STUDIES OF HYPOXEMIC/REOXYGENATION INJURY: WITHOUT AORTIC CLAMPING

V. Role of the L-arginine–nitric oxide pathway: The nitric oxide paradox

This study tests the hypothesis that nitric oxide, which is endothelial-derived relaxing factor, produces reoxygenation injury via the L-arginine–nitric oxide pathway in hypoxemic immature hearts when they are placed on cardiopulmonary bypass. Twenty 3-week-old piglets undergoing 2 hours of hypoxemia (oxygen tension about 25 mm Hg) on a ventilator were reoxygenated by initiating cardiopulmonary bypass (oxygen tension about 400 mm Hg). Five animals were not treated, whereas the pump circuit was primed with the nitric oxide–synthase inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME, 4 mg/kg) in five piglets. L-Arginine, the substrate for nitric oxide, was administered in a fivefold excess (20 mg/kg), together with L-NAME in five piglets (L-NAME and L-arginine), and given alone in five other piglets (L-arginine). Five normoxemic, instrumented piglets served as a control group, and five others underwent 30 minutes of cardiopulmonary bypass without preceding hypoxemia. Left ventricular contractility was determined as end-systolic elastance by pressure-dimension loops. Myocardial conjugated dienes were measured as a marker of lipid peroxidation, and the antioxidant reserve capacity (malondialdehyde production in tissue incubated with *t*-butylhydroperoxide) was measured. Nitric oxide level was determined in coronary sinus plasma as its spontaneous oxidation product, nitrite. Cardiopulmonary bypass per se did not alter left ventricular contractility, cause lipid peroxidation, or lower antioxidant capacity. Reoxygenation without treatment depressed cardiac contractility (end-systolic elastance $38\% \pm 12\%$ of control*), raised nitric oxide (127% above hypoxemic values), increased conjugated dienes (1.3 ± 0.2 vs 0.7 ± 0.1 , control*), and reduced antioxidant reserve capacity (910 ± 59 vs 471 ± 30 , control*). Inhibition of nitric oxide production by L-NAME improved end-systolic elastance to $84\% \pm 12\%$,** limited conjugated diene elution (0.8 ± 0.1 vs 1.3 ± 0.2 , no treatment**), and improved antioxidant reserve capacity (679 ± 69 vs 910 ± 59 , no treatment**). Conversely, L-arginine counteracted these beneficial effects of L-NAME, because left ventricular function recovered only $24\% \pm 6\%$,* conjugated dienes were 1.2 ± 0.1 ,* and antioxidant reserve capacity was 826 ± 70 .* L-Arginine alone caused the same deleterious biochemical changes as L-NAME/L-arginine and resulted in 60% mortality. The close relationship between postbypass left ventricular dysfunction (percent end-systolic elastance) and myocardial conjugated diene production ($r = 0.752$) provides in vivo evidence that lipid peroxidation contributes to myocardial dysfunction after reoxygenation. These results suggest the L-arginine–nitric oxide pathway, which is normally of

Kiyozo Morita, MD,^a Michael P. Sherman, MD,^b Gerald D. Buckberg, MD,^a Kai Ihnken, MD,^a Georg Matheis, MD,^a Helen H. Young, PhD,^a and Louis J. Ignarro, PhD,^c Los Angeles, Calif.

From The Departments of Surgery,^a Pediatrics,^b and Pharmacology,^c University of California at Los Angeles School of Medicine, Los Angeles, Calif.

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Address for reprints: Gerald D. Buckberg, MD, UCLA Medical

Center, Department of Surgery, B2-375 CHS, P.O. Box 951741, 10833 Le Conte Ave., Los Angeles, CA 90095-1741.

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physiologic benefit by producing endothelial-derived relaxing factor, is involved in the pathogenesis of myocardial reoxygenation injury (* $p < 0.05$ vs control; ** $p < 0.05$ vs no treatment, analysis of variance). (J THORAC CARDIOVASC SURG 1995;110:1200-11)

Low output syndrome still contributes to perioperative morbidity and mortality,^{1,2} and early ventricular dysfunction may develop or persist despite successful anatomic correction of cyanotic congenital defects.³⁻⁵ We have shown previously^{6,7} that "unintended" reoxygenation injury occurs when hypoxemic myocardium is placed on cardiopulmonary bypass (CPB) and may contribute to postbypass myocardial dysfunction. This damage may offset the immediate benefits of surgical correction.

It is accepted generally that the highly reactive hydroxyl radical ($\cdot\text{OH}$) causes oxidant injury to the myocardium.⁸ Nitric oxide ($\cdot\text{NO}$) is a physiologic modulator of vascular tone (known as endothelium-derived relaxing factor [EDRF])⁹ and limits neutrophil activation and platelet aggregation.⁷ The interaction of $\cdot\text{NO}$ and superoxide is well established,^{9,10} but a biochemical byproduct of this reaction has only recently been implicated in tissue injury.¹¹⁻¹³ Beckman and colleagues¹¹ have suggested that generation of $\cdot\text{OH}$ via the Haber-Weiss pathway may be limited in vivo and proposed that $\cdot\text{NO}$ reacts with superoxide anion (O_2^-) in pathologic states to produce peroxynitrite anion (OONO^-) and $\cdot\text{OH}$. Hogg and colleagues¹⁴ also demonstrated that simultaneous generation of $\cdot\text{NO}$ and O_2^- can lead to production of $\cdot\text{OH}$. This chemical reaction may be viewed as an alternate pathway of $\cdot\text{OH}$ generation that causes reoxygenation damage.

Our preliminary results show that myocardial dysfunction occurs after reoxygenation of hypoxemic immature hearts on the initiation of CPB and that the L-arginine- $\cdot\text{NO}$ pathway is partly involved in its pathogenesis.⁶ The present study provides further evidence that in vivo reoxygenation injury of hypoxemic immature heart is mediated in part by the $\cdot\text{NO}$ pathway and shows that this damage can be (1) limited by blocking $\cdot\text{NO}$ synthase with an L-arginine analog, N^G -nitro-L-arginine methyl ester (L-NAME), and (2) worsened by increasing $\cdot\text{NO}$ production by adding L-arginine.

Material and method

Thirty immature, 2- to 3-week-old Yorkshire-Duroc piglets (3 to 5 kg) were premedicated with 0.5 mg/kg diazepam intramuscularly, anesthetized with 30 mg/kg

pentobarbital intraperitoneally followed by 5 mg/kg intravenously each hour, and supported by a volume-limited respirator (Servo 900D, Siemens-Elema, Solna, Sweden) via a tracheostomy. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

The surgical preparation for cardiopulmonary bypass, including vessel cannulation for blood sampling and monitoring, is similar to that described previously.⁷

Evaluations

Hemodynamics. Hemodynamic measurements were made during control conditions, each 15 minutes during hypoxemia, and 15 and 30 minutes after CPB was discontinued. Cardiac output was determined by duplicate injections of 1 ml of 4°C cold saline solution into a central venous catheter. Systemic vascular resistance index (SVRI) was calculated after using the following equation:

$$\text{SVRI (mm Hg} \cdot \text{min} \cdot \text{ml}^{-1} \cdot \text{kg)}$$

$$= (\text{MAP} - \text{CVP}) (\text{mm Hg}) \cdot \text{CO}^{-1} (\text{ml/min}) \cdot \text{BW (kg)}$$

where MAP is mean aortic pressure, CVP is central venous pressure, and CO is cardiac output in liters per minute.

Left ventricular performance. Left ventricular pressure (LV) and ultrasonic crystal signals from sonomicrometer crystals placed on the anteroposterior LV epicardial surface were amplified and digitalized to inscribe LV pressure-dimension loops. A series of pressure-dimension loops under variable loading conditions were generated by transient occlusion of the inferior vena cava during a 7-second period of apnea, during control conditions, and 30 minutes after discontinuation of CPB.¹⁵ The end-systolic pressure volume relationship (ESPVR) was analyzed by an interactive videographics data analysis computer program (Spectrum, Bowman Gray School of Medicine, Winston-Salem, N.C.) on an 386/33 MHz IBM computer (IBM, Armonk, N.Y.), and LV performance was described as the slope of linear regression (end-systolic elastance [Ees]), as described previously.¹⁶ Post-bypass LV contractility was assessed by percent recovery of prebypass control value (percent Ees).

Tissue injury due to oxidants

Conjugated dienes. Myocardial conjugated dienes (CDs) were measured as a marker of lipid peroxidation. At the end of each study, LV endocardial biopsy specimens were frozen immediately and stored in liquid nitrogen, and tissue levels of hydroxy CDs were determined as described by Lesnfsky and coworkers.¹⁷

Antioxidant reserve capacity. In vitro lipid peroxidation was measured by the method of Godin and coworkers¹⁸ to assess the antioxidant state or vulnerability to subsequent oxidant stress as described previously.⁷

·NO in coronary sinus blood. Direct measurements of ·NO cannot be made because of its short half-life.^{9, 10} Thus ·NO was determined in plasma as its spontaneous oxidation product, nitrite (NO_2^-), which was reconverted to ·NO and quantitated with a sensitive chemiluminescence assay using a nitrogen oxide analyzer (DASIBI Environmental Corp., model 2108, NO_x Analyzer, Glendale, Calif.). The method was modified to increase the sensitivity of the detector to 0.8 parts per billion (ppb) of ·NO (1 pmol/0.1 ml of test sample). Plasma samples were obtained from the coronary sinus before hypoxemia, immediately before reoxygenation, and 30 minutes after reoxygenation. Plasma concentrations taken during CPB were adjusted for dilutional effects of the volume in the extracorporeal circuit.

Experimental groups

Nonhypoxemic studies

Observation without CPB. Five piglets were anesthetized, instrumented, and observed over 5 hours while breathing inspired oxygen fraction (oxygen tension [FiO_2] about 0.6 [Po_2] about 250 mm Hg) to define the stability of an open-chest preparation and provide baseline function and biochemical data.

CPB without hypoxemia or reoxygenation. Five piglets underwent 30 minutes of CPB at Po_2 about 400 mm Hg without preceding hypoxemia, followed by 30 minutes of observation with FiO_2 adjusted to keep Po_2 about 100 to 150 mm Hg after the discontinuation of CPB to determine the effects of CPB per se (CPB).

Hypoxemia and reoxygenation on CPB

Twenty piglets underwent hypoxemia, which was produced by lowering inspired FiO_2 to 6% to 7%, resulting in a Po_2 of 20 to 30 mm Hg for up to 120 minutes, followed by reoxygenation on CPB (Po_2 about 400 mm Hg). Reoxygenation before 120 minutes of hypoxemia was begun if mean arterial pressure fell below 30 mm Hg. Arterial pH was maintained above 7.3 by NaHCO_3 bolus intravenous infusions.

No treatment. Five piglets underwent reoxygenation without additives to the prime of the extracorporeal circuit.

L-NAME. In five piglets the CPB prime was supplemented with the L-NAME (4 mg/kg) to block ·NO synthase.

L-NAME and L-arginine. In five piglets the CPB prime was supplemented with L-NAME (4 mg/kg) and a fivefold excess of L-arginine (20 mg/kg).

L-Arginine. In five piglets the CPB prime was supplemented with L-arginine only (20 mg/kg).

A 30-minute period of reoxygenation on CPB was followed by 30 minutes of post-CPB observation with FiO_2 adjusted to keep Po_2 about 100 to 150 mm Hg before final functional assessments were performed and specimens for biochemical analyses were obtained. At the end of each experiment, the brain and thoracic and intraabdominal

organs were inspected visually to confirm closure of the foramen ovale and arterial ductus and to determine if gross evidence of damage occurred.

Statistics. Data were analyzed with the use of the StatView V2.0 program (Abacus Concepts Inc., Berkeley, Calif.) on a Macintosh IIfx computer (Apple, Inc., Cupertino, Calif.). Analysis of variance was used for intergroup comparison, and the paired Student *t* test was used for comparison of variables within experimental groups. The relationship between functional impairment (percent Ees) and lipid peroxidation (CDs) was tested by linear regression analysis. Differences were considered significant at the probability level of $p < 0.05$. Group data were expressed as mean plus or minus standard error of the mean.

Results

Hemodynamic values did not change in nonhypoxemic piglets observed over a 5-hour period without CPB. Final hemodynamic and biochemical values were unchanged from their baseline levels and were similar in piglets undergoing CPB without hypoxemia/reoxygenation.

Hemodynamics. Hypoxemia increased cardiac index and stroke work by 39% and 64% initially, followed by a gradual decline over an average tolerated hypoxemic period of 91 ± 4 minutes. Premature institution of CPB because of hemodynamic deterioration was needed in 12 of 20 piglets. There was no difference in the mean duration of hypoxemia among the groups. Hypoxemia caused systemic vasodilatation in all groups (Fig. 1), with SVRI decreasing further to 66% of prehypoxemic values during reoxygenation on CPB after hypoxemia in the no treatment and L-arginine groups. The addition of L-NAME to the CPB pump circuit raised SVRI to 167% of prehypoxemic values during CPB, and this vasoconstriction was reversed by the addition of L-arginine to the circuit with L-NAME. CPB could be discontinued in only two of five piglets treated with L-arginine because of severe hypotension that was unresponsive to volume infusion (60% mortality). The lowest SVRI after CPB was noted in the two survivors that received L-arginine.

NO in coronary sinus blood. CPB at Po_2 about 400 mm Hg without preceding hypoxemia raised ·NO 280% above control values; ·NO returned to pre-CPB values when CPB was discontinued and Po_2 was lowered to 100 to 150 mm Hg (Fig. 2). Hypoxemia reduced plasma ·NO 20% and reoxygenation on CPB raised ·NO $127\% \pm 50\%$ above hypoxemic values, but ·NO during CPB remained substantially lower than without preceding hypoxemia ($p < 0.01$) despite a comparable Po_2 level.

Fig. 1. Effects of hypoxemia-reoxygenation and modification of L-arginine-NO pathway on SVRI. No Rx, Reoxygenation CPB without treatment; L-NAME, N^G-nitro-L-arginine methyl ester (4 mg/kg); L-NAME and L-arginine, L-NAME (4 mg/kg) and L-arginine (20 mg/kg); L-Arg, L-arginine (20 mg/kg) added to the extracorporeal circuit priming fluid.

The addition of L-NAME to the extracorporeal circuit reduced the extent of ·NO rise after reoxygenation compared with no treatment ($89\% \pm 18\%$ vs $127\% \pm 15\%$ of hypoxemic values, $p < 0.05$) (Fig. 3). Conversely, adding a fivefold excess of L-arginine with L-NAME and L-arginine alone augmented ·NO production markedly during reoxygenation, resulting in values of $204\% \pm 45\%$ and $202\% \pm 43\%$ above hypoxemic levels, respectively ($p < 0.05$ vs no treatment).

LV performance. Complete functional recovery (Ees = 98% of pre-CPB values) followed CPB without preceding hypoxemia. Reoxygenation on CPB without altering the pump prime (no treatment) was associated with profound post-CPB myocardial dysfunction; Ees recovered only $38\% \pm 12\%$ of prehypoxemic control values ($p < 0.05$ vs CPB). LV contractility recovered $84\% \pm 12\%$ after adding L-NAME to the CPB circuit prime ($p < 0.05$ vs no treatment). Conversely, adding a fivefold excess of L-arginine counteracted these beneficial effects of ·NO-synthase inhibition because Ees recovered only $24\% \pm 6\%$. The most severe post-CPB mechanical dysfunction occurred after the use of L-arginine

alone; only two of five piglets survived (60% mortality rate), and Ees recovered only 32% and 19%, respectively (Fig. 4).

Conjugated dienes. LV subendocardial muscle CD concentration (Fig. 5) was similar in nonhypoxemic piglets placed on CPB to those in normoxemic, instrumented piglets (control). Reoxygenation on CPB (no treatment) increased myocardial tissue CD 89% above control values (0.7 ± 0.1 vs 1.3 ± 0.2 , $p < 0.05$). The addition of L-NAME reduced CD compared with no treatment (0.8 ± 0.1 vs 1.3 ± 0.2 , $p < 0.05$), and L-arginine counteracted these beneficial effects of L-NAME. Tissue CD values in piglets receiving L-arginine alone were comparable with those in piglets that were either untreated or received L-NAME and L-arginine. A negative linear relationship existed between the degree of myocardial dysfunction and lipid peroxidation ($r = 0.752$) (Fig. 6).

Antioxidant reserve capacity. The antioxidant state of myocardial tissue was unchanged by CPB (Fig. 7). Conversely, reoxygenation while on CPB reduced antioxidant reserve capacity, because malondialdehyde increased significantly in myocardial

Fig. 2. Coronary sinus $\cdot$ NO during CPB with and without hypoxemia. Data are expressed as percentage of individual control value in piglets undergoing CPB without hypoxemia (CPB) and reoxygenation without treatment (No Rx).

homogenates incubated with the oxidant *t*-butylhydroperoxide. Reoxygenation-induced reduction in antioxidant reserve capacity was limited by adding L-NAME to the extracorporeal circuit. Supplementation of the CPB prime with L-arginine alone or in combination with L-NAME resulted in a antioxidant reserve capacity comparable with untreated piglets.

Postmortem examination showed pancreatic damage ranging from petechia to frank hemorrhage in all piglets treated with L-NAME alone. No pancreatic alteration occurred in the absence of L-NAME or when L-arginine was added. Macroscopic injury of other organs did not occur, and the foramen ovale and ductus arteriosus were always occluded.

Discussion

This study confirms the occurrence of reoxygenation cardiac dysfunction in the intact immature hypoxemic heart. The internal consistency of the functional and biochemical findings (NO level, LV function, evidence of lipid peroxidation, and re-

duced antioxidant reserve capacity) provides additional evidence that the L-arginine $\cdot$ NO pathway is involved in its pathogenesis. We interpret the protective effect of a potent $\cdot$ NO synthase inhibitor, *N*^G-nitro-L-arginine methyl ester (L-NAME), and the deleterious effects of L-arginine, a $\cdot$ NO donor, to strengthen our conclusion concerning the role of this pathway. These findings are consistent with a report by Baccaro and coworkers,¹⁹ which showed that L-arginine methylester limits cardiac reoxygenation injury in the isolated guinea-pig heart model.

Ventricular dysfunction in cyanotic congenital heart disease may develop or persist after successful anatomic correction with CPB (e.g., arterial repair of transposition of the great arteries^{3,5} and tetralogy of Fallot without residual pulmonary stenosis).⁴ Several factors have been implicated: (1) chronic exposure to hypoxemia, (2) abnormal loading of the ventricle before correction, and (3) injury induced by the operative repair itself (i.e., global ischemia). Furthermore, experimental studies imply that

Fig. 3. Effects of altering L-arginine→NO pathway on coronary sinus ·NO. Hypoxemic value reflects percent of prehypoxemic control values in all groups. Values at 5 and 30 minutes on CPB are expressed as percent of individual hypoxemic values.

Fig. 4. Postbypass myocardial contractility. LV Ees after discontinuation of CPB, expressed as percentage of individual prehypoxemic values. Data in L-arginine group include two survivors (32% and 19%).

Fig. 5. CD content in reoxygenated LV subendocardial muscle. Biopsy samples taken 30 minutes after discontinuation of CPB. Dotted bar shows range of values $\pm$ standard error (absorbance 233/mg total lipid) in normoxemic instrumented control piglets.

chronic cyanosis may increase the vulnerability to intraoperative ischemia by accelerating depletion of high-energy phosphates.^{20, 21} Our findings suggest that reoxygenation injury is an additional causative factor and implicates the L-arginine–NO pathway in its development.

Hydroxy CDs are free fatty acid oxidation products that are possible markers of lipid peroxidation,^{17, 22} and studies in isolated heart models document the association of lipid peroxidation and myocardial dysfunction^{23, 24} after reoxygenation. Our studies in intact piglets confirm this correlation (Fig. 6). Del Nido and colleagues²⁵ reported myocardial lipid peroxidation, quantified by increased production of CDs in preischemic right ventricular biopsy samples from cyanotic children placed on CPB, suggesting the presence of clinical reoxygenation myocardial injury. Teoh and colleagues²⁶ reported recently that myocardial glutathione peroxidase activity was reduced in patients undergoing repair of tetralogy of Fallot, which suggests reduction in natural oxygen defense mechanisms. This study, together with others,^{23, 24} documents reduced antioxidant reserve capacity that would increase the susceptibility to ischemic damage during aortic clamping needed for repair.

Clinical²⁷ and experimental²⁸ studies imply that

free radicals are involved in the pathogenesis of postischemic myocardial dysfunction (stunning), and a burst of free radicals has been detected during reperfusion by electron paramagnetic resonance spectroscopy and the spin trap α -phenyl *N*-tert-butyl nitron in a canine model of stunning.²⁸ Recently peroxynitrite (ONOO^-), a byproduct of the interaction of $\cdot\text{NO}$ and superoxide anion (O_2^-), has been implicated in the pathogenesis of oxidative injury.¹¹⁻¹³ Peroxynitrite causes lipid peroxidation by at least three mechanisms: (1) hydrogen ion-catalyzed homolytic cleavage to form hydroxyl radical and nitrogen dioxide,¹¹ (2) direct oxidative reaction with sulfhydryl groups,¹² and (3) reaction with metal ions to form a potent nitrating agent. Our current findings suggest that $\cdot\text{NO}$ contributes to reoxygenation injury possibly through interaction with O_2^- to form ONOO^- , as shown previously by Beckman and coworkers¹¹ and Hogg and colleagues.¹⁴

Our results also provide in vivo proof that $\cdot\text{NO}$ production is Po_2 dependent. $\cdot\text{NO}$ was determined in plasma as its spontaneous oxidation product, nitrite (NO_2^-), because of its short half-life.^{9, 10} The biologic reaction (inactivation) of $\cdot\text{NO}$ under physiologic conditions can be accounted for by spontaneous oxidation of $\cdot\text{NO}$ to NO_2^- . Recent studies show that 86% of infused $\cdot\text{NO}$ is metabolized to

Fig. 6. Relationship between functional impairment and lipid peroxidation. Relationship between functional recovery assessed by percent Ees and myocardial CDs 30 minutes after discontinuation of CPB (see “text” for description).

nitrite after single passage through the coronary system.⁹ Coronary sinus $\cdot\text{NO}$ was reduced 20% during hypoxemia and increased markedly after abrupt reoxygenation on CPB. Endothelial $\cdot\text{NO}$ production by $\cdot\text{NO}$ synthase, a calmodulin-activated enzyme, requires reduced nicotinamide adenine dinucleotide phosphate and oxygen to oxidize arginine to $\cdot\text{NO}$ and L-citrulline.²⁹ Myocardial $\cdot\text{NO}$ synthesis thereby becomes reduced during hypoxemia, and reoxygenation may stimulate rapid $\cdot\text{NO}$ synthesis, because intracellular Ca^{2+} and other substrates (arginine and reduced nicotinamide adenine dinucleotide phosphate) remain available during hypoxemia.

The $\cdot\text{NO}$ values reflect nitrite alone and thereby underestimate true $\cdot\text{NO}$ production, because these studies were performed before the methodology to measure nitrate and nitrite that yields a $\cdot\text{NO}$ level that approximates the true value more precisely. Our findings documenting the relationship between $\cdot\text{NO}$ and Po_2 during hypoxemia/reoxygenation are

consistent with a recent study³⁰ showing that V_{max} values for $\cdot\text{NO}$ synthase are related directly to Po_2 . This raises the possibility of controlling $\cdot\text{NO}$ production by modifying how rapidly Po_2 is raised when CPB is initiated. This implies that our use of Po_2 about 400 mm Hg may have biased results unfavorably and that a lower Po_2 strategy may have been more advantageous. We reported previously that hypoxemia reduces pulmonary $\cdot\text{NO}$ production,³¹ and perhaps contributes to hypoxemic pulmonary vasoconstriction. The lung is ischemic during CPB so that restoration of pulmonary blood flow may reflect a form of lung reperfusion injury, which causes endothelial dysfunction with consequent inability of the pulmonary endothelium to generate sufficient $\cdot\text{NO}$ to modulate post-CPB vascular tone. Consequently, the beneficial effects of $\cdot\text{NO}$ after CPB may produce an exogenous source of $\cdot\text{NO}$ to compensate for the limited endothelial production.³² Therefore, there may be a $\cdot\text{NO}$ paradox, like the calcium and oxygen paradox,³³ whereby an important physiologic substance can be

Fig. 7. Antioxidant reserve capacity. Vulnerability of hypoxemic and reoxygenated myocardium to subsequent oxidant stress is determined by exposing cardiac homogenates to 0 to 4 mmol/L *t*-butylhydroperoxide (*t*-BHP) for 30 minutes at 37° C (see "text" for description).

deleterious depending on when and in what concentration it is administered.

CPB without preceding hypoxemia (Fig. 2) enhanced $\cdot\text{NO}$ production more than reoxygenation after hypoxemia, but this overproduction of $\cdot\text{NO}$ did not cause lipid peroxidation, because antioxidant reserve capacity was unaltered by CPB alone. This finding is consistent with the observation that CPB is safe in the immature heart, as has been shown in the adult animal.²² One explanation for the lesser rise in $\cdot\text{NO}$ (assessed by NO_2^-) and greater degree of lipid peroxidation (at a comparable Po_2 level) in piglets subjected to CPB with preceding hypoxemia may be that $\cdot\text{NO}$ was inactivated by superoxide anion (O_2^-) during the reoxygenation process rather than becoming oxidized to nitrite (NO_2^-). Consequently, the $\cdot\text{NO}$ reacts with O_2^- -generated intermediate peroxynitrite (OONO^-), which decomposes homolytically to $\cdot\text{OH}$ and NO_2 ¹²⁻¹⁴ without intermediate formation of nitrite¹⁰ and formed this highly toxic $\cdot\text{OH}$, which produced the lipid peroxidation quantified by the increased CDs.

L-Arginine, a precursor for $\cdot\text{NO}$, did not increase lipid peroxidation despite further enhancement of $\cdot\text{NO}$ generation and its deleterious effects on LV

function. The extent of oxidant damage was assessed by post-CPB biopsy samples of CD content of LV subendocardial muscle. We envision reoxygenation injury as an ongoing process that is kindled at the time molecular O_2 is reintroduced. Conceivably, the severe systemic vasodilatation that precluded discontinuation of CPB in nonsurvivors may have compromised assessment of the full extent of oxidant damage because CPB could be discontinued in only two of five piglets. L-NAME limited $\cdot\text{NO}$ production as SVRI rose substantially during CPB, and this was confirmed by the $\cdot\text{NO}$ measurements in coronary sinus blood.

Although L-NAME limited cardiac reoxygenation injury, $\cdot\text{NO}$ synthase inhibition may superimpose ischemic damage to other organs because macroscopic evidence of pancreatic damage ranging from petechia to hemorrhage occurred in all L-NAME-treated piglets. Pancreatic damage is a complication of CPB in human subjects, and its pathogenesis seems partially related to low regional perfusion.³⁴ $\cdot\text{NO}$ is a critical modulator of vascular tone so that the dose-response relationship must be performed to define the type and concentration of $\cdot\text{NO}$ synthase inhibitor that minimizes oxidant injury and

endothelial disruption while maintaining the beneficial physiologic actions of nitric oxide. The importance of *in vivo* studies is reaffirmed by these observations, because the adverse effects of $\cdot\text{NO}$ inhibition would not be disclosed in *in vitro* studies. Our findings of improved myocardial recovery by $\cdot\text{NO}$ synthase inhibition in the hypoxemic/reoxygenation model differ with the recent observations of Johnson and colleagues,³⁵ showing that $\cdot\text{NO}$ is cardioprotective in the regional ischemia/reperfusion model. Their observations of the benefits of $\cdot\text{NO}$ donors and precursors in the setting of ischemia/reperfusion have been confirmed.³⁶⁻³⁸ They speculate that $\cdot\text{NO}$ may be beneficial by inhibiting accumulation and adherence of circulating blood components (i.e., neutrophils and platelets). Blood flow is stagnant in regional ischemia, so that neutrophils can adhere to vascular endothelium, plug capillaries, become activated during reperfusion, and cause myocardial stunning and no reflow phenomenon on reperfusion.³⁹ Coronary flow is fixed in *in vitro* studies of hypoxemia in which coronary vessels dilate maximally and thereby lower perfusion pressure^{40, 41} and may cause regional hypoperfusion. Furthermore, the high coronary flow rate during hypoxemia in the *in vivo* model avoids blood stagnation and may prevent accumulation of lactate that may act as a reducing equivalent to limit oxidant damage when molecular O_2 is reintroduced.⁴² Furthermore, our recent study shows that hypoxemic/reoxygenation damage is a more severe insult than ischemic/reperfusion stress of comparable duration (i.e., 30 minutes), suggesting that a different pathophysiology may occur in each process.⁴³

Depressed cardiac function related to $\cdot\text{NO}$ overproduction that is reversed by competitive initiation of $\cdot\text{NO}$ synthesis occurs also in studies of septic shock and various cytokines.^{44, 45} These observations emphasize the importance of the experimental model in defining mechanisms of injury and support the contention that enhanced $\cdot\text{NO}$ generation may be either salutary (e.g., ischemia/reperfusion) or detrimental depending on clinical or experimental conditions.

We measured antioxidant reserve capacity to assess the vulnerability to subsequent oxidant stress (e.g., exposure to a standard oxidant stimulus) as Godin and workers¹⁸ showed this measurement reflects the status of endogenous defense mechanisms (e.g., glutathione). Severe reduction of endogenous antioxidants persisted after reoxygenation on CPB, and this is consistent with the recent report

of Teoh and colleagues,²⁶ showing reduced glutathione peroxidase in cyanotic patients placed on extracorporeal circulation. Our findings of postreoxxygenation cardiac dysfunction in untreated piglets are comparable with those isolated heart preparations,^{23, 24} in which hypoxemia depletes endogenous antioxidant stores (i.e., superoxide dismutase, glutathione, and thiol groups) and further reduction in thiol protein and glutathione follows O_2 restoration.^{23, 24} The rate of restoration of endogenous antioxidants can be modified by the energy state after reoxygenation, as Tribble and Jones⁴⁶ documented that reduced nicotinamide adenine dinucleotide phosphate supply from reduced nicotinamide adenine dinucleotide via transhydrogenation in mitochondria is essential for maintaining the reduced glutathione pool. The antioxidant reserve capacity in L-NAME-treated group, coupled with less lipid peroxidation, may thereby reflect the protective effects on mitochondrial metabolism.

The limitations of the acute hypoxemic model are acknowledged because chronic cyanosis results in myocardial metabolic adaptations^{47, 48} that are absent in the acute preparation.⁷ However, the relevance of the acute model when severe hemodynamic deterioration occurred at Po_2 about 25 mm Hg to the clinical situation at higher Po_2 , is shown by the similarity of our results to clinical observations in biopsy findings of cyanotic patients placed on CPB.^{7, 26, 49-51} These data lead us to conclude that induction of CPB may cause an "unintended" reoxygenation injury resulting in myocardial dysfunction and that this can be modified favorably by controlling the method of reoxygenation. Our findings imply that the L-arginine- $\cdot\text{NO}$ pathway is involved in the pathogenesis of myocardial reoxygenation injury of hypoxemic immature piglet heart placed on cardiopulmonary bypass. Further studies are needed to see if reduced $\cdot\text{NO}$ and/or O_2^- generation will limit this damage.

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