Developmental changes in cardiac recovery from anoxia-reoxygenation

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Sedmera, David, Pavel Kucera, and Eric Raddatz. Developmental changes in cardiac recovery from anoxia-reoxygenation. Am J Physiol Regulatory Integrative Comp Physiol 283: R379–R388, 2002. First published May 10, 2002; 10.1152/ajpregu.00534.2001.—The developing cardiovascular system is known to operate normally in a hypoxic environment. However, the functional and ultrastructural recovery of embryonic/fetal hearts subjected to anoxia lasting as long as hypoxia/ischemia performed in adult animal models remains to be investigated. Isolated spontaneously beating hearts from Hamburger-Hamilton developmental stages 14 (14HH), 20HH, 24HH, and 27HH chick embryos were subjected to 30 or 60 min of anoxia followed by 60 min of reoxygenation. Morphological alterations and apoptosis were assessed histologically and by transmission electron microscopy. Anoxia provoked an initial tachycardia followed by bradycardia leading to complete cardiac arrest, except for the youngest heart, which kept beating. Complete atrioventricular block appeared after 9.4 ± 1.1, 1.7 ± 0.2, and 1.6 ± 0.3 min at stages 20HH, 24HH, and 27HH, respectively. At reoxygenation, sinoatrial activity resumed first in the form of irregular bursts, and one-to-one atrioventricular conduction resumed after 8, 17, and 35 min at stages 20HH, 24HH, and 27HH, respectively. Ventricular shortening recovered within 30 min except at stage 27HH. After 60 min of anoxia, stage 27HH hearts did not retrieve their baseline activity. Whatever the stage and anoxia duration, nuclear and mitochondrial swelling observed at the end of anoxia were reversible with no apoptosis. Thus the embryonic heart is able to fully recover from anoxia/reoxygenation although its anoxic tolerance declines with age. Changes in cellular homeostatic mechanisms rather than in energy metabolism may account for these developmental variations.

chick embryo; cardiogenesis; oxygen deprivation; ultrastructure; apoptosis

Vertebrate embryos and fetuses develop normally in a rather hypoxic microenvironment, and their tissues, in particular myocardium, display a relatively low oxidative metabolism (25, 39). Although energy can be produced both by anaerobic glycolysis and mitochondrial oxidations, early cardiogenesis is severely affected by hypoxia (20), and the embryonic cardiac oxidative function can be rapidly impaired by O₂ lack (15, 30, 34).

Because of the clinical relevance, numerous studies were performed in various animal models in vitro or in vivo to better understand the mechanisms of myocardial injury induced by O₂ deprivation and readmission, including functional and ultrastructural disturbances (8, 17, 41). Myocardial ischemia/hypoxia notably leads to energy deficit, which induces deleterious metabolic consequences and loss of structural integrity at the cell level (10, 11). However, substantially less is known about the functional and structural disturbances induced by anoxia-reoxygenation in the immature myocardium (15). In the context of the fetal pathology associated with transient uteroplacental ischemia (9) and the recent advances in perinatal and intrauterine surgery (4, 5, 24), this knowledge is of increasing importance to achieve adequate strategies of myocardial protection.

Using a recently developed in vitro preparation of isolated, spontaneously beating embryonic chick heart (34, 36), we found that this heart responds rapidly and reversibly to 1-min anoxia followed by reoxygenation. Moreover, similarities as well as differences appear to exist between embryonic and adult heart with respect to the role that disturbances of glycolytic metabolism (47), pH regulation (28), and Ca²⁺ homeostasis (46) may play in the postanoxic dysfunction.

The aim of this work was to investigate the capacity of the developing hearts to recover from long anoxic episodes, similar to those used in adult animal models, i.e., 30 and 60 min. Thus a functional and ultrastructural approach was carried out throughout early cardiogenesis, from looped tubular heart to septating trabeculated heart. Chrono-, dromo-, and inotropic responses of isolated embryonic hearts to anoxia-reoxygenation were quantified, and integrity of the cellular components was tested using transmission electron microscopy.

Materials and Methods

Medium

The standard culture medium was composed of (in mmol/l) 99.25 NaCl, 4 KCl, 0.30 NaH₂PO₄·2H₂O, 10 NaHCO₃, 0.79

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MgCl₂·6H₂O, 0.75 CaCl₂·2H₂O, and 8 μl (+)-glucose; the pH was maintained at 7.4 by equilibration with selected gas containing 2.31% CO₂ (HCO₃⁻/CO₂-buffered medium). The buffering capacity of this medium was 22 μmol H⁺·ml⁻¹·pH unit⁻¹.

Preparation and Mounting of the Hearts

Fertilized eggs of Warren strain hens were incubated at 38°C and high humidity for 48, 72, 96, and 120 h to obtain embryos at Hamburger-Hamilton developmental stages 14 (14HH), 20HH, 24HH, and 27HH (see Ref. 16), respectively. The entire hearts were then carefully excised from explanted embryos by section at the level of the ventral aorta and the sinus venosus at stage 14HH and at the level of the truncus arteriosus as well as between the sinus venosus and the atrium at stages 20HH to 27HH.

The isolated, spontaneously beating heart was placed in the culture compartment (300 μl) of an airtight stainless steel chamber provided with two glass windows for observation and measurements and maintained under strictly controlled metabolic conditions on the thermostabilized stage of an inverted microscope (IMT2 Olympus, Tokyo, Japan) as described previously (36). Briefly, the culture compartment was separated from the gas compartment by a thin (15 μm) transparent and gas-permeable silicone membrane (RTV 141, Rhône-Poulenc, Lyon, France). The hearts were slightly flattened by the silicone membrane, and the resulting thickness of the myocardial tissue facing the gas compartment was ≈300 μm. Because it was technically difficult to measure PO₂ directly within the embryonic heart without damage, the profiles of PO₂ levels within the myocardium during anoxia-reoxygenation had been previously determined using mathematical models of O₂ diffusion and consumption and discussed in great detail (34, 36). Computer simulations indicated that myocardial absolute anoxia was reached in <15 s, and O₂ concentration became steady again after 1 min of reoxygenation. Moreover, vascularization and myoglobin that could buffer intracellular PO₂ were absent at the investigated developmental stages. Thus PO₂ at the tissue level could be strictly controlled and rapidly modified by flushing humidified high-grade gas (purity ≥99.99%) of selected composition through the gas compartment, i.e., air + 2.31% CO₂ for normoxia, and nitrogen + 2.31% CO₂ for anoxia. Accordingly, normoxic values of PO₂ and PCO₂ were 138 and 15.6 mmHg under our experimental conditions, respectively.

Functional Recordings

Contractions at the level of the atrium and the apex of the ventricle were recorded simultaneously using a computerized microphotometric technique as previously published (34). Briefly, two adjustable phototransistors were positioned over the projected image of the contracting atrium and ventricle and were connected to a Macintosh computer via an analog-to-digital converter. Thus contractions were optically detected as edge motion of the myocardial wall. The temporal resolution of acquisition sampling was 0.01 s.

From the simultaneous recordings, it was possible to continuously determine 1) the atrial and ventricular beating rate [heart rate (HR), beats/min], 2) the mean velocity of the atrioventricular (AV) propagation of the contraction (PV, mm/s), which was obtained from the actual distance of the selected regions divided by the time interval between the peaks of the maximal contraction velocity in atrium and in ventricle, and 3) the actual amplitude of the ventricular shortening at the apex (S, μm), which was determined from video recordings. Moreover, the efficiency of the AV propagation was calculated as the ratio of the ventricular beating rate to the atrial beating rate and is expressed as a percentage.

Experimental Protocol

After a period of 30 min of stabilization under normoxia, the hearts were submitted to 30 or 60 min of anoxia followed by 60 min of reoxygenation. The chron-, dromo-, and inotropic parameters were determined continuously throughout the experiments.

Measurement of Extracellular pH

To distinguish the detrimental effects of anoxia-reoxygenation per se from concurrent acidosis, we measured extracellular pH throughout the experiment at stage 24HH. Variations of extracellular pH were measured photometrically in the immediate vicinity (50 μm) of the ventricular wall at the apex using phenol red (Sigma) as optical probe diluted in the culture medium as previously described (28).

Sampling and Morphological Evaluation

For morphological evaluation, the hearts were sampled as follows: 1) normal hearts (freshly isolated from the embryos, n = 4), 2) controls (after 2 h of culture under normoxic conditions, n = 5), 3) at the end of 30 (n = 5) or 60 (n = 5) min of anoxia, and 4) after 5, 30, and 60 min of reoxygenation (n = 6, 6, and 5, respectively).

For histology, the hearts (n = 2 per stage and protocol) were fixed in buffered 4% formalin, processed into paraffin, cut at 5 μm, and stained with hematoxylin-eosin. Apoptotic or necrotic cell death was evaluated on stage 24HH specimens fixed for 1 h in Carnoy’s fixative using methods of in situ tailing or in situ nick translation as previously described (14).

For transmission electron microscopy, hearts (n = 2–4 per stage and protocol) were fixed with 2% glutaraldehyde-1% formaldehyde in cacodylate buffer adjusted to 280 mosmol/kgH₂O with NaCl for 1 h on ice, rinsed in cacodylate with 4% sucrose, postfixed with osmium tetroxide, and routinely processed into Epon. Ultrathin sections stained with uranyl acetate-lead citrate method (38) were examined under Zeiss CM 12 transmission electron microscope and photographed at ×8,000 primary magnification.

Myocardial Protein Content and Lactate Production

In a separate set of experiments, protein content and lactate production of the rapidly growing hearts were determined throughout the investigated period of development as previously described (39). Protein content of the entire hearts was determined according to Lowry et al. (26) using bovine serum albumin as a standard. Lactate produced by the hearts in the culture medium under normoxia or after 30 or 60 min of anoxia was measured spectrophotometrically according to Rosenberg and Rush (40).

Statistical Analysis

All values are reported as means ± SE. For the sake of simplicity of graphical representation, some values were normalized as a percentage of the preanoxia values. The significance of any differences between investigated developmental stages was assessed with one-way ANOVA with Tukey post hoc test. The statistical significance was defined by a value of P < 0.05.

This investigation fully conforms with the **Guiding Principles for Research Involving Animals and Human Beings** of the American Physiological Society.
RESULTS
Stability of the Preparation Under Normoxia

Under normoxia, the activity of the isolated chick embryonic heart was stable in culture for at least 2 h. However, 4% of the investigated hearts at stage 24HH presented spontaneous second-degree AV block, and 30% of the stage 27HH hearts developed irreversible tachycardia with impaired AV propagation. These irregular hearts were discarded from experimentation. Values of HR, PV, and S under steady-state normoxia from stages 14HH to 27HH are reported in Table 1.

Cardiac Growth and Lactate Production

Developmental changes in protein content showing the rapid growth of the heart and the myocardial lactate production under normoxia and anoxia are presented in Table 2. Normoxic and anoxic lactate production leveled off from stage 24HH onward.

Anoxia

At stage 14HH, regular but slowed HR persisted during the entire duration of anoxia (Fig. 1). From stage 20HH to stage 24HH, irregular tachycardic bursts persisted throughout anoxia with gradually decreasing frequency and high interindividual variability (not shown). By contrast, at stage 27HH, the hearts reacted rapidly (within seconds) to anoxia by a transient tachycardia, followed rapidly by bradycardia and finally by total cessation of atrial activity (Fig. 1). The time to drop to 80% of preanoxic HR was 3.0 ± 0.2 (n = 10), 1 ± 0.1 (n = 10), 2.3 ± 0.2 (n = 15), and 0.75 ± 0.1 (n = 10) min at stages 14HH, 20HH, 24HH, and 27HH, respectively (P < 0.05, except stage 20HH was not different from stages 24HH and 27HH).

No conduction disturbances, apart from a slightly slowed propagation velocity, were observed at stage 14HH. However, in older hearts the first episode of complete block of AV conduction resulted after a period depending on developmental stage: 9.4 ± 1.1, 1.7 ± 0.2, and 1.6 ± 0.3 min at stages 20HH, 24 HH, and 27HH, respectively (P < 0.001 at stage 20HH vs. stage 24HH or 27HH). The complete AV block (Fig. 2B) was sometimes preceded by a brief period of second-degree block (3:2, 2:1) at stages 24HH and 27HH.

PV decreased progressively during anoxia at stages 14HH and 20HH and dropped to zero due to development of complete AV block after 1–3 min at stages 24HH and 27HH (Figs. 1C and 2B). Occasionally, bursts of atrial activity were transmitted to the ventricle (in 1:1 pattern) during the first 10–20 min of anoxia at stages 20HH, 24HH, and 27HH only. No ventricular ectopic bursts were observed at any stage, in agreement with our previous observations (28, 47).

From stage 20HH onward, S diminished rapidly after the onset of anoxia before development of total AV block (Figs. 1D and 2B). Indeed, the time to drop to 80% of preanoxic S was 4.0 ± 0.2 (n = 10), 0.4 ± 0.05 (n = 10), 0.5 ± 0.05 (n = 15), and 0.6 ± 0.06 min (n = 10) at stages 14HH, 20HH, 24HH, and 27HH, respectively (P < 0.001, at stage 14HH vs. stages 20HH, 24HH, or 27HH).

Reoxygenation

Chronotropic response. From stage 20HH to stage 27HH, after 30 min of anoxia, there was initially a period of about 30 s with no activity upon reoxygenation (Figs. 2C and 3), even when there were some residual atrial bursts at the end of anoxia. By contrast, at stage 14HH most often near-normal activity was present despite a slight bradycardia (Fig. 3). The first cardiac chamber to recover was always the atrium, but its initial activity was mostly irregular in the form of bursts of contraction of small amplitude (Fig. 2C). The latter were soon replaced by regular atrial activity, and HR gradually increased, even exceeding preanoxic values in some hearts, and finally normalized in most cases after ~15 min (Fig. 3). After 60 min of anoxia, the types of disturbances induced by reoxygenation lasted longer as illustrated by a series of ventricular bursting activity still observed 16 min after O2 readmission (Fig. 4). The latter were much more marked than after 30 min of anoxia (Fig. 2G).

Table 1. Physiological parameters of isolated hearts at normoxia

<table>
<thead>
<tr>
<th>Hamburger-Hamilton Stage</th>
<th>HR (beats/min)</th>
<th>PV (mm/s)</th>
<th>S (μm)</th>
<th>AV Distance (mm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>14HH</td>
<td>118 ± 9</td>
<td>14.6 ± 1.0</td>
<td>20 ± 1</td>
<td>0.98 ± 0.05</td>
<td>10</td>
</tr>
<tr>
<td>20HH</td>
<td>152 ± 4</td>
<td>12.1 ± 0.6</td>
<td>11 ± 2</td>
<td>1.64 ± 0.08</td>
<td>10</td>
</tr>
<tr>
<td>24HH</td>
<td>164 ± 3</td>
<td>15.6 ± 0.7</td>
<td>14 ± 3</td>
<td>2.37 ± 0.06</td>
<td>20</td>
</tr>
<tr>
<td>27HH</td>
<td>180 ± 4</td>
<td>19.9 ± 1.2</td>
<td>36 ± 7</td>
<td>3.25 ± 0.09</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of hearts investigated. HR (beats/min), mean atrioventricular propagation velocity (PV), ventricular shortening (S), and actual distance between investigated atrial and ventricular regions (AV distance) from Hamburger-Hamilton developmental stage 14HH to stage 27HH under normoxic conditions. HR increased significantly from one stage to another (P < 0.04). PV was the same at stages 14HH and 24HH but was the lowest at stage 20HH and the highest at stage 27HH (P < 0.02). S decreased from stages 14HH to 20HH (P < 0.001), was unchanged at stage 24HH, and increased at stage 27HH (P < 0.01). AV distance varied according to the increasing size of the developing heart. ANOVA was used for statistical analyses.

Table 2. Cardiac growth and lactate production

<table>
<thead>
<tr>
<th>Hamburger-Hamilton Stage</th>
<th>Protein/heart (μg)</th>
<th>Normoxic Lactate (nmol/h)</th>
<th>Anoxic Lactate (nmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11HH</td>
<td>3.1 ± 0.5(3)</td>
<td>2.5 ± 0.7(3)</td>
<td>7.1 ± 0.8(3)</td>
</tr>
<tr>
<td>20HH</td>
<td>18.3 ± 0.5(9)</td>
<td>23.3 ± 2.1(5)</td>
<td>26.5 ± 2.8(3)</td>
</tr>
<tr>
<td>24HH</td>
<td>67.3 ± 7.0(9)</td>
<td>43.0 ± 8.8(4)</td>
<td>44.3 ± 1.3(3)</td>
</tr>
<tr>
<td>27HH</td>
<td>168.4 ± 14.9(9)</td>
<td>49.4 ± 0.8(5)</td>
<td>63.2 ± 2.7(3)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of determinations. Developmental changes in myocardial protein content and lactate production under normoxia and anoxia. Protein content increased from one stage to another (P < 0.01). The anoxic lactate production tended to be higher than aerobic production with no statistical significance, whatever the stage investigated (ANOVA). *Higher than at stage 11HH; †higher than at stage 20HH (P < 0.05).
Dromotropic response. From stage 20HH onward, the efficiency of AV coupling increased progressively from 0% (complete AV block) through variable values of second-degree block to 100% at resumption of 1:1 conduction (Fig. 3). Pp was still slowed down (an equivalent of first-degree AV block) early after this complete resumption and recovered to preanoxic values within the next 10 min at stages 20HH and 24HH, irrespective of duration of preceding anoxia, but remained lower than normal at stage 27HH. The rates of recovery demonstrated clearly the differences between stages, with the younger hearts recovering more rapidly. The duration of preceding anoxia had no significant effect on persistence of arrhythmias until stage 24HH. However, at stage 27HH, a significant difference in persistence of AV block was observed between 30 and 60 min of anoxia (Figs. 3 and 5).

Inotropic response. After an anoxia of 30 min, S recovered progressively and reached or transiently exceeded preanoxic values within ~30 min of reoxygenation at all stages except stage 27HH. After an anoxia of 60 min, only stages 14HH and 20HH recovered total control values of S. The amplitude of S varied during the period of second-degree AV block with a typical phenomenon reminiscent of postrest potentiation (Fig. 2, G and H, and Fig. 4).

Variation of Extracellular pH

The time course of extracellular pH in the vicinity of the ventricle during anoxia-reoxygenation is illustrated in Fig. 6. During anoxia, there was an initial acidification at a rate of ~0.05 pH U/min followed by a slowing down of pH decline at a rate of 0.01 pH U/min until the end of anoxia, the nadir being at pH 7.12 ± 0.07 (n = 3). Then, reoxygenation resulted in a slow realkalinization at ~0.005 pH/min.

Morphological Analysis

Two hours of normoxic culture did not cause any gross or ultrastructural injury to the cells whatever the investigated stage (Fig. 7, a and b). At stages 14HH and 20HH, there were no detectable changes throughout anoxia/reoxygenation (Fig. 7c), whatever the anoxia duration. The most important histological and ultrastructural changes were observed at the end of 30 or 60 min of anoxia from stage 24HH onward and consisted in cellular and nuclear edema with occasional chromatin marginalization (Fig. 7d). However, we did not observe any increase of DNA fragmentation typical for either apoptotic or necrotic cell death using in situ tailing or nick translation reactions (data not shown). The cellular membranes as well as myofibrillar structures remained intact, whereas mitochondria were subject to significant swelling, which disappeared gradually during reoxygenation (Fig. 7f). No differences in ultrastructural changes were observed in atrium, AV canal, compact and trabeculated ventricular myocardium, or conotruncus.
All morphological alterations were fully reversible after 60 min of reoxygenation, whatever the stage investigated.

DISCUSSION

Normoxia

Values of the functional parameters (HR, Pv, and S) under normoxic steady state at the investigated stages correspond well to those found in vivo (19) or in vitro (2, 28, 34, 39). The fact that S and Pv were higher at stage 14HH than at stage 20HH could be explained by a different pattern of contraction, which is peristaltoid at stage 14HH, the heart having a form of looped tube with no trabeculations (44). Similarly, Pv results from the combination of a rapid conduction in atria and ventricle and a slow conduction in the region of the AV canal, the length of which varies throughout cardiogenesis (2, 48). Between stages 20HH and 27HH, AV distance doubled, HR and AV delay increased by 20%, while Pv increased by 65%, showing that adequate AV synchronization is assured in the rapidly growing heart.

Anoxia

The general pattern of response to anoxia was similar among the stages investigated. However, the cessation of regular activity during anoxia was faster and the negative chrono-, dromo-, and inotropic responses much more marked in older hearts. This suggests that anaerobic metabolism contributes to energy production more efficiently in the early myocardium (39) and that developmental changes in contractile protein profile and myofilaments properties (12, 27) could also play a role.

Concerning the initial shift of the baseline after the onset of anoxia (Fig. 2B), we have previously shown that it corresponds to a transient ventricular contraction, i.e., an incomplete relaxation, which is significantly attenuated by the L-type Ca2+ channel antagonist verapamil (46).
Particularly sensitive to anoxia was the AV canal, which functions in a manner similar to the adult AV node to maintain out-of-phase ventricular contraction. However, at stage 14HH, this region is probably not yet fully differentiated (2), and the overall myocardial slow conduction velocity is sufficient to ensure the delay, necessary for maintaining unidirectional blood flow. Furthermore, more uniform spread of excitation and contraction is well suited for the peristaltoid mode of contraction. This could partly explain the absence of AV uncoupling at this stage. In contrast, activity of the sinoatrial cardiomyocytes showed an appreciable resistance to O2 deprivation even at more advanced stages. This might well be correlated with regional variations of metabolic (39), ultrastructural (22, 23), and molecular phenotypes (12, 29) of the cardiomyocytes.

Reoxygenation

We found previously that reoxygenation-induced cardioplegia and AV block (the so-called oxygen paradox) appear after an anoxia as short as 1 min at stages 20HH and 24HH (34, 47). In this study, arrhythmias observed at reoxygenation had a characteristic pattern depending on the developmental stage. At stage 14HH, there were only changes in HR (bradycardia \(\rightarrow\) tachycardia \(\rightarrow\) normalization). In older stages, there was block of AV conduction reminiscent of temporary conduction disturbances, which affect AV bundle in adult human hearts. The persistence of slowed propagation velocity observed for several minutes after resumption of 1:1 conduction, an equivalent of first-degree AV block, corresponds well with these results. Interestingly, there were two principal patterns of second-
degree block: one in typical regular form \( N:1 \) or \( N:(N - 1) \), e.g., 2:1 (Fig. 2, E and F) or 5:4 (Fig. 2G) and the other in the form of bursts of 1:1 conduction interrupted by periods of complete block (Fig. 4). Conduction disturbances (AV block) associated with hypoxia were already noted by previous investigators (2); however, this is the first study that characterizes and quantifies them and makes developmental and dose-response correlations under strictly controlled levels of oxygenation.

Ventricular shortening above preanoxic values was observed during reoxygenation after 60 min of anoxia at stages 14HH and 20HH and after 30 min of anoxia at stage 24HH. Such a positive inotropic effect could be related to \( \text{Ca}^{2+} \) accumulation, which we found at stage 17HH during anoxia and reoxygenation in recent pilot experiments (unpublished observations) and was also reported in isolated rat cardiomyocytes (45), resulting subsequently in alteration of \( \text{Ca}^{2+} \) handling. The incomplete recovery of the contractile function observed after 30 min of anoxia at stage 27HH, or after 60 min of anoxia at stage 24HH, might well be attributed to deleterious perturbations of energy metabolism related to anoxia-reoxygenation and/or to subtle injury to contractile machinery not perceived by transmission electron microscopy and partly due to reactive oxygen species production enhanced by lactate accumulation (35).

Morphological Changes

The reversibility of ultrastuctural disturbances correlates with the generally good functional recovery and points out the relative resistance of the developing myocardium to anoxia-reoxygenation. The reversible nuclear and mitochondrial changes are similar to those observed in animal models of adult myocardium after ischemia-reperfusion. In contrast, dense mitochondrial inclusions, characteristic of irreversible anoxic injury in adult cardiac conducting cells (3), were not observed. This reinforces the notion that functional recovery is better in developing heart. However, in the adult heart, marginalization of chromatin and mitochondrial alterations, including amorphous matrix densities, appear to persist for a longer period during reperfusion (18).
The absence of detectable modifications of myofibrillar apparatus could be due, at least partly, to cessation of contractile activity during anoxia as a protective effect (energy sparing), to a lesser susceptibility of the scanty immature sarcomeres to metabolic alterations, and to the ability of the embryonic cardiomyocytes to use glycolytically derived ATP even under O₂ deprivation to maintain cellular homeostasis. This is similar to glycogen-rich, myofibrill-poor adult conduction system cells, which seem more tolerant of anoxia than the working myocardium (3).

Mitochondrial swelling could be related to anoxia-induced Ca²⁺ overload, perturbed ionic balance, and/or to increased production of reactive oxygen species upon reoxygenation (35).

Programmed cell death, or apoptosis, plays an important role in heart morphogenesis (7, 32). However, it is found predominantly in the mesenchymal tissues such as cardiac cushions and not in the myocardium except the outflow tract (7, 33). This seems to be true for both birds and mammals (21), and the paucity of cell death in the prenatal myocardium was apparent only at stage 24HH (d), but myofibrils remained intact. e And f: after 30 min of reoxygenation, alterations are fully reversed. In all cases, cardiomyocytes from left ventricular apical trabeculae are shown. Scale bar, 1 μm.

Fig. 7. Ultrastructural changes during anoxia-reoxygenation at stage 14HH (left; a, c, and e) and stage 24HH (right; b, d, and f). a And b: specimen submitted to 2 h of normoxic culture shows normal nuclear (N), mitochondrial (Mi), and myofibrillar (My) morphology. c And d: after 30 min of anoxia, nuclear and mitochondrial edema was apparent only at stage 24HH (d), but myofibrils remained intact. e And f: after 30 min of reoxygenation, alterations are fully reversed. In all cases, cardiomyocytes from left ventricular apical trabeculae are shown. Scale bar, 1 μm.
Variations of External pH and Lactate Production

Because carbon dioxide produced by the heart was freely diffusible across cellular and silicone membranes and was continuously removed by the flux of gas in the chamber, the decrease of pH observed during anoxia reflected metabolic rather than respiratory acidosis.

Between stages 11HH and 27HH, lactate production of the heart measured under normoxic and anoxic conditions increased 20- and 9-fold, respectively, while its protein content increased 54-fold (Table 2). Thus, within 80 h of development, normalized lactate production decreased from about 0.8 to 0.3 nmol h⁻¹ μg⁻¹ under normoxia (as we previously observed, Ref. 39) and from about 2 to 0.4 nmol h⁻¹ μg⁻¹ under anoxia. In our experimental conditions, the highest lactate concentration found in the culture medium was 2 mM after 60 min of anoxia at stage 27HH. Taking into account the volume of the chamber and the buffering capacity of the medium, the theoretical drop of external pH due to lactic production would be 0.01 at most. Under anoxia, besides lactate production, hydrolysis of ATP and catabolic processes contribute also to tissue acidosis, which is known to depress contractile activity also in the embryonic chick heart when pH reaches abruptly low values. For example, a controlled rapid drop to pH 6.5 during an anoxia of 1 min worsens reoxygenation-induced dysfunction and delays recovery at stage 24HH (28). However, under our experimental conditions, the lowest pH reached progressively at the end of 30 min of anoxia was 7.1 (Fig. 6), which should be well supported by the preparation because contractile function of the developing myocardium is known to be more resistant to acidosis than that of the adult (13, 31). Furthermore, we have previously shown (28, 46) that hearts (stage 24HH) submitted to brief anoxia (1 min) at pH 7.4 display the same types of reoxygenation-induced disturbances as those observed in the present work, although pH drop and ATP depletion are negligible during such a short anoxic episode. This suggests that reoxygenation-induced arrhythmias and depression of contractility were due to consequences of oxygen deprivation-readmission rather than the pH drop during the preceding anoxia.

We previously discussed that diffusion barriers for O₂ are negligible in our preparation and that Po₂ levels return rapidly (few seconds) to normal values (34, 36). On the contrary, restoration of cellular homeostasis (e.g., pH, ionic balance, and redox status) necessary for normal pacemaking activity, conduction, and contractility is much more delayed. With regard to the important role played by such homeostatic mechanisms, it is relevant to note that inhibition of L-type Ca²⁺ channels, inactivation of bicarbonate transport systems, or exogenous antioxidants improve the functional recovery of embryonic chick hearts submitted to anoxia-reoxygenation at stage 24HH (28, 37, 46).

Conclusion

Embryonic hearts reacted rapidly and reversibly to anoxia lasting as long as hypoxia-ischemia performed in adult animal models. However, the rate of postanoxic recovery declined progressively from tubular heart to trabeculated septating heart. Thus oxygen dependency of the cardiac activity increases with development while the myocardial oxidative capacity is known to remain unchanged throughout early cardiogenesis (39). This apparent contradiction suggests that the anoxia/reoxygenation-induced disturbances observed in this work were not directly related to differentiation of the aerobic energy metabolism but rather to developmental changes in cellular homeostatic mechanisms (e.g., pH-, ion-, and redox-regulating systems). Thus the effectiveness of protective strategies of fetal cardiomyocytes submitted to hypoxia-reoxygenation might significantly differ according to myocardial maturation.

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Preliminary results of this work have appeared in abstract form (42).

REFERENCES


12. Franco D, Lamers WH, and Moorman AFM. Patterns of expression in the developing myocardium: towards a morpholog-
EMBRYONIC MYOCARDIAL RECOVERY FROM ANOXIA-REOXYGENATION


23. Knaapen MWM, Vrolijk BCM, and Wenink ACG. Nuclear and cellular size of myocytes in different segments of the developing rat heart. Anat Rec 244: 118–125, 1996.


