Glutathione Reverses Endothelial Damage From Peroxynitrite, the Byproduct of Nitric Oxide Degradation, in Crystalloid Cardioplegia

Masanori Nakamura, MD, PhD; Vinod H. Thourani, MD; Russell S. Ronson, MD; Daniel A. Velez, MD; Xin-Liang Ma, MD, PhD; Sara Katzmak, BS; Jill Robinson, BS; L. Susan Schmarkey, BS; Zhi-Qing Zhao, MD, PhD; Ning-Ping Wang, MD; Robert A. Guyton, MD; Jakob Vinten-Johansen, PhD

Background—NO has been advocated as an adjunct to cardioplegia solutions. However, NO undergoes a rapid biradical reaction with superoxide anions to produce peroxynitrite (ONOO\(^-\)). ONOO\(^-\) in crystalloid cardioplegia solution induces injury to coronary endothelium and to systolic function after cardioplegia and reperfusion. However, ONOO\(^-\) may be degraded to less lethal or cardioprotective intermediates with glutathione (GSH) in reactions separate from its well-known antioxidant effects. We hypothesized that GSH detoxifies ONOO\(^-\) and reverses defects in endothelial function and systolic function when present in crystalloid cardioplegia.

Methods and Results—In anesthetized dogs on cardiopulmonary bypass, a 45-minute period of global normothermic ischemia was followed by 60 minutes of intermittent cold crystalloid cardioplegia (Plegisol) and 2 hours of reperfusion. The cardioplegia solution contained 5 \(\mu\)mol/L authentic ONOO\(^-\); catalase was included to attenuate the potential antioxidant effects of GSH and to unmask the effects on ONOO\(^-\). In 1 group (CP+GSH, n=5), the cardioplegia contained 500 \(\mu\)mol/L GSH, whereas 1 group received crystalloid cardioplegia without GSH (CCP, n=6). There were no group differences in postcardioplegia left ventricular systolic function (end-systolic pressure-volume relation, impedance catheter: CCP 10.0±2.4 versus CP+GSH 10.6±1.3 mmHg/mL) or diastolic chamber stiffness (β-coefficient: CCP 0.35±0.2 versus CP+GSH 0.31±0.18). Myocardial neutrophil accumulation (myeloperoxidase activity) was attenuated in CP+GSH versus CCP (2.2±0.7 versus 5.4±1.2, P<0.05). In postexperimental coronary arteries, maximal endothelium-dependent relaxation was greater in CP+GSH than in CCP (118±6% versus 92±5%, P<0.05), with a smaller EC\(_{50}\) value (−7.10±0.05 versus −6.98±0.03, respectively, P<0.05). Smooth muscle relaxation was complete in both groups. The adherence of neutrophils to postexperimental coronary arteries as a measure of endothelial function was less in CP+GSH than in CCP (98±18 versus 234±36 neutrophils/mm\(^2\), P<0.05). Nitrosoglutathione, a byproduct of the reaction between ONOO\(^-\) and GSH, was greater in CP+GSH than in CCP (4.1±2.3 versus 0.4±0.2 \(\mu\)g/mL, P<0.05).

Conclusions—GSH in crystalloid cardioplegia detoxifies ONOO\(^-\) and forms cardioprotective nitrosoglutathione, resulting in attenuated neutrophil adherence and selective endothelial protection through the inhibition of neutrophil-mediated damage. (*Circulation*. 2000;102[suppl III]:III-332-III-338.)

Key Words: cardioplegia • nitric oxide • endothelium

**N** NO was recently advocated as an adjunct to cardioplegia. NO is a potent cardioprotective agent that attenuates neutrophil-mediated damage.\(^1,2\) Accordingly, NO has been shown to attenuate neutrophil function and neutrophil–endothelial cell interactions\(^3-4\) which initiate the inflammatory cascade and sequelae of contractile dysfunction and infarction. Accordingly, NO therapy in cardioplegia solutions during experimental cardiac surgery has been associated with a reduction in infarct size, improved coronary artery endothelial function, attenuated neutrophil accumulation, and better contractile function.\(^5,6\)

NO reacts with superoxide anions at a diffusion-limited rate to produce peroxynitrite (ONOO\(^-\)). Superoxide anions are abundantly produced during ischemia and reperfusion, whereas NO is produced by the coronary artery endothelium and neutrophils. Accordingly, ONOO\(^-\) has been reported to be generated by both vascular endothelium and neutrophils.\(^7,8\) Hence, the close proximity of NO and superoxide anions in the vascular space may promote ONOO\(^-\) generation during and after the periods of ischemia encountered during cardiac arrest as induced with chemical cardioplegia solutions that contain therapeutic concentrations of NO donor agents. NO

From the Emory University School of Medicine, Atlanta, Ga and the Division of Cardiothoracic Surgery, Carlyle Fraser Heart Center of Emory University, Cardiothoracic Research Laboratory, Atlanta, Ga.

Reprint requests to Dr Jakob Vinten-Johansen, Cardiothoracic Research Laboratory, 550 Peachtree St, NE, Atlanta, GA 30365-2225. E-mail jvinten@emory.edu

© 2000 American Heart Association, Inc.

*Circulation* is available at http://www.circulationaha.org

III-332
therapy has had varying results, with some investigators reporting cardioprotective effects,5,6 whereas others have reported cardiotoxic effects.9,10 This variability may be related to the generation of ONOO\(^-\) and its subsequent physiologic effects on coronary vascular endothelium and myocardium. Ronson et al11 reported that ONOO\(^-\) demonstrated deleterious effects in crystalloid cardioplegia in a canine model of normothermic ischemia followed by cardioplegic arrest. In contrast, ONOO\(^-\) in a blood environment (blood cardioplegia solution) demonstrated a beneficial effect,11 consistent with the observations of Lopez et al10 and Nossuli et al.12

The cardioprotective effects of ONOO\(^-\) may be related to the presence of endogenous thiol-containing substances such as glutathione (GSH), albumin, and cysteine. These thiol-containing molecules potentially convert ONOO\(^-\) to less harmful or even cardioprotective byproducts. In addition to its potent antioxidant effects through the conversion of hydrogen peroxide to molecular oxygen and water via GSH peroxidase, GSH converts ONOO\(^-\) into potential NO donors such as S-nitrosoglutathione (GSNO) and S-nitroglutathione,13,14 which exhibit physiological and cardioprotective effects similar to those of NO.12

Because ischemically injured myocardium is significantly depleted of tissue GSH compared with normal myocardium, ONOO\(^-\) generated by NO-enhanced cardioplegia solutions delivered to ischemic myocardium in which superoxide anions may be coincidentally generated may cause damage to myocardium and vascular endothelium, rather than the cardioprotection intended by the inclusion of NO. In the present study, we tested the hypotheses that GSH, added to crystalloid cardioplegia that contains authentic ONOO\(^-\), attenuates the deleterious effects of ONOO\(^-\) on postcardioplegic endothelial dysfunction and cardiodynamic dysfunction.

Methods

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH publication No. 80-23, revised 1985).

Neutrophil Isolation

After anesthesia was induced and arterial access was gained, 200 mL of peripheral arterial blood was harvested for postexperimental analysis of coronary artery endothelial function with neutrophil (neutrophil) adherence properties. The blood was mixed with 45 mL of anticoagulation agents, which included 1.6% citric acid and 2.5% sodium citrate (pH 5.4) and 100 mL of 6% dextran solution in buffered Hanks’ balanced salt solution (HBSS). Neutrophils were isolated with the use of the Ficoll-Paque (Sigma Chemical Co) technique described in detail elsewhere.2 The cells were placed in Ca\(^{2+}\) and Mg\(^{2+}\)-free solution, counted, and adjusted to \(\sim 9 \times 10^8\) cells/mL. Final suspensions contained 94±1% neutrophils, and cell viability averaged 99±0.5% as determined by trypan blue exclusion.

Heartworm-free dogs of either sex that weighed 22.6 to 35.4 kg (mean weight 28.4±1.1 kg) were premedicated with subcutaneous morphine sulfate (4 mg/kg) followed by initial anesthesia with intravenous 2.5% thiopental (20 mg/kg). After endotracheal intubation, mechanical ventilation was begun, and the respiratory rate was adjusted to maintain pH 7.35 to 7.45, PaO\(_2\) of >100 mm Hg, and PaCO\(_2\) of 35 to 45 mm Hg. Deep anesthesia was maintained with the continuous infusion of fentanyl citrate (0.4 \(\mu\)g · kg\(^{-1}\) · min\(^{-1}\)) and diazepam (0.003 mg · kg\(^{-1}\) · min\(^{-1}\)). The right femoral artery and vein were cannulated for arterial blood sampling and for fluid administration, respectively. After median sternotomy, the azygous vein was ligated, and the pericardium was incised and tented to form a cradle. Millar MPC-500 solid-state catheters (Millar Instruments) were placed in the proximal aorta through the right internal mammary artery to measure aortic pressure and into the left ventricle (LV) through an apical stab incision to measure instantaneous LV pressure. The left atrium was cannulated for the infusion of hypertonic saline (9%) to measure parallel conductance. After heparinization (300 U/kg), a 7F octapolar impedance catheter (Webster) was inserted into the LV through the right internal carotid artery to measure instantaneous LV conductance and blood volume for analysis of systolic and diastolic properties with pressure-volume analysis as previously described.11

The left subclavian artery was cannulated for arterial inflow, and the superior and inferior vena cavae were transternally cannulated to harvest venous return. Cardiopulmonary bypass was instituted with a Cobe Optima membrane oxygenator (Cobe Cardiovascular, Inc) primed with 1.5 L hetastarch (Hespan; DuPont Pharmaceutical). Total cardiopulmonary bypass was initiated, and the left and right ventricles were ventilated through direct cannulation and gravity drainage. Finally, a pressure-monitoring aortic root cannula (DLP, Inc) was inserted into the proximal aorta for the delivery of cardioplegia. Thermistor probes were placed in the anterior and posterior wall to continuously monitor intramyocardial temperature.

Experimental Protocol

After the initiation of cardiopulmonary bypass and acquisition of baseline measurements, the aorta was cross-clamped for 45 minutes of normothermic global ischemia. Subsequently, hypothermic (4 °C), multidose (every 20 minutes) crystalloid cardioplegia (CCP) solution (Plegisol; Abbott Laboratories) was infused into the aortic root at 50 mm Hg with the Myocardial Protection System delivery device (Quest Medical, Inc). Then, 600 mL was administered for both induction and terminal cardioplegia, and 400 mL was delivered during intermittent infusions.11 Iced saline slush was topically applied to maintain myocardial temperature during cardiac arrest. During the delivery of crystalloid cardioplegia, the coronary sinus effluent was harvested from the right ventricle and discarded to avoid systemic effects of ONOO\(^-\) or its decomposition products and direct effects of cumulative concentrations of ONOO\(^-\) exerted during reperfusion. Systemic mean arterial pressure was kept at 70 mm Hg during reperfusion.

The animals were randomized to receive either 5 mmol/L authentic ONOO\(^-\) (CCP, 100 mL) or ONOO\(^-\) (5 mmol/L) supplemented with 500 mmol/L GSH (CCP+GSH, 100 mL) solution (Plegisol; Abbott Laboratories) for delivery of cardioplegia for both groups of both groups. The concentration of ONOO\(^-\) exerted during cardiac arrest was determined in vitro pilot studies (data not shown). Because ONOO\(^-\) is readily decomposed at relatively neutral pH, 0.1 N NaOH was added to the ONOO\(^-\) stock solution to enhance stability, and the appropriate concentration of ONOO\(^-\) was mixed with Plegisol immediately before the delivery of cardioplegia. The concentration of ONOO\(^-\) was measured immediately before the first delivery and again after the final delivery of cardioplegia solution, and the percent decomposition was calculated as \(1-(\text{final absorbance/base absorbance})\) × 100. The percent decomposition at the final delivery of cardioplegia was comparable between the groups (CCP 7.7±2.2%, CCP+GSH 5.8±2.2%). The delivery of ONOO\(^-\) was also confirmed through an analysis of tissue nitrotyrosine, the footprint of ONOO\(^-\) (described later).

After the final delivery of cardioplegia and systemic rewarmin to 37 °C, systemic pressure was reduced to 50 mm Hg and the aortic cross-clamp was removed. After electromechanical resuscitation, the mean arterial pressure was gradually increased from 50 to 150 mm Hg with fluid boluses until the left ventricular pressure and coronary perfusion pressure were re-established.
10 mm Hg. Ventricular fibrillation was counteracted with direct-current countershocks of 10 to 20 W-s. The heart was maintained in the total vented bypass state for the initial 30 minutes of reperfusion, after which cardiopulmonary bypass was discontinued, and functional data were collected every 30 minutes for the next 90 minutes off bypass. After the final data collection, euthanasia was accomplished with the administration of 100 mg/kg pentobarbital, and the hearts were excised and immediately immersed in cold Krebs-Henseleit (K-H) buffer for excision of the coronary arteries to determine vascular function (see later).

**Experimental End Points**

**LV Performance and Chamber Stiffness**

LV performance was described with the load-independent end-systolic pressure-volume relationship (ESPVR) from gradually decreasing pressure-volume loops acquired during transient bicaval occlusion. ESPVR values were calculated with the equation $P_a - E_{sp}(V_a - V_s)$, where $P_a$ is the end-systolic pressure, $E_{sp}$ is the slope of the linear ESPVR (elastance), $V_a$ is the end-systolic volume, and $V_s$ is the volume axis intercept when $P_a = 0$ mm Hg. LV chamber stiffness was determined by fitting the gradually declining end-diastolic pressure-volume points to the exponential relation $P_a = \alpha e^{\beta V_d}$, where $P_a$ and $V_d$ are the end-diastolic pressure and volume points, respectively; $\alpha$ is the $P_a$ intercept at $V = 0$ mL; and $\beta$ is the unitless modulus of chamber stiffness used to describe the degree of curvature of the $P_a - V_d$ relation.

**Plasma Creatine Kinase Activity**

Arterial blood samples (3 mL) were withdrawn at baseline, at the end of normothermic ischemia, at the end of cardioplegic arrest, and at 2 hours of reperfusion. The samples were centrifuged at 2500g and 4°C for 10 minutes. The plasma was analyzed spectrophotometrically for creatine kinase (CK) activity (Sigma Diagnostics) and for protein concentration as described previously. Plasma CK activity was expressed as international units per gram of protein.

**Tissue Water Content**

Tissue water content was determined through desiccation of subepicardial and subendocardial samples of the LV free wall at 85°C for 48 hours. Tissue water content was calculated with the formula [(wet weight−dry weight)/wet weight]×100.

**Myeloperoxidase Activity for Neutrophil Accumulation in Cardiac Tissue**

Myeloperoxidase (MPO), an enzyme that is specific for neutrophils, was analyzed spectrophotometrically at 460 nm in postexperimental LV tissue samples as described elsewhere.

**Postexperimental Coronary Artery Endothelial Function**

**Isolated Coronary Artery Rings**

Agonist-stimulated endothelial relaxation responses to agonist stimulators of NO synthase were determined in postexperimental coronary artery rings as a bioassay of endothelial function as described in detail previously.

Indomethacin (10 μmol/L) was added to organ chambers to inhibit prostaglandin effects. The coronary rings were subsequently preconstricted with the thromboxane A2 mimetic and subsequently preconstricted with the thromboxane A2 mimetic chamber to inhibit prostaglandin effects. The coronary rings were described in detail previously.

**Neutrophil Adherence Assay**

Basal endothelial function related to the inhibition of neutrophil adherence by endogenously released NO was assessed according to the adherence of fluorescently labeled (PKH26 vital fluorescent dye; Sigma Chemical Co) neutrophils to postexperimental coronary artery endothelium with the use of epifluorescence microscopy as described in detail previously.

**Nitrosoglutathione Concentration of Cardioplegic Solution**

GSH reacts with ONOO− to form GSNO. The concentration of GSNO was analyzed spectrophotometrically in samples of cardioplegic solution withdrawn from the aortic root and the coronary sinus during the delivery of cardioplegia.

**Quantification of Tissue Nitrotyrosine in LV Myocardium**

The quantification of LV free wall nitrotyrosine levels as an estimate of ONOO− delivery was performed with ELISA with a mouse IgG monoclonal anti-nitrotyrosine primary antibody (Upstate Biotechnology) as previously published. Tissue levels of nitrotyrosine were compared with nitrated protein solution (0.04% BSA) prepared as a standard. The amount of nitrotyrosine content in tissue samples was calculated with use of the standard curve generated from nitrated BSA that contains known amounts of nitrotyrosine and expressed as nanogram of nitrotyrosine per milligram of protein.

**NO Levels in Cardioplegia Solution Estimated by Nitrate/Nitrite Concentration**

The major decomposition products of NO (potentially regenerated from GSNO) are nitrate and nitrite. The concentration of nitrate/nitrite in effluent cardioplegia was measured according to the vanadium reduction technique reported previously and is expressed in micromoles per liter per liter.

**Statistical Analysis**

Time-related differences and group-time interactions were analyzed by 2-way ANOVA for repeated measures. Single-event, nonrepeated variables were compared between groups by standard $t$ test or nonparametric tests based on the normality of the distribution of data. Relaxation responses in postexperimental rings were expressed as the percent change in tension from the preconstricted levels, and these data were compared at each concentration between groups by standard $t$ test or nonparametric test if data were not normally distributed. EC50, the dose of the drug required to effect 50% of maximum relaxation, was calculated and expressed as the negative log of the drug concentration. A value of $P<0.05$ was considered statistically significant. All data are presented as mean±SEM.

**Results**

One animal in each of the CP+GSH and CCP groups was excluded due to technical intraoperative complications. In addition, 1 animal in the CCP group was unable to be weaned from cardiopulmonary bypass due to severe LV dysfunction. Five animals in each group were included in the final analysis.

Blood gas data obtained before and after stabilization on bypass were not significantly different between groups at any time point. Anterior and posterior myocardial temperatures during antecedent global ischemia and cardioplegic arrest were comparable between groups throughout these periods.

Aortic cross-clamp time (CCP 112±1.7 minutes versus CP + GSH 112±0.7 minutes), cardioplegic arrest time (CCP 65.8±0.9 minutes versus CP + GSH 66.6±0.4 minutes), and total cardiopulmonary bypass times (CCP 163±2.3 minutes versus CP + GSH 164±4.8 minutes) were comparable between groups. Hematocrit values before (CCP 35±1.2% versus CP + GSH 34±5.7%) and during CPB (CCP 19±1.4% versus CP + GSH 19.6±0.6%) were also comparable between the groups. Both groups required at least 1 direct-current countershock to convert ventricular fibrillation without differences in the number of cardioversions during the early period of reperfusion. PO2 and PCO2 of the cardioplegia solutions were comparable between groups.
TABLE 1. Postbypass LV Systolic Contractile Performance and Diastolic Chamber Stiffness by Pressure-Volume Analysis

<table>
<thead>
<tr>
<th>Cardiodynamic Variable</th>
<th>Reperfusion, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>( E_{\text{ss}} ), mm Hg/mL</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>8.2±2.2</td>
</tr>
<tr>
<td>CP+GSH</td>
<td>9.0±0.8</td>
</tr>
<tr>
<td>( V_{\text{ss}} ), mL</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>31±8.5</td>
</tr>
<tr>
<td>CP+GSH</td>
<td>23±2.9</td>
</tr>
<tr>
<td>( \beta )-Coefficient</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>0.26±0.11</td>
</tr>
<tr>
<td>CP+GSH</td>
<td>0.32±0.07</td>
</tr>
</tbody>
</table>

There were no time or group differences.

Steady-State Hemodynamic Parameters
There were no group differences in steady-state mean arterial blood pressure, heart rate, or LV end-diastolic pressure at any time during the experiment.

LV Systolic Performance and Chamber Stiffness
Table 1 shows postbypass LV performance and chamber stiffness. After the discontinuation of cardiopulmonary bypass, \( E_{\text{ss}} \), \( V_{\text{ss}} \), and diastolic chamber stiffness (\( \beta \)-coefficient) were comparable between groups throughout the reperfusion phase. In addition, there was no significant difference between any preischemic and posts ischemic cardiodynamic variable in either group.

Plasma CK Activity
Baseline plasma CK activities were comparable between groups (Figure 1). There was no significant increase in plasma CK activity after global ischemia. At 90 minutes after the discontinuation of cardiopulmonary bypass, plasma CK level tended \((P=0.12)\) to be greater in the CCP group than in the CP+GSH group. However, these differences did not reach statistical significance. These data are consistent with the LV performance data.

Tissue Water Content
Postexperimental transmural LV tissue water contents were comparable between the 2 groups (CP 79.5±0.6% versus CP+GSH 79.6±0.3%, \( P=0.8 \)). These data are consistent with the lack of group differences for diastolic chamber stiffness data.

MPO Activity in LV Tissue
Postexperimental LV MPO activity in the CP+GSH group (2.2±0.6 absorbance units/min) was significantly lower than that in the CCP group (5.4±1.1 absorbance units/min, \( P<0.05 \)), suggesting that neutrophil accumulation in the postcardioplegic myocardium was attenuated by the inclusion of GSH in ONOO⁻-enhanced crystalloid cardioplegia.

Postexperimental Coronary Artery Endothelial Function

Agonist-Stimulated Vascular Reactivity
There were significant differences in concentration-dependent relaxation responses to the endothelium-dependent, receptor-dependent vasodilator acetylcholine in postexperimental coronary arteries (Figure 2). Compared with the CP+GSH group, the concentration-response curves to acetylcholine were shifted significantly to the right in the CCP group, with a reduction in the maximum relaxation (92±5% versus 118±6%, \( P=0.02 \) overall, Figure 2) and an increase in EC\(_{50}\) (−6.98±0.03 versus −7.10±0.05, \( P=0.04 \)). There was no overall decrease in relaxation with the endothelium-dependent receptor independent vasodilator A23187; there were no group differences in maximal relaxation or EC\(_{50}\) (CCP −7.15±0.04 versus CP+GSH −7.18±0.05). Responses to the endothelium-independent vasodilator sodium nitroprusside showed no group differences in maximum relaxation or EC\(_{50}\) between CCP (−7.15±0.04) and CP+GSH (−7.18±0.05). These data suggest that adjunct GSH protects the receptor-mediated function of the postcardioplegic coronary vascular endothelium from damage induced by ONOO⁻.

Basal Postexperimental Neutrophil Adherence
The adherence of unstimulated neutrophils to postexperimental coronary artery endothelium was significantly less in the CP+GSH group (98±18 versus 234±36 neutrophils/mm², \( P<0.05 \)). These data demonstrate that neutrophil adherence to postexperimental coronary artery endothelium, related to basal production of NO, in CCP was attenuated by GSH.

GSNO Concentration
There was an increased concentration of GSNO in the CP+GSH cardioplegia solution compared with the CCP group \((P<0.05)\) (Figure 3, left). In the coronary sinus effluent, the concentration of GSNO was comparable in both groups; there was a significant trascardiac difference in the CP+GSH group, whereas there was no such difference in the CCP group, suggesting an uptake of GSNO by the myocardium in the CP+GSH group.

Nitrate/Nitrite Concentration in Cardioplegia Effluent
Nitrate/nitrite concentrations in cardioplegia effluent and plasma during early reperfusion are summarized in Table 2. There was a trend toward a greater concentration of nitrate/nitrite in coronary sinus effluent during the delivery of the second cardioplegia in the CP+GSH group, but this did not

Figure 1. CK activity in systemic blood throughout experiment. Base indicates baseline; ISC, 40 minutes after normothermic ischemia by aortic cross-clamp; CP, terminal cardioplegia delivery before release of aortic cross-clamp; R15, 15 minutes of reperfusion after release of aortic cross-clamp; and 90 min, 90 minutes after discontinuation of cardiopulmonary bypass.
reach statistical significance. Nitrate/nitrite concentrations at all other time points were comparable between the two groups. These data suggest that GSNO was not converted to free NO.

**Tissue Nitrotyrosine**
Figure 4 demonstrates that there was a comparable level of nitrotyrosine in LV myocardium in both CCP and CP+GSH groups. These data suggest that comparable amounts of ONOO\(^{-}\) were delivered to the myocardium in both groups or that the protective mechanisms of GSH did not attenuate the nitration of tyrosine in the CP+GSH group.

**Discussion**
ONOO\(^{-}\) is a degradation product generated by the biradical reaction between NO and superoxide anions. Due to the 1:1 stoichiometry, the rate of ONOO\(^{-}\) appearance increases in proportion to the concentration of its 2 substrates. Hence, ONOO\(^{-}\) levels may increase when superoxide anion and NO generation increase during reperfusion\(^{20,21}\) or with stimulation of the endothelium\(^7\) in an environment in which NO is simultaneously produced.\(^{22}\) Although NO has been reported to be cardioprotective in many in vivo studies,\(^1,23\) it has been demonstrated to be deleterious in crystalloid (in vitro) environments.\(^{21,24}\) It has been suggested that these deleterious effects of NO in crystalloid environments may be mediated directly via ONOO\(^{-}\), via ground-state peroxynitrous acid,\(^{25}\) or via the hydroxyl radical–like metabolite of ONOO\(^{-}\).\(^{26}\)

Accordingly, Ronson et al\(^{11}\) reported that 5 \(\mu\)mol/L ONOO\(^{-}\) added to crystalloid cardioplegia markedly reduced postcardioplegia systolic functional recovery, increased neutrophil accumulation in LV myocardium, and increased tissue edema. In contrast, this same concentration of ONOO\(^{-}\) in blood cardioplegia had cardioprotective effects. In the present study with a model similar to that of Ronson et al,\(^{11}\) 500 \(\mu\)mol/L GSH was added to crystalloid cardioplegia that contained 5 \(\mu\)mol/L authentic ONOO\(^{-}\). In support of a reaction between ONOO\(^{-}\) and GSH, the reaction product GSNO was generated in the GSH-enhanced cardioplegia group, consistent with other reports\(^{13,27}\) but not in the group without GSH. However, we could not demonstrate that GSNO was further broken down to authentic NO, because there was no group difference in nitrate/nitrite levels in effluent cardioplegia solution. GSH-enhanced crystalloid cardioplegia had no effect on postcardioplegia systolic or diastolic function or tissue water content. However, GSH did reduce neutrophil accumulation in postcardioplegia LV myocardium and was associated with significantly better coronary artery endothelial function (agonist-stimulated relaxation and basal neutrophil adherence properties). Hence, GSH added to crystalloid cardioplegia that contained ONOO\(^{-}\) reversed the deleterious effects of this anion, possibly by generating GSNO. These data suggest that GSH may be useful in preventing the deleterious effects of ONOO\(^{-}\) derived from NO if NO donors are used as adjuncts to crystalloid cardioplegia solutions in which detoxifying thiol agents are absent.

In the present study, 45 minutes of ischemia was imposed to sensitize the myocardium to injury. In this model of severe LV injury, GSH could act either to reduce the oxidant species (hydrogen peroxide to water and molecular oxygen via GSH peroxidase activity or to react with ONOO\(^{-}\) to form GSNO. This antioxidant action may partially account for the blunting of the deleterious actions of ONOO\(^{-}\). To separate the antioxidant actions of GSH from those of GSNO, catalase was added to the cardioplegia solution in each experimental group in a concentration that favored a rapid reaction with oxidant species. The rate constant for the reaction of catalase

<table>
<thead>
<tr>
<th>Group</th>
<th>CP 1</th>
<th>CP 2</th>
<th>CP 3</th>
<th>Reperfusion 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCP</td>
<td>16.9±.8.9</td>
<td>19.6±6.8</td>
<td>24.7±3.8</td>
<td>23.3±8.6</td>
</tr>
<tr>
<td>CP+GSH</td>
<td>18.5±6.8</td>
<td>41.8±19.7</td>
<td>25.6±11.3</td>
<td>20.7±5.1</td>
</tr>
</tbody>
</table>

**Table 2. Nitrate/Nitrite Concentrations in Each Cardioplegia Effluent and 5 Minutes After Release of Cross-Clamp**
with hydrogen peroxide is 10 times faster than the corresponding value for GSH. Therefore, catalase should outcompete GSH for hydrogen peroxide and, hence, its antioxidant activity. The general antioxidant effect of catalase in both groups may be responsible for the excellent recovery of postcardioplegia systolic and diastolic function in both groups, consistent with the observations of others, compared with the marked dysfunction observed by Ronson et al in an identical model in which catalase was not added to cardioplegia solution. Hence, a protective effect on functional recovery could not be exerted in the absence of injury. Although we cannot be sure that the antioxidant effects of GSH were completely eliminated with catalase, the conversion of ONOO− to GSNO was strongly supported by our data (Figure 3).

In the present study, 5 μmol/L ONOO− was added to the crystalloid cardioplegia solution to represent potential ONOO− derived from exogenously added NO donor agents. Under normal conditions, the generation of NO is relatively low (1 to 20 nmol/L). However, tissue NO concentrations can increase 1000-fold with activation of the inducible form of NO synthase or activation of neutrophils or with parenteral administration of NO donor agents. A coincident increase in superoxide anion would provide substrates for ONOO− production. A high concentration of ONOO− may be achieved during early reperfusion when NO is derived from both the vascular endothelium and activated neutrophils or when NO levels are augmented by exogenous NO donors. Carreras et al reported an increase in ONOO− generation coincident with the generation of superoxide anions and NO in activated neutrophils. Therefore, with the assumption of a 1:1 stoichiometry between NO and superoxide anions, the 5 μmol/L concentration of ONOO− used in the present study may be relevant to cases in which NO donors are used in cardioplegia solutions.

The mechanisms of vasculoprotection by GSH and reduction in neutrophil accumulation are not clear. The improved coronary artery endothelial function may have been related to the attenuation of neutrophil adherence to endothelium and consequent endothelial injury, observed by Nossuli et al, which were mediated by GSNO. In the presence of ONOO−, GSH has been shown by others to (1) cause vasorelaxation, (2) stimulate the levels and activity of guanylate cyclase in endothelial cells, (3) attenuate ONOO−-induced hemolysis, and (4) inhibit platelet aggregation. The effects of GSH in the presence of ONOO− can be inhibited by hemoglobin or inhibitors of guanylate cyclase. The physiological effects of GSH in the presence of ONOO− are consistent with the actions of an NO-like substance. Indeed, GSH reacts with ONOO− to form S-GSNO12 or S-nitroglutathione (GSNO27), both of which are purported NO donors, or further breakdown of these molecules may generate authentic NO itself. Recently, Balazy et al showed that the reaction of ONOO− with GSH produced GSNO2, which released NO and demonstrated potent vascular relaxant activity. Nossuli et al observed a concentration-dependent appearance of GSNO in an in vitro reaction between GSH and 10 to 100 μmol/L ONOO−, which is within the range of ONOO− used in the present study. In fact, GSNO was found in the present study to appear in the delivered cardioplegia solution of the CP+GSH group, and this GSNO was absent from the coronary venous effluent, which implies myocardial uptake of the nitrosothiol. The vascular protection and reduction in neutrophil accumulation in LV tissue when GSH was present in ONOO− cardioplegia may therefore be related to the generation GSNO or other products of thiol nitrosation.

In summary, with a clinically relevant model of cardioplegia myocardial protection in which 5 μmol/L ONOO− conferred significant contractile and endothelial dysfunction, the addition of GSH attenuated postcardioplegia vascular endothelial dysfunction and attenuated myocardial neutrophil accumulation. GSH did not, however, improve postcardioplegia dysfunction, potentially via a reduction in oxidant-mediated injury to the contractile process by catalase added to both groups. For surgeons who consider using NO-related therapy in a crystalloid cardioplegia environment to reduce ischemic-reperfusion injury during surgical revascularization, the addition of GSH to the cardioplegia solution may detoxify ONOO− derived from NO donors or NO precursors and thereby allow full advantage to be taken of the potent cardioprotection afforded by NO therapy.

Acknowledgments
This work was supported by a grant-in-aid from the American Heart Association, National Center, to Dr Vinten-Johansen and by contributions from the Carlyle Fraser Heart Center of Emory University. We appreciate the support of Quest Medical, Inc (Allen, Tex) for cardioplegia delivery supplies. The authors are grateful for the assistance of Gail H. Nechtman in the preparation of the manuscript.

References

Figure 4. Nitrotyrosine levels (ng/mg tissue protein) in postexperimental LV myocardium. Comparable levels of tissue nitrotyrosine suggest equal delivery of ONOO−.
26. Beckman JS, Beckman TW, Chen J, et al. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA. 1990;87:1620–1624.
Glutathione Reverses Endothelial Damage From Peroxynitrite, the Byproduct of Nitric Oxide Degradation, in Crystalloid Cardioplegia

Masanori Nakamura, Vinod H. Thourani, Russell S. Ronson, Daniel A. Velez, Xin-Liang Ma, Sara Katzmark, Jill Robinson, L. Susan Schmarkey, Zhi-Qing Zhao, Ning-Ping Wang, Robert A. Guyton and Jakob Vinten-Johansen

_Circulation_. 2000;102:Iii-332-Iii-338
doi: 10.1161/01.CIR.102.suppl_3.III-332

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/suppl_3/Iii-332

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/