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Calcium-Induced Cell Death: Susceptibility of Cardiac Myocytes Is Age-Dependent

Abstract. *Rat hearts perfused with calcium-free media exhibit an age-dependent response when a normal calcium concentration is restored to the perfusate. The response of 15- and 26-day hearts is an explosive cell death which includes immediate cessation of contractile activity, as well as release of large amounts of creatine phosphokinase activity and myoglobin. Between birth and 15 days of age, the sensitivity of the heart to this calcium-induced cellular disruption changes from a relative insensitivity before 6 days of age to a slight but constant sensitivity from 7 to 11 days of age, and to full sensitivity at 15 days of age. This pattern of sensitivity may be due to the rapid increase in complexity and quantity of the membrane systems of cardiac myocytes during the first 15 days of growth.*

Calcium ions are important in the maintenance of normal cell structure and function. They are thought to be integral constituents of the cell membrane, both within the lipid bilayer and in the adjacent basement membrane (*1*). The cardiac myocyte is dependent on the presence of calcium ions in the extracellular fluid for excitation-contraction coupling (*2–4*) and for the structural integrity of its surface membrane (*5–7*). A sufficient concentration of calcium ions (approximately 50 μM) must be present in the extracellular fluid to preserve the structural integrity of the adult cardiac sarcolemma and its adjacent basement membrane (*7–10*). Perfusion of adult hearts with calcium-deficient solutions results in alterations in the capacity of individual myocytes to control the flux of calcium ions across their surface membrane (*7, 9, 11*). In a typical experiment, perfusion of the heart with calcium-free solution, followed by reperfusion with solutions containing normal plasma levels of calcium, leads to rapid cessation of contractile activity, to contracture of the heart, and to massive release of intracellular components such as creatine phosphokinase (CPK) and myoglobin (*9, 10, 12–15*).

This explosive cell death, termed the "calcium paradox" by Zimmerman and colleagues (*12, 13*), has been studied extensively in perfused preparations of adult hearts. The sensitivity of young hearts to calcium-deficient solutions has not been studied by perfusion techniques. However, myocytes prepared from neonatal hearts according to standard procedures (with calcium-free media and selective proteolytic enzymes) do survive when subsequently cultured in calcium-containing medium (*16*). Since myocytes prepared from adult hearts do not survive long when cultured in calcium-containing media (*17*) we speculated that the neonatal heart has a different sensitivity to a transient exposure to calcium-free medium than does the adult heart. Therefore, we applied the nonrecirculating Langendorff perfusion technique to hearts from 3- to 26-day-old rats and examined the development of sensitivity to calcium-induced cell death. We report here that the sensitivity of the rat heart to calcium-induced cell death is absent before 7 days of age and is fully developed by 15 days. From 7 to 11 days the heart exhibits a constant but minor sensitivity to changes in extracellular calcium concentrations.

As shown in Fig. 1, the 26-day hearts showed rapid tissue disruption and contracture when calcium was reintroduced into the perfusing medium after 20 minutes of calcium-free perfusion. The amounts of CPK activity and myoglobin released into the perfusing medium during the calcium repletion period represented 36 percent of the total CPK activity and 35 percent of the total myoglobin released by the 26-day hearts during the 70-minute collection period used in our study (Table 1).

In contrast to the adult heart (*13, 15*) and the 26-day heart, the 3- to 6-day hearts resumed and maintained weak rhythmic beating during the entire 20-minute calcium repletion period. The 3- to 6-day hearts did not undergo contracture or release CPK activity and myoglobin during the calcium repletion period (Fig. 1 and Table 1). The total amounts (per milligram of heart, dry weight) of CPK activity and myoglobin released by the 3- to 6-day hearts were not significantly different from the total amounts released by hearts of rats in the other age groups studied (Table 1). If hearts from all of the age groups were affected similarly by the calcium depletion-repletion procedure, the amount of CPK activity released (per milligram of heart, dry weight) during calcium repletion would be the same for each heart studied. Thus the data in Table 1 indicate that, unlike the 26-day hearts, the 3- to 6-day hearts are not sensitive to a transient depletion of extracellular calcium.

Of the 7- to 11-day hearts, 65 percent resumed very weak beating during the calcium repletion period. The beating differed for each heart studied; that is, the beating was unstable with respect to rate, rhythm, and strength and often did not continue for the entire calcium repletion period. Although these hearts did not undergo contracture, they did release small amounts of CPK activity during calcium repletion (Table 1 and Fig. 1). The release of CPK activity (Table 1) by the 7-, 8- to 10-, and 10- to 11-day hearts suggests that a slight sensitivity to the depletion of extracellular calcium first appears in the rat heart at 7 days of age, and that this sensitivity remains unchanged from 7 to 11 days of age. The sensitivity was characterized as slight between 7 and 11 days, since less than 5 percent of the total CPK activity and no detectable myoglobin were released by these hearts during the calcium repletion period (Table 1).

The 15-day hearts developed severe contracture and released large amounts of CPK activity and myoglobin at the start of the calcium repletion period (Fig.

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1). Of the total CPK activity and total myoglobin released by the 15-day hearts during the perfusion sequence, 32 and 24 percent, respectively, were released during calcium repletion (Table 1). The amounts of CPK activity and myoglobin released by the 15-day hearts during the calcium repletion period were not significantly different from the amounts released by the 26-day hearts (Table 1), indicating that the 15- and 26-day hearts are affected similarly by the calcium depletion-repletion protocol. The type of damage (contracture, time course, and severity of the release of intracellular components) sustained by the 15- and 26-day hearts during the calcium repletion period was comparable to the damage previously reported for adult hearts subjected to similar experimental calcium depletion and repletion procedures.

Table 1. The effect of calcium depletion and repletion on CPK and myoglobin release by neonatal rat heart. The perfusion procedure was as outlined in Fig. 1. After the final 10-minute washout period with standard perfusion fluid (SPF), the ventricles were dissected free, dried at 110°C for 16 hours, and weighed. CPK units are nanomoles of creatine formed per minute at 37°C. The total heart content of releasable CPK activity and myoglobin was assumed to be the total released during the 70-minute collection period. The averages of the total amounts of CPK activity and myoglobin released for all of the hearts studied were 2834 ± 55 CPK units per milligram of heart weight and 32 ± 0.6 μg of myoglobin per milligram of heart weight. During the growth period from 3 to 26 days, the dry weight of the heart was related linearly to the total amounts of CPK activity (r = .96) and myoglobin (r = .98) released by each heart. The perfusion periods were (1) calcium depletion, 20 minutes; (2) calcium repletion, 20 minutes; (3) Triton X-100, 10 to 15 minutes; (4) total released during the 70-minute collection period; and (5) calcium controls, 20 minutes. The number of hearts in each group is shown by N. Values are means ± standard error.

Age (days)	N	Body weight (g)	Heart weight (dry, mg)	CPK units released per milligram of heart weight					Myoglobin (μg) released per milligram of heart weight		
				$-Ca^{2+}$ (1)	$+Ca^{2+}$ (5)	$+Ca^{2+}$ (2 or 5)	TX-100 (3)	Total (4)	$+Ca^{2+}$ (2 or 5)	TX-100 (3)	Total (4)
3 to 6	16	10.51 ± 0.34	3.1 ± 0.1	13 ± 6.3		0	2617 ± 85	2629 ± 83	0	31.6 ± 0.7	31.6 ± 0.7
7	4	14.80 ± 0.38	4.8 ± 0.3	45 ± 20		47 ± 23	2793 ± 168	2886 ± 192	0	29.6 ± 0.5	29.6 ± 0.5
8 to 10	7	21.5 ± 0.5	5.6 ± 0.1	38 ± 10		89 ± 21	2758 ± 132	2884 ± 134	0	31.5 ± 2.4	31.5 ± 2.4
10 to 11	5	26.3 ± 0.5	6.6 ± 0.2	8 ± 5.4		32 ± 10	3018 ± 179	3057 ± 179	0	34.3 ± 2.4	34.3 ± 2.4
15	3	42.3 ± 1.5	12.7 ± 0.5	8 ± 4.6		923 ± 94	1905 ± 64	2835 ± 117	9.1 ± .7	29.4 ± 2.7	38.5 ± 3.4
26	3	80.3 ± 2.3	25.5 ± 0.2	2 ± 1.7		1041 ± 264	1812 ± 178	2855 ± 134	12.6 ± 2.8	22.5 ± 1.2	35.1 ± 2.8
				Control experiments							
3	3	9.6 ± 0.5	3.1 ± .2		0	0	2731 ± 80	2731 ± 80	0	34.1 ± 1.5	34.1 ± 1.5
11 to 12	4	28.6 ± 0.6	7.6 ± .3		0	0	3293 ± 194	3293 ± 194	0	33.5 ± 1.9	33.5 ± 1.9

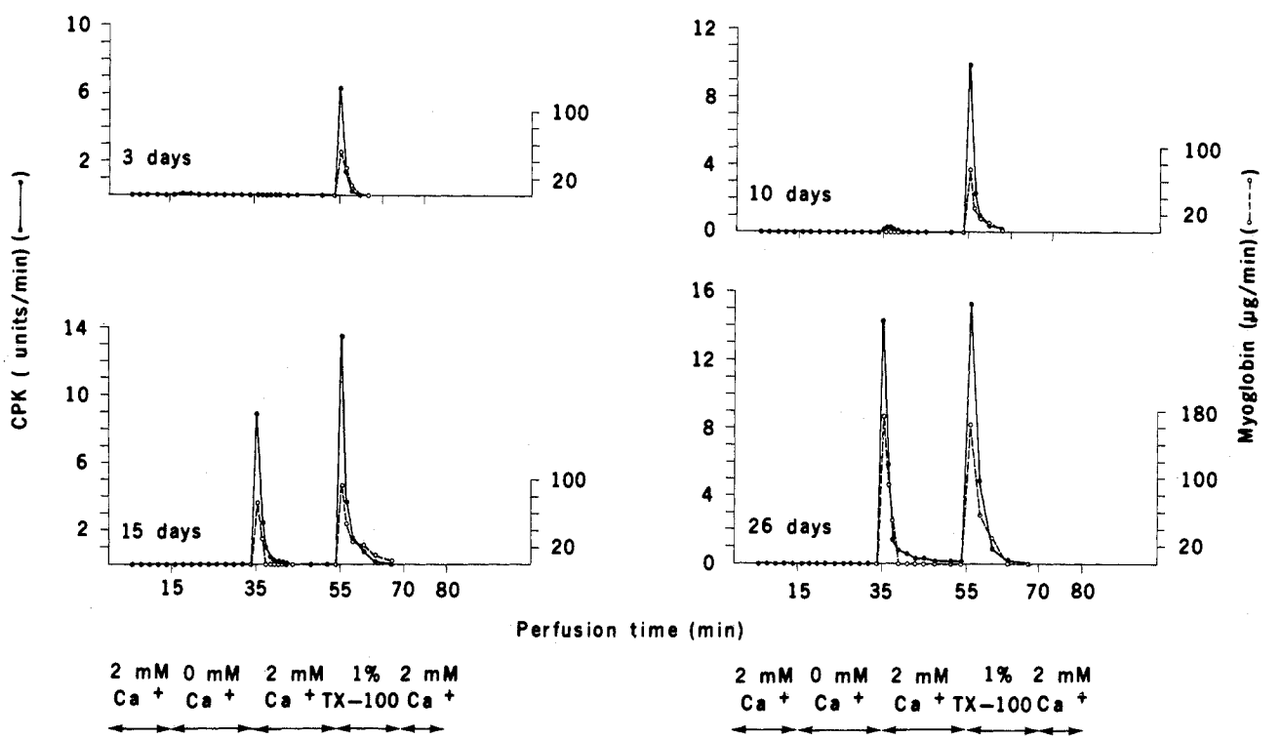

Fig. 1. Typical release of CPK activity and myoglobin from hearts of 3-, 10-, 15-, and 26-day-old rats perfused according to the following procedure: 30 minutes with standard perfusion fluid (SPF) (two sequential 15-minute periods), 20 minutes with SPF minus calcium (calcium depletion period) (*27*), 20 minutes with SPF (calcium repletion period), 10 to 15 minutes with SPF plus 1 percent Triton X-100 (Triton X-100 period), and 10 minutes with SPF. Control hearts were treated according to the same procedure, except that SPF was substituted for SPF minus calcium. The SPF was Krebs-Henseleit bicarbonate buffer pH 7.4, containing 2.0 mM calcium and 11 mM glucose. The perfusion temperature was 36° to 37°C, and the perfusion pressure ranged from 60 to 100 cm of H_2O, depending on the age of the animal (*28*). Hearts that did not sustain a beating rate ≥ 180 beats per minute during the first 15-minute equilibration period were discarded. Samples for measurement of CPK activity and myoglobin were collected continuously from the start of the second 15-minute equilibration period (time zero in the above figures) to the end of the Triton X-100 period. The CPK activity was determined by a modification of Sigma colorimetric procedure No. 520, which measures the amount of creatine formed in 30 minutes at 37°C. The CPK units are micromoles of creatine formed per minute at 37°C. Myoglobin was measured by the benzidine method (*29*).

The CPK activity data indicate that 3-to 6-day hearts are not sensitive to changes in extracellular calcium. However, the observation that, in this age range, the strength and the number of contractions during the calcium repletion period did not return to the normal type observed during the 30-minute equilibration period leads us to suspect that there was some damage to the mechanisms that control ion fluxes across the sarcolemma or to the contractile apparatus. The 3- to 6-day myocardium is thus probably not immune to injury caused by changes in the extracellular calcium concentration, but is resistant to the more destructive effects which occur in adult hearts.

In the adult heart, these destructive effects may depend on the structural and functional complexity of the surface membrane system and of the sarcoplasmic reticulum. During perfusion of adult hearts with calcium-depleted medium, minor structural changes occur in the sarcolemma, the basement membrane (glycocalyx), the intercalated disks, and the transverse tubules (*5–7, 10, 11, 13–15, 18*). The most frequently noted alteration is the formation of vacuoles between the basement membrane and the sarcolemma and in the intercalated disk area. The two opposing sides of the intercalated disk also become slightly separated, and the T tubules become somewhat dilated. These changes have been correlated with the loss of the capacity of the sarcolemma to control calcium movement into and out of the myocyte (*6, 7, 9, 11*). Thus, when calcium is reintroduced into the perfusing medium, the massive influx of calcium into the cytosol results directly, as well as indirectly by stimulating release of calcium from the sarcoplasmic reticulum (*3*), in cytoplasmic calcium overload. Calcium reperfusion also decreases the calcium-binding and uptake capacities of the sarcoplasmic reticulum (*19*). The resulting calcium overload causes rapid and uncontrolled contraction of the myofibrils, with subsequent rupture of the intercalated disks and surface membranes (*10, 11, 13, 14, 18*) and thus release of many intracellular components into the perfusing medium. Mitochondria react to the calcium overload by increased uptake and binding of calcium (*19*), which results in internal calcium deposits and disruption of mitochondrial function.

We speculate that the lower sensitivity of the 3- to 6-day myocardium to changes in extracellular calcium concentration is related to the incomplete development of parts of the surface membrane system (transverse tubules, glycocalyx, and intercalated disks), of the sarcoplasmic reticulum, and of the myofibrils in the neonatal heart. Before 7 days of age, the cardiac myocyte has an underdeveloped sarcoplasmic reticulum and surface membrane system; that is, the T tubules are rudimentary, the intercalated disks are just forming and are not interdigitated, and the sarcoplasmic reticulum is sparse and less active in uptake and release of calcium than is the adult sarcoplasmic reticulum (*3, 20–24*). During the 7- to 14-day period the surface membrane system rapidly increases in complexity and surface area, especially the T tubules and intercalated disks. The sarcoplasmic reticulum develops as a honeycomb network around the myofibrils at this time (*20–24*). By 3 weeks of age, the cardiac myocyte has acquired transverse tubules with a glycocalyx covering, intercalated disks, and a sarcoplasmic reticulum, all of which are structurally and functionally similar to those of the adult cardiac myocyte (*3, 14, 21, 24*). The initial 21-day period is also characterized by an increase in size, organization, and calcium sensitivity of the myofibrils of the cardiac myocytes.

During this 3-week period of rapid membrane development, the neonatal myocytes appear to change from having a neonatal type of control over the level of calcium-ion in the cytoplasm to having an adult type of control (*4, 20, 25*). Langer *et al.* (*4*) reported that cultured myocytes prepared from 2-day rat hearts resume strong rhythmic beating in 1 m*M* calcium medium after being subjected to 10 minutes in calcium-free medium. Although the accumulation of intracellular La^{3+} ion demonstrated that these myocytes had lost some control over calcium fluxes, sufficient control remained to regulate the contraction-relaxation cycle and prevent the destruction that occurs in adult myocytes subjected to similar calcium depletion experiments.

Neonatal cardiac myocytes, by being smaller and rounder in transverse section, are less tightly packed than adult cardiac myocytes (*21, 23, 26*). This difference in cell shape and packing, plus the lack of fully developed membrane systems within and between the neonatal myocytes, confers a structural flexibility upon the neonatal heart that the adult heart does not have. Thus, between 3 and 15 days of age, the pattern of sensitivity to calcium-induced cellular disruption in the rat cardiac myocyte suggests that neonatal and adult cardiac myocytes control calcium movements by different mechanisms. Further, the parallel between this pattern of sensitivity and the time course of development and maturation of the membrane systems which control calcium movements in the cardiac myocyte suggests that the neonatal mechanism for calcium control may not depend on membrane components that the adult mechanism depends on.

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27. Five minutes of calcium-free perfusion is sufficient to cause calcium-induced cell death in the adult heart. We used a 20-minute period of calcium-free perfusion because the ultrastructural damage that occurs in the calcium-depleted heart becomes more pronounced at 20 minutes than at 5 minutes. Thus, by maximizing the damage to each myocyte, we should be able more accurately to determine the ages at which the neonatal heart becomes slightly sensitive

and fully sensitive to calcium-induced cell death. The age at which the heart becomes fully sensitive to calcium-induced cell death may be extended beyond 15 days by decreasing the time of calcium-free perfusion, but we think that maximum sensitivity would still be expressed at 3 to 4 weeks of age. At this age, the structural and functional complexity of the surface membrane has matured, plus the calcium binding and release properties of the sarcoplasmic reticulum and the calcium sensitivity of the myofilaments have both reached their adult levels (*3*).

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Atherosclerosis: Prevention by Agents Not Affecting Abnormal Levels of Blood Lipids

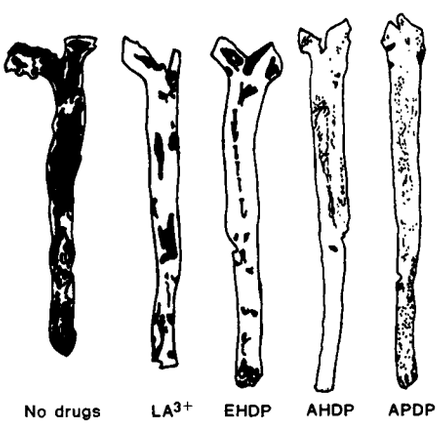
Abstract. *Diet-induced atherosclerosis in macaque monkeys was suppressed by anticalcifying agents without changing abnormal levels of blood cholesterol and lipoprotein. The agents included inhibitors of arterial calcium deposition (diphosphonates) and a calcium ion antagonist (lanthanum). The study suggests that regulation of calcium flux and extracellular deposition in arteries may offer new principles of treatment for cardiovascular disease.*

Cardiovascular disease is still the leading cause of death in industrial nations (*1*). The principal underlying disorder is atherosclerosis (*1*), especially the fibrotic atheromatous plaque (*2*). Studies involving the use of low-fat diets (*1*) or antilipemic drugs (*3*) have not provided convincing evidence that lowering of blood cholesterol concentrations prevents heart disease or protects against atherosclerosis (*4*). It therefore appears desirable to search for alternative methods of treatment.

Recent studies on rabbits (*5*) indicate that agents which inhibit excessive deposition of calcium into arterial walls also inhibit diet-induced atherosclerosis despite high levels of serum cholesterol. The agents include the anticalcifying agents ethane-1-hydroxy-1,1-diphosphonate (EHDP), azacycloheptane-2,2-diphosphonate (AHDP), amino-1-hydroxypropane-1,1-diphosphonate (APDP), and the specific calcium ion antagonist lanthanum (La^{3+}). In the present study these substances were tested for their ability to suppress atherosclerotic plaque formation in monkeys.

Adult male *Macaca fascicularis* monkeys were randomly assigned to one of four groups (eight monkeys per group). One group was placed on a normal diet and three groups on an atherogenic diet containing (by weight) 10 percent butter and 0.1 percent cholesterol. One of the latter three groups received the high-fat diet alone; the other two also received either $LaCl_3$ or EHDP. Two additional groups of three monkeys each received the same atherogenic diet with added AHDP or APDP. Daily dosages were as follows: EHDP and $LaCl_3$, 120 mg/kg for 6 months and 40 mg/kg thereafter; AHDP and APDP, 40 mg/kg. Blood samples were taken bimonthly and analyzed for total cholesterol in plasma, high-density lipoprotein, low-density plus very low density lipoprotein, and total and ionized calcium in serum (*5*). The various cholesterol lipoprotein fractions were separated by magnesium phosphotungstate precipitation (*6*). After 24 months the monkeys were killed by an overdose of pentobarbital. At autopsy, the aortas were overlaid with clear plastic foil, the aortic contours and lesions were outlined in ink, and the percentage of intima affected by atherosclerosis was determined by point counting (*7*). Cross sections were then removed from the left and right proximal coronary arteries and the opened aortas for histological examination. Whole aortic intima and media were analyzed biochemically for the content of collagen, elastin, cholesterol, and calcium by methods previously described (*5*). DNA content was measured by the method of Burton (*8*).

In monkeys on the atherogenic diet the concentration of total cholesterol in plasma rose from the control level (132 ± 43 mg/dl, mean ± standard deviation) to atherogenic levels regardless of whether or not anticalcifying drugs were given: for untreated monkeys 468 ± 134 mg/dl; for monkeys treated with La^{3+}, 425 ± 123 mg/dl; with EHDP, 434 ± 125 mg/dl; with AHDP, 448 ± 135 mg/dl; and with APDP, 511 ± 208 mg/dl. These increases were due to increases in low-density plus very low density lipoprotein. The mean concentration of high-density lipoprotein in each of the experimental groups was not significantly different from the control value (52 ± 15 mg/dl). Likewise, concentrations of total and ionized calcium in serum were about



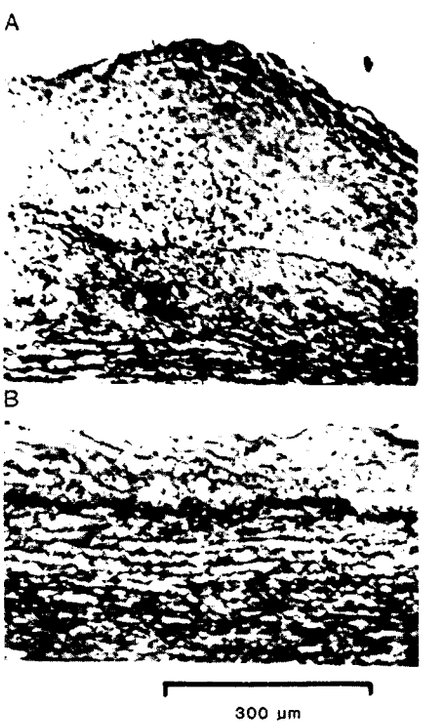


Fig. 1 (left). Tracings of aortas from monkeys that received the atherogenic diet with or without one of several anticalcifying agents. Black areas represent intimal atherosclerotic lesions. Fig. 2 (right). Micrographs of atherosclerotic lesions. (A) Plaque from aorta of untreated monkey on atherogenic diet. The intima is markedly raised (top half of micrograph) by proliferated lipid-laden foam cells (large clear cells) surrounded by a capsule of collagen accumulations (gray area). There is destruction of intimomedial elastica (black bands), and collagen and elastica changes reach into the inner aortic media (bottom half). (B) Aortic lesion from La^{3+}-treated monkey on atherogenic diet. The intima is only slightly raised by a few layers of foam cells; there is no collagen accumulation or elastica derangement in the intima or media. Aortic lesions from monkeys treated with the other anticalcifying agents showed similar morphology. Some were even more superficial.

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