kilogram range) is reported both to enhance myocardial contractility and to po-

Fig. 7. Calcium Transients and left ventricular pressure.

tentiate myocardial relaxation. In the present study, it seems that in the calcium-loaded heart preparation this relaxant effect may have been suppressed, and that therefore the contractile effect became dominant.

The CPK level in the perfusion medium did not change immediately after isoproterenol application, but gradually rose after the application with a peak occurring at 45 min. Thus, in contrast to the Ca-paradox phenomenon⁹, in which the CPK outflow increases immediately after calcium loading, the increase in CPK outflow took place gradually in our present experiment.

In this study, we investigated the intracellular calcium kinetics of the whole heart using Fura2/AM. Fluorescent calcium indicators like Fura-2 allow the intracellular free calcium concentration to be monitored continuously. In the present experiment, we simultaneously measured the left ventricular pressure and the intracellular calcium concentration in a Langendorff rat preparation.

We found that an increase in the calcium concentration of the perfusion medium augmented the Ca-transient, and that it was further enhanced by the addition of isoproterenol to the perfusion medium.¹⁰ The X-ray spectroscopic analysis clearly revealed that the mitochondrial calcium content was increased 1 min after the preparation was loaded with 5.5 mM calcium.¹¹

The above results suggest that in the presence of an increased intracellular calcium concentration, even a small amount of isoproterenol can induce myocardial injury.

REFERENCES

1. ZIMMERMANS ANE, HULSMANN WC: Paradoxical influence of calcium ions on the permeability of the cell membranes of the isolated rat heart. *Nature* 1996; **211**: Aug. 6, 646
2. SHEN AC, JENNINGS RB: Myocardial calcium and magnesium in acute ischemic injury. *Am J Pathol* 1972; **67**: 417
3. KANEKO N, TAKEISHI M: New theory on the Pathogenesis of acute myocardial infarction. *Jpn Circ J* 1986; **50**: 198
4. RONA G, CHAPPEL CI, BALAZS T, GAUDRY R: An infarct-like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat. *Arch Pathol* 1959; **67**: 443
5. SOMANI P, LADDU AR, HARDMAN HF: Nutritional circulation in the heart. III. Effects of isoproterenol and beta adrenergic blockage on myocardial hemodynamics and rubidium-86 extraction in the isolated supported heart preparation. *J Pharmacol Exp Ther* 1970; **175**: 577
6. RONA G, BOUTET M, HUTTNER I: Membrane permeability alterations as manifestation of early cardiac muscle cell injury. In *Recent Advances in Studies on Cardiac Structure and Metabolism. Pathophysiology and Morphology of Myocardial Cell Alterations*, edited by A. Fleckenstein, G. Rona, vol. 6, p. 439. Baltimore, University Park Press, 1975
7. FLECKENSTEIN A, JANKE J, DORING HJ, PACHINGER O: Ca overload as the determinant factor in the production of catecholamine-induced myocardial lesions. In *Recent advances in studies on cardiac structure and metabolism, Cardiomyopathies*, edited by E. Bajusz et. al. vol. 2, p. 455, Baltimore, University Park Press, 1973
8. KANEKO N, HOSODA S, TERAOKA K, KAWAKAMI M: Myocardial cell death caused by calcium overload. XIth European Congress: p. 213, 1989
9. HEARSE DJ, BAKER JE, HUMPHREY SM: Verapamil and the calcium paradox. *J Mol Cell Cardiol* 1980; **12**: 733
10. KANEKO N, UCHIDA T, HORIKAWA Y, HOSODA S, TERAOKA K, TAKEISHI M, MATSUDA R: Ca accumulation in the mitochondria of the rat hearts. *Jpn Circ J* 1990; 54 annual scientific meeting, 287
11. KANEKO N, TERAOKA K, HORIKAWA Y, UCHIDA T, MATSUDA R, HOSODA S, NAKAMURA S: Pathophysiological function of the mitochondria as a calcium reservoir: Quantitative X-ray microanalysis: Microbeam analysis 1990; 408