Oxidative and Glycogenolytic Capacities within the Developing Chick Heart

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ABSTRACT

Cardiac morphogenesis and function are known to depend on both aerobic and anaerobic energy-producing pathways. However, the relative contribution of mitochondrial oxidation and glycogenolysis, as well as the determining factors of oxygen demand in the distinct chambers of the embryonic heart, remains to be investigated. Spontaneously beating hearts isolated from stage 11, 20, and 24HH chick embryos were maintained *in vitro* under controlled metabolic conditions. O₂ uptake and glycogenolytic rate were determined in atrium, ventricle, and conotruncus in the absence or presence of glucose. Oxidative capacity ranged from 0.2 to 0.5 nmol O₂/(h·μg protein), did not depend on exogenous glucose, and was the highest in atria at stage 20HH. However, the highest reserves of oxidative capacity, assessed by mitochondrial uncoupling, were found at the youngest stage and in conotruncus, representing 75 to 130% of the control values. At stage 24HH, glycogenolysis in glucose-free medium was 0.22, 0.17, and 0.04 nmol glucose U/(h·μg protein) in atrium, ventricle, and conotruncus, respectively. Mechanical loading of the ventricle increased its oxidative capacity by 62% without altering glycogenolysis or lactate production. Blockade of glycolysis by

iodoacetate suppressed lactate production but modified neither O₂ nor glycogen consumption in substrate-free medium. These findings indicate that atrium is the cardiac chamber that best utilizes its oxidative and glycogenolytic capacities and that ventricular wall stretch represents an early and major determinant of the O₂ uptake. Moreover, the fact that O₂ and glycogen consumptions were not affected by inhibition of glyceraldehyde-3-phosphate dehydrogenase provides indirect evidence for an active glycerol-phosphate shuttle in the embryonic cardiomyocytes. (*Pediatr Res* 49: 363–372, 2001)

Abbreviations

DNP, 2,4-dinitrophenol
G3PDH, glyceraldehyde-3-phosphate dehydrogenase
GU, glucose unit
HH, embryonic stage
HR, heart rate
IAA, iodoacetate
SR, sarcoplasmic reticulum

The embryonic/fetal heart operates and develops normally in a relatively hypoxic microenvironment and shows both aerobic and anaerobic energy-producing capacities. In such a heart, by contrast with the adult, mitochondria convert fatty acids to energy at a very low rate (1), whereas glycolysis contributes significantly to ATP production (2, 3) and glycogen turnover is rapid (4). Such metabolic features confer a benefit to the developing heart, because glucose requires less O₂ than fatty acids to produce a given amount of ATP, which is advantageous specially when O₂ availability is limited (5, 6).

The embryonic myocardium (7–9) displays also a glycogen concentration 10–20-fold higher than in the adult (3) and can tolerate a transient depletion of exogenous substrates in normoxia (10) or even recover rapidly from substrate-free anoxic episodes (11, 12). Similar characteristics are found in fetal and

neonatal hearts (13–15). However, in avian embryos (16) and mammalian fetuses (17), the cardiovascular response to O₂ lack is very rapid despite important glycolytic capacities, indicating that under physiologic conditions, the embryonic/fetal heart works at the upper limit of its function curve. Indeed, during early development, the heart grows rapidly, its hemodynamic performances such as developed pressure and mechanical work increase significantly (18), and its O₂ demand increases at the same time (19), making cardiac activity critically dependent on O₂. Moreover, because extrinsic cardiac innervation is absent at early stages, regulation of the cardiovascular function is directly dependent on intrinsic mechanisms at the tissue level. For example, alterations of loading conditions of the ventricle affect directly and significantly the cardiovascular performances (20). Such alterations should also induce changes in myocardial energy demand and production, which deserve to be investigated. Given the recent progress in fetal cardiology and the lack of information about mechanoeenergetics of the developing heart, the knowledge of the main determinants of the O₂ consumption, the ratio of basal to total

Received April 26, 2000; accepted October 26, 2000.

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Supported in part by the Swiss National Science Foundation Grant 31-37668.93.

metabolism, and the mechanical efficiency throughout early cardiogenesis is of importance. In particular, the spatiotemporal pattern of metabolic activity remains to be characterized.

Glucose catabolism along two distinct pathways (glycolysis and mitochondrial oxidation) is essential to ensure normal cardiac morphogenesis and function. In the embryonic chick heart (21–23) and in the fetal mammal heart (2, 3, 24), lactic production and the pentose-phosphate pathway are especially active and appear to be primarily dependent on exogenous glucose rather than on glycogen. Whether glucose derived from glycogen is preferentially oxidized in comparison with exogenous glucose, as it is in adult cardiomyocytes (25), has to be explored.

The present study was undertaken 1) to determine the relative contribution of the aerobic and glycolytic pathways to energy production during early cardiogenesis, 2) to find out whether atrium, ventricle, and conotruncus show differences of oxidative and/or glycogenolytic capacities, and 3) to investigate the effects of mechanical loading and beating rate on energy metabolism of the embryonic myocardium.

Preliminary results of this study have appeared as abstracts (11, 26).

METHODS

Chemicals and Media

d(+)Glucose and l-epinephrine were purchased from Fluka, IAA and 2,3-diphosphoglycerate from Sigma Chemical Co., DNP from Serva, and verapamil from Knoll AG Germany (Isoptin).

The standard culture medium was composed of (in mmol/L): NaCl 99.25; KCl 2.01; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 10.67; KH_2PO_4 2.65; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.79; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.75; d(+)glucose 8.0; pH measured 7.35; 235 mosmol/kg H_2O . Buffering capacity, 6.93 $\mu\text{mol H}^+ / (\text{mL} \cdot \Delta\text{pH} = 1)$.

The O_2 source was purified human Hb diluted at a final monomeric concentration of 1 mmol/L in phosphate buffer (Sørensen) at pH 6.8 and in the presence of 0.5 mmol/L 2,3-diphosphoglycerate to decrease affinity for O_2 (19). This solution was equilibrated with a gas mixture of 8% O_2 and 92% N_2 to maintain Hb at approximately 50% saturation.

Dissection of Embryos and Mounting of Isolated Hearts

Fertilized eggs from Warren hens were incubated 40, 72, and 96 h at 38°C and 90% relative humidity to obtain embryos at stages 11 (heart rudiments fused), 20 (S-shaped heart), and 24HH (septating trabeculated heart) according to Hamburger and Hamilton (27), respectively. Entire hearts were carefully excised from explanted embryos by section at the level of the ventral aorta and the sinus venosus at stage 11HH and at the level of the truncus arteriosus as well as between the sinus venosus and the atrium at stage 20 and 24HH. In a set of experiments, isolated hearts were divided into atria (left + right), ventricle, and conotruncus, the cutting being performed at the level of the atrioventricular canal and at the level of the ventriculoconal junction.

As described elsewhere (19), the isolated spontaneously beating heart was placed in the culture compartment (40 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 stage of an inverted microscope (IMT-2 Olympus, Tokyo, Japan) equipped with a thermostabilized incubator at 37.5°C. The culture compartment was separated from the O_2 source compartment (12 μL , 0.7-mm thick) by a thin (15 μm) transparent and gas-permeable silicone membrane (RTV141, Rhône-Poulenc, Lyon, France). The compartment of the O_2 source (oxyhemoglobin) was perfused at a standard rate of 56 $\mu\text{L}/\text{min}$, and the composition of the culture medium could be modified using remote-controlled push-pull syringes.

Measurement of Regional O_2 Consumption

The regional O_2 uptake was measured according to the spectrophotometric technique previously published (19) with few modifications. Briefly, the principle of the method was to use oxyhemoglobin as the O_2 donor as well as the indicator of respiration. In stop-flow condition, the O_2 uptake of the tissue was proportional to the rate of desaturation of oxyhemoglobin measured photometrically ($\Delta\text{OD}/\Delta t$). The light source (442 nm) was the beam of a helium cadmium laser (Liconix, U.S.A.) controlled and stabilized using an acoustico-optic modulator. The measuring beam was focalized on and passed through a selected region (50 μm in diameter) of the slightly flattened and rather transparent embryonic heart. The output signal from the phototransistor of the photometer attached to the trinocular of the microscope was A/D converted, recorded, and processed using an Apple Macintosh.

The heart was allowed to equilibrate in the chamber for 30–40 min before the first measurement. Then, O_2 consumption was determined alternately at the level of atrium, ventricle, and conotruncus. Recordings of the transmural O_2 flux expressed as $\text{nmol O}_2 / (\text{h} \cdot \text{mm}^2)$ in a given region were performed during short periods of stop-flow (30 s) and systematically duplicated at 1-min intervals. The reproducibility of these measurements was fully satisfactory with a coefficient of variation of 2%. O_2 consumption was corrected for protein content to obtain the oxidative capacity expressed as $\text{nmol O}_2 / (\text{h} \cdot \mu\text{g})$. Renewal of the culture medium or changing O_2 delivery by varying the rate of Hb perfusion within the range of 28–112 $\mu\text{L}/\text{min}$ did not alter O_2 uptake.

Determination of Glycogen Content, Glycogenolysis, and Lactate Production

Glycogen content was determined spectrofluorometrically (28) using an automated setup and expressed as GU.

Before dissection and storage at -20°C , all the excised hearts were thoroughly rinsed at $0-4^\circ\text{C}$ (60 min on a rotary shaker) in glucose-free solution to remove extracellular glucose remaining in cardiac cavities that could interfere with ulterior measurements. Washing hearts under these conditions had no effect on glycogen content. Atria, ventricles, and conotruncus were pooled at stage 20 (20 hearts) and 24HH (10 hearts). Glycogen and protein in the different cardiac regions

were determined in the same pools. For determination of the total protein and glycogen content, 4, 2, and 1 entire hearts were used at stage 11, 20, and 24HH, respectively. After thawing, all the samples were sonicated (3×2 s on an ice bath) for biochemical determinations.

For determination of glycogenolysis, isolated hearts were dissected either immediately after removal from the embryos or after 2 h of incubation in normoxic glucose-free medium at 37.5°C. Glycogenolytic rate was obtained as the difference between glycogen content determined before and after incubation. It was tested that glycogen content decreased linearly with respect to time, indicating that the rate of glycogenolysis was constant throughout this experiment.

The lactate produced by the hearts in the culture medium within 2 h was measured spectrophotometrically according to Rosenberg and Rush (29).

The aerobic and glycolytic production of ATP was calculated on the stoichiometric basis of 6 ATP generated per molecule of O_2 consumed (aerobic) and 1 ATP generated per molecule of lactate produced (glycolytic).

Morphometry

The end-diastolic external wall area of atrium, ventricle, and conotruncus, the ventricular perimeter, and shortening at the apex of the ventricle were obtained from frames of video recordings of contracting hearts performed before and after each series of measurements. The end-diastolic and end-systolic external perimeters of the ventricle were calculated from the distance between the middle of the inner curvature (localized between atrioventricular cushions and conotruncal ridges) and the apex. Because the hearts were slightly flattened by the silicone membrane, the morphometric analysis, based on video recordings, was performed on the assumption that the preparation could be considered as rather two-dimensional.

Experiments

Effects of oxidizable exogenous substrates on myocardial O_2 uptake. O_2 consumption of the whole heart and in each cardiac region was determined in the absence and presence of exogenous glucose (8 mmol/L, control). The O_2 uptake of the empty contracting heart (no pressure generated) corresponded to basal O_2 consumption plus suprabasal O_2 requirement for mechanical deformation (measurable shortening due to cross-bridge cycling) and ionic activation.

Basal oxidative metabolism of the loaded hearts. The basal oxidative metabolism of the nonbeating loaded embryonic heart includes aerobic energy cost of proliferation, differentiation, maturation, and maintenance of ionic gradients and cellular integrity. To determine the corresponding basal O_2 consumption, electromechanical work (ionic activation + cross-bridge cycling) was minimized by maintaining the hearts in diastolic arrest by use of the calcium antagonist verapamil at a final concentration of 1 μ M (30).

Effects of beating rate and mechanical loading on myocardial O_2 uptake. To investigate whether myocardial O_2 consumption was related to beating rate, epinephrine (11 μ M) was added to accelerate HR. The relationship between ventricular

O_2 uptake and HR was determined only at stage 24HH, as catecholamines have significant positive chronotropic (31) and inotropic (32) effects from this stage onward. The consequences of exposure to verapamil or epinephrine on enzymatic activities, glucose transport, and glycogenolysis were not investigated in the present study.

In a set of separate experiments to mimic loaded conditions at stage 20 and 24HH, the ventricle of isolated hearts was filled with viscous deformable biocompatible and transparent silicone oil (Silbione V1000, Rhône-Poulenc, Lyon, France) according to our previous study (33). To adjust the load for different heart sizes, oil was gently introduced using a glass micropipette under visual control until diastolic and systolic diameters of the ventricle reached physiologic values determined *in ovo* (34). The drop of silicone, ovoid in shape, was squeezed at each ventricular contraction, partially bulged into atrioventricular and predominantly ventriculoconal canals, and then returned to its initial shape during diastole. Such reversible geometric modifications show that the volume of the ventricle varied according to cardiac cycle and that pressure was generated, because the radii of curvature at the extremities of the drop were shorter during systole (Laplace law).

The coefficient of O_2 solubility in this silicone oil is much higher than in the culture medium, *i.e.* 8.29 versus 0.982 nmol O_2 /(μ L·760 mm Hg) at 37°C. For this reason, in contrast with unloaded conditions, a correction factor of 1.11 was used to calculate the actual O_2 consumption of loaded ventricles.

The metabolic and functional changes after addition of glucose, drugs, and inhibitors were followed during a period of at least 40 min. All the solutions were adjusted to pH 7.35. Protein content was determined according to Lowry *et al.* (35) using bovine serum albumin as standard.

An index of mechanical activity of the ventricle per unit of time was calculated as follows: [perimeter shortening (mm)/end-diastolic perimeter (mm)] · [end-diastolic wall area (mm²)] · (beats/min). This index reflects the total movement of myocardial wall per minute (mm²/min).

Inhibition and stimulation of glycolysis and mitochondria. Glycolysis was blocked at the level of the G3PDH by using IAA (100 μ M). The maximal oxidative capacity was assessed by activating specifically and maximally the mitochondrial electron transport chain with uncoupling agent DNP (200 μ M).

Normalization and Statistics

Unless otherwise stated, O_2 uptake, lactate production, and glycogenolysis were corrected for the mean protein content of the distinct cardiac regions and of the whole heart.

Values are given as mean $\pm$ SEM. Statistical analysis was performed using 1-way ANOVA, Tukey-Kramer, and unpaired or paired *t* test. Statistical significance was defined by a value of $p \leq 0.05$.

The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

RESULTS

Biochemical, Morphometric, and Functional Parameters of the Isolated Heart

Myocardial wall area, protein content, and glycogen store are reported in Table 1. The protein content of the whole heart increased approximately 22-fold, whereas the end-diastolic area increased by 8-fold between stage 11 and 24HH (*i.e.* within 56 h), reflecting essentially the rapid thickening of the growing myocardial wall. The protein/area ratio increased in all the regions and corresponded to the quantity of protein constituting the myocardial wall under 1 mm² of external surface. Moreover, this ratio was useful to take into consideration the thinning of the ventricular wall when it was stretched by mechanical loading.

Throughout development, normalized glycogen remained rather stable in atrium and ventricle but decreased markedly in conotruncus and was much higher in atrium than in conotruncus at stage 24HH (Table 2). Under our experimental conditions, the end-diastolic perimeter and perimeter shortening of the unloaded ventricle as well as HR increased significantly from stage 11 to 24HH (Table 3).

The index of mechanical activity was 2.8, 7.5, and 11.9 mm²/min in unloaded hearts at stage 11, 20, and 24HH, and 15.5 and 22.4 mm²/min in loaded hearts at stage 20 and 24HH, respectively.

Metabolic Characteristics within the Developing Heart

The transmural O₂ flux measured along the heart tube ranged from 0.6 to 1.0 at stage 11HH and from 1.5 to 2.1 nmol O₂/(h·mm²) at stage 20–24HH. The corresponding unloaded oxidative metabolism ranged from 0.2 to 0.5 nmol O₂/(h·μg protein), did not depend on exogenous glucose, and was the highest in atria (Table 4).

Reserves of oxidative capacity assessed by mitochondrial uncoupling were important in the youngest hearts and in conotruncus (Fig. 1). Values of O₂ uptake determined 3 to 6 min after addition of DNP could be lower than the actual peak

of O₂ uptake reached just before the first measurement. Indeed, at stage 20 and 24HH, the fall in O₂ uptake during the first 30 min of exposure to DNP could be fitted using an exponential curve with a correlation coefficient (*r*²) ranging from 0.90 to 0.99 (Fig. 1a). Thus, the extrapolated values of ventricular transmural O₂ flux at the very beginning of exposure to DNP were 2.8 ± 0.15 (*n* = 3) and 2.4 ± 0.2 (*n* = 3) nmol O₂/(h·mm²) at stage 20 and 24HH, respectively, and were 10% higher than the values determined experimentally. These extrapolated values correspond to an oxidative capacity of 0.53 and 0.24 nmol O₂/(h·μg) at stage 20 and 24HH, respectively, and most likely represent the lowest limits of the actual peak values. Mitochondrial uncoupling initially accelerated the hearts and then resulted in cessation of contractions within 18 ± 5 (*n* = 3) and 19 ± 1 (*n* = 3) min at stage 20 and 24HH, respectively, but surprisingly did not affect contractions of the younger stage (stage 11HH) (Fig. 1a).

From stage 11 to stage 24HH, glycogenolysis provided glucose at a rate greatly sufficient to maintain cellular oxidations, IAA-inhibitable lactate production (Table 5), and contractile activity for hours in substrate-free medium (assuming that 6 O₂ are used to fully oxidize 1 GU and that 2 mol of lactate are derived from 1 GU). The contribution of another endogenous store such as fatty acids to ATP production was not considered, because their rate of oxidation appears to be negligible up to stage 24HH (1).

Normalized glycogenolysis almost tripled in the whole heart between stage 11 and 24HH but varied regionally, the highest value being found in atria and the lowest in conotruncus (Table 4).

In the unloaded beating hearts, the contribution (30%) of glycolytically derived ATP, assessed by lactate production, remained stable throughout early cardiogenesis (Table 5).

Effects of Exogenous Glucose

Whatever the stage investigated, 1) O₂ consumption in atria, ventricle, and conotruncus was independent of exogenous glucose, 2) glucose prevented glycogen degradation, and 3) glucose stimulated aerobic lactate production (Table 5). Ventric-

Table 1. Changes in morphometric and biochemical parameters showing rapid myocardial growth during early embryogenesis

	Wall area mm ²	Protein content μg	Glycogen store nmol GU
Stage 11HH			
A	0.46 ± 0.02(31)	nd	nd
V	0.44 ± 0.02(31)	nd	nd
CT	0.24 ± 0.01(31)	nd	nd
t	1.14 ± 0.04(31)	3.1 ± 0.5[3]	1.3 ± 0.1[11]
Stage 20HH			
A	1.28 ± 0.04(33)	5.1 ± 0.3[8]	3.9 ± 0.2[4]
V	1.96 ± 0.05(33)	10.3 ± 0.5[8]	6.7 ± 1.1[4]
CT	1.10 ± 0.04(33)	3.5 ± 0.3[8]	1.9 ± 0.4[4]
t	4.34 ± 0.11(33)	18.6 ± 0.6[11]	14.3 ± 0.7[25]
Stage 24HH			
A	2.70 ± 0.09(32)	16.8 ± 1.5[10]	16.3 ± 1.7[4]
V	4.34 ± 0.12(32)	44.3 ± 2.6[10]	27.8 ± 4.1[4]
CT	1.92 ± 0.04(32)	12.8 ± 1.4[10]	4.3 ± 0.8[4]
t	8.97 ± 0.21(32)	68.3 ± 5.0[13]	63.1 ± 2.2(53)

Myocardial end-diastolic wall area (mm²), protein content (μg), and glycogen store (nmol GU/heart) in atrium (A), ventricle (V), conotruncus (CT), and in the whole heart (t). Mean ± SEM; (), no. of individual hearts; [], no. of determinations on pooled hearts; unloaded conditions; nd, not determined.

Table 2. Regional normalized glycogen content (nmol GU/ μ g protein) at stage 20 and 24HH

	Atrium	Ventricle	Conotruncus
Stage 20HH	0.74 $\pm$ 0.03	0.70 $\pm$ 0.03	0.60 $\pm$ 0.03
Stage 24HH	0.91 $\pm$ 0.12*	0.59 $\pm$ 0.09	0.32 $\pm$ 0.05§

Both glycogen and protein contents were determined in the very same hearts. Mean $\pm$ SEM. Four determinations for each region. * $p < 0.05$ vs CT at stage 24HH; § $p < 0.01$ vs CT at stage 20HH (ANOVA).

Table 3. Morphometric and functional parameters of the empty and loaded contracting ventricle

Wall area mm ²		End-diastolic perimeter mm		Perimeter shortening μ m		HR beats / min	
unloaded	loaded	unloaded	loaded	unloaded	loaded	unloaded	loaded
11HH 0.44 $\pm$ 0.02(31)	nd	0.87 $\pm$ 0.02	nd	67.6 $\pm$ 3.0	nd	83 $\pm$ 4	nd
20HH 1.95§ $\pm$ 0.05(33)	2.23* $\pm$ 0.10 (7)	1.93§ $\pm$ 0.03	2.07* $\pm$ 0.01	57.2§ $\pm$ 8.9	112.2* $\pm$ 24.4	130§ $\pm$ 2	128 $\pm$ 6
24HH 4.39¶ $\pm$ 0.13(29)	5.54*¶ $\pm$ 0.17(24)	3.37¶ $\pm$ 0.06	3.66*¶ $\pm$ 0.06	76.2¶ $\pm$ 5.0	92.3 $\pm$ 6.2	120 $\pm$ 5	160*¶ $\pm$ 6

External wall area, end-diastolic perimeter, perimeter shortening, and HR were measured after stabilization in the culture chamber. Values obtained with and without glucose were pooled because they were not significantly different. (), no. of hearts investigated; mean $\pm$ SEM; * vs unloaded ($p \leq 0.03$); § vs stage 11HH ($p < 0.05$); ¶ vs stage 20HH ($p \leq 0.004$); unpaired t test.

Table 4. Oxidative (ox; nmol O₂ $\cdot$ h⁻¹ $\cdot$ μ g protein⁻¹) and glycogenolytic (gg; nmol GU $\cdot$ h⁻¹ $\cdot$ μ g protein⁻¹) capacities in atrium, ventricle, conotruncus, and in whole hearts

stage	Atrium		Ventricle		Conotruncus		Whole heart	
	ox	gg	ox	gg	ox	gg	ox	gg
11HH	nd	nd	nd	nd	nd	nd	0.30 $\pm$ 0.02	0.10
20HH	0.44§ $\pm$ 0.02	0.17	0.39§ $\pm$ 0.01	0.17	0.46*§ $\pm$ 0.02	0.10	0.43§ $\pm$ 0.01	0.14
24HH	0.29*† $\pm$ 0.01	0.22	0.19 $\pm$ 0.01	0.17	0.25* $\pm$ 0.01	0.04	0.24 $\pm$ 0.01	0.26

At each stage, values of ox were obtained from 31–33 unloaded hearts. All values of gg were obtained from four determinations (see 'Material and Methods'). Mean $\pm$ SEM. * vs ventricle; † vs conotruncus; § vs stage 11 or 24HH (ANOVA).

ular end-diastolic perimeter and shortening and HR were also independent of exogenous substrate. At stage 24HH, HR was 120 $\pm$ 7 ($n = 19$) and 136 $\pm$ 10 ($n = 14$) beats/min in the absence and presence of glucose, respectively.

Effects of Loading and HR on Myocardial Metabolism

Mechanical loading 1) augmented ventricular end-diastolic perimeter (Table 3), 2) accelerated HR by 30% at stage 24HH only (Table 3), and 3) increased O₂ uptake and shortening at stage 20 and 24HH (Fig. 2a). At stage 24HH, additional work due to loading altered neither glycogen remaining in ventricle after 2 h of activity in glucose-free medium [15.0 $\pm$ 0.8 ($n = 13$; loaded) versus 16.5 $\pm$ 1.3 ($n = 12$; unloaded) nmol GU/ventricle] nor lactate production of the whole heart [11.6 $\pm$ 1.6 ($n = 11$; loaded) versus 11.6 $\pm$ 0.9 ($n = 9$; unloaded) nmol lactate $\cdot$ h⁻¹ $\cdot$ heart⁻¹].

A linear relationship was found at stage 24HH between O₂ uptake of the loaded ventricle and HR in the absence (Fig. 2b) but not in the presence of glucose (large dispersion of data points, not shown) with r^2 of 0.67 and 0.12, respectively. This relationship reflects the actual dependency of mechanical activity on oxidative metabolism, because some data points reported in Figure 2b were obtained from hearts beating spontaneously at low (75 beats/min) or high (up to 200 beats/min) rate without any pharmacologic interventions. Addition of epinephrine accelerated the loaded heart from 140 $\pm$ 6 to 180 $\pm$ 10 beats/min, increased the ventricular oxidative metabolism from 0.32 $\pm$ 0.02 to 0.36 $\pm$ 0.01 nmol O₂/(h $\cdot$ μ g) ($n = 3$; $p < 0.04$) but had no significant effect on ventricular end-diastolic perimeter, shortening, and wall area.

The basal O₂ uptake of the nonbeating loaded ventricle was 0.23 $\pm$ 0.02 ($n = 3$) nmol O₂/(h $\cdot$ μ g protein) and did not depend on exogenous glucose.

Inhibition of G3PDH

Whatever the stage investigated and glucose concentration, O₂ uptake was not modified by blockade of glycolysis during at least 40 min (Fig. 3 and Table 5). However, in nine hearts out of 16, O₂ decreased by approximately 30% after 80 min of exposure to IAA, whereas it was stable in the rest. In substrate-free medium, all the hearts tested beat regularly for at least 50 min after addition of IAA. By contrast, in the presence of glucose, hearts were stopped by IAA after 25.7 $\pm$ 1.2 min ($n = 3$) and 14.8 $\pm$ 1.2 min ($n = 4$) at stage 20 and 24HH, respectively. At stage 11HH, however, neither O₂ consumption nor contractions were altered by IAA (Fig. 3). Glycogen content determined after 2 h of activity in substrate-free medium was not affected by IAA at stages 11 and 20HH and was only slightly increased at stage 24HH (Table 5).

DISCUSSION

Metabolic Conditions In Vitro

At the investigated stages, the hearts lack vascularization and all the myocardial demands are met by diffusion. Under our *in vitro* conditions, although epicardium progressively covers the heart from stage 19 to 26HH (36, 37), the cardiac tissue was adequately oxygenated, taking into account that 1) O₂ consumption was not dependent on the perfusion rate and increased significantly after mitochondrial stimulation, 2) Po₂

was buffered by Hb, 3) the silicone membrane had a high permeability to O₂ and CO₂, and 4) HR remained constant during stop-flow periods, which is not observed when the hearts become hypoxic (30, 33). Accumulation of metabolites during stop-flow had no effect on O₂ uptake, because renewal of the culture medium did not alter O₂ uptake and buffering capacity was sufficient as discussed elsewhere (16, 19). Furthermore, HR as well as O₂ consumption of atria, ventricle, and conotruncus did not vary for at least 2 h in the presence or

absence of substrate, attesting the remarkable metabolic stability of the hearts contracting spontaneously in the culture chamber.

Regional Differences in Myocardial Oxidative Capacities

Our results show that oxidative capacity within the embryonic heart was higher in atria, was independent of exogenous glucose, and culminated at stage 20HH. The findings that normalized glycogen content and glycogenolytic rate were the highest in atria clearly indicate that sinoatrial tissue could better tolerate combined O₂/substrates deprivation. This is supported by the observations that sinoatrial spontaneous pace-making activity can persist under anoxia, whereas ventricle and conotruncus stop (12, 33, 38). Thus, rhythmic activity of the sinoatrial region could be related to glycogenolytic metabolism, whereas myocardial contractility could depend preferentially on oxidative phosphorylation because mechanical loading increased O₂ consumption without modifying glycogenolysis and lactate production. High glycogen concentration has also been reported in the atrium of the 4–12-wk-old human embryonic heart (9).

The lowest glycogenolytic rate and the highest reserve of oxidative capacity were found in conotruncus. This surprising finding could be explained at least partly by the fact that during early cardiogenesis, the primitive myocardial cells of the conotruncus wall differentiate into fibroblast phenotype or smooth muscle (39), which is known to display a high glycolytic metabolism with exogenous glucose as the main substrate (40). It should also be mentioned that conotruncus is a zone in which electromechanical delay and contraction time are the longest (41) and in which extensive programmed cell death (42) could result in mitochondrial dysfunction. Furthermore, expression of the energy-consuming SR Ca²⁺-ATPase is the lowest in the conotruncal region (43). By contrast, in atrial and ventricular myocardium, cells continue to differentiate into functional aerobic cardiomyocytes and should use optimally their oxidative capacities and glycogen stores to improve their mechanical performances, especially under loaded conditions.

Figure 1. The unloaded embryonic heart displayed high reserves of oxidative capacity. (a) Ventricular transmurial O₂ flux (left-hand ordinate, closed circles) and beating rate (right-hand ordinate, open squares) before and after mitochondrial uncoupling with DNP in representative experiments. (b) Increase of O₂ uptake induced by DNP was expressed as percent of control values. A indicates atrium; V, ventricle; CT, conotruncus. Values are mean $\pm$ SEM. Three hearts have been investigated at each stage; glucose, 8 mmol/L; ¶, vs stage 11HH ($p < 0.05$); *, vs A or V ($p < 0.05$) (ANOVA).

Table 5. Developmental changes in metabolic parameters and effects of blockade of glycolysis in presence or absence of glucose

Stage	CONTROL			IAA		
	11HH	20HH	24HH	11HH	20HH	24HH
glucose 8 mmol/L						
Oxidative capacity nmol O ₂ /(h·μg)	0.29 $\pm$ 0.06 (3)	0.40 $\pm$ 0.01 (4)	0.23 $\pm$ 0.01 (4)	0.29 $\pm$ 0.06(3)	0.39 $\pm$ 0.02 (4)	0.21 $\pm$ 0.02 (4)
Lactate production nmol/(h·μg)	0.87 $\pm$ 0.16 [4]	1.20 $\pm$ 0.09 [7]	0.64 $\pm$ 0.10 [5]	0.52 $\pm$ 0.19[4]	0.13 $\pm$ 0.06*[4]	0.11 $\pm$ 0.03* [4]
Aerobic ATP nmol/(h·μg)	1.81 $\pm$ 0.10(31)	2.55 $\pm$ 0.07(33)	1.46 $\pm$ 0.05 (32)	nd	nd	nd
Glycolytic ATP nmol/(h·μg)	0.81 $\pm$ 0.23 [3]	1.25 $\pm$ 0.11 [5]	0.63 $\pm$ 0.13 [4]	nd	nd	nd
no substrate						
Glycogen content after 2 h nmol GU/μg	0.23 $\pm$ 0.03[28]	0.49 $\pm$ 0.02[42]	0.42 $\pm$ 0.01(127)	0.16 $\pm$ 0.03[6]	0.52 $\pm$ 0.02[17]	0.54 $\pm$ 0.03*(31)
Lactate production nmol/(h·μg)	0.32 $\pm$ 0.06 [9]	0.23 $\pm$ 0.02[17]	0.27 $\pm$ 0.02 [23]	0.26 $\pm$ 0.03[4]	0.06 $\pm$ 0.02*[5]	0.04 $\pm$ 0.004*[6]

Mean $\pm$ SEM; IAA, 100 μM; (), no. of individual hearts; [], no. of determinations on pooled hearts; *vs control ($p < 0.003$, t test).

Figure 2. Oxidative capacity of the ventricle was related to wall stretch and HR. (a) Effects of mechanical loading. Ventricular oxidative capacity and shortening varied according to the load (values obtained in absence and presence of glucose were pooled because they were not significantly different). Beating rate of the loaded hearts was 128 ± 6 and 160 ± 6 at stage 20 and 24HH, respectively. Mean $\pm$ SEM; (), number of hearts investigated; *, $p \leq 0.006$ vs unloaded (paired t test). (b) Effects of beating rate. Values of ventricular oxidative capacity ($n = 26$) were determined at stage 24HH in seven loaded individual hearts deprived of exogenous substrate. Equation of the linear regression is $y = 0.001x + 0.232$, with a r^2 of 0.67 (y and x are expressed as nmol O₂ / (h·μg) and beats/min, respectively). The values obtained at 0, 150, and 200 beats/min were significantly different ($p \leq 0.03$).

Determinants of the Ventricular O₂ Uptake

At stage 24HH, loading accelerated the heart up to 160 beats/min, which corresponds to the *in ovo* HR (44), and increased the ventricular oxidative metabolism by 75%. This HR acceleration (30%) could partly be explained by wall

Figure 3. Blockade of glycolysis did not affect myocardial O₂ uptake but could prevent contractions. Representative ventricular transmural O₂ flux (left-hand ordinate, closed circles) and beating rate (right-hand ordinate, open squares) are reported before and during exposure to IAA (100 μmol/L). Oxidative and contractile activities were dissociated by IAA at stages 20 and 24HH in contrast with stage 11HH. Glucose, 8 mmol/L; unloaded ventricle.

distension (attested by the increase of wall area and end-diastolic perimeter), resulting in stimulation of stretch-activated ion channels known to be present at this stage (45). According to the relationship in Figure 2b, such an acceleration would increase ventricular O₂ uptake by only 13%. Consequently, the specific effect of mechanical loading on oxidative metabolism represented 62%, which is less than in mammalian neonatal ventricle (46). Although changes in volume and developed pressure were not measurable in our experimental conditions, it is reasonable to consider that loading the ventricle increased wall stretch up to physiologic level. This is attested by end-diastolic and end-systolic perimeters (Table 3) comparable to values determined *in ovo* (34). Whether length-dependent activation of the sarcomeres occurs in embryonic cardiomyocytes is still unknown. Our data, however, show that myocardial fiber tension is also a major determinant of the O₂ consumption in the embryonic heart. Furthermore, at stage 20–24HH, the ventricle contracting in the loaded state appears to use its maximal oxidative capacity, because its transmural O₂ flux was comparable to that reached after mitochondrial uncoupling. By comparison, adult heart can use up to 90% of its maximal oxidative capacity (47).

Loading the ventricle increased the index of mechanical activity by 106 and 88% and increased concomitantly oxidative metabolism by 25 and 75% at stages 20 and 24HH, respectively (Fig. 2a). Thus, at stage 24HH, aerobic ATP synthesis seems to be mostly related to mechanical activity as in the adult. By contrast, at stage 20HH, the dissociation between aerobic ATP synthesis and mechanical activity could be partly explained by the fact that the myocardial capacity to produce glycolytic ATP from exogenous glucose is 2-fold higher than at stage 24HH (Table 5).

The fact that increasing HR augmented O₂ uptake only when glucose was lacking (Fig. 2b) suggests that under physiologic conditions, *i.e.* working in the presence of glucose, glycolysis rather than mitochondrial oxidations should provide extra energy when the heart accelerates. In the embryonic chick heart, end-systolic pressure increases with HR (48), suggesting that wall stress and energy consumption should increase when the heart accelerates *in vivo*. Such a relationship between energy expenditure and cardiac frequency is also illustrated in Figure 2b. As in our preparation, increasing HR does not alter myocardial suprabasal O₂ consumption in isolated immature rabbit heart perfused in the presence of glucose (49). Thus, during the

embryonic/fetal life, the capability of the working myocardium to use preferentially energy derived from glycolysis could also protect the cardiovascular function against occasional hypoxic stress. Furthermore, utilization of exogenous glucose is energetically more advantageous than endogenous carbohydrate. We have previously shown that oxidizable substrates such as palmitate or pyruvate alter neither glycogenolysis nor O_2 consumption of the embryonic heart and that glucose derived from glycogen appears to be the sole substrate used by the embryonic myocardium when glucose is lacking (11, 26).

In the loaded heart at stage 24HH, the ratio of basal to total ventricular O_2 consumption was rather large (68%) compared with the ratio of 30 to 50% found in neonatal (49) and adult hearts (50–52). This could be partly explained by the important energy consumed by biosynthetic activity in the rapidly growing heart. Indeed, the latter passed from 19 to 68 μg of protein within 24 h between stages 20 and 24HH. Reducing equivalents required for these biosyntheses are likely provided by the pentose-phosphate pathway, which is especially active in the rapidly growing avian (21–23) and mammalian (3) hearts.

Developmental Changes in Sensitivity to Metabolic Inhibitors

At stage 11HH, the remarkable stability of beating rate and amplitude of contraction throughout exposure to metabolic inhibitors (IAA or DNP) could be related to developmental differences in excitation-contraction coupling (43, 53) in tolerance to acidosis (54, 55) or in utilization of a preexisting pool of intracellular ATP (19). By contrast, at stage 20–24HH, the IAA-induced dissociation between O_2 consumption and contractile activity was rapidly achieved only in the presence of glucose. This effect of glucose could result from deleterious upstream accumulation of phosphorylated glycolytic intermediates. Indeed, the latter are known to alter SR calcium release through ryanodine receptors (56) that appear to be functional at these stages (30, 43). Inhibition of glycolysis by IAA is also known to suppress spontaneous beating and contractile activities more rapidly in fetal than in adult heart (57).

It is generally accepted that the rapid respiratory rate of mitochondria in the active state (state 3) can increase further by approximately 50% in the presence of a specific uncoupling agent such as DNP, although ATP synthesis is no longer coupled to respiration. Thus, at stage 11HH, myocardial mitochondria appeared to be less active because the DNP-induced stimulation of respiration is higher than at stage 20–24HH, *i.e.* 80 to 130% *versus* 25 to 80% (Fig. 1). Furthermore, the conotruncus systematically displayed the highest DNP-induced stimulation of O_2 uptake whatever the stage. At stage 20–24HH, the biphasic change in HR induced by DNP (Fig. 1) should not affect the biphasic change in O_2 uptake because there was no correlation between HR and O_2 uptake under the experimental conditions, *i.e.* in the presence of glucose.

Regarding the dissociation between O_2 uptake and contractile activity induced by DNP, it is difficult to interpret our results apart from the facts that ATP could be rapidly depleted and/or that DNP could hyperpolarize the membrane and suppress action potentials, possibly through activation of ATP-

sensitive channels that play a major role in cellular excitability during early cardiogenesis (58). Maximalization of mitochondrial respiration also enhances generation of reactive O_2 species (59) that could hasten contractile dysfunction.

Metabolic Pathways in the Embryonic Myocardium

Inhibition of G3PDH by IAA almost totally suppressed lactate production but altered neither O_2 consumption nor glycogenolytic rate. These surprising findings provide indirect evidence that another pathway than glycolysis is involved in O_2 consumption and glycogen utilization. Indeed, when G3PDH is inhibited, dihydroxyacetone-phosphate could accumulate upstream and represent a substrate for the glycerol-phosphate shuttle pathway. The latter may contribute to transport of reducing equivalents into mitochondria of the embryonic chick myocardium as it does in neonatal mammalian heart (60) and compensate for the low activity of the Krebs cycle and malate-aspartate shuttle (2). Such a concept is also supported by the fact that at stage 24HH, the theoretical O_2 uptake dependent on the glycerol-phosphate shuttle corresponds to the actual value of 0.25 nmol $O_2/(h \cdot \mu g)$ (assuming that glucose derived from glycogen is entirely transformed into dihydroxyacetone-phosphate and that 1 atom of oxygen is consumed per mole of reducing equivalent transported via the glycerol-phosphate shuttle).

Our findings indicate also that mechanical activity is dependent on aerobic metabolism early during cardiogenesis but can be temporarily sustained by glycolytic ATP synthesis. Indeed, inhibition of the respiratory chain (61, 19) and mitochondrial uncoupling (this study) suppress contractions of myocytes in 2- to 10-d-old embryonic chick hearts after a delay of 10–30 min even in the presence of glucose. Nevertheless, oxidative capacity and normalized lactate production varied in parallel and both culminated at stage 20HH (Table 5), suggesting that cellular oxidations and glycolytic activity are tightly coupled when glucose is present. Indeed, in the embryonic chick heart, we found that about 30% of the total ATP could be derived from aerobic lactate production whatever the stage investigated. This proportion is close to the value of 22% found in fetal guinea pig heart (2) but much higher than in the adult heart, *i.e.* 5% (62). This feature could explain the fact that in embryonic cardiomyocytes, ATP concentration can remain unaffected by rather low Po_2 (63).

Mechanical Efficiency of the Embryonic Heart

Between stage 11 and 24HH, the peristaltic-contracting heart tube is transformed into contracting compartments, the geometry of which changes dramatically. Our data show that in loaded hearts, the total energy expenditure (aerobic + glycolytic) per beat decreased from 0.6 to 0.3 pmol ATP $\cdot$ beat $^{-1} \cdot \mu g_{\text{protein}}^{-1}$, whereas the index of mechanical activity increased by approximately 50% between stage 20 and 24HH, and, besides, cardiac work is known to increase markedly from stage 24HH onward (18). Consequently, as suggested in our previous article (19), the mechanical (contractile) efficiency of the cardiac pump does increase with development and could be correlated with maturation of mitochondrial machinery, tran-

sition from random arrangement of myofibrils to organized sarcomeres, increased density of myofilaments (64), and changes in types of contractile proteins (65). Furthermore, changes in organization of the myofibrillar system, as those described in the young developing heart (64), are thought to control the activity of glycolytic enzymes associated with actin (66). It has also been shown in chick embryo cardiomyocytes that glycolytic enzymes (e.g. aldolase and G3PDH) are associated with actin filaments (66), suggesting that ATP necessary to assure rapid cross-bridge cycling may be provided preferentially by glycolytic activity associated with myofibrils. Such a metabolic microcompartmentation, also proposed in neonatal ventricular myocytes (67), could contribute to improve efficiency.

In conclusion, this study has established for the first time the topography of both the oxidative and glycogen metabolism in the developing chick heart in the unloaded and loaded state. It emerges from our results that the efficiency of the myocardium to use its oxidative and glycogenolytic capacities is the highest in atrium and that ventricular wall stretch represents an early determinant of the myocardial O_2 uptake. Our results also provide indirect evidence for an active glycerol-phosphate shuttle involved in the transport of reducing equivalents into mitochondria of the embryonic myocardium.

Acknowledgments. The authors thank Michel Jadé for the construction of the miniaturized culture chamber and Christian Häberli and André Singy for the construction of the electronic hardware.

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