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Fetal mouse hearts: A model for studying ischemia

(ATP content/lysosomal enzymes/cardiac ultrastructure/hypoxia)

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ABSTRACT **A new experimental model for the study of two important aspects of ischemia, namely, oxygen and substrate deprivation, is proposed: the intact, beating fetal mouse heart in organ culture. This model offers long-term stability, ease and reproducibility of preparation, and the ability to manipulate experimental conditions. Hearts deprived of oxygen and glucose ceased beating immediately. After 3–4 hr of deprivation, biochemical and ultrastructural changes consistent with ischemic injury were evident. These include depletion of ATP and glycogen levels, loss of cytoplasmic enzymes, and extensive swelling and disruption of mitochondrial structure. Glucose and insulin partially protected against ATP depletion. Upon resupply of oxygen and glucose, beating resumed immediately, ATP levels rapidly increased to control levels and, consistent with this, mitochondrial structure returned toward normal. During the recovery phase autophagic vacuoles containing damaged mitochondria and myofibrils were seen, indicating that repair mechanisms were activated. Consistent with this, the proportion of lysosomal enzymes that were present in the nonsedimentable fraction of the tissue homogenate increased. We conclude that the cultured fetal mouse heart is a model useful for studying myocardial responses to anoxia and/or substrate deprivation and for assessing interventions designed to limit damage or to stimulate repair after ischemic injury.**

Definition of the biochemical and ultrastructural responses of the myocardium to hypoxia and ischemia has obvious clinical importance. If the sequence of biochemical events leading to necrosis were defined, it is possible that rational interventions could be designed to salvage damaged cells and to stimulate repair. Traditional approaches for studying cardiac hypoxia and ischemia have utilized either intact animals, with the attendant disadvantage of rather poorly controlled and poorly defined conditions, or *in vitro* models such as Langendorff-perfused hearts or papillary muscle strips, which can be controlled more precisely but which are stable for only brief periods. In the present report we describe an alternative experimental model, the fetal mouse heart in organ culture, which combines the advantages of long-term stability, ease and reproducibility of preparation, and the ability to manipulate experimental conditions. Intact beating hearts from fetal mice can be maintained in organ culture for weeks, and they respond to cardioactive drugs and hormones in a manner similar to adult myocardium (1, 2). We have reported that depriving cultured fetal mouse hearts of oxygen and glucose results in loss of beating function, ATP depletion, and partial loss of intracellular enzymes such as lactic dehydrogenase and creatine phosphokinase (3). Cooling the hearts during deprivation protects against ATP and enzyme depletion while elevated temperatures accelerate cell necrosis (4).

The experiments reported here define the ultrastructural and biochemical responses of fetal mouse hearts to two conditions associated with ischemia: transient anoxia and transient anoxia coupled with substrate deprivation. Ischemia *in vivo* involves a third factor, namely, the accumulation of metabolites due to insufficient perfusion in the affected region of the heart. Thus, the fetal mouse heart model reproduces only two of the three important components of true ischemia. Biochemical and ultrastructural responses of the fetal hearts subjected to oxygen and substrate deprivation ("ischemia") are correlated, and the results represent the average response of all myocardial cells. That is, this is a model of global rather than focal "ischemia." Hearts from 15- to 22-day-old fetal mice were used in this study. The metabolic responses of fetal hearts of different stages of maturation differ (5). In this context, it should be emphasized that fetal cardiac metabolism may differ from that of the adult heart; therefore, extrapolating results obtained using fetal hearts to the adult myocardium must be done with this in mind.

Biochemical and ultrastructural changes in the fetal heart after resupply of oxygen and substrate are also presented. During this "recovery" phase, the appearance of increased numbers of autophagic vacuoles and changes in the intracellular distribution of lysosomal enzymes demonstrate stimulation of repair processes. This model permits assessment of interventions designed to stimulate repair processes and to salvage damaged cells.

Abbreviation: MEM, Minimum Essential Medium.

MATERIALS AND METHODS

Fetal Mouse Heart Organ Culture. Intact beating hearts from 15- to 22-day fetal mice were maintained on stainless steel grids at an air (95% O_2 with 5% CO_2)–medium interface in organ culture dishes (Falcon) as described (1). Media used were Minimum Essential Medium (MEM), Medium 199, and Earle's Salt Solution (Grand Island Biologicals). Each experiment was performed at 37° using hearts from matched littermates maintained in culture for 1 day.

Experimental Conditions. Control cultures were maintained in 95% O_2 with 5% CO_2. Cultures were made anoxic by replacing the culture medium with argon- or nitrogen-saturated MEM or Earle's Salt Solution and incubating them in sealed culture jars continuously, or in some cases periodically, flushed with 95% N_2 with 5% CO_2. Oxygen content of the medium was measured using a model 213 gas analyzer (Instrumentation Laboratories, Inc.) and was found to be <5 mm of Hg. (670 Pa). Cultures were made "ischemic" by using glucose-free MEM or Earle's Salt Solution in addition to depriving the cultures of oxygen. There was no flow of the medium in either control or experimental cultures. Rather, a reservoir of constant volume (0.6 ml) permits exchange of metabolites by diffusion. At the end of the period of anoxia or ischemia, cultures were either harvested for ultrastructural and biochemical analyses or were resupplied with oxygen and glucose-containing medium for recovery studies. Medium in culture dishes containing control hearts was also replaced.

Electron Microscopy. Hearts were fixed by immersion in 5% glutaraldehyde in phosphate buffer, pH 7.3, at 4° immediately on removal from the culture dish. The atria were removed and tissue from the ventricles was minced and al-

Table 1. Percentage of control ATP levels remaining in fetal mouse hearts after 3–4 hr of anoxia and/or glucose deprivation

Conditions		Age of fetal mouse hearts		
Oxygen	Glucose	15–16 Days	17–20 Days	21–22 Days
+	—	—	94 ± 3.1% (9)	99 ± 5.0% (2)
—	+	81 ± 4.6% (4)**	57 ± 3.2% (17)*	34 ± 0.7% (3)*
—	—	47 ± 8.3% (5)*	44 ± 3.0% (23)*	18 ± 0.5% (7)*

Control ATP levels (41 ± 4.0 μmol/mg of protein) were measured in fetal mouse hearts cultured for 1 day in either MEM or Earle's Salt Solution (with glucose). The data are given as the average percentage of control ±1 SEM; the numbers in parentheses represent the number of litters assayed. An asterisk denotes values of $P < 0.001$ that the experimental group is the same as the control, and a double asterisk values of $P < 0.03$ obtained using Student's t test for paired data.

lowed to fix for 4 hr. After post-fixation in 1% osmium tetroxide, the tissue was dehydrated in acetone and embedded in Araldite for electron microscopy. At least two blocks of tissue were selected randomly for sectioning from each heart. Thick sections (1 μm) stained with toluidine blue were examined with the light microscope. Thin sections stained with uranyl acetate and lead citrate were examined in a Zeiss 9A electron microscope.

Other Methods. ATP content was measured in hearts homogenized in cold 0.4 N perchloric acid using the firefly luciferase method as described (3, 6). The lysosomal enzymes cathepsin D, acid phosphatase, and β-acetylglucosaminidase were assayed, and total activities and the ratios of nonsedimentable enzyme to total enzyme activities were determined as described (7, 8). Creatine phosphokinase activity was assayed by the method of Szasz (9). Protein content was measured using the method of Lowry *et al.* (10) and glycogen, using the anthrone reaction (11). The proportion of adenine nucleotides present was determined by a modification of the high-pressure liquid chromatography method of Brown (12, 20). Statistical analyses were made using Student's t test for paired data.

RESULTS AND DISCUSSION

Myocardial metabolism

Availability of oxygen and glucose are important determinants of cell viability and function in cardiac tissue. The ATP content of fetal mouse hearts subjected to transient ischemia appears to be related to cell viability as measured by intracellular enzyme depletion and restoration of beating function (3, 4). Experiments were performed to compare the effects of transient anoxia and transient "ischemia" (oxygen and glucose deprivation) on ATP levels in fetal mouse hearts in culture. Three age groups were studied. Hearts from 15- to 16-day fetal mice are innervated and show high glycolytic capacity (5). Hearts from 21- to 22-day fetal mice (near term) are large (>6 mg wet weight) and not ideal for culturing but nonetheless beat and survive in culture for several days. Glycolytic capacity in these hearts is significantly reduced compared to younger hearts and mitochondrial substrate oxidation is relatively more important (5). Hearts from 17- to 20-day fetal mice are well-differentiated and are most suited for culturing.

Table 1 shows the effect of oxygen and/or glucose deprivation on ATP levels in fetal mouse hearts from matched littermates maintained in culture for 1 day. The ATP content in hearts of all ages was unaffected by 3–4 hr of glucose deprivation in the presence of oxygen. The amino acids present in MEM and preformed intracellular glycogen, fat, and protein stores were sufficient to maintain normal ATP levels via oxidative metabolism, as well as normal beating function. Deprivation of oxygen in the presence of glucose for 4 hr resulted in immediate loss of beating function and in a significant reduction in ATP levels for all ages. Older hearts with the lowest capacity for glucose utilization showed the greatest ATP depletion. ATP levels in 15- to 16-day fetal mouse hearts were 81% of control values (41 ± 4 nmol/mg of protein) while ATP levels in 21- to 22-day fetal mouse hearts were depressed to 34% of control values. Anoxia coupled with glucose deprivation for 3–4 hr resulted in a further reduction in ATP content for all age groups. The ATP content of 15- to 20-day fetal mouse hearts was 45% of control after 4 hr of oxygen and glucose deprivation; the ATP content of 21- to 22-day fetal mouse hearts was only 18% of control.

As has been shown in models using adult heart (13, 14), the proportion of adenine nucleotides changed as a result of ischemia. In control fetal hearts the proportion of ATP:ADP:AMP is 89:10:1; after 4 hr of ischemia the proportion is 50:42:8.

Glycogen levels were also decreased during anoxia and ischemia. At the end of 4 hr of ischemia, glycogen was 13.0 ± 1.7% of control levels (9.1 ± 1.5 mg/g wet weight, $n = 8$) in hearts from matched littermates. Hearts subjected to anoxia but supplied glucose were only partially protected against glycogen depletion (J. S. Ingwall and S. R. Gross, unpublished results).

Therefore, alterations in ATP content, adenine nucleotide distribution, and glycogen stores are similar to those described in other models and are consistent with reduced ATP synthesis by mitochondrial substrate oxidation. These results show that fetal mouse hearts in organ culture are responsive both to anoxia and to anoxia coupled with glucose deprivation and that they can be used as hypoxic and ischemic models.

Upon resupply of oxygen and glucose after 3–4 hr of deprivation, ATP levels rapidly returned to control levels. As shown in Fig. 1, ATP levels were the same as in controls after 4 hr of resupply of oxygen and glucose. The ability of hearts to utilize substrates other than glucose during recovery was also tested. Hearts deprived of oxygen and glucose for 4 hr were resupplied oxygen with or without glucose for 4 or 24 hr. ATP levels 4 or 24 hr post-ischemia were the same in hearts supplied either glucose and amino acids (complete MEM) or amino acids only (MEM without glucose). At 4 hr during the recovery period, ATP levels in hearts supplied with complete MEM and glucose-free MEM were 86 ± 6.6% ($n = 6$) and 90 ± 12% ($n = 4$) of control ATP values, respectively. At 24 hr during recovery the corresponding values were 88 ± 4.0% ($n = 14$, complete MEM) and 88 ± 3.0% ($n = 5$, glucose-free MEM).

These results show that continued glucose deprivation does not prevent resynthesis of ATP if oxygen is restored to the cultures. Indeed, ATP levels are near control values in hearts with and without exogenous glucose. This is due to at

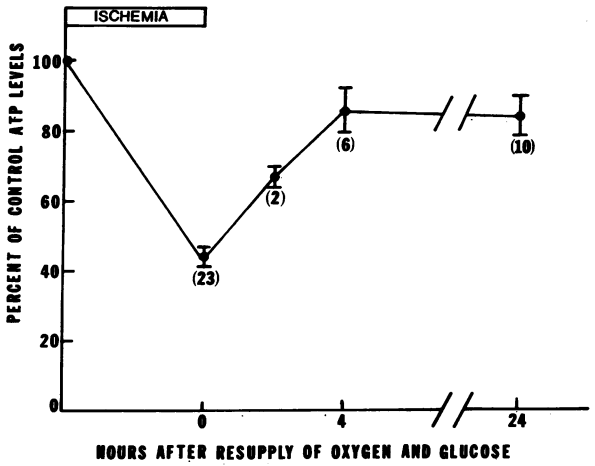

FIG. 1. Changes in ATP levels in 17- to 20-day fetal mouse hearts in organ culture subjected to 4 hr of oxygen and glucose deprivation with time after resupply of oxygen and glucose. Data is expressed as percentage of control ATP levels ± 1 SEM; the numbers in parentheses represent the number of matched litters tested. Control ATP content was 41 ± 4 μmol/mg of protein.

least three factors. First, hearts can utilize non-glucose sources such as lactate produced during the insult and amino acids supplied in the medium or from degraded proteins. Second, glycogen is not completely depleted after 4 hr of ischemia and may be used for new ATP synthesis. Last, and probably most important, adenine nucleotides are still present in the cells, permitting rapid resynthesis of ATP. The ability of the cells to carry out oxidative phosphorylation is not irreversibly altered by the ischemic insult. Moreover, beating function, which ceased upon imposition of anoxia, resumed even before ATP levels returned to control values. The ability of hearts to recover, i.e., to resume beating function and ATP synthesis, may be due to the fact that the hearts are not beating during the insult. Quiescent heart cells require less energy than beating cells, and accordingly they are better able to survive partial ATP depletion.

The fetal mouse heart model is well-suited to assess interventions designed to salvage damaged cells and to stimulate repair processes both during ischemic and hypoxic insults and during recovery. As an example of this, the effects of glucose and insulin in medium containing potassium (10 mM) on the extent of ATP depletion after 4 hr of anoxia were tested. Using matched littermates, anoxic hearts supplied glucose (5.5 mM) and insulin (50 or 500 μg/ml) in MEM contained 68 ± 3.3% of control ATP levels compared to only 40 ± 4.0% in hearts deprived of oxygen, glucose and insulin ($n = 5$, $P < 0.001$). Glucose is clearly the more important component. Anoxic hearts supplied glucose in MEM had 67 ± 3.0% of control ATP levels ($n = 10$), the same as anoxic hearts supplied both insulin and glucose. Insulin alone showed a small but significant effect. Hearts supplied insulin in glucose-free MEM had 57 ± 6.7% control ATP levels after 4 hr of anoxia, while hearts deprived of insulin and glucose had 43 ± 4.4% of control ATP levels ($n = 11$, $P < 0.01$).

Myocardial ultrastructure

Ultrastructural changes in fetal mouse hearts subjected to 1–3 hr of "ischemia" followed by resupply of oxygen and substrate have been assessed and correlate well with the biochemical observations described. The myocytes in control hearts (Fig. 2A) were generally oval to slightly elongated. The extent of development of the myofilaments varied widely, with some cells containing only a few sarcomeres, while in others the myofibrils had assumed a parallel arrangement with Z bands in register. Large numbers of mitochondria of variable size and shape were seen in the perinuclear region and between the myofibrils. The mitochondria were compact and the cristae were intact. Occasional tubules of rough sarcoplasmic reticulum, studded with ribosomes, and a prominent Golgi apparatus were seen in the perinuclear region. Numerous tubules of smooth sarcoplasmic reticulum were scattered throughout the cytoplasm. Transverse tubules were not identified with certainty in most of the cells. An abundance of glycogen granules was present throughout the cytoplasm. Oval to elongated nuclei frequently contained multiple nucleoli, and an occasional cell was undergoing mitosis. A few cells in the central region of the heart had undergone degenerative changes, but the vast majority appeared normal. A comparison of the ultrastructure of fetal mouse hearts maintained in culture for 1 or 2 days with hearts immediately excised from the fetus has been reported (15).

Following the onset of ischemia (Fig. 2B), the most consistent alterations occurred in the mitochondria, where swelling and decreased matrix density were readily apparent in some cells within 1 hr. The cristae become separated and displaced from their usual orientation. An increased number of lipid vacuoles was found in the cytoplasm and margination of the nuclear chromatin was seen in many cells. The myofibrils remained intact; however, the Z bands were less prominent and an increased number of broad aberrant forms were seen. After 3 hr of ischemia most of the cells showed these changes. Numerous prominent vesicles, which appear to be empty, were seen in the cytoplasm by this time. Whether these vesicles were dilated sarcoplasmic reticulum or represent increased pinocytosis has not been established. Abundant glycogen granules and ribosomes were still present in many of the cells, while in others reduction of glycogen was apparent. With progressively longer periods of ischemia, a greater number of cells showed obvious degenerative changes and some were frankly necrotic (16).

After resupply of oxygen and glucose the ultrastructure

Table 2. Percentage of control lysosomal enzyme activities remaining in 20-day fetal mouse hearts after 3.5–4 hr of "ischemia" and during recovery

	End of "ischemia"	2-hr recovery	24-hr recovery
β-Acetylglucosaminidase	98 ± 3.1% (8)	98 ± 2.7% (8)	74 ± 2.1% (6)*
Cathepsin D	89 ± 3.6% (8)*	88 ± 2.8% (8)*	77 ± 4.8% (6)*
Acid phosphatase	101 ± 1.6% (8)	102 ± 1.5% (8)	88 ± 2.0% (6)*

Control levels for β-acetylglucosaminidase (263 ± 6.8 nmol of nitrophenol per hr/mg of protein), cathepsin D (49.6 ± 1.5 μg of tyrosine per hr/mg of protein), and acid phosphatase (295 ± 5.0 nmol of nitrophenol per hr/mg of protein) were measured in fetal mouse hearts cultured in 95% oxygen. The data are given as the average percentage of litter-matched controls ±1 SEM; the numbers in parentheses represent the number of litters assayed. An asterisk denotes values of $P < 0.05$ obtained using Student's t test for paired data.

FIG. 2. (A) Control. The nucleus (N) has evenly dispersed chromatin. The cristae of the mitochondria (M) are closely spaced with a normal matrix density. The sarcomeres are demarcated by well-defined Z bands (Z). The Golgi apparatus (G) is prominent in the perinuclear zone. Numerous glycogen granules are seen. ×10,100 magnification. (B) Three hours of ischemia. There is margination and clumping of nuclear chromatin. The mitochondria (M) are swollen, the cristae are displaced, and there is a decreased matrix density. The Z bands (Z) are less prominent. Numerous dilated vesicles (v) are seen in the cytoplasm. Interstitial space (IS). ×10,100 magnification. (C) After 2.5 hr of reoxygenation. Clumping of nuclear chromatin is less pronounced. Mitochondria (M) resume normal appearance. Myofilaments are readily seen but Z bands are obscure. A prominent autophagic vacuole (A) containing damaged mitochondria and filaments is seen in the perinuclear zone. Interstitial space (IS). Desmosome (D). Nucleus (N). ×9600 magnification.

returned toward normal (Fig. 2C). Nuclear chromatin dispersed and margination was less apparent. The mitochondria resumed a normal condensed appearance and cristae became realigned. Of particular interest is the formation of autophagic vacuoles containing damaged mitochondria and myofilaments in the cytoplasm of many of the cells. These were often surrounded by a double membrane and found both in the perinuclear region and in the cell periphery. These vacuoles were seen as early as 1 hr after reoxygenation of the ischemic hearts and suggest that they represent the sequestration and isolation of irreversibly damaged organelles, indicating that repair mechanisms have been activated. Except for the damaged organelles within the vacuoles, the remainder of the cell cytoplasm appeared normal. The number of totally necrotic cells present increased with the duration of ischemia.

The biochemical and ultrastructural responses of fetal mouse hearts to transient ischemia demonstrate reversible loss of mitochondrial structure and function. The rapid return of ischemic mitochondria to normal appearance coupled with rapid resynthesis of ATP show that 4 hr of ischemia does not irreversibly damage most mitochondria.

Lysosomal enzymes

The appearance of autophagic vacuoles suggests that repair mechanisms were activated during recovery of the hearts. To examine this further, the activities and distributions of several lysosomal enzymes were analyzed in hearts from 20-day fetuses subjected to 3.5–4 hr of "ischemia." This ischemic insult was sufficient to damage and/or cause necrosis in many myocardial cells, as indicated by a 40 ± 8.6% (n = 6) loss of creatine phosphokinase activity 24 hr after the injury (control values were 1.28 ± 0.10 μmol of ATP per min/mg of protein). Nevertheless, at the end of the ischemic period there was little or no alteration in the total activities of lysosomal enzymes (Table 2) and, perhaps more important, no redistribution of the enzyme activity between the sedimentable (particulate) fraction and the nonsedimentable (supernatant) fraction of the tissue homogenate (Table 3). After 2 hr of recovery, on the other hand, a small but significant redistribution of enzyme activity occurred, leaving more of the total activity in the nonsedimentable fraction (Table 3).

Table 3. Percentage of total lysosomal enzyme activity present in the non-sedimentable (supernatant) fraction compared with the total (non-sedimentable plus sedimentable) activity in 20-day fetal mouse hearts after 3.5–4 hr of "ischemia" and during recovery

	β-Acetylglucosaminidase	Cathepsin D
Control, day 1	55 ± 1.5% (13)	48 ± 1.2% (6)
End of ischemia	55 ± 1.5% (13)	48 ± 1.4% (6)
2-hr recovery	58 ± 1.9% (13)*	51 ± 2.4% (6)*
Control, day 2	56 ± 1.2% (7)	
24-hr recovery	60 ± 1.1% (7)*	

Assays were performed as described for Table 2 and in *Materials and Methods*. The data are given as the mean ±1 SEM; the numbers in parentheses represent the number of litters assayed. An asterisk denotes values of $P < 0.05$ compared to matched controls obtained using Student's t test for paired data.

After 24 hr total activities were reduced (along with creatine phosphokinase activity) and the fraction that was nonsedimentable remained increased (Tables 2 and 3).

Increases in nonsedimentable lysosomal enzyme activity are usually assumed to reflect increased fragility or "lability" of lysosomes and increased availability of the enzymes to endogenous substrates. Such changes have been often correlated with the appearance of large lysosomal structures, particularly autophagic vacuoles, which are assumed to be more susceptible to breakage during homogenization. Thus, the lysosomal changes noted biochemically in hearts recovering from ischemia probably reflected the presence of large autophic vacuoles during the repair process as demonstrated by electron microscopy.

Increases in nonsedimentable activity have also been postulated to reflect damage of lysosomes *in situ* and release of lysosomal enzymes into the cytoplasm. This concept underlies the "lysosomal hypothesis" of ischemic myocardial necrosis. It suggests that potentially reversible injury progresses to irreversible infarction because lysosomal hydrolytic enzymes have been activated and released from damaged organelles during ischemia (17–19). Compatible with this hypothesis, some studies have demonstrated increased nonsedimentable activities in infarcted hearts (17, 18); however, it has been difficult to know whether such lysosomal changes precede and cause the necrosis as the "lysosomal hypothesis" would require, or are merely the nonspecific result of cell death (19). Our results suggest that in fetal hearts *in vitro*, ischemic damage and/or necrosis of some cells is sufficient to cause creatine phosphokinase depletion but does not require significant release of lysosomal enzymes or increases in nonsedimentable activity during the injury. Rather, such changes seem more characteristic of later repair processes.

Conclusions

In summary, fetal mouse hearts in organ culture can be used to study the response of myocardial cells both to anoxia and to anoxia coupled with glucose deprivation. Responses to anoxia include immediate cessation of beating function and depletion of ATP and glycogen. Deprivation of oxygen and glucose resulted in a further depression of ATP and glycogen levels and loss of cytoplasmic enzymes. In spite of extensive alterations in mitochondrial structure, hearts recovered beating function and normal ATP synthesis upon resupply of oxygen. Longer periods of deprivation and/or elevated temperatures may lead to irreversible damage.

In hearts subjected to transient ischemia, lysosomal autophagic vacuoles containing damaged mitochondria and myofibrils were observed during recovery, indicating that repair mechanisms were activated. These ultrastructural changes were accompanied by small increases in the proportion of lysosomal enzymes that were present in the nonsedimentable fraction of the tissue homogenate, reflecting increased organelle fragility and/or increased enzyme availability. It is concluded that cultured hearts of fetal mice provide a useful model for studying responses to hypoxia and substrate deprivation and for assessing interventions designed to limit damage or to stimulate repair after ischemic injury.

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