

# Myocardial Sulfhydryl Pool Alterations Occur During Reperfusion After Brief and Prolonged Myocardial Ischemia In Vivo

Edward J. Lesnefsky, Ira M. Dauber, and Lawrence D. Horwitz

Myocardial sulfhydryl (SH)-containing compounds, including reduced glutathione (GSH), are both defenses against and potential markers of reactive oxygen metabolite injury during ischemia and reperfusion. We examined the alterations in GSH and other myocardial SH pools during reperfusion in anesthetized dogs exposed to brief (15 minutes,  $n=7$ ) or prolonged (90 minutes,  $n=6$ ) regional ischemia caused by occlusion of the left anterior descending artery. Ninety minutes of ischemia followed by 5 hours of reperfusion, which resulted in myocardial necrosis of  $43.9 \pm 4.0\%$  of the area at risk, caused a 22% reduction in total myocardial SH groups ( $p < 0.01$ ), a 57% decrease in nonprotein myocardial SH groups ( $p < 0.01$ ), a 56% decrease in GSH ( $p < 0.01$ ), and a 62% decrease in non-GSH, nonprotein SH groups ( $p < 0.02$ ). However, protein SH groups were not significantly reduced (12% decrease,  $p = \text{NS}$ ). Also, myocardial release of GSH and oxidized glutathione (GSSG) into the coronary venous effluent occurred during early reperfusion. In contrast, 15 minutes of ischemia, followed by 30 minutes of reperfusion, did not alter myocardial total SH groups, protein SH groups, or GSH (9% decrease,  $p = \text{NS}$ ); nor was there reperfusion release of GSH or GSSG. However, even with brief ischemia, nonprotein SH groups decreased 23% ( $p < 0.05$ ), due mainly to a 59% decrease in the non-GSH, nonprotein SH pool ( $p < 0.05$ ). These changes after brief ischemia occurred without alterations in myocardial GSSG or the GSH/GSSG ratio. Thus, the pattern of depletion of myocardial SH pools during reperfusion differs depending on the duration of the preceding ischemia. The decreases in nonprotein SH and in non-GSH, nonprotein SH pools did not occur (9% decrease and 3% decrease, respectively; both  $p = \text{NS}$ ) in dogs subjected to brief occlusion ( $n=6$ ) that were treated before coronary occlusion with 500 mg/kg i.v. dimethylthiourea, an intracellular scavenger of  $\text{H}_2\text{O}_2$  and  $\cdot\text{OH}$ . Since only the non-GSH, nonprotein SH pool decreased significantly after brief ischemia and since this decrease could be prevented by antioxidant intervention, this pool appears to be the most sensitive SH indicator of reactive oxygen metabolite-induced myocardial ischemia/reperfusion injury. (*Circulation Research* 1991;68:605–613)

**R**eactive oxygen metabolites produced during myocardial ischemia and reperfusion appear to contribute to the postischemic myocardial contractile dysfunction ("stunning") caused by brief ischemia,<sup>1–3</sup> as well as the myocardial necrosis following prolonged periods of ischemia.<sup>4–6</sup> Myocardial sulfhydryl (SH) compounds are important

defenses against injury caused by reactive oxygen metabolites,<sup>7–10</sup> and alterations in myocardial SH pools may be markers of this injury. The most frequently studied SH pool is glutathione (GSH), the cofactor for the enzyme glutathione peroxidase. GSH is oxidized to GSSG during metabolism of hydrogen peroxide<sup>7</sup> and lipid peroxides<sup>11</sup> by glutathione peroxidase. GSH also decreases membrane lipid peroxide formation,<sup>12,13</sup> reacts with superoxide<sup>14</sup> and other free radicals,<sup>15</sup> and maintains protein SH groups in their reduced state.<sup>16–18</sup> Thus, GSH can protect against reactive oxygen metabolite-induced myocardial injury by several mechanisms.

Although previous investigation<sup>9,19–21</sup> has focused on the role of GSH, changes in other myocardial SH pools could occur, either in concert with or independent of changes in GSH. The role of other SH pools

From the Division of Cardiology, University of Colorado Health Sciences Center, Denver.

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Address for reprints: Edward J. Lesnefsky, MD, Division of Cardiology, University Hospitals of Cleveland, 2074 Abington Road, Cleveland, OH 44106.

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during regional ischemia is unknown, since these previous studies using *in vivo* regional ischemia and reperfusion were limited to measurements of total glutathione levels in models of prolonged ischemia. We measured GSH and other myocardial SH pools to compare the relative changes in myocardial SH pools during *in vivo* regional ischemia and reperfusion. In addition, we compared the changes in SH pools that occurred in two models of ischemia/reperfusion injury: a "brief" 15-minute occlusion without myocardial necrosis and a "prolonged" 90-minute occlusion involving myocardial necrosis. Differential changes in SH pools in these two models could reflect different magnitudes of oxidative injury during reperfusion, perhaps influenced by the duration of the preceding ischemic insult. Furthermore, any SH pools that are reduced after brief ischemia should be sensitive SH indexes of ischemia/reperfusion injury.

### Materials and Methods

#### *Canine Model of Ischemia/Reperfusion*

Mongrel dogs of either sex (18–27 kg) were anesthetized with thiamylal sodium (8 mg/kg) and chloralose (100 mg/kg), with supplemental chloralose given as needed. Dogs were intubated and ventilated with 35% O<sub>2</sub>-65% N<sub>2</sub> to maintain arterial oxygenation in the physiological range at Denver's altitude. A left lateral thoracotomy was performed, and the heart was suspended in a pericardial cradle. Catheters were inserted into the distal aorta via the femoral artery and into the left atrium. The left anterior descending artery (LAD) was dissected free 2–3 cm distal to its origin, and a snare occluder was placed around it. In a subgroup of dogs, a 20-gauge angi catheter was inserted into the coronary sinus with the tip at the orifice of the great cardiac vein to obtain venous blood from the LAD region.<sup>10,22</sup> This allows sampling of blood from the ischemic/reperfused LAD zone, although there is some admixture from nonischemic myocardium.

LAD occlusion was performed by tightening the snare for either 15 minutes (brief occlusion)<sup>1–3</sup> or 90 minutes (prolonged occlusion).<sup>4–6</sup> All dogs developed a visible area of regional cyanosis and dyskinesia during LAD occlusion. Reperfusion was accomplished by release of the snare and resulted in transient ventricular tachycardia and resolution of cyanosis in all dogs. In dogs in which myocardial GSH and GSSG release was measured, regional myocardial blood flow was determined at 10 (brief) or 75 (prolonged) minutes of occlusion by the injection of 15- $\mu$ m-diameter radiolabeled microspheres.<sup>23</sup> Reperfusion was continued for 300 minutes in prolonged-occlusion dogs and for 30 minutes in brief-occlusion dogs. Area at risk and infarct size were measured by a postmortem dual-perfusion method using Evans blue and triphenyl tetrazolium chloride.<sup>4</sup>

A separate group of dogs was used for tissue measurements. Transmural biopsies weighing 50–100 mg were obtained by use of a hollow biopsy drill

(Alko Diagnostic Corp., Holliston, Mass.) with a 4-mm drill bit, as previously described.<sup>24</sup> Biopsies are frozen within 2 seconds by this instrument, which immerses the biopsy in isopentane (–150°C) cooled by a liquid nitrogen jacket. All biopsies obtained at the end of reperfusion were taken within 2 minutes, while arterial blood pressure and oxygenation were maintained. Biopsies obtained sequentially during the experiment were closed by purse-string epicardial sutures using pledgets. All LAD region biopsies were obtained in a region of the heart located between the LAD and one of its diagonal branches from myocardium that visually appeared dyskinetic. We used transmural biopsies so that we could use a rapid-freezing technique that minimizes the SH group oxidation that has hampered measurements of these groups in previous studies.<sup>20</sup> Tissue was stored at –70°C until analysis.

Infarct size was determined in the 90-minute-occlusion dogs, which were reperfused for 5 hours, by incubation with triphenyl tetrazolium chloride. Risk area could not be determined by the dual perfusion method because of biopsy wounds in the myocardium; therefore, it was determined by injection of technetium-99m-radiolabeled 15- $\mu$ m albumin microspheres (3M Co., Minneapolis, Minn.) at 75 minutes of occlusion, followed by subsequent autoradiography.<sup>5,25,26</sup> Because of the need for rapid sample processing to prevent SH group oxidation, we were not able to obtain both tissue biopsy and coronary sinus samples simultaneously in the same dogs during early reperfusion. In the 15-minute-occlusion dogs, the absence of myocardial infarcts in all dogs was confirmed by tetrazolium staining; risk area was not measured because of biopsy wounds in the myocardium. A reduction in tissue ATP, previously described in this model,<sup>27</sup> was used to confirm that LAD zone ischemia occurred during coronary occlusion. Six dogs received therapy before 15 minutes of ischemia with dimethylthiourea (DMTU) (500 mg i.v. in 500 ml normal saline over one-half hour) to test if SH group changes were reduced when a reactive oxygen metabolite scavenger was given.<sup>2,5</sup> Three additional dogs underwent 90 minutes of sham LAD coronary "occlusion" followed by 5 hours of "reperfusion" as time controls.

#### *Measurement of Coronary Sinus GSH and GSSG Release*

Samples were obtained and processed as we have previously described.<sup>10</sup> Coronary sinus and paired aortic samples were obtained before the occlusion, at the end of the occlusion period, and at 2, 5, 7, 10, 15, and 30 minutes of reperfusion. One-milliliter blood samples were obtained simultaneously from arterial and coronary sinus catheters and then immediately divided (0.5 ml each) into separate tubes containing GSH and GSSG collection buffer. GSH collection buffer consisted of 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 3 mM dinitrothiobenzene (DTNB) and 5 mM EDTA. Superna-

tants for GSH assay were frozen at  $-70^{\circ}\text{C}$  until analysis. GSSG collection buffer was 0.3 ml of 0.1 M potassium phosphate buffer at pH 6.5 with 10 mM *N*-ethylmaleimide (NEM) and 5 mM EDTA. NEM reacts with reduced SH groups and prevents their detection in the assay. Supernatants for GSSG analysis were then processed to remove unreacted NEM. Supernatants (0.4 ml) were passed through Sep-Pak C18 cartridges (Waters Associates, Milford, Mass.), followed by 1 ml potassium phosphate buffer. The eluate was frozen and stored at  $-70^{\circ}\text{C}$  until analysis.

An adaptation of the assay<sup>10</sup> devised by Tietze<sup>28</sup> and modified by Adams et al<sup>29</sup> was used to measure GSH and GSSG using a multiwell plate-reader (Biotek, Highland Park, Vt.). For GSH assay, each well contained 50  $\mu\text{l}$  of 5 mM EDTA and 3 mM DTNB in 0.1 M potassium phosphate buffer, 10  $\mu\text{l}$  glutathione reductase (0.25 mg/ml, Sigma type III), 50  $\mu\text{l}$  sample, and 100  $\mu\text{l}$  potassium phosphate buffer. For GSSG assay, reactants were similar, except 150  $\mu\text{l}$  sample was used and the 100  $\mu\text{l}$  potassium phosphate was omitted. Standard curves were used with concentrations of 0–500 pmol/well of both GSH and GSSG. The reaction was started by the addition of 40  $\mu\text{l}$  of 1.25 mM NADPH (cofactor for glutathione reductase). Total well volume was 250  $\mu\text{l}$ . Absorbance (optical density [OD]) at 412 nm ( $\text{OD}_{412}$ ) was followed at 2-minute intervals at  $28^{\circ}\text{C}$ . Standard curves of concentration versus net  $\text{OD}_{412}$  (OD of the sample – OD of sample blank) were constructed at each time. All chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis.

Grossly hemolyzed samples occurred rarely (<1%) and were discarded. The recovery of added GSH (10–100 nmol/ml) and GSSG (0.5–5 nmol/ml) averaged 93% and 96%, respectively. Less than 0.4% of added GSH was oxidized to GSSG during processing.

#### Measurement of Myocardial GSH and GSSG

Analyses were performed by the method of Ishikawa and Sies.<sup>30</sup> Approximately 100 mg tissue for GSH assay was pulverized at liquid nitrogen temperature, weighed, and homogenized at  $4^{\circ}\text{C}$  in 6 vol of 1 M perchloric acid with 2 mM EDTA. The homogenate was centrifuged, and the supernatant was neutralized to pH 7.0 with 2 M KOH in 0.3 M MOPS. The supernatant was then re-centrifuged, and the resulting supernatant was assayed by the plate-reader method described above. Tissue for GSSG analysis was homogenized in 6 vol of 1 M perchloric acid with 2 mM EDTA and 10 mM NEM. The supernatant was neutralized to pH 6.2 by 2 M KOH and 0.3 M MOPS. One milliliter was applied to a previously unused Sep-Pak C18 column to extract unreacted NEM. An additional 1.0 ml of 0.1 M potassium phosphate eluted the sample. The combined eluate was used for GSSG assay using the glutathione reductase assay described above. The recovery of added GSH averaged 89%, and recovery of added GSSG averaged 85%. DMTU did not alter measured GSH levels,

although it interfered with measurement of GSSG, probably by an effect on the Sep-Pak columns.

#### Measurement of Myocardial Total SH and Nonprotein SH Groups

Myocardial SH groups were determined by a modification of the method of Sedlak and Lindsay,<sup>31</sup> which uses the spectrophotometric end point of the reaction of SH groups with DTNB. Approximately 100 mg tissue was homogenized in 2 ml of 0.02 M EDTA at  $4^{\circ}\text{C}$ . For assay of total SH groups, 125  $\mu\text{l}$  supernatant was combined with 375  $\mu\text{l}$  of 0.2 M Tris, pH 8.2; 25  $\mu\text{l}$  of 0.01 M DTNB in methanol; and 1,975  $\mu\text{l}$  methanol. The methanol is used as a protein-denaturing agent. The reaction was allowed to stand for 15 minutes; samples were then centrifuged at 3,000g for 15 minutes, and  $\text{OD}_{412}$  of the supernatant was measured. A molar absorbance of 13,100  $\text{cm}^{-1}\cdot\text{mol}^{-1}$  was used to calculate SH group concentration. Nonprotein SH groups were measured using 1,250  $\mu\text{l}$  original supernatant. To this, 1 ml deionized water and 250  $\mu\text{l}$  of 50% trichloroacetic acid, to precipitate proteins, was added. The tube was agitated, allowed to stand for 15 minutes, and centrifuged for 15 minutes at 3,000g. Five hundred microliters of the resulting supernatant was added to 1 ml of 0.4 M Tris, pH 8.9, and 0.1 M DTNB in phosphate buffer. The mixture was agitated,  $\text{OD}_{412}$  was measured, and the extinction coefficient was used to determine SH group concentration. GSH was used as the SH group standard. Protein SH groups were calculated as the difference between total and nonprotein SH levels. Non-GSH, nonprotein SH groups were calculated as the difference between nonprotein SH and GSH. DMTU did not alter SH levels measured by these assays.

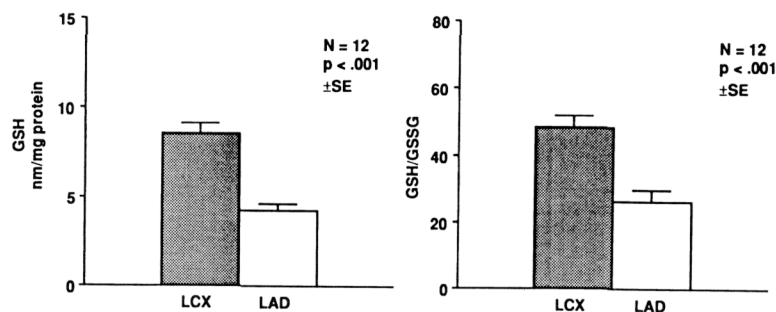
#### Other Biochemical Assays

Protein was measured by the Coomassie brilliant blue dye binding method of Bradford<sup>32</sup> using bovine serum albumin as a standard. Tissue ATP was measured by the fluorometric method of Lowry and Passonneau.<sup>33</sup> This method measures ATP using the hexokinase-catalyzed phosphorylation of glucose to glucose-6-phosphate by ATP. Glucose-6-phosphate was quantitated by the reduction of NADP to NADPH, catalyzed by glucose-6-phosphate dehydrogenase. NADPH was measured by emission at 380 nm after excitation at 469 nm. Tissue samples were calculated using a standard curve of ATP standards.

#### Statistical Analysis

Differences over time within groups were compared by two-way analysis of variance (ANOVA) using the Student-Newman-Keuls test of multiple comparisons. Differences between two groups were compared by one-way ANOVA. Differences between ischemic/reperfused and normal myocardium were compared by *t* test with paired analysis.<sup>34</sup> A difference of  $p < 0.05$  was considered significant.

## PROLONGED OCCLUSION MODEL



## BRIEF OCCLUSION MODEL

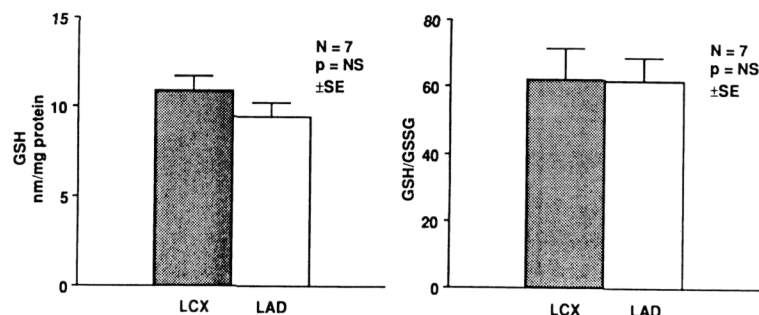


FIGURE 1. Bar graphs showing reduced glutathione (GSH) and the GSH/oxidized glutathione (GSSG) ratio in tissue during reperfusion after brief (15-minute) and prolonged (90-minute) ischemia. In the prolonged-occlusion model, GSH decreased in ischemic/reperfused left anterior descending (LAD) compared with normal circumflex (LCX) myocardium, with a decrease in the GSH/GSSG ratio. In the brief-occlusion model, LAD and LCX myocardial levels measured after 30 minutes of reperfusion were similar.

## Results

## Tissue SH Levels: 90-Minute Ischemia (Prolonged)

Tissue GSH and GSSG were measured in the ischemic/reperfused LAD zone and the normal circumflex (LCX) zone. Tissue GSH was markedly reduced in the LAD region compared with the LCX region at 300 minutes of reperfusion (Figure 1, top panels). Tissue GSSG was similar in both regions (LAD,  $18.4 \pm 1.3$  nmol/g tissue; LCX,  $21.9 \pm 1.8$  nmol/g tissue;  $n=12$ ,  $p=NS$ ). The GSH/GSSG ratio, an index of myocardial oxidative stress,<sup>30,35</sup> was reduced in the LAD zone (Figure 1).

Serial tissue biopsies were obtained from the LAD zone to evaluate the time course of GSH and GSSG changes. Although there was a trend toward a slightly higher GSSG before ischemia, myocardial GSSG did not change significantly during ischemia or reperfusion (GSSG preocclusion,  $20.9 \pm 1.8$  nmol/g tissue;

90-minute occlusion,  $16.5 \pm 1.6$  nmol/g tissue; 30-minute reperfusion,  $17.9 \pm 3.7$  nmol/g tissue; 120-minute reperfusion,  $14.7 \pm 2.2$  nmol/g tissue; 300-minute reperfusion,  $16.6 \pm 2.1$  nmol/g tissue;  $n=5$ ,  $p=NS$ ). Myocardial GSH decreased significantly after 90 minutes of ischemia, decreased further during the initial 120 minutes of reperfusion, and remained depressed at 300 minutes of reperfusion (Figure 2).

As previously described,<sup>10</sup> prolonged coronary occlusion caused release of GSH and GSSG into the coronary sinus during early reperfusion. Levels in the coronary sinus were elevated compared with corresponding levels in the aorta, as well as compared with preocclusion coronary sinus levels (Figures 3 and 4, left panels). This release of GSH coincides with the decrease in myocardial GSH seen at end occlusion and during reperfusion (Figure 2). GSSG release occurred without a detectable change in myocardial GSSG.

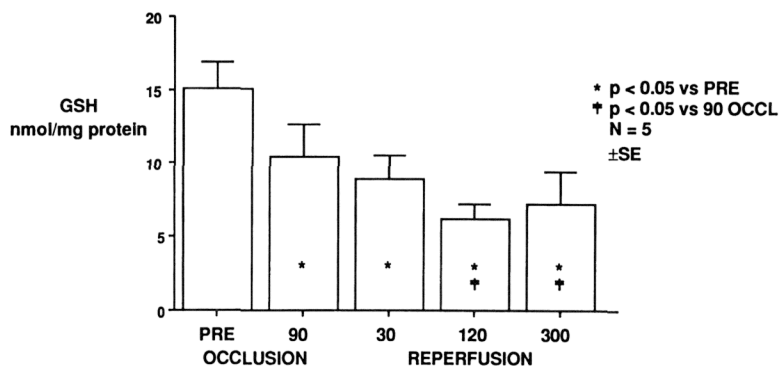


FIGURE 2. Bar graph showing reduced glutathione (GSH) in tissue during ischemia and reperfusion with 90 minutes of ischemia (90 OCCL). PRE, preocclusion. GSH levels measured in sequential biopsies decreased in ischemic/reperfused myocardium in the left anterior descending zone at the end of 90 minutes of ischemia, with a further decrease during reperfusion.

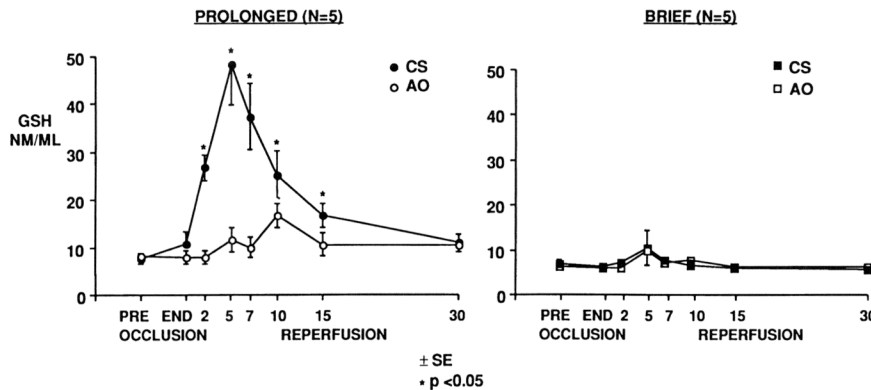


FIGURE 3. Graphs showing reduced glutathione (GSH) release during reperfusion. GSH levels increased in the coronary sinus (CS) compared with aorta (AO) during reperfusion after 90, but not 15, minutes of ischemia.

Total tissue SH groups were decreased in the LAD region compared with the LCX zone when measured at 300 minutes of reperfusion ( $n=6$ ,  $p<0.01$ ) (Figure 5). This was due mainly to a decrease in the nonprotein SH groups ( $p<0.01$ ), which include GSH. The fall in the nonprotein SH pool was due to significant decreases in both GSH and non-GSH, nonprotein SH groups ( $p<0.02$ ). There was a modest trend toward a decrease in protein SH groups, but this did not reach significance ( $p=0.20$ ) (Figure 5). Total tissue protein was similar in both groups (LAD,  $131\pm3$  mg protein/g tissue; LCX,  $136\pm7$  mg protein/g tissue;  $n=6$ ,  $p=NS$ ).

Infarct size in our experiments as a percent of area at risk (infarct size÷area at risk) averaged  $43.9\pm4.0\%$  ( $n=17$ ). Area at risk as a percent of the left ventricle (area at risk÷left ventricular area) was  $25.7\pm1.7\%$  ( $n=17$ ). The extent of myocardial infarction is comparable with that previously reported in untreated dogs with 90 minutes of ischemia and subsequent reperfusion.<sup>5,6,25</sup> Heart rate increased at the end of reperfusion, whereas the heart rate–blood pressure product and aortic pressure were unchanged during ischemia and reperfusion (Table 1).

*Tissue SH Levels: 15-Minute Ischemia (Brief)*

After 15 minutes of ischemia, both myocardial GSH (Figure 1, lower panels) and GSSG (LAD,  $20.7\pm2.6$  nmol/g tissue; LCX,  $23.8\pm2.4$  nmol/g tissue;  $n=7$ ,  $p=NS$ ), measured at 30 minutes of reperfusion, were unchanged in the LAD region compared with the LCX region. The GSH/GSSG ratio was also unchanged at 30 minutes of reperfusion (Figure 1). In two experiments, there was no change in GSH, GSSG, or the GSH/GSSG ratio at the end of the ischemic period. Thus, in contrast to the prolonged

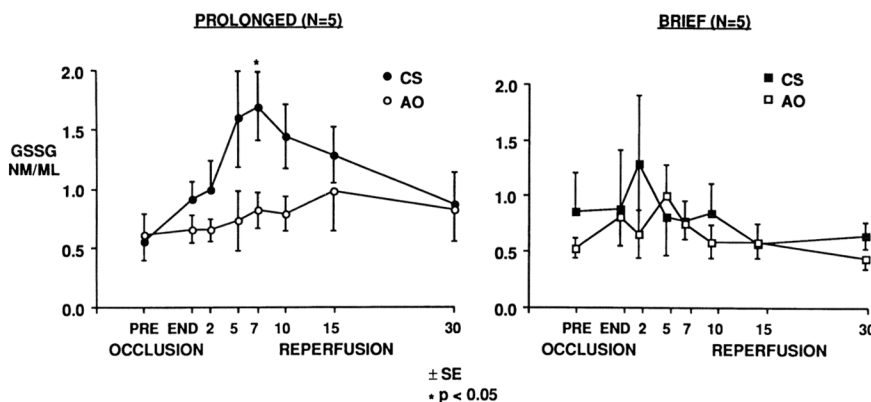


FIGURE 4. Graphs showing oxidized glutathione (GSSG) release during reperfusion. GSSG levels increased in the coronary sinus (CS) compared with the aorta (AO) during reperfusion after 90, but not 15, minutes of ischemia.

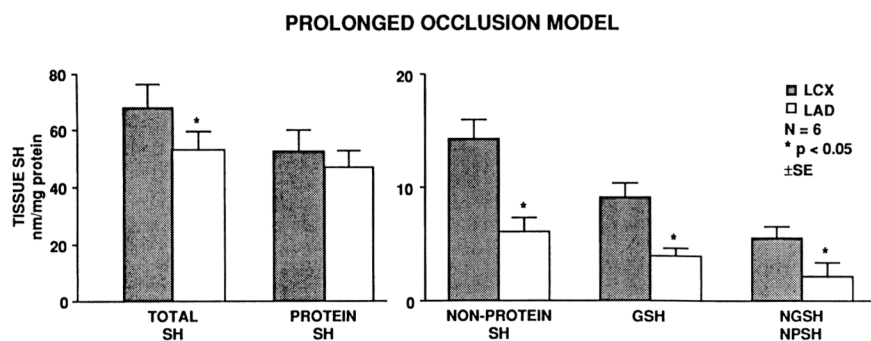


FIGURE 5. Bar graphs showing tissue sulfhydryl (SH) pools during reperfusion after 90 minutes of ischemia. At 300 minutes of reperfusion, total SH, nonprotein SH, reduced glutathione (GSH), and non-GSH, nonprotein SH (NGSH NPSH) pools were decreased in the ischemic/reperfused left anterior descending (LAD) zones compared with the normal circumflex (LCX) zones, without a significant difference in protein SH groups.

TABLE 1. Hemodynamics During Brief and Prolonged Occlusion

	Preocclusion	Occlusion		Reperfusion	
		15 min	90 min	30 min	300 min
<i>Prolonged ischemia (n=6)</i>					
RPP (beats/min×mm Hg×10 <sup>4</sup> )	11.6±1.3	12.9±1.8	11.7±1.7	11.8±1.7	14.6±1.0
HR (beats/min)	110±7	123±9	112±12	123±16	167±15*
AO (mm Hg)	104±8	103±8	103±7	95±5	89±4
<i>Brief ischemia (n=7)</i>					
RPP (beats/min×mm Hg×10 <sup>4</sup> )	13.0±1.5	12.6±1.8	...	11.8±1.3	...
HR (beats/min)	111±6	113±6	...	109±4	...
AO (mm Hg)	115±10	110±11	...	107±9	...
<i>Brief ischemia+DMTU (n=6)</i>					
RPP (beats/min×mm Hg×10 <sup>4</sup> )	16.0±1.4	15.9±1.2	...	16.2±1.5	...
HR (beats/min)	138±19	138±17	...	138±17	...
AO (mm Hg)	119±7	118±6	...	120±6	...

Values are mean $\pm$ SEM. RPP, rate-pressure product; HR, heart rate; AO, aortic pressure; DMTU, dimethylthiourea.

\* $p < 0.05$  vs. preocclusion.

occlusion model, brief coronary occlusion-reperfusion did not alter tissue GSH or the GSH/GSSG ratio.

Brief occlusions also did not cause release of GSH or GSSG during reperfusion (Figures 3 and 4, right panels). Plasma levels of GSH and GSSG measured in the coronary sinus in the brief-occlusion dogs were similar to levels in the aorta. LAD regional myocardial blood flow during occlusion was similar in the prolonged-occlusion dogs (endocardium,  $0.16 \pm 0.07$  ml/min/g; epicardium  $0.33 \pm 0.08$  ml/min/g;  $n=5$ ) and brief-occlusion dogs (endocardium,  $0.14 \pm 0.08$  ml/min/g; epicardium,  $0.25 \pm 0.04$  ml/min/g;  $n=5$ ,  $p=NS$ ).

Total SH and protein SH groups were similar in LAD and LCX regions in the brief-occlusion model (Figure 6). However, nonprotein SH groups in the LAD region were decreased compared with the LCX zone (Figure 6). This was due to a significant decrease in the non-GSH, nonprotein SH pool (Figure 6) while GSH was unchanged. Therefore, although most SH groups are preserved, even with brief coronary occlusions there is a selective decrease in low molecular weight SH compounds other than GSH.

ATP was reduced in transmural biopsies from the LAD region ( $4.3 \pm 0.5$   $\mu$ mol/g,  $n=7$ ) compared with LCX ( $6.0 \pm 0.4$   $\mu$ mol/g,  $n=7$ ;  $p=0.05$ ) (Table 2). The

decrease of 23% in transmural ATP is similar to changes previously reported with 15 minutes of regional ischemia followed by reperfusion.<sup>27</sup>

Six additional dogs were treated with DMTU (500 mg/kg i.v. over 20 minutes) before 15 minutes of occlusion and 30 minutes of reperfusion to test whether the decrease in non-GSH, nonprotein SH could be reduced by therapy with a scavenger of reactive oxygen metabolites. DMTU was chosen since it is a cell-permeable scavenger of  $H_2O_2$  and  $\cdot OH$  that reduced canine myocardial injury after both brief<sup>2</sup> and prolonged<sup>5</sup> periods of ischemia. ATP decreased by 28% in the LAD region (Table 2), similar to the decrease in untreated controls. Total SH and protein SH were unchanged after ischemia and reperfusion (Table 2). GSH decreased 11% ( $p=NS$ ), similar to the decrease in untreated animals. DMTU reduced the decrease in nonprotein (9% decrease) and non-GSH, nonprotein SH pools (3% decrease) that occurred in untreated controls (Table 2). Thus, these two sensitive SH pools were protected by a cell-permeable scavenger of  $H_2O_2$  and  $\cdot OH$ . Heart rate-blood pressure product, heart rate, and aortic pressure were unchanged during ischemia and reperfusion and were not significantly different from the other groups.

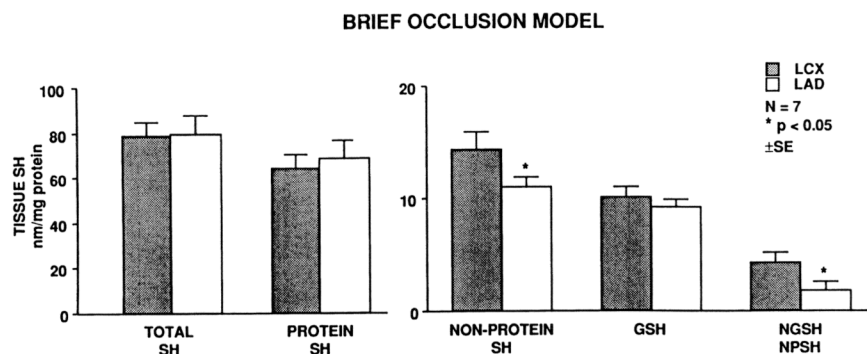


FIGURE 6. Bar graphs showing tissue sulfhydryl (SH) pools during reperfusion after 15 minutes of ischemia. At 30 minutes of reperfusion, total SH groups, as well as protein SH, and reduced glutathione (GSH) were similar in ischemic/reperfused left anterior descending (LAD) and normal circumflex (LCX) myocardium. Nonprotein SH groups were decreased, due to a decrease in non-GSH, nonprotein SH groups (NGSH NPSH), compared with normal myocardium.

TABLE 2. Sulfhydryl Pools During Brief Occlusion

	Untreated (n=7)		DMTU (n=6)	
	LAD	LCX	LAD	LCX
Total SH (nmol/mg protein)	79.4±8.3	78.4±6.6	78.5±2.3	79.3±2.3
Protein SH (nmol/mg protein)	68.4±8.2	64.0±6.1	67.1±2.2	66.9±2.2
Nonprotein SH (nmol/mg protein)	11.1±0.9*	14.4±1.5	11.9±0.8	13.1±0.8
GSH (nmol/mg protein)	9.2±0.7	10.1±1.0	8.5±0.8	9.5±0.6
Non-GSH nonprotein SH (nmol/mg protein)	1.8±0.8*	4.3±0.9	3.4±1.1	3.5±0.6
ATP (μmol/g)	4.3±0.5*	6.0±0.4	4.3±0.3*	6.0±0.3

Values are mean±SEM. DMTU, treated with dimethylthiourea; LAD, left anterior descending myocardium; LCX, normal circumflex myocardium; SH, sulfhydryl; GSH, reduced glutathione.

\**p*<0.05 vs. corresponding LCX.

### Tissue SH Groups in Time Controls

Tissue SH groups at the end of the reperfusion period were similar in time controls and the normal LCX zone in the 90-minute occlusion model (Table 3). Tissue GSSG at the end of reperfusion was also similar to the GSSG levels measured throughout reperfusion in the 90-minute occlusion model.

### Discussion

Reactive oxygen metabolites appear to contribute to in vivo myocardial ischemia/reperfusion injury.<sup>1-6</sup> Support for this concept is predominantly based on experiments in which oxygen metabolite scavengers reduce evidence of myocardial injury in brief (15-minute) and prolonged (90-minute) models of coronary occlusion. The prolonged occlusion model involves myocardial necrosis<sup>4-6</sup>; the brief occlusion model does not.<sup>1-3</sup> Superoxide dismutase (a scavenger of superoxide anion),<sup>1,4</sup> DMTU (a scavenger of hydrogen peroxide and the hydroxyl radical),<sup>2,5</sup> and deferoxamine (a blocker of iron-catalyzed hydroxyl generation)<sup>3,6</sup> reduce injury in both models. Despite apparent reductions in infarct size measured at 6 hours of reperfusion or improved recovery of regional function with scavenger treatment, there is little insight into the oxidative alterations that occur with these two differing degrees of ischemia/reperfusion injury.

We found that during reperfusion after a 90-minute coronary occlusion there was a decrease in myocardial total SH levels. This decrease in SH groups involved mainly nonprotein SH groups, in-

cluding both GSH and the non-GSH, nonprotein pool. Protein SH groups were relatively well preserved, even with this severe ischemic insult. Decreases in most SH pools with release of GSSG from myocardium are consistent with substantial oxidative injury in the prolonged occlusion model. In contrast, brief periods of ischemia, although similar with respect to the degree of blood flow reduction, are not associated with such striking changes in SH groups. Myocardial tissue levels of GSH, GSSG, total, and protein SH groups were unchanged. However, even this brief period of ischemia followed by reperfusion caused a significant decrease in the nonprotein SH pool, due to a fall in non-GSH, nonprotein SH groups.

The non-GSH, nonprotein SH pool may be a sensitive marker of the injury that occurs with brief occlusions. This pool is composed of the SH-containing amino acid cysteine, its derivatives such as cysteamine, and other low molecular weight SH groups. These include acetyl coenzyme A, ergothioneine, and lipoic acid.<sup>36</sup> Ergothioneine is a naturally occurring dietary SH molecule that is present in animal tissues.<sup>36</sup> Most animal tissues, including heart, have a total non-GSH, nonprotein SH level of 0.3–0.7 μmol/g tissue,<sup>36</sup> which is in excellent agreement with the myocardial levels we obtained in the normal region. The decrease in the non-GSH, nonprotein SH pool in the brief model occurred while myocardial GSH, GSSG, and other SH pools were unchanged. The decrease was prevented by treatment with DMTU, an intracellular scavenger of H<sub>2</sub>O<sub>2</sub> and ·OH, further suggesting that the decrease is due to alterations induced by reactive oxygen metabolites. The decrease in non-GSH, nonprotein SH groups appears to be a sensitive indicator of mild oxidative injury. Mild oxidative injury probably decreases the non-GSH, nonprotein SH pool before GSH or GSSG are changed because, although glutathione reductase reduces GSSG to GSH, it cannot reduce the oxidized forms of non-GSH, nonprotein SH groups.<sup>28</sup>

Canine myocardial ischemia longer than 45 minutes is associated with myocyte membrane disruption in the ischemic zone.<sup>37</sup> Since GSH is normally secreted only by the liver,<sup>7</sup> the myocardial GSH release during reperfusion after 90 minutes of ischemia

TABLE 3. Tissue Sulfhydryl Groups in Time Controls

	Time control (n=3)	90-min LCX (n=6)
Total SH (nmol/mg protein)	66.9±2.6	67.1±7.7
Protein SH (nmol/mg protein)	50.9±2.0	52.9±7.6
Nonprotein SH (nmol/mg protein)	16.1±0.9	14.3±1.7
GSH (nmol/mg protein)	9.0±0.4	9.1±1.3
Non-GSH nonprotein SH (nmol/mg protein)	7.1±0.7	5.5±1.0

Values are mean±SEM. LCX, normal circumflex zone; SH, sulfhydryl; GSH, reduced glutathione.

probably requires severe membrane injury. The lack of GSH release after 15 minutes of ischemia is consistent with preserved membrane integrity after brief ischemia. The decrease in myocardial GSH after 90 minutes of ischemia can partly be accounted for by reperfusion GSH release due to membrane disruption, as well as GSH conversion to GSSG with subsequent myocardial release. Also, direct reactions between GSH and superoxide or other radicals that destroy GSH and produce products other than GSSG could have occurred.<sup>14,15</sup>

Tissue GSSG has not been previously measured during in vivo regional ischemia/reperfusion.<sup>9,19-21</sup> Previous in vitro studies in buffer-perfused hearts demonstrated small increases in tissue GSSG as well as GSSG release with reperfusion injury.<sup>38</sup> In contrast, we found that GSSG did not accumulate in myocardium during ischemia or reperfusion, despite myocardial necrosis and detectable release into venous effluent. Our data suggests that in vivo, severely injured myocardium releases GSSG at a rate that prevents significant tissue accumulation. GSSG can be released by membrane pumps from intact oxidatively stressed cells<sup>30</sup> or by the membrane disruption present in severely injured cells.<sup>39</sup> It appears that sampling of venous effluent for GSSG is a more sensitive test of potential oxidative injury than tissue sampling in vivo. The absence of myocardial GSSG release in the brief occlusion model suggests that GSSG release occurs predominantly from cells with membrane injury.

GSH and other nonprotein SH groups help prevent the oxidation of protein SH groups.<sup>7,31</sup> Protein SH groups are important to protein structure and function.<sup>16-18</sup> We found that protein SH groups were maintained even with the severe degree of myocardial injury after prolonged occlusion, despite significant decreases in the other SH pools. Consistent with our observations in vivo, in the buffer-perfused rabbit heart, protein SH groups decreased during ischemia but exhibited recovery toward preocclusion levels during the first 30 minutes of reperfusion.<sup>40</sup> Protein SH groups appear to be spared at the expense of nonprotein SH groups.

A decrease in SH groups could predispose to oxidative injury. Consistent with this hypothesis, prior chemical depletion of GSH increases subsequent myocardial injury.<sup>21,41</sup> It appears likely in view of our results that depletion of SH pools other than GSH may also be important in ischemia/reperfusion injury, especially after brief durations of ischemia that do not cause necrosis. Administration of exogenous *N*-(2-mercaptopropionyl)glycine, a source of exogenous SH groups, reduced postischemic myocardial dysfunction (stunning) after brief coronary occlusion<sup>42</sup> and infarct size after prolonged coronary occlusion.<sup>43</sup> *N*-Acetylcysteine, another SH-containing compound, reduced ischemia/reperfusion injury in the buffer-perfused rabbit heart.<sup>44</sup> In vivo, *N*-acetylcysteine administered after 30 minutes of ischemia in a 90-minute LAD occlusion model did not reduce

infarct size but did improve LAD zone contractile function measured during reperfusion.<sup>20</sup> Infusion of GSH has increased total myocardial GSH levels and reduced infarct size.<sup>21</sup> Although previous interest has focused on GSH, it appears likely in view of our study that exogenous SH-group therapy<sup>20,21,44</sup> may reduce myocardial ischemia/reperfusion injury by decreasing oxidation of both GSH and the non-GSH, non-protein SH pool.

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