Differential contribution of mitochondria, NADPH oxidases, and glycolysis to region-specific oxidant stress in the anoxic-reoxygenated embryonic heart

Eric Raddatz,1 Anne-Catherine Thomas,1 Alexandre Sarre,1,2 and Messod Benathan3

1Department of Physiology, Faculty of Biology and Medicine, and 2Cardiovascular Assessment Facility, University of Lausanne, Lausanne; and 3Department of Plastic and Reconstructive Surgery, University Hospital, Lausanne, Switzerland

Submitted 20 August 2010; accepted in final form 22 December 2010

Raddatz E, Thomas AC, Sarre A, Benathan M. Differential contribution of mitochondria, NADPH oxidases, and glycolysis to region-specific oxidant stress in the anoxic-reoxygenated embryonic heart. Am J Physiol Heart Circ Physiol 300: H820–H835, 2011. First published December 30, 2010; doi:10.1152/ajpheart.00827.2010.—The ability of the developing myocardium to tolerate oxidative stress during early gestation is an important issue with regard to possible detrimental consequences for the fetus. In the embryonic heart, antioxidant defences are low, whereas glycolytic flux is high. The pro- and antioxidant mechanisms and their dependency on glucose metabolism remain to be explored. Isolated hearts of 4-day-old chick embryos were exposed to normoxia (30 min), anoxia (30 min), and hyperoxic reoxygenation (60 min). The time course of ROS production in the whole heart and in the atria, ventricle, and outflow tract was established using lucigenin-enhanced chemiluminescence. Cardiac rhythm, conduction, and arrhythmias were determined. The activity of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase as well as the content of reduced and oxidized glutathione were measured. The relative contribution of the ROS-generating systems was assessed by inhibition of mitochondrial complexes I and III (rotenone and myxothiazol), NADPH oxidases (diphenylene iodonium and apocynine), and nitric oxide synthases (N-monomethyl-l-arginine and N-iminoethyl-l-ornithine). The effects of glycolysis inhibition (isoacacetate), glucose deprivation, glycogen depletion, and lactate accumulation were also investigated. In untreated hearts, ROS production peaked at 10.8 ± 3.3, 9 ± 0.8, and 4.8 ± 0.4 min (means ± SD; n = 4) of reoxygenation in the atria, ventricle, and outflow tract, respectively, and was associated with arrhythmias. Functional recovery was complete after 30–40 min. At reoxygenation, 1) the respiratory chain and NADPH oxidases were the main sources of ROS in the atria and outflow tract, respectively; 2) glucose deprivation decreased, whereas glycogen depletion increased, oxidative stress; 3) lactate worsened oxidant stress via NADPH oxidase activation; 4) glycolysis blockade enhanced ROS production; 5) no nitrosative stress was detectable; and 6) the glutathione redox cycle appeared to be a major antioxidant system. Thus, the glycolytic pathway plays a predominant role in reoxygenation-induced oxidative stress during early cardiogenesis. The relative contribution of mitochondria and extramitochondrial systems to ROS generation varies from one region to another and throughout reoxygenation.

reactive oxygen species; glutathione redox cycle; glycogen; nitrotyrosine; antioxidant enzymes
the availability of glucose-6-phosphate deriving from either external glucose (a major energy substrate) or glycogen stores (58). The latter is 10 times higher in the embryonic/fetal myocardium than in the adult heart (86) and is critical for normal heart development (54). It is not known whether glucose deprivation, glycogen depletion, or lactate accumulation, mimicking ischemic conditions, can influence ROS production in the stressed embryonic heart.

We first aimed to characterize the myocardial endogenous antioxidants and determine the time course of ROS production in the atria, ventricle, and outflow tract of the embryonic heart model throughout anoxia-reoxygenation. Second, we assessed the relative contribution of the mitochondrial and extramitochondrial sources of oxyradicals and the implication of the glycolysis pathway in reoxygenation-induced oxidative stress.

**MATERIALS AND METHODS**

**Drugs and Chemicals**

Lucigenin chloride, diphenylene iodonium (DPI; flavoprotein inhibitor), apocynine (APO; NADPH oxidase inhibitor), rotenone (ROT; inhibitor of mitochondrial complex I), myxothiazol (MYXO; inhibitor of mitochondrial complex III), iodoacetate (IAA; blocker of GAPDH), l-arginine [nitric oxide (NO) synthase (NOS) substrate], N-(2-mercaptopropionyl)glycine (MPG; antioxidant), N-acetyl-L-cysteine (NAC; antioxidant), and all other standard chemicals were purchased from Sigma-Aldrich. N-monomethyl-L-arginine (L-NMMA; NOS inhibitor) and N-iminoethyl-L-ornithine (L-NIO; NOS inhibitor) were from Alexis. SOD, CAT, NADH, and NADPH were from Roche. All pharmacological agents used and their respective targets are shown in Fig. 1.

**Preparation and In Vitro Mounting of the Heart**

This investigation fully conformed with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society and was approved by the veterinary authorities of the State of Vaud, Switzerland.

Fertilized eggs from Lohman Brown hens were incubated during 96 h at 38°C and 95% relative humidity to obtain stage 24HH embryos according to Hamburger and Hamilton (34). Hearts were carefully excised from the embryo and dissected to isolate the atria, ventricle, and outflow tract (conotruncus). Spontaneously beating hearts or isolated parts were placed in the culture compartment of a stainless steel airtight chamber. The latter was equipped with two windows for observation and measurements and was maintained...
under controlled conditions on the thermostabilized stage (37.5°C) of an inverted microscope (IMT2 Olympus, Tokyo, Japan) as previously described in detail (55, 61). Briefly, the culture compartment (40 μl) was separated from the gas compartment by a 15-μm transparent and gas-permeable silicone membrane (RTV 141, Rhône-Poulenc, Lyon, France). Hearts or dissected parts were slightly flattened by the silicone membrane, and the resulting thickness of the myocardial tissue facing the gas compartment was ~300 μm. Thus, PO2 at the tissue level could be strictly controlled and rapidly modified (within <5 s) by flushing high-grade gas of selected composition through the gas compartment. At the investigated stage, the heart lacks coronary vascularization, and the myocardial oxygen requirement is met exclusively by diffusion.

The HCO3/CO2-buffered medium was composed of (in mmol/l) 99.25 NaCl, 0.3 NaH2PO4, 10 NaHCO3, 4 KCl, 0.79 MgCl2, 0.75 CaCl2, and 8 D-glucose. This culture medium was equilibrated in the chamber with 21% O2-2.3% CO2-balance N2 (normoxia), 97.7% O2-2.3% CO2 (hyperoxia), or 97.7% N2-2.3% CO2 (anoxia), yielding a pH of 7.4.

Functional Recording

The ECG and shortening at the level of the atria and ventricle (apex) were recorded simultaneously in vitro using a computerized technique as previously described (62, 66). Briefly, the ECG, which presented the P, QRS, and T components, was used to determine atrioventricular conduction and characterize arrhythmias while contractions of the atria and ventricle were optically detected as edge motion of the myocardial wall.

**Anoxia-Reoxygenation Protocol**

Hearts were allowed to stabilize for 45 min under normoxia and then subjected to 30 min of anoxia followed by 60 min of hyperoxia. Hearts were made hyperoxic upon reoxygenation to exacerbate all possible intracellular prooxidant systems. Under our experimental conditions, catabolites, such as lactate and protons, accumulated in the medium during anoxia, but the PCO2 remained stable as there was a continuous flushing of the gas compartment by 97.7% N2-2.3% CO2.

**Lucigenin-Enhanced Chemiluminescence**

Freshly isolated hearts were loaded in lucigenin (0.1–10 mM) for 1 h at room temperature and then mounted in the thermostabilized (37.5°C) culture chamber as previously described (55, 61).

**Detection of Oxidizing Reactions During Anoxia-Reoxygenation**

The time course and spatial pattern of myocardial production of ROS [mainly superoxide anion (O2·−)] were determined by lucigenin-enhanced chemiluminescence (CL) using a home-made thermostabilized luminometer well adapted to the culture chamber (Fig. 2A). This equipment was perfectly light tight, and all manipulations were performed from the exterior without total darkness in the laboratory. The photon counter was a highly sensitive photomultiplier tube (PMT sensor module with an embedded microcontroller and RS-232-C interface, type HC135-01, Hamamatsu) and was connected to a computer for data acquisition and processing. The PMT box was cooled at 4°C, and the abutting box containing the preparation...

![Fig. 2. Oxidant stress in the anoxic-reoxygenated embryonic heart detected by lucigenin-enhanced chemiluminescence (CL). A: time course of CL (in photons/s) obtained with the standard protocol of anoxia-reoxygenation. Three hearts at stage 24HH were pooled in the culture chamber (10 mM lucigenin). B: CL measured under preanoxia, at the peak, and during the hyperoxic steady state as well as the integrated CL during the first 60 min of reoxygenation were linearly related to the number of hearts pooled in the culture chamber. Values were corrected for background. C: peak and integrated CL normalized for protein content were linearly related to lucigenin concentration ranging from 0.1 to 10 mmol/l. Three hearts were pooled in the culture chamber. Numbers in parentheses are numbers of determinations. r², Coefficient of determination.](http://ajpheart.physiology.org/)

---

**H822 OXIDANT STRESS IN THE ANOXIC-REOXYGENATED EMBRYONIC HEART**

**AJP-Heart Circ Physiol** • **VOL 300** • **MARCH 2011** • www.ajpheart.org
maintained at 37°C. The input window of the photon detector was placed close (~1 cm) to the window of the chamber containing the spontaneously contracting hearts, and the counts were integrated over 1-s periods (sampling rate of 1/s). The background signal (dark current + lucigenin activity) was 18 ± 7 photons/s (mean ± SD; n = 400 data). To manipulate the preparation in the ambient light without overloading the photomultiplier, a rotary shutter separated the detector window from the culture chamber. One of the main advantages of this setup, besides its sensitivity, is that the Po2 at the tissue level as well as the composition of the culture medium could be continuously and strictly controlled. The chamber (was connected to a system of light-tight tubings, allowing the gas compartment of the chamber to be flushed with high-grade gas of selected composition and the culture medium to be replaced using remote-controlled pull-push syringes. Lucigenin was added to the medium at a final concentration ranging from 0.1 to 10 mM and did not interfere with the cardiac activity. The standard lucigenin concentration used was 10 mM to significantly increase the signal-to-noise ratio. Generally, each determination of CL was performed with either three hearts, six atria, three ventricles, or eight outflow tracts pooled in the culture chamber to investigate comparable amounts of myocardial tissue. All reported CL values were corrected for the background signal obtained under anoxia.

That the CL signal was proportional to the mass of cardiac tissue present in the culture chamber at a given concentration of lucigenin shows that it precisely reflected the flux of ROS generated by the hearts (Fig. 2f). Furthermore, the fact that the CL signal normalized for protein was linearly related to lucigenin concentration (Fig. 2C) indicates that the amplifying reaction was convenient under our experimental conditions.

**Determination of Endogenous Antioxidant Systems**

**CAT activity.** CAT activity was measured spectrophotometrically according to Aebi (1) with minor modifications. Because of their minute size, 70–70 hearts at stage 24HH were pooled, homogenized in phosphate buffer using a pestle, sonicated, and then centrifuged (1 h, 30,000 rpm) at 0–4°C. The decrease of absorbance initiated by the addition of the supernatant sample was measured at a wavelength of 240 nm with H2O2 as the substrate. A standard curve was established using CAT from beef liver, and linearity of the method was tested with increasing volumes of supernatant.

CAT activity was reported as millimoles per microgram of myocardial protein.

**SOD activity.** SOD activity (SOD1, SOD2, and SOD3) was determined spectrophotometrically according to Paoletti and Mocali (52). Briefly, 40 hearts were pooled, homogenized, and sonicated in triethanolamine-diethanolamine (TEA-DEA) buffer (pH 7.4) and centrifuged (1 h, 30,000 rpm), and the supernatant dialyzed for 4 h against phosphate buffer. All procedures were performed at 0–4°C. The decrease of absorbance after the addition of the sample and mercaptooethanol was measured at 340 nm at room temperature in TEA-DEA buffer containing NADPH, EDTA, and MnCl2.

SOD activity was reported as millimoles per microgram of myocardial protein.

**Glutathione reductase cycle.** To assess the components of the glutathione reductase cycle present in the embryonic heart, glutathione peroxidase (GPx) activity, GRx activity, GSH, GSSG, and total glutathione were determined.

Glutathione-related enzymes were tested as previously described (10). Four embryonic hearts were extracted in 200 μl of cold 0.125 M phosphate and 0.625 mM EDTA buffer (pH 7.2) using a tissue homogenizer. After centrifugation, 50 μl of supernatant were tested for each enzyme. Test mixtures for the determination of GRx activity contained 0.17 mM NADPH and 2.2 mM glutathione disulphide and were incubated at 37°C. GPx activity was tested at 25°C in the presence of 0.34 U/ml GRx from baker’s yeast (Sigma), 5 mM EDTA, 3.75 mM sodium azide, 5 mM GSH, 0.28 mM NADPH, and 75 mM H2O2. The oxidation of NADPH was followed at 340 nm using a DU-65 Beckmann spectrophotometer equipped with a thermostated Auto 6 sampler. Enzyme activities were estimated from the linear part of the kinetic curves. One milliunit of enzyme activity corresponds to one nanomole of NADPH oxidized per minute. Specific activities are expressed as milliunits of enzyme activity per milligram of protein.

GSH was determined after four hearts were extracted in 200 μl of cold 0.25 M perchloric acid. After centrifugation of the protein precipitate, the supernatant was tested by HPLC (Hewlett-Packard Series 1050) with electrochemical detection (BAS LC-4C). GSH was separated from cysteine using phase II ODS 3 mm (100 × 3.2 mm) and 0.1 M monochloroacetic acid and 3.3 mM 1-heptanesulphonic acid (pH 2.6). The flow rate of the mobile phase was 0.7 ml/min. Detection was performed with a Hg-Au electrode set at a working potential of +150 mV versus an Ag/AgCl reference electrode. Results are expressed as nanomoles of GSH per milligram of protein.

Total glutathione and GSSG were determined using the recycling assay of Tietze (84). Ten hearts were extracted with 200 μl of cold 0.1 M hydrochloric acid using a sonicator. After centrifugation, the supernatant (1 volume) was mixed with 5% 5-sulphosalicylic acid (5 min). A 5% trichloracetic acid solution in water (1 volume) of the protein precipitate was separated by centrifugation. For both assays, 50 μl of supernatant were determined against a calibration curve of 8–40 nmol GSH/ml or 4–20 nmol GSSG/ml.

**Nitrotyrosine Immunoblot Analysis**

It is well known that the combination of O2− and NO produced during reoxygenation can result in the formation of peroxynitrite (ONOO−), which, in turn, leads to the nitration of protein tyrosine and formation of nitrotyrosine (an index of ONOO−-induced protein alteration). The tendency of embryonic cardiac tissue to form nitrotyrosine under oxidative and/or nitrosative stress was assessed by exposing hearts to either hyperoxia (97% O2, 150 min) in the presence of a NO donor [diethylentriamine (DETA)-NONOate, 100 μM] or anoxia-reoxygenation in the absence or presence of a NOS inhibitor (N-NO, 5 mM). After each experimental protocol, three hearts were pooled, sonicated (3 × 3 s at 4°C) in sample buffer (2.4 mg protein/ml), and stored at −20°C for a subsequent immunoblot assay according to Baechtold et al. (5) with minor modifications. Briefly, heart homogenates were fractionated by SDS-PAGE, and Western blot analysis was performed using anti-nitrotyrosine mouse monoclonal IgG incubated overnight at 4°C as the primary antibody and goat anti-mouse IgG horseradish peroxidase conjugate (goat polyclonal IgG, Upstate Biotechnology) as the secondary antibody. The negative control was BSA, and the positive control was nitrated BSA, nitrated bovine SOD, and nitrated dimer of SOD. Immunodetection and quantification were performed by enhanced CL and using Image Master Total Lab Software (Amersham Biosciences).

**Myocardial Glycogen Depletion**

To know whether the postanoxic ROS production depended on glycogen content, hearts were depleted in glycogen by normoxic preincubation during 2 h at 37.5°C in substrate-free medium, whereas control hearts were preincubated in 8 mM glucose. Control or depleted hearts were then mounted in the culture chamber without glucose and subjected to anoxia and reoxygenation to determine the time course of ROS production.

**Protein, Glycogen, and Lactate Determination**

Protein, glycogen, and lactate were determined according to our previous work with slight modifications (58). Briefly, protein content was determined according to Lowry et al. (44) using BSA as the standard. Glycogen content was determined spectrofluorometrically using an automated setup and expressed as glucose units according to Nahorski and Rogers (49). Before dissection and storage at −20°C, all

**References**

H823 AJP-Heart Circ Physiol • VOL 300 • MARCH 2011 • www.ajpheart.org
excised hearts were thoroughly rinsed at 0–4°C (60 min on a rotary shaker) in glucose-free solution to eliminate all traces of glucose remaining in the cardiac cavities and that could interfere with ulterior measurements. Glycogen and protein were determined in the same hearts or cardiac regions. After being thawed, all samples were sonicated (3 × 2 s on an ice bath) for biochemical determinations. Lactate produced by the hearts in the culture medium after anoxia-reoxygenation was measured spectrophotometrically according to Rosenberg and Rush (59) and normalized for protein content.

Statistical Analysis

All values are reported as means ± SD. The significance of any difference between two groups was assessed with an unpaired Student’s t-test. Differences between regions combined with treatments were determined using one-way ANOVA completed by the Tukey test. Statistical significance was defined by P values of ≤0.05.

RESULTS

ROS Production During Normoxia, Hyperoxia, and Anoxia-Reoxygenation

A typical recording of the production of oxyradicals throughout normoxia, anoxia, and hyperoxic reoxygenation is shown in Fig. 2A. Preanoxic, peak, integrated, and O2 steady-state values of CL were systematically corrected for the anoxic background. At the end of reoxygenation, the rapid return to the background signal induced by flushing N2 through the gas compartment of the chamber showed that ROS production depended strictly on O2 availability. Regardless the time point investigated, the CL signal was proportional to the number of hearts mounted in the chamber (Fig. 2B), and the peak and integrated CL, once normalized for protein, were linearly related to the concentration of lucigenin (Fig. 2C).

The low basal CL under normoxia as well as the CL during hyperoxia (Fig. 3, A and B) were constant for hours after stabilization. Hearts were also submitted to a normoxia-hyperoxia transition to assess the effect of sole hyperoxia independently of preceding anoxia (Fig. 3C). When hearts were submitted to an anoxia-normoxia transition, a modest but detectable transient increase of CL was observed (Fig. 3D), whereas an anoxia-hyperoxia transition strongly boosted ROS production (Fig. 3E). Unexpectedly, when anoxia was preceded by hyperoxia (Fig. 3F) instead of normoxia (Fig. 3E), the burst of ROS provoked by reoxygenation was attenuated. Table 1 shows that peak, integrated, and O2 steady-state CL values determined during a normoxia-hyperoxia transition represented only 38%, 31%, and 23% of those determined during an anoxia-hyperoxia episode, respectively (see Fig. 3, C and

Fig. 3. Dependence of oxidant stress on level of oxygenation. A–F: characteristic lucigenin-enhanced CL of embryonic hearts subjected to steady normoxia (A), steady hyperoxia (B), and various combinations of normoxia, hyperoxia, and anoxia (C–F). The levels of oxygenation are indicated in the inset. Stabilization is the period of time necessary to reach 37°C and metabolic steady-state in the culture chamber. Glucose was used at 8 mM. Stage 24HH hearts were used.
E). Furthermore, the contribution of MYXO-inhibitable ROS production (mainly mitochondrial) ranged from 46% to 88% of the total ROS generation, regardless the type of transition (Table 1).

None of the treatments of hearts modified CL during preanoxia relative to control hearts. Hearts exposed to DPI, APO, MPG, NAC, SOD, l-NMMA, l-NIO, NADH, and NADPH or deprived of exogenous glucose continued to beat normally under normoxia for hours. In contrast, ROT and MYXO completely stopped cardiac activity within 10–20 min, and IAA completely stopped cardiac activity within ~40 min.

In a separate series of experiments, we found that the reoxygenation-induced peak of ROS did not vary between the primary heart tube (stage 11HH, 40 h) and the septating heart (stage 25HH, 108 h) and was 0.77 ± 0.28 (n = 4), 1.07 ± 0.44 (n = 6), 0.74 ± 0.21 (n = 10), and 0.63 ± 0.08 (n = 5) photons s⁻¹ μg⁻¹ at stages 11HH, 20HH, 24HH, and 25HH, respectively.

Whatever the experimental protocol, the protein content of the whole heart at stage 24HH was 74.1 ± 9.4 μg (n = 26) and the protein content in the atria, ventricle, and outflow tract was 16.9 ± 3.6 (n = 12), 46.5 ± 4.8 (n = 12), and 12.2 ± 1.8 μg (n = 12), respectively.

Nitrosative Stress Was Not Detectable in the Embryonic Chick Heart

Nitrotyrosine, an RNS of DNT and a marker of protein nitrosylation by ONOO⁻, was detectable by immunoblot analysis already in the freshly isolated heart. However, hyperoxia (150 min, 98% O₂) combined with a NO donor at a rather high concentration (100 μM DETA-NONOate) or anoxia-reoxygenation without or with inhibition of inducible ROS by l-NIO did not alter the pattern of the basal immunoblot (not shown).

Furthermore, ROS production during reoxygenation was not altered when ROS activity was inhibited, i.e., 1,962 ± 346 (control, n = 6) versus 2,100 ± 462 (1 mM l-NMMA, n = 6) photons/μg protein, and the addition of l-arginine (10 mM) had no effect on CL relative to control.

Myocardial Antioxidant Systems

The activity of antioxidant enzymes and content of GSH, GSSG, and total glutathione were determined in whole hearts just dissected from 4-day-old chick embryos (in vivo), after 150 min of normoxia in vitro, and after anoxia-reoxygenation. Normalized activities of SOD, CAT, GRx, and GPX were 2.0 ± 0.7 (n = 5) μmol/μg, 4.4 ± 1.3 (n = 4) μmol/μg, 12.5 ± 1.5 (n = 6) μmol/mg, and 11.7 ± 1.7 (n = 6) μmol/mg, respectively. The contents of GSH and GSSG were 16.5 ± 3.1 (n = 5) and 2.7 ± 0.7 (n = 5) mmol/mg, respectively, and total GSH + GSSG was 20.0 ± 4.9 (n = 7) mmol GSH/mg. None of the components of the glutathione redox cycle was significantly modified after 150 min under normoxia or after anoxia-reoxygenation.

Functional Disturbances and Recovery

Anoxia-reoxygenation induced mainly bradycardia, atrial ectopy, atrioventricular blocks, bursting activity, and myocardial stunning, as previously described elsewhere (62). The major functional electromechanical disturbances and highest incidence of arrhythmias were associated with the burst of ROS, but cardiac activity fully recovered and arrhythmias totally disappeared after ~30 min, when ROS production stabilized (see Fig. 2A, O₂ steady state). The presence of lucigenin affected neither the baseline values nor the rate of postanoxic functional recovery compared with our previous work (66).

Mitochondrial Versus Extramitochondrial ROS Production

We established the bell-shaped relationship between reoxygenation-induced ROS production and the preceding duration of anoxia, indicating that the reintroduction of O₂ by itself may not be the only determinant of the degree of oxidative stress since the longer the preceding anoxia, the higher the ROS production (Fig. 4A). Blockade of mitochondrial complexes I or III did not totally suppressed the oxidative burst (Fig. 4B). ROS production upon reoxygenation was significantly reduced by the flavoprotein inhibitor DPI alone or combined with MYXO (Fig. 4C) and by the NADPH oxidase inhibitor APO [1,885 ± 481 (control, n = 6) vs. 962 ± 115 (5 mM APO, n = 6) photons/μg, P < 0.03]. These results indicate that extramitochondrial sources of ROS noticeably contribute to reoxygenation-induced oxidative stress. NAC (5 mM, n = 4), MPG (5 mM, n = 4), and SOD (5,000 U/ml, n = 4) significantly reduced ROS production by 26%, 36%, and 31%, respectively (P < 0.03). Additionally, APO reduced ROS production the most efficiently (49%) relative to NAC, MPG, and SOD. No extracellular NAD(P)H-dependent enzymes involved in ROS production were expected to be present in the preparation since the addition of NADH (100 μM, n = 3) or NADPH (100 μM, n = 3) in the medium affected CL neither during preanoxia nor during reoxygenation (not shown).

In a separate series of experiments, to assess the possible influence of 10 mM lucigenin on the studied mechanisms, hearts were also exposed to anoxia-reoxygenation in 100 μM lucigenin without (control) and with MYXO. The integrated CL during reoxygenation was 471 ± 104 (n = 4) and 152 ± 64 (n = 4) photons/μg protein in control and MYXO-treated hearts.
Fig. 4. Mitochondrial and extramitochondrial sources of oxyradicals. A: a bell-shaped relationship was established between the integrated CL during 60 min of reoxygenation and the duration of the preceding anoxia. B: inhibition of the mitochondrial complex I (ROX) or complex III (MYXO) reduced both the peak of ROS production (arrows) and time to peak by ~50%. C: the peak of ROS production at reoxygenation depended on both mitochondrial and extramitochondrial sources. Mitochondrial (MYXO inhibitable) and extramitochondrial (mainly DPI inhibitable) sources contributed to the burst of ROS production at reoxygenation. The vertical brackets indicate DPI-inhibitable ROS production. DPI was used at 10 μM, MYXO was used at 10 μM, and glucose was used at 8 mM. *P < 0.02 vs. control or MYXO treatment. Values are means ± SD. Numbers in parentheses are numbers of determinations.

Table 2. Glycogen content and lactate production after anoxia-reoxygenation depended on glucose availability and mitochondrial activity (myxothiazol inhibitable)

<table>
<thead>
<tr>
<th>Glucose (8 mM)</th>
<th>No Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated hearts</td>
</tr>
<tr>
<td>Glycogen content, nmol GU/μg</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>Lactate production, nmol·h⁻¹·μg⁻¹</td>
<td>0.43 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3–4 determinations of 5–8 hearts each. GU, glucose units. *P < 0.03 vs. untreated hearts; †P < 0.05 vs. glucose (8 mM)- and myxothiazol (10 μM)-treated hearts.
OXIDANT STRESS IN THE ANOXIC-REOXYGENATED EMBRYONIC HEART

Table 3. Glycogen content in the atrium, ventricle, and outflow tract after anoxia-reoxygenation

<table>
<thead>
<tr>
<th>Glycogen, nmol GU/µg</th>
<th>Atrium</th>
<th>Ventricle</th>
<th>Outflow tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated hearts</td>
<td>1.11 ± 0.10</td>
<td>0.64 ± 0.07*</td>
<td>0.36 ± 0.04†</td>
</tr>
<tr>
<td>Myxothiazol-treated hearts</td>
<td>0.69 ± 0.08†</td>
<td>0.40 ± 0.01‡</td>
<td>0.18 ± 0.05‡‡</td>
</tr>
<tr>
<td>Diphenylene iodonium-treated hearts</td>
<td>0.90 ± 0.15</td>
<td>0.56 ± 0.04*</td>
<td>0.34 ± 0.01†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4 determinations for each condition. Unlike diphenylene iodonium (10 µM), myxothiazol (10 µM) had a depleting effect on glycogen in the presence of 8 mM glucose whatever the region investigated. Glycogen content was determined at the end of reoxygenation. *P < 0.05 vs. the atrium; †P < 0.01 vs. the atrium or ventricle; ‡P < 0.01 vs. untreated hearts (by ANOVA).

Fig. 5. Effects of hyperoxia, glucose, and lactate on ROS-induced CL before and after 30 min of anoxia (peak, integration, and O2 steady state). A: when anoxia was preceded by hyperoxia (H; solid bars) instead of normoxia (N; open bars), CL increased during preanoxia but was significantly reduced during reoxygenation (peak, integration, and O2 steady state; see also Fig. 3, E and F). The latter effect was the most pronounced in the presence of 8 mM glucose. ROS production was reduced in glucose-free medium. B: CL was reduced by the removal of glucose and increased by the addition of lactate alone. Lactate-induced ROS production was abolished by DPI (inhibitor of flavin-dependent enzymes). Values are means ± SD. Numbers in parentheses are numbers of determinations. *P < 0.05; #P < 0.01 vs. no substrate.
4-day-old embryonic chick heart were of the same order as in newborn (6, 32, 78) and adult (17, 67, 95) cardiac tissue. SOD activity, although comparable with that in cardiomyocytes isolated from 10-day-old chick embryos (91), was about five-fold lower than in newborn (6, 78) or adult (8, 16, 23, 72, 88, 90) hearts, whereas CAT activity appeared to be slightly lower than in the newborn or adult myocardium.

The baseline ratio of GSH to GSSG, an indicator of cell redox status and antioxidant defense capacity, was 6.1 in the embryonic heart, which is quite comparable with the ratio in the adult heart, ranging between 5 and 9 (68, 69, 95), or even higher than in adult ventricular myocytes (27). This finding indicates that the embryonic myocardium may cope with the strong and transient oxidative stress at reoxygenation predominantly through an active glutathione redox cycle. Intracellular levels of GSH and GSSG can also regulate mitochondrial ROS production (70), which could affect the degree of reoxygenation-induced oxidant stress. In cultured cardiomyocytes from 11-day-old chick embryos, the GSH-to-GSSG ratio under normoxia was surprisingly high, i.e., 70 (57). In the latter, however, ischemia-reperfusion drastically reduced the GSH-to-GSSG ratio, whereas in our preparation as well as in the piglet heart (32), this ratio did not vary significantly after anoxia-reoxygenation. This observation suggests that the glutathione redox cycle is more efficient in the early intact heart than in cultured cardiomyocytes to maintain a stable GSH-to-GSSG redox cycle is more efficient in the early intact heart than in adult ventricular myocytes (27). This finding indicates that the embryonic myocardium may cope with the strong and transient oxidative stress at reoxygenation predominantly through an active glutathione redox cycle. Intracellular levels of GSH and GSSG can also regulate mitochondrial ROS production (70), which could affect the degree of reoxygenation-induced oxidant stress. In cultured cardiomyocytes from 11-day-old chick embryos, the GSH-to-GSSG ratio under normoxia was surprisingly high, i.e., 70 (57). In the latter, however, ischemia-reperfusion drastically reduced the GSH-to-GSSG ratio, whereas in our preparation as well as in the piglet heart (32), this ratio did not vary significantly after anoxia-reoxygenation. This observation suggests that the glutathione redox cycle is more efficient in the early intact heart than in cultured cardiomyocytes to maintain a stable GSH-to-GSSG redox cycle is more efficient in the early intact heart than in adult ventricular myocytes (27). This finding indicates that the embryonic myocardium may cope with the strong and transient oxidative stress at reoxygenation predominantly through an active glutathione redox cycle. Intracellular levels of GSH and GSSG can also regulate mitochondrial ROS production (70), which could affect the degree of reoxygenation-induced oxidant stress. In cultured cardiomyocytes from 11-day-old chick embryos, the GSH-to-GSSG ratio under normoxia was surprisingly high, i.e., 70 (57).

Thus, in the embryonic heart, GSH appears to be a major intracellular reductant, although other antioxidant systems, such as thioredoxins, thioredoxin reductase, and glutaredoxins, which catalyze disulfide reduction in the presence of NADPH, are already present in the myocardium of mouse embryos before embryonic day 8 (18, 39). GSH also serves as an important intracellular antioxidant in the heart during early postnatal development (22, 46). In contrast, myoglobin, which can also play an antioxidant role in the mature heart (29), is not yet expressed in the embryonic chick heart at stage 24HH.

### Oxidative and Nitrosative Stresses

**Oxidant stress.** Neither normoxic basal ROS production nor steady hyperoxic ROS production (Fig. 3, A and B) affected the activity of the isolated embryonic heart for at least 2 h in vitro (not shown). The normoxia-hyperoxia transition provoked a moderate increase of ROS followed by a sustained ROS production comparable with that observed under steady hyperoxia (Fig. 3, B and C). Furthermore, the three-fold increase of the integrated amount of ROS generated during the anoxia-hyperoxia transition relative to the normoxia-hyperoxia transition (Fig. 3, C and E, and Table 1) most likely reflects the combination of exacerbating and depressing effects of the preceding 30 min of anoxia on prooxidant and ROS-scavenging systems, respectively. The temporal pattern of ROS production during anoxia-reoxygenation (Figs. 2 and 3E) was quite comparable with recordings performed in the ischemic-reperfused mouse adult heart also using lucigenin-enhanced CL (29). In addition, we found that the capacity of the embryonic myocardium to produce ROS at reoxygenation was constant throughout early cardiogenesis (i.e., during 70 h between the primary heart tube to the septating heart), suggesting that the redox balance is maintained during this critical period of development.

During anoxia, the CL signal did not differ from the background signal, showing that there was no ROS production, which is consistent with our previous work (61) performed...
using the intracellular fluorescent probe DCFH. The bell-shaped relationship between reoxygenation-induced ROS production and duration of the preceding anoxia shows that the peak was reached after an anoxia lasting for 45 min, which is similar to the relationship between superoxide production at reperfusion and ischemic duration in the newborn heart (71). It is conceivable that the strict lack of O₂ beyond 45 min sufficiently alters the myocardial prooxidant systems to temporarily attenuate their activity upon O₂ reintroduction. Such alterations may be attributed to exposure of mitochondrial

Fig. 7. Heterogeneity of the oxidant stress in the embryonic heart during reoxygenation after 30 min of anoxia. A: spontaneously beating heart isolated from a 4-day-old chick embryo (stage 24HH) and mounted in the culture chamber. The dashed lines indicate the levels of cutting to prepare the isolated atria, ventricle, and outflow tract. B: ROS-enhanced CL during reoxygenation in 6 pooled atria, 3 ventricles, and 8 outflow tracts. Arrows indicate the peak of ROS production. The slope of the dotted lines (first derivative) represents the maximal acceleration of ROS. C: time to peak and normalized maximal acceleration of ROS production in the distinct cardiac parts. Values are means ± SD. Numbers in parentheses are numbers of determinations. *P < 0.05 vs. the atria and ventricle.

Fig. 8. Effects of inhibition of mitochondrial complex III (MYXO) and flavoenzymes (DPI) on ROS production during reoxygenation in the atria, ventricle, and outflow tract. The inhibitor of mitochondrial complex III (MYXO; 10 μM) and the inhibitor of flavin-dependent enzymes (DPI; 10 μM) were used alone or in combination. The effects of these agents on ROS-induced CL were different in the atria, ventricle, and outflow tract. Four to five determinations were used per condition. ★P < 0.05 vs. the untreated preparation.
complexes and NADPH oxidases to strong acidification during a prolonged anoxia followed by the slow recovery of pH during reoxygenation, as previously described (66).

Preanoxic hyperoxia increased ROS production compared with preanoxic normoxia but significantly reduced the subsequent reoxygenation-induced oxidative stress (Figs. 3, E and F, and 5A), suggesting a potentially protective effect. This observation is in line with data obtained in the adult heart showing that prooxidant hyperoxic exposure or pharmacological stimulation of mitochondrial ROS production before ischemia induces preconditioning and attenuates postischemic injury (30, 51, 60).

During reoxygenation, the residual ROS production observed after inhibition of both mitochondrial complex III and flavoenzymes, including NADPH oxidases (MYXO + DPI; Figs. 4C and 8), ranged between 11% and 47% of the total production, depending on the region investigated (the lowest percentage was in the atria). The finding that ROS production was not completely inhibited by the pharmacological agents used suggest that other O2-dependent prooxidant systems are implicated.

Nitrosative stress. Even though inducible NOS is strongly expressed and active in the embryonic chick heart and there is a significant overproduction of NO during anoxia-reoxygenation (82), it appears that the nitrosative stress was insignificant (or neutralized by effective turnover or repair) since no extra formation of nitrotyrosine was detected, even when the generation of ONOO− was pushed up using a NO donor combined with hyperoxia. Furthermore, inhibition of NOS or addition of L-arginine (NOS substrate) did not affect ROS production under our experimental conditions, suggesting that there was no uncoupling of NOS induced by anoxia-reoxygenation. These observations are corroborated by our previous work (61) using the DCFH probe, which is known to be sensitive not only to H2O2 and OH● but also to ONOO−, and show that the production of ONOO− resulting from a possible reaction between NO and O2− is negligible in the embryonic heart subjected to anoxia-reoxygenation.

Alternatively, basal NO production determined in the embryonic myocardium during normoxia (82) may lead to protein S-nitrosylation, a process known to contribute to cardioprotection, e.g., through protection of cysteine residues against oxidative stress (76). Also, physiological levels of NO protect cardiomyocytes isolated from 10-day-old chick embryo against ischemia-reperfusion-induced oxidant stress (37) and improve the postanoxic recovery of excitation-contraction coupling in the ventricle of the embryonic chick heart at stage 24HH (47).

Functional Alterations Associated With Reoxygenation-Induced Oxidative Stress

The antioxidant systems of the atria, ventricle, and outflow tract are likely the most solicited and overwhelmed during the first minutes of reoxygenation when acceleration of the ROS flux is maximal before the peak (Fig. 7), a critical period of myocardial vulnerability to oxidative injury. After the peak, steady ROS production progressively established, reflecting a possible balance between pro- and antioxidant mechanisms. Once this steady state was reached, arrhythmias ceased and electromechanical function fully recovered, suggesting that all the parts of the embryonic heart can cope with hyperoxic conditions only after achieving a new redox equilibrium. This hypothesis is strongly supported by the similarity between the values of the GSH-to-(GSH + GSSG) ratio determined at the end of reoxygenation and after 150 min of steady normoxia, i.e., 0.67 and 0.65, respectively. The remarkable tolerance of the embryonic myocardium to oxidative stress is also illustrated by the fact that the external H2O2 concentration must be as high as 1 mM to significantly impair cardiac function (31) and that the chick embryo adapts efficiently to an episode of hyperoxia during its in ovo development (74). In comparison, cultured fetal mouse cardiac myocytes stop beating in the

---

Fig. 9. Model of the relative contribution of mitochondrial and extramitochondrial sources to oxidative stress throughout reoxygenation. A: percent contribution of mitochondrial (MYXO inhibitable) and extramitochondrial (partly DPI inhibitable) ROS to the peak, integrated, and O2 steady-state CL in the atria, ventricle, and outflow tract. B: model of the time course of mitochondrial and extramitochondrial contributions to reoxygenation-induced total ROS production in the atria, ventricle, and outflow tract based on the present findings. n = 4–5.
In the presence of 50 μM H$_2$O$_2$ (50). Furthermore, the facts that glucose worsens ROS production (Fig. 5) and is arrhythmogenic at reoxygenation (85) and that the duration of ventricular arrhythmias was shortened by the antioxidant vitamin C at 10 mM (unpublished data) clearly indicate that transient oxidative stress contributes to the temporary reoxygenation-induced dysrhythmias. Endogenous vitamin C is present in the yolk sac membrane early during development of the chick (77) and may afford protection for the embryo against oxidative stress.

ROT and MYXO, as well as antimycin A (55), stopped cardiac activity within 10–30 min, showing that energy derived from anaerobic glycolysis assessed by lactate production (Table 2) cannot efficiently compensate for energy produced by oxidative phosphorylation. Thus, mitochondria play a pivotal role in the functioning of the embryonic myocardium, despite a high glycolytic capacity (58), which can meet the energy requirement only for a short period under anoxia. Additionally, the modulatory role of mitochondria in ROS production is illustrated by the fact that activation of the mitochondrial ATP-sensitive K$^+$ (K$_{ATP}$) channel augments ROS production in the embryonic ventricle upon reoxygenation (61). It has been proposed that ROS produced during ischemia-reperfusion provoke oscillations in mitochondrial metabolism and action potential duration (via sarcoplaemal K$_{ATP}$ channels), provoking arrhythmias (3). This phenomenon might also occur in the anoxic-reoxygenated embryonic heart since we recently observed that the QT interval (reflecting the duration of ventricle depolarization) is markedly altered during the phase of arrhythmic activity (63) and that a K$_{ATP}$ channel antagonist (glibenclamide) tends to prolong arrhythmias (14).

**Sources of ROS in the Atria, Ventricle, and Outflow Tract Throughout Reoxygenation**

Inhibition of mitochondrial complexex I or III partially reduced ROS production during the first 30 min of reoxygenation. It is generally accepted that ROT and MYXO inhibit ROS release by complexes I and III, respectively, although the degree of inhibition depends on the respiratory state and/or substrate of the mitochondria (79). Furthermore, inhibition of flavoenzymes by DPI (including NADPH oxidases, complex I, cytochrome P-450 reductase, and three isoforms of NOS; Fig. 4C) and inhibition of NADPH oxidases by APO reduced the oxidative burst by 30–50%. In our study, APO was used as a NADPH oxidase inhibitor; however, other studies have shown that it also has potential antioxidant activity (36) and has to undergo H$_2$O$_2$-induced dimerization to interact with the components of the NADPH oxidase complex and block its activity (73).

The generation of superoxide by the whole heart during reoxygenation was partly (25–35%) quenched by a large excess of SOD and by the antioxidant agents MPG and NAC. MPG and NAC are thiol-containing compounds and could afford protection by preserving the thiol redox status in the anoxic-reoxygenated embryonic heart, reducing the damaging effect of oxidative stress. NAC, which enhances glutathione biosynthesis by providing cystein residues, also reacts with OH$^*$ and H$_2$O$_2$, and MPG scavenges ONOO$^-$ and OH$^*$.

Regardless of the limited specificity of DPI and APO (13), these findings clearly indicate that extramitochondrial ROS-generating systems also exist in the embryonic heart and are specially active during the early phase of reoxygenation, which is corroborated by the shorter time to peak in the presence of ROT or MYXO (Fig. 4B).

Interestingly, the relative contribution of mitochondrial and extramitochondrial ROS sources to reoxygenation-induced oxidant stress varied from one region to another and throughout reoxygenation (Figs. 8 and 9). The time course of ROS production was comparable in the atria and ventricle but notably differed from that in the outflow tract (conotruncus). At the stage investigated, the outflow tract is subjected to extensive and physiological programmed cell death (89), is a hypoxic zone (75, 96), is highly sensitive to oxidative stress induced by H$_2$O$_2$ (28), and contains ~2.5-fold less glycogen than the atrium and ventricle (58). It is conceivable that the capacity of ROS detoxification in the intensively remodeling outflow tract is lower than in the atria and ventricle, with the antioxidant systems being rapidly overwhelmed upon reoxygenation when acceleration of ROS production is maximal (Fig. 7). Furthermore, the fact that the peak of ROS at reoxygenation was reached more rapidly in the outflow tract than in other parts is highly compatible with our finding that the lower the glycogen content, the higher the burst of ROS (Fig. 6, C and D). Alternatively, the facts that APO had the strongest inhibitory effect (50%) on ROS production in the whole heart and that DPI had the strongest inhibitory effect on ROS production in the outflow tract (Fig. 8, integration) suggest that NADPH oxidases can be regarded as the predominant extramitochondrial prooxidant system in the reoxygenated embryonic heart, especially in the conotruncal tissue. The decreasing gradient along the heart tube of the mitochondrial contribution to reoxygenation-induced oxidant stress is in line with our previous data showing that the atria present the highest oxidative phosphorylation, glycogen content, and glycolgenolytic capacity, whereas the outflow tract exhibits the lowest glycogen content and glycogenolytic rate (58). Furthermore, the facts that under normoxia the mitochondrial O$_2$ consumption of the atria, ventricle, and outflow tract is not altered by increasing O$_2$ delivery or by removing glucose (58), whereas ROS production was considerably increased under hyperoxia relative to normoxia, especially in the presence of glucose (Fig. 5A, preanoxia), confirm that extramitochondrial systems significantly contribute to oxidative stress. In this context, it should be mentioned that NADPH oxidases can also be activated by mitochondrial ROS, which might intensify reoxygenation-induced oxidant stress (20).

Regarding our results obtained with DCFH, it should be mentioned that this fluorescent probe, in contrast to lucigenin, is known to be preferentially oxidized by H$_2$O$_2$ or OH$^*$ but poorly by O$_2^•$- (with the short-lived superoxide being rapidly transformed to H$_2$O$_2$) (97). We (61) have previously shown that H$_2$O$_2$ and/or OH$^*$ production is predominant in the anoxia-normoxia transition (using DCFH). Our present data show that O$_2^•$- production was predominant in the anoxia-hyperoxia protocol but negligible in the anoxia-normoxia protocol (using lucigenin).

At the stage investigated, the contribution of noncardiomyocyte systems to ROS generation can be considered negligible in the atria and ventricle, which are mostly composed of proliferating and differentiating cardiomyocytes (e.g., absence of vascularization, smooth muscle cells, fibroblasts, endothelium, and extrinsic innervation), whereas it can be important in the
mentioned that myocardial O2 consumption is not altered by formation at the onset of reoxygenation (Fig. 1). It is likely that this change in redox state provides reducing equivalents resulting Ca2+/Mg2+ receptors (38), which are functional in our model (81). The developing heart and to transfer cytosolic reducing equivalents into mitochondria (at the complex II level via FADH2) to maintain the erol-phosphate shuttle, which is known to be highly active in the developing heart under anoxia but to be arrhythmogenic at reoxygenation (85), worsened oxidative stress during reoxygenation with no effect under normoxia (Fig. 5). Paradoxically, inhibition of GAPDH exacerbated ROS production upon reoxygenation, more markedly in the presence of glucose (Fig. 6B). GAPDH inhibition prevented the downstream formation of oxidizable pyruvate, leading to an upstream accumulation of glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate (Fig. 1). The fact that such glycolytic intermediates can be deleterious is supported by our previous observation that IAA stops hearts faster in the presence of glucose than in substrate-free medium (58). On the other hand, dihydroxyacetone-phosphate represents a substrate for the glycerol-phosphate shuttle, which is known to be highly active in the developing heart and to transfer cytosolic reducing equivalents into mitochondria (at the complex II level via FADH2) to maintain efficient oxidative phosphorylation when GAPDH is blocked (58, 65). The accumulation of phosphorylated glycolytic intermediates alter the sarcoplasmic reticulum Ca2+ release through ryanodine receptors (38), which are functional in our model (81). The resulting Ca2+ overload might increase mitochondrial ROS generation and finally lead to cardiac arrest within <1 h. It should be mentioned that myocardial O2 consumption is not altered by either IAA, exogenous pyruvate, palmitate, or glucose withdrawal (58). Alternatively, the shortage of pyruvate resulting from GAPDH inhibition may induce the conversion of lactate to pyruvate by lactate dehydrogenase, which, in turn, could increase the NAD(P)H-to-NAD(P)+ ratio and superoxide formation via the activation of NADPH oxidases (see Fig. 1).

In substrate-free medium, the reoxygenation-induced peak of ROS was significantly increased after glycogen depletion (Fig. 6, C and D). With glycogen being the sole source of energy substrate to be oxidized in this condition (58), a possible interpretation is that there is less glycogen-derived glucose-6-phosphate available for the pentose phosphate pathway to operate optimally, which could decrease the production of crucial reducing equivalents (NADPH) necessary for the activity of antioxidant enzymes such as GPx and GRx (see Fig. 1). Consequently, the myocardial endogenous defense against oxyradicals could be weakened when the glycogen store decreases. When lactate accumulated under anoxia in the presence of glucose or when lactate was externally added, the lactate-to-pyruvate concentration ratio increased and consequently also increased the NAD(P)H-to-NAD(P)+ ratio. It is likely that this change in redox state provides reducing equivalents to NADPH oxidases and results in DPI- or APO-inhibitable ROS formation at the onset of reoxygenation (Fig. 1).

In comparison, in the adult heart, glycolysis is protective, whereas IAA and glycogen depletion disturb mechanical activity under ischemia in rats (4) and lactate stimulates both basal and postischemic ROS production, which is partly controlled by the tissue pyruvate-to-lactate concentration ratio, in guinea pigs (7).

**Characteristics and Limitations of Lucigenin-Enhanced CL**

The principle, specificity, optimization, and limitations of lucigenin-enhanced CL have been critically reviewed in detail (12, 33, 42, 80, 94). Being aware of the difficulties in measuring low-level CL, we have taken these works into consideration for the adaptation of the method to our own model and for the interpretation of data regarding ROS/RNS. Indeed, it has been emphasized that CL should be considered as a “useful general assay” of oxidative stress (33) rather than a method allowing discrimination between radical species. In our study, lucigenin used at a high concentration to increase the signal-to-noise ratio can be regarded as a valid chemiluminescent probe for assessing ROS production and did not interfere with cardiac activity. However, the unavoidable artifacts due to redox cycling and autoxidation undergone by the molecule (94) as well as its interaction with other oxidant species than O2·− cannot be ruled out. Nevertheless, lucigenin appears to be more specific for O2·− in the isolated heart (19), cultured neonatal cardiomyocytes (24), and myocardial homogenates (56), and the use of exogenous SOD significantly reduced the CL signal by 32% in the embryonic heart. Since CL was determined with a high temporal resolution, it was possible to investigate the first seconds of reoxygenation-induced oxidative stress, a critical period of time for the reason that the antioxidant systems can be transiently overwhelmed by an abrupt burst of potentially damaging oxyradicals (as assessed by the acceleration of ROS production).

**Conclusions**

The fact that exogenous glucose, lactate accumulation, glycogen depletion, and inhibition of GAPDH significantly enhanced oxyradox production emphasizes the predominant role played by the glycolytic pathway in reoxygenation-induced disturbances observed in the embryonic heart. Furthermore, the relative contribution of mitochondria and extramitochondrial systems to ROS generation vary from one region to another and throughout reoxygenation. Our findings provide new insights into pro- and antioxidant mechanisms by which the different parts of the developing heart respond to an anoxic episode occurring during a critical period of cardiogenesis.

**Acknowledgments**

The authors thank Michel Jadé for the construction of the thermostabilized luminometer and Christian Häberli and André Singy for the construction of the electronic hardware. The authors thank Dr. Michèle Markert and Martine Vaglio for performing nitrotyrosine Western blots. Dr. Pavel Kucera is specially acknowledged for support and stimulating discussions.

**GRANTS**

This work was partly supported by Swiss National Science Foundation Grant 3100A0-105901.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**

Oxidant Stress in the Anoxic-Reoxygenated Embryonic Heart

H833


14. Cand F, Verdiotti J. Superoxide dismutase, glutathione peroxidase, cat-


69. Servidio G, Di Venosa N, Federici A, D’Agostino D, Rollo T, Prigallo F, Altomare E, Fiore T, Vendemiale G. Brief hypoxia before normoxic reperfusion (postconditioning) protects the heart against isch-


