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Exogenous GSH Protection during Hypoxia-Reoxygenation of the Isolated Rat Heart: Impact of Hypoxia Duration

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The objective of this study was to determine the interaction between duration of myocardial hypoxia and presence of exogenous glutathione (GSH) on functional recovery upon subsequent reoxygenation. Isolated perfused rat hearts were subjected to 20, 30, 40, or 50 min hypoxia (HYP), which resulted in a progressive decline in the amount of contractile recovery (% of normoxic rate–pressure product (RPP) and developed pressure) during 30 min reoxygenation. Supplementation with 5 mM GSH throughout normoxia, hypoxia, and reoxygenation significantly improved contractile recovery during reoxygenation after 20 and 30 min hypoxia ($p < 0.05$), but had no effect after longer durations of hypoxia when contractile recovery was typically below 40% of RPP and significant areas of no-reflow were observed. ECG analysis revealed that GSH shifted the bell-shaped curve for reperfusion ventricular fibrillation to the right resulting in attenuated fibrillation after 20 and 30 min hypoxia then increased incidences after 40 min when Control hearts were slow to resume electrical activity. ECG conduction velocity was well preserved in all hearts after 20 and 30 min hypoxia, but GSH administration significantly attenuated the decline that occurred with longer durations. GSH supplementation did not attenuate the 35% decline in intracellular thiols during 30 min of hypoxia. When 5 mM GSH was added only during 40 min of hypoxia, RPP recovery after reoxygenation was improved compared to unsupplemented Controls (73% vs. 55% of pre-hypoxia value, $p < 0.05$). Administration of GSH only during reoxygenation following 40 min of hypoxia did not alter RPP recovery compared to Control hearts. We conclude that cardioprotection by exogenous GSH is dependent on the duration of hypoxia and the functional parameter being evaluated. It is not due to an enhancement of intracellular GSH suggesting that exogenous GSH acts extracellularly to protect sarcolemmal proteins against thiol oxidation during the phase of hypoxia when oxidative stress is a major contributor to cardiac dysfunction. Furthermore, if enough damage accrues during oxygen deprivation, supplementing with GSH during reoxygenation will not impact recovery.

Keywords: Oxidative stress, myocardial stunning, thiols, free radicals, antioxidants

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INTRODUCTION

Reduced glutathione (GSH) has been hypothesized to play an important role in protecting against oxidative stress and mechanical dysfunction subsequent to myocardial ischemia or hypoxia.[1–5] Indirect support for this hypothesis is derived from findings that myocardial ischemia or hypoxia alone[1–3,5] or followed by reperfusion[1,2,4] results in a significant duration dependent decline in intracellular GSH concentration. However, investigations designed specifically to address the relationship between GSH status and myocardial functional recovery have been equivocal. Some investigators reported that prior depletion of intracellular GSH does not affect postischemic contractile function[6] or arrhythmias[7] in rat hearts, or cytosolic enzyme release in cat hearts.[8] Conversely, others have reported larger myocardial infarct size in GSH-depleted pig hearts[9] and decreased contractile recovery in GSH-depleted hearts from rats,[10] cats [8] and pigs.[9] Exogenous supplementation of GSH to GSH-depleted hearts resulted in improved reperfusion contractile function after short-term global ischemia in rat hearts[10] and coronary artery occlusion in pigs.[9] We have reported that GSH supplementation to normal rat hearts during brief, intermittent hypoxia also results in accelerated recovery of contractile function.[11] Conversely, Tani[12] reported that supplementation of GSH or other sulfhydryl donor agents to normal rat hearts had no impact on mechanical function or calcium uptake following global ischemia.

Differences among earlier studies in insult duration and amount of tissue damage, as well as differences in models and selection of functional evaluation have contributed to the uncertainty surrounding the impact of GSH as a cardioprotective agent. It is well known that the extent of reperfusion injury is related to the duration of the preceding ischemic bout.[13,14] Furthermore, Henry *et al.*[15] have observed that the magnitude of postischemic oxygen radical generation is also dependent on ischemic duration in a bell-shaped manner; low radical production after short and long lengths of ischemia and highest at intermediate lengths. Thus, in the present paper, we have examined the impact of duration of insult on exogenous GSH protection of several functional parameters in the isolated rat heart subjected to hypoxia and reoxygenation. Substrate-free hypoxia was employed primarily to allow delivery of GSH at all times throughout varying lengths of oxygen deprivation. This model also minimizes the impact of variables other than the delivery of oxygen and substrate. Separate groups of hearts were exposed to one of four insult durations: 20, 30, 40, or 50 min, which provided a progressive increase in the severity of the hypoxic stress. Functional parameters selected were typically evaluated individually at a single length of ischemia or hypoxia in previous GSH studies and included: ventricular mechanical properties, coronary flow, and electrocardiographic characteristics.

METHODS

Animals

Male Sprague–Dawley rats were purchased from the breeding colony maintained by the Animal Resources Center at The University of Texas at Austin. This investigation was approved by the University's Animal Care and Use Committee and conforms with the *Guide for the Care and Use of Laboratry Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Age at the time of sacrifice was between 56 and 67 days and body weight was 250–325 g.

Isolated Heart Perfusions

All hearts were perfused according to the isolated, non-recirculating, Langendorff heart preparation. Rats were anesthetized with an IP injection

of 0.5 ml/kg body weight of anesthesia cocktail consisting of: ketamine (100 mg/ml), xylazine (20 mg/ml) and acepromazine (10 mg/ml). An intravenous injection of heparin (100 IU) was administered immediately prior to heart excision. The heart was rapidly excised, lightly blotted with a Kimwipe, and immersed in a beaker of cold 0.9% NaCl resting on an electronic balance for the determination of gross heart weight. The aorta was mounted on a stainless steel cannula for retrograde perfusion at 80 cm hydrostatic pressure with normoxic Krebs–Henseleit bicarbonate buffer (KHB) maintained at 37°C. During the initial minutes of perfusion, the heart was trimmed of non-cardiac tissue, which was weighed and subtracted from the gross weight. The heart was enclosed for the duration of the experiment within a water jacketed chamber (37°C) sealed with parafilm. The normoxic KHB was gassed with O_2–CO_2 (19 : 1) and contained (in mM) NaCl 118.5, KCl 4.7, $CaCl_2$ 3.0 (2.1 for free Ca^{++}), $MgSO_4$ 1.0, Na_2EDTA 0.5, $NaHCO_3$ 24.7, glucose 10 and insulin 12 IU/l. Substrate-free hypoxia was induced by perfusion with modified KHB containing 10 mM mannitol instead of glucose and gassed with N_2–CO_2 (19 : 1).

GSH Exposure and Length of Perfusion Periods

All hearts were initially perfused with normoxic buffer for 30 min, made hypoxic for various lengths of time, and then reoxygenated for 30 min. Preliminary studies were carried out to determine a range of hypoxic durations that resulted in myocardial dysfunction after reoxygenation ranging from mild but significant stunning, about 20% loss of mechanical function, to complete absence of contractile function. Based on these experiments, hearts were exposed to one of four durations of hypoxia: 20, 30, 40, or 50 min. In GSH-treated hearts, the normoxic and hypoxic buffers were supplemented with 5 mM reduced glutathione (Sigma Chemical Co., St. Louis, MO) so that GSH was present throughout the initial normoxic baseline period, hypoxia, and reoxygenation. The pH of all perfusion buffers was 7.4.

A second study was carried out to determine the impact of GSH when administered only during hypoxia or only during reoxygenation following a hypoxic duration that caused significant dysfunction. Due to the time lag between the two studies, preliminary experiments were again performed in Control hearts to determine the duration of hypoxia that would produce up to a 50% decline in mechanical function after recovery without creating areas of no-reflow, which would trap GSH administered during hypoxia and prevent its delivery during reoxygenation. Based on these trials, a 40 min duration of hypoxia was selected; in 6 Control hearts, no significant residual no-reflow was observed after 40 min of hypoxia and 30 min of reoxygenation (0% in 4 hearts, $< 5\%$ in 2 hearts). For this timing-of-application study, hearts were randomly assigned to one of four hypoxial/reoxygenation groups. Control hearts were not supplemented at any time. In two groups, exogenous 5 mM GSH was administered either during hypoxia (HYP) only or during reoxygenation (REOX) only. In a separate group of hearts, 2.5 mM GSSG was substituted for GSH during the hypoxic period to determine whether the protective effect of GSH during hypoxia was specific to the reduced form of the molecule. Hypoxia hearts received GSH only during the first 37 min of the 40 min hypoxic perfusion period. A 3 min GSH-free hypoxic perfusion washout period preceded reoxygenation to minimize the possible contribution of GSH during the initial minutes of reoxygenation.

Functional Measurements

Ventricular beating rate and intraventricular systolic and diastolic pressures were monitored by inserting a fluid filled, 20 gauge hypodermic needle into the left ventricular lumen through the apex. In the Langendorff preparation ventricular

filling occurs during diastole via thebesian veins at a pressure proportional to the perfusion pressure[16] allowing for reperfusion-related changes in end-diastolic pressure and developed pressure to be monitored with an open catheter or needle.[11,12,16] The needle was attached via a 6 inch pressure monitoring line (model MX570, Medex, Hilliard OH) to a Gould DTX pressure transducer (Gould Cardiovascular Products, Oxnard, CA) interfaced with a Gould oscillographic recorder (Gould Recording Systems, Cleveland OH). Heart rate was determined from ventricular pressure traces. Rate–pressure product (RPP), a measure of ventricular work, was determined as the product of ventricular beating rate and intraventricular developed pressure (peak systolic–diastolic pressure). Coronary flow was determined by weighing timed collections (20 s) and normalized for heart weight. Electrocardiograms were collected using a bi-polar lead configuration with the stainless steel cannula serving as the grounded lead and the apical needle serving as the active lead. ECG data were acquired using modifications of a method described by Combs *et al.*[17] This method produced time domain data accurate to the 1/1000 second for the determination of changes in ECG segment duration before and after hypoxia, and the determination of rhythm status of the heart. Real-time ECG monitoring was performed in oscillographic mode. Ten to fifteen second ECG traces were recorded to a hard drive after 30 min of normoxic perfusion and again following the hypoxic insult and 30 min of reoxygenation for subsequent analysis.

Reoxygenation

Upon reoxygenation, some hearts entered into periods of ventricular fibrillation. In order to compare fibrillation incidence between GSH and untreated groups, no attempt was made to defibrillate the heart during the first 10 min of reoxygenation. Coronary flow, heart rate, systolic and diastolic pressure were determined after 3, 5, and 10 min of reoxygenation. Hearts fibrillating at the time of these measurements were assigned a developed pressure value of zero, and fibrillation incidence was recorded in binary fashion. After 10 min of reoxygenation, fibrillating hearts were countershocked using an electrical stimulator (Phipps and Bryd model 611) by delivering a single square wave pulse of 80 V and 20 ms duration, across the heart via the aortic cannula and intraventricular needle. In some cases, multiple single pulses were administered over several minutes until sinus rhythm was reestablished. In 3 hearts exposed to 20 min of hypoxia (2 untreated and 1 GSH-treated), fibrillation persisted well beyond 10 min of reoxygenation despite attempts to defibrillate. Contractile measurements from these hearts were excluded from mean values for the affected time points. Functional measurements were taken after 30 min of reoxygenation, then all hearts were perfused for an additional 3 min with GSH-free medium to rinse out interstitial and intravascular GSH in GSH-treated hearts prior to freeze-clamping. Thus total reoxygenation time for both GSH-treated and Control hearts was 33 min. Hearts were freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen and then stored at −100°C until analyzed.

No-Reflow Determination

Instead of freeze-clamping, hearts were injected with 0.3% phthalocyanine blue pigment (Monastral Blue, Sigma Chemical, St. Louis, MO) at the reoxygenation pressure through a sidearm of the aortic cannula. Injection was continued for 5 s after the first appearance of blue dye dripping from the heart. The perfusate in-flow line was immediately clamped, the heart cut from the cannula, rinsed in 0.9% NaCl and fixed in formalin. After 24 h fixation hearts were sliced in 1 mm sections perpendicular to the long axis and photographed on a white opaque Plexiglas under lighted platform. Color photographic negatives were projected and digitized (Sigma Scan, Jandel

Scientific, Sausalito, CA) for the determination of no-reflow (dye-free) area, calculated as a percentage of total cross-sectional area. Three non-apical slices were digitized to determine mean no-reflow area in each heart.

Intracellular Thiol Measurement

To determine if exogenous GSH supplementation impacted gross intracellular thiol concentration, approximately 150 mg portions of ventricle were ground under liquid nitrogen and homogenized in 0.3 M perchloric acid, 5 mM EDTA. After centrifugation, the extract containing acid soluble thiols was neutralized to pH 7.0 (6.85–7.15) via micro-titration with KOH, centrifuged to remove the neutralization precipitate and frozen in liquid nitrogen after gassing with N_2 to remove dissolved oxygen. Within three hours of extraction all extracts were thawed to 0–4°C and assayed for acid soluble thiols using a modification of the DTNB binding method.[18] We have previously determined that this fraction is composed of approximately 90% GSH by comparing the non-specific colorimetric thiol determination with the GSH-specific enzymatic recycling method of Tietze.[19]

Statistical Analysis

All statistical analyses were performed using SPSS for Windows™. As it was hypothesized that exogenous GSH would attenuate myocardial stunning, the one-tailed Student's *t*-test was employed for comparisons of flow, contractile and electrical responses in GSH vs. untreated groups at each duration of hypoxia. The Mann–Whitney *U* test was used to compare fibrillation incidence in GSH-treated and -untreated Control hearts. For the timing-of-application study, a one-way ANOVA was used to compare functional parameters in the four treatment groups at each time point. In cases where ANOVA indicated group differences, *post hoc* comparisons were made using Duncan's Multiple Range test. A p value ≤ 0.05 was considered statistically significant for all analyses.

RESULTS

Functional Responses during Normoxia and Hypoxia

Table I displays the baseline functional status of the hearts immediately prior to hypoxia. Baseline coronary flow and electrical conduction velocity during 30 min of normoxic perfusion were similar in GSH-treated hearts and untreated hearts. Heart rate was slightly, but significanfly ($p < 0.05$), lower in GSH-treated hearts after 30 min of normoxic perfusion. However, developed pressure was proportionally higher so that ventricular work (RPP) was not significantly altered by the presence of exogenous GSH.

RPP decline during hypoxia was virtually identical between GSH-treated and Control hearts (Figure 1, panel A). All hearts were akinetic after 15 min of hypoxia (range 8–12 min) and were in contracture at the time of reoxygenation as indicated by elevation of intraventricular diastolic

TABLE I Pre-hypoxia functional characteristics of isolated perfused hearts

Functional parameter	Control	Glutathione (5 mM)
Coronary flow (ml/min/g)	10.6 ± 0.2	11.0 ± 0.2
Heart rate (bpm)	315 ± 6	286 ± 8.5*
Developed pressure (mmHg)	68 ± 1.1	73 ± 1.3*
Rate–pessure product	21,400 ± 590	20,800 ± 500
PR interval (ms)	23.9 ± 0.88	24.8 ± 1.04
QRS duration (ms)	17.9 ± 0.43	17.1 ± 0.52

Data are means ± SE for 26–37 hearts. Control and GSH groups from all four subsequent hypoxic durations were combined. Hearts were perfused by the Langendorff mode as described in Methods. *$p < 0.01$ vs. Control value.

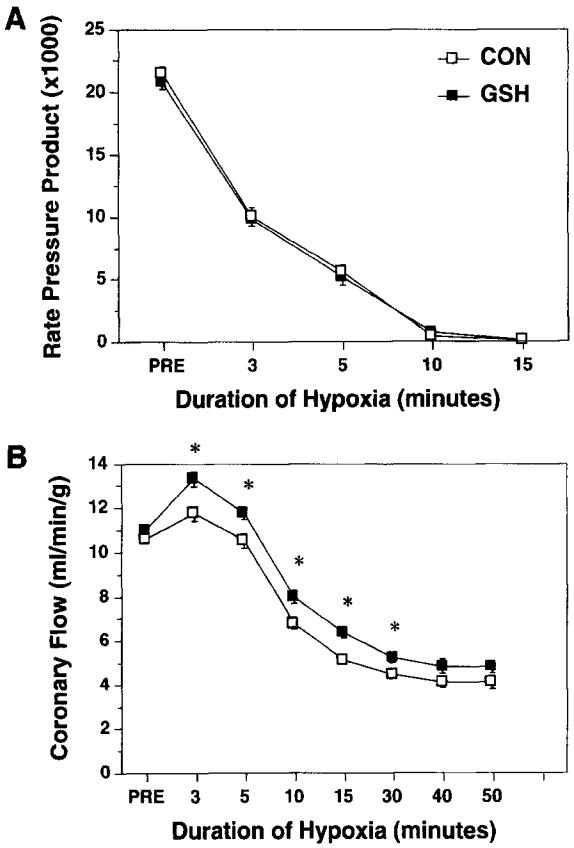

FIGURE 1 Effect of exogenous GSH on rate of functional decline during hypoxia. Panel A: Contractile decline (heart rate × developed pressure) during hypoxia. Values represent means ± SE for combined hearts from all hypoxic durations in each treatment group, Con $n = 37$, GSH $n = 34$. There were no significant differences in RPP at any time point. Panel B: Effect of exogenous GSH on the coronary flow changes during hypoxia. Data are combined means ± SE for all groups. Therefore $n = 30$–35 hearts at each time point through 15 min of hypoxia. At 30 min, $n = 25$–26. At 40 min, $n = 15$, at 50 min $n = 5$ in each group. *$p < 0.05$ vs. Con at same time point.

pressure. Coronary flow (Figure 1, panel B) was transiently elevated during the first 5 min of hypoxia and then declined steadily in concert with the decline in RPP and the increase in intraventricular diastolic pressure. The initial hyperemic effect of hypoxia onset was augmented in hearts perfused with GSH, and remained significantly elevated compared to Control hearts through 30 min of hypoxia.

Impact of Hypoxia Duration on Functional Responses during Reoxygenation (GSH Present during Normoxia, Hypoxia and Reoxygenation)

Coronary Flow

Coronary flow responses during reoxygenation are depicted separately for each hypoxic duration in Figure 2. As hypoxic duration was increased, coronary flow at the onset of reoxygenation was progressively decreased. After 20 min of hypoxia coronary flow rebounded rapidly, but with increasing hypoxic duration progressively failed to recover until finally (after 50 min of hypoxia) actually deteriorated during reoxygenation. Coronary flow responses in GSH-treated hearts were significantly different from Control hearts only in the first 5 min of reoxygenation following 20 or 30 min of hypoxia. This difference was small and disappeared by 10 min of reoxygenation.

Electrical Rhythm Responses

All hearts were in a stable, sinus rhythm throughout normoxia as verified by pressure traces and ECG recordings. Hypoxia induced a rapid change in ECG characteristics. Initially, bradycardia progressively developed, followed by PR interval elongation and, finally, complete AV blockade. Ultimately, ventricular electrical activity disappeared completely, followed by the disappearance of atrial electrical activity. After 20 min of hypoxia, all hearts were electrically silent and remained so until reoxygenation.

Rhythm responses during reoxygenation were highly dependent upon the preceding duration of hypoxia (Table II). After 20 min of hypoxia, all hearts in Control and GSH-treated groups were in ventricular fibrillation at 3 min of reoxygenation; however, hearts treated with GSH spontaneously defibrillated more rapidly resulting in a reduced fibrillation incidence by 10 min of reoxygenation ($p < 0.05$). After 30 min of hypoxia, fibrillation incidence remained high and was not significantly altered by GSH supplementation.

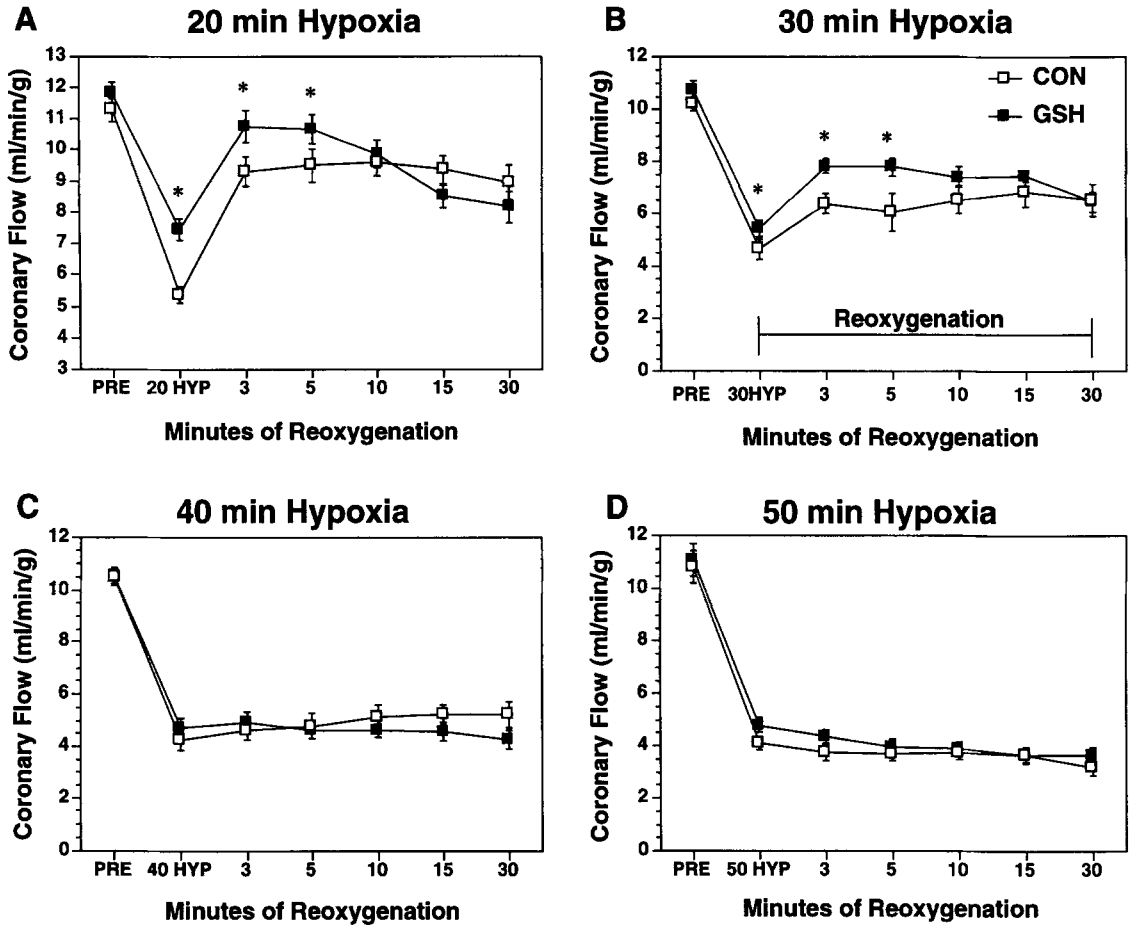

FIGURE 2 Effect of hypoxic duration and exogenous GSH on coronary flow recovery during reoxygenation. Data are presented as means ± SE for 9–11 hearts at each data point, except for panel D which represents means for 5 hearts. Graphs depict baseline coronary flow, flow at the end of hypoxia, and flow at specified time points during reoxygenation. Panel A: 20 min hypoxia, Panel B: 30 min hypoxia, Panel C: 40 min hypoxia, Panel D: 50 min hypoxia. *$p < 0.05$ vs. untreated hearts at same time point.

After 40 min of hypoxia, Control hearts were much slower to resume electrical activity compared to the previous hypoxic duration (data not shown) and fibrillation was absent in all hearts throughout reoxygenation. In contrast, 6 of 10 hearts treated with GSH had fibrillation incidences after 40 min of hypoxia ($p < 0.05$ vs. time-matched Control hearts), a frequency similar to that observed in Control hearts made hypoxic for only 30 min. Finally, after 50 min of hypoxia, all Control and GSH-supplemented hearts were electrically silent throughout all or the majority of the reoxygenation period.

Electrical Conduction Velocity

After sinus rhythm was restored, comparisons of pre-hypoxic ECGs with those acquired after 30 min of reoxygenation revealed that electrical conduction velocity was well preserved after 20 or 30 min of hypoxia, despite significant declines in contractile and vascular function. PR interval (Figure 3, panel A), QRS duration (Figure 3, panel B), and total conduction time (atrial depolarization onset to end of ventricular depolarization, Figure 3, panel C) were not significantly different in Control or GSH-treated hearts. In contrast,

TABLE II Fibrillation incidence during post-hypoxic reoxygenation

Hypoxia (min)	Treatment	n	3 min Reox	5 min Reox	10 min Reox
20	CON	10	10	10	6
20	GSH	10	10	8	2*
30	CON	11	4	8	8
30	GSH	10	7	9	4
40	CON	10	0	0	0
40	GSH	10	3	6*	6*
50	CON	5	0	0	0
50	GSH	5	0	0	0

n, number of hearts in each group. Data represent the number of hearts fibrillating for each group. Electrocardiograms of perfused hearts were analyzed as described in Methods.
*$p < 0.05$ vs. Control (GSH-free) group at same time point. Reox = reoxygenation.

40 min of hypoxia resulted in a significant increase in AV conduction delay (PR interval), QRS duration, and total conduction time in Control hearts. The elongation of PR interval was absent in hearts perfused with GSH. The increase in QRS duration and total conduction time tended to be reduced in GSH hearts as well. However, these differences were not statistically significant. After 50 min of hypoxia, electrical disruption was so severe (total absence of QRS or persistent AV conduction block) that segment durations could not be quantified in either the GSH or Control group.

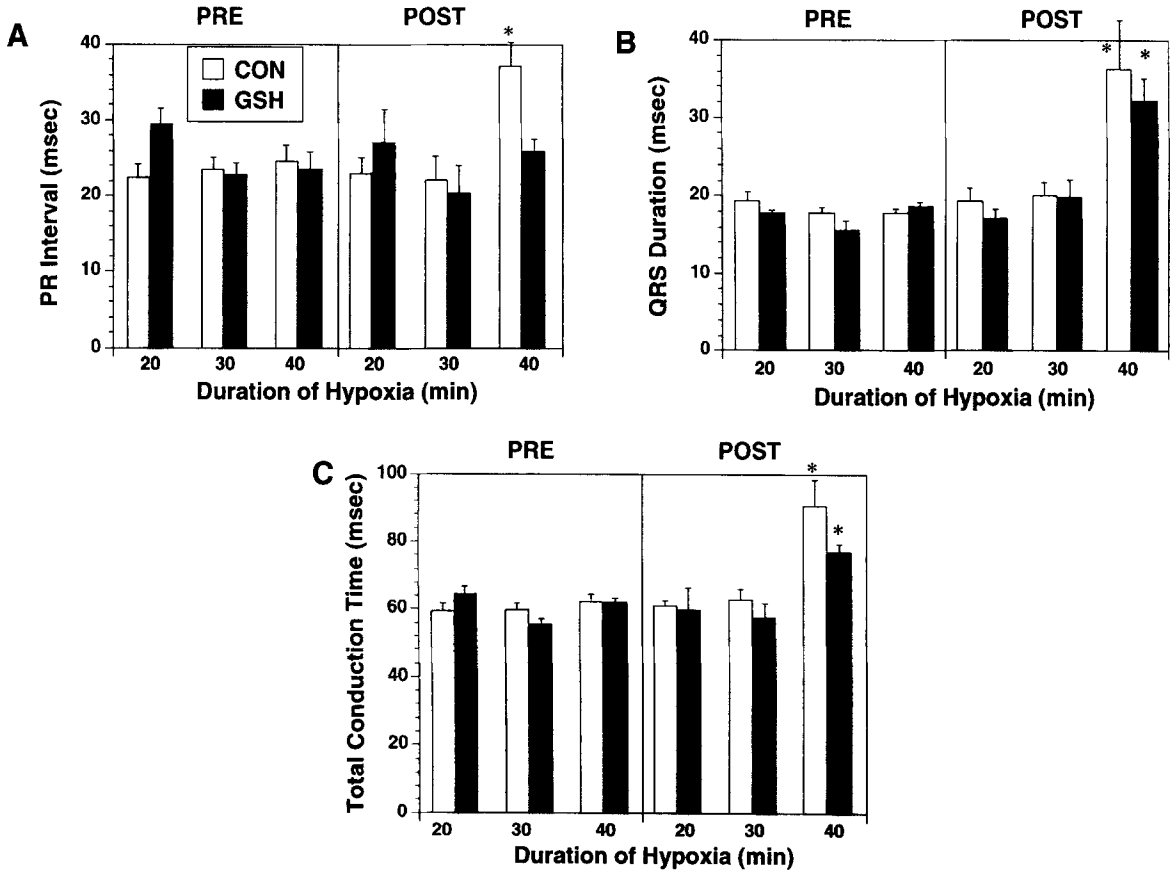

FIGURE 3 Effect of duration of hypoxia and exogenous GSH on electrical conduction recovery after hypoxia. Panel A: PR interval (ms). Panel B: QRS duration (ms). Panel C: Total conduction time (atrial depolarization onset to end ventricular depolarization). ECG recordings were made after 30 min of normoxia (pre) and after 30 min of reoxygenation following the hypoxic insult (post). In each panel, the left side of the graph depicts paired values under normoxic conditions for each hypoxic group. The right side of each panel depicts the same groups measured following hypoxia and reoxygenation. Bars and error bars represent means ± SE for 6–10 hearts in each group. *$p < 0.05$ compared to same group before hypoxic insult.

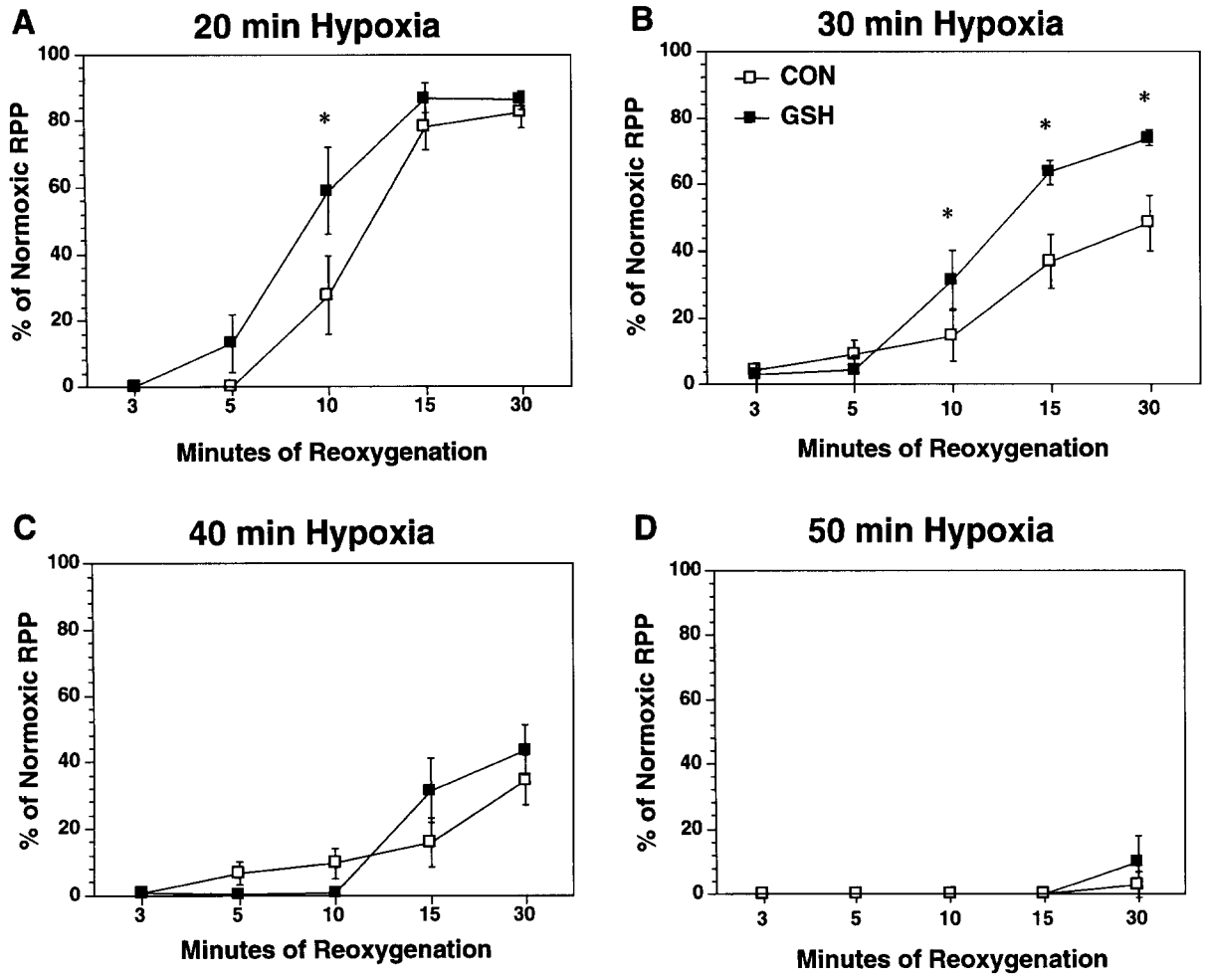

FIGURE 4 Effect of exogenous 5 mM GSH on recovery of mechanical function (heart rate × developed pressure, RPP) during reoxygenation. Values represent means ± SE. All hearts were at zero RPP at the moment of reoxygenation. RPP recovery following 20 (Panel A), 30 (Panel B), 40 (Panel C), or 50 (Panel D) minutes of hypoxia is depicted separately for each condition. Recovery is expressed as a percentage of RPP measured during normoxia prior to insult in each group. *$p < 0.05$ vs. Control at same time point.

Contractile Recovery

Increasing hypoxia duration resulted in a progressive decline in contractile recovery (% recovery of RPP) upon reoxygenation. After 20 min of hypoxia, quantification of early contractile recovery was prevented by ventricular fibrillation. As a consequence of decreased fibrillation incidence by 10 min of reoxygenation, RPP recovery was significantly elevated in GSH hearts compared to Control. However, after 15 min of reoxygenation, approximately 80% contractile recovery was observed in both groups. No further recovery was observed from 15 to 30 min of reoxygenation (Figure 4, panel A).

GSH supplementation significanfly improved both the initial rate of contractile recovery and its final magnitude following 30 min of hypoxia (Figure 4, panel B). After the reoxygenation period, Control hearts regained only 48 ± 8% of pre-hypoxic RPP compared to 74 ± 2% by the GSH-treated hearts ($p < 0.05$). After 40 min of hypoxia, contractile recovery was reduced to < 40% of baseline normoxic RPP and was not improved

by GSH supplementation at the 0.05 level of significance (Figure 4, panel C). After 50 min of hypoxia, contractile recovery was absent or nearly absent in all hearts, independent of GSH supplementation (Figure 4, panel D).

Intracellular Thiol Status

Figure 5 depicts the impact of hypoxia and reoxygenation on intracellular thiol concentration in Control and GSH-supplemented hearts. Sixty minute of normoxic perfusion of Control hearts did not alter intracellular thiol levels compared to hearts rinsed for 5 min with normoxic KHB. However, increasing hypoxia duration in Control hearts resulted in a progressive decline in total soluble thiols after reoxygenation until they were only about 40% of normoxic thiol levels after 50 min of hypoxia. This decline was not prevented by GSH supplementation in 20 or 30 min hypoxic hearts. However, measured thiols were markedly elevated in hearts reoxygenated after 40 or 50 min of hypoxia. This increase in measured thiols is attributable to the presence of exogenous GSH trapped within "no-reflow" regions of myocardial tissue. Myocardial no-reflow was absent after 20 or 30 min of hypoxia, but was evident in hearts subjected to the longer hypoxic durations based on a separate group of hearts inspected after dye-injection.

Impact of Timing of GSH Application on Functional Responses

Coronary Flow

When GSH was administered during hypoxia only, coronary flow was higher throughout most of the 40 min hypoxic period and the subsequent reoxygenation period (Figure 6). Coronary flow in hearts exposed to exogenous GSH only during reoxygenation did not differ from untreated Control hearts at any time during hypoxia or reoxygenation ($p > 0.05$). In contrast to the observations on hearts that were hypoxic for 40 min in the duration study, dye-injection of 6 Control hearts revealed no significant residual no-reflow (0 in 4 hearts, $\leq 5\%$ in 2 hearts) after 40 min of hypoxia and 30 min of reoxygenation.

Contractile Recovery

The impact of timing of GSH application on the rate and magnitude of mechanical recovery

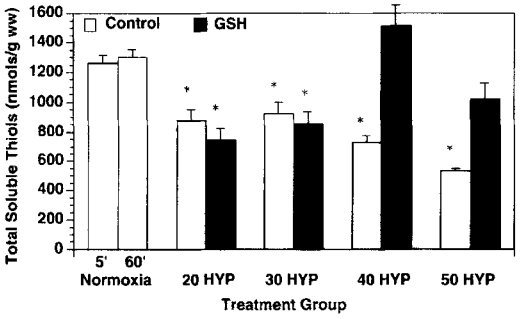

FIGURE 5 Effect of hypoxia of increasing duration, and reoxygenation on intracellular thiols with and without exogenous GSH supplementation. Values represent means ± SE for 5–8 hearts per group. Gray bars depict values for hearts subjected to either 5 or 60 min of normoxic perfusion only ($n = 4$ in each group). *$p < 0.05$ compared to normoxic perfusion only. The apparent elevation in intracellular GSH in 40 and 50 min hypoxic GSH groups is attributable to trapped GSH in non-reperfused vasculature, based on separate dye-injection experiments.

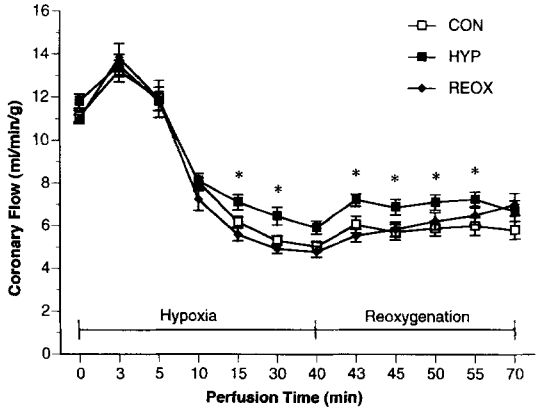

FIGURE 6 Effect of timing of GSH application on coronary flow during hypoxia and reoxygenation. Data are presented as means ± SE for 8–11 hearts at each data point. HYP, 5 mM GSH administered only during hypoxia; REOX, 5 mM GSH administered only during reoxygenation; CON, unsupplemented Control. *HYP significantly higher than other groups, $p < 0.05$.

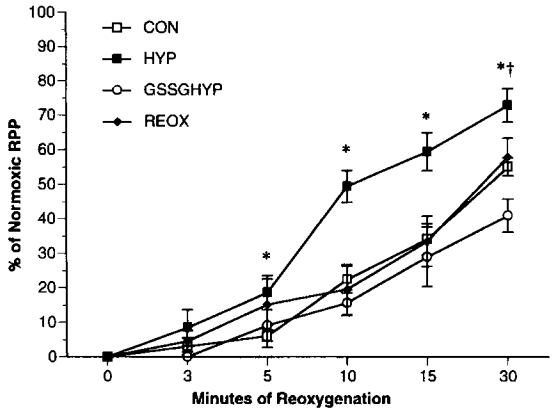

FIGURE 7 Effect of timing of GSH application on recovery of mechanical function during reoxygenation. Data are presented as means ± SE for 8–11 hearts at each data point, except GSSGHYP where $n = 4$. HYP, 5 mM GSH administered only during hypoxia; GSSGHYP, 2.5 mM GSSG administered only during hypoxia; REOX, 5 mM GSH administered only during reoxygenation; CON, unsupplemented Control. *HYP significantly higher than CON and GSSGHYP at 5 min and all other groups at later time points, $p < 0.05$ vs. CON. †GSSGHYP significantly lower than all other groups at same time point, $p < 0.05$.

during 30 min of reoxygenation is depicted in Figure 7. By 5 min of reoxygenation, the group that received GSH only during the preceding 40 min hypoxic period was found to have significantly greater recovery of RPP and it remained greater throughout the reoxygenation period ($p < 0.05$). Providing GSH only during reoxygenation did not affect mechanical recovery following 40 min of hypoxia. Differences in recovery of intraventricular developed pressure accounted primarily for the differences in recovery of RPP among the groups. The trends in developed pressure were the same as for mechanical recovery ($p < 0.05$ for hypoxia vs. Control) and heart rates were the same in all groups ($p > 0.05$) (data not shown). The protective effect of GSH during hypoxia was specific to the reduced form of GSH, as it could not be replicated by substituting GSSG for GSH (Figure 7). Recovery in hearts treated with 2.5 mM GSSG during hypoxia was not only significantly worse than those treated with GSH, but also significantly lower than unsupplemented hearts after 30 min reoxygenation.

DISCUSSION

Previous studies have been inconclusive regarding GSH efficacy as a cardioprotectant; both no effect[6–8,12] and a beneficial effect[9–11] have been reported. Differences in several important model and methodological variables may have been responsible for these equivocal results. In the present study we used a single model, the isolated hypoxic rat heart, which is a widely used model to sensitively examine intrinsic cardiac function. A broad range of contractile dysfunction was generated by separately examining four different durations of hypoxia. We observed that GSH administration: (1) significantly improved contractile function after 20 and 30 min of hypoxia when the amount of contractile recovery was typically above 50% of normal function, but had no effect when the duration of hypoxia was long enough to cause more extensive cardiac dysfunction resulting in contractile recoveries typically below 40% (Figure 4); (2) shifted the classical "bell-shaped" curve for reperfusion ventricular fibrillation incidences to the right resulting in attenuated ventricular fibrillation after 20 and 30 min of hypoxia, but increased incidences after 40 min when Control hearts were slow to resume electrical activity (Table II); and (3) attenuated delays in electrical conduction pathways (Figure 3). Thus, the results of this study demonstrate that exogenous GSH provides a significant cardioprotective effect and that the effect is dependent on the duration of hypoxic insult.

The concentration of GSH chosen (5 mM) is within the range of exogenous GSH concentrations used in other studies[10–12] and is approximately equal to the cytosolic concentration in rat hearts. Consistent with our earlier study,[11] 5 mM GSH did not alter normoxic cardiac function (Table I). It was deemed important to use a concentration similar to that used by others in order to better clarify reasons for discrepancies among those studies by adjusting other variables, i.e., hypoxia duration, severity of damage, choice of functional measurement, etc. Also, the finding

that this relatively high exogenous level of GSH could not attenuate the decline in cytosolic levels during hypoxia reinforces that exogenous GSH is not easily taken into the myocyte and, thus provides evidence for speculation regarding on the site of protection which will follow later in this discussion.

Tani[12] did not observe a protective effect of exogenous GSH on the ability of the Langendorff-perfused rat heart to regain contractile function following 30 min of zero-flow ischemia. Our results suggest that a reason GSH failed to attenuate reperfusion injury in Tani's study is that the amount of myocardial injury incurred after the bout of global ischemia was too severe. In his study, GSH in concentrations of 2, 5, and 10 mM was administered to the heart for 20 min prior to ischemia and during 30 min of reperfusion. Recovery of RPP was only 25%, left ventricular end-diastolic pressure remained over 40 mmHg, and reperfusion calcium uptake was high indicative of irreversible injury. We also did not find exogenous GSH to be effective in hearts with RPP this low and end-diastolic pressure this high. Further support for our contention that the degree of injury in Tani's study was too great for antioxidants to be effective is that he found superoxide dismutase (SOD) plus catalase to be ineffective at concentrations eliciting improved recovery in another model of ischemia/reperfusion.[20]

Alternatively, another reason that Tani did not find that antioxidants did not affect reperfusion injury is that the antioxidants were not administered during oxygen deprivation. As the length of ischemia increases, oxygen radical generation during reperfusion eventually decreases,[15] yet the amount of injury increases.[13] This indicates that damage does occur during oxygen deprivation and, therefore, adding a cardioprotective agent after damage has been done will not prevent the prior damage. Several investigators have reported that GSH declines during hypoxia or ischemia[1–3,5] indicating that oxidative stress can occur during oxygen deprivation. Consistent with this idea, we observed that GSH administered only during 40 min of hypoxia attenuated myocardial dysfunction upon subsequent reoxygenation with unsupplemented buffer, but adding GSH only during reoxygenation following this length of hypoxia did not affect recovery (Figure 7).

Previous studies have reported that postischemic functional recovery or arrhymogenesis is not affected by decreased intracellular GSH levels.[6,7] Consistent with these studies, the cardioprotection by exogenous GSH observed herein does not appear to be dependent on the maintenance or increase in intracellular thiol concentration. In the present study, exogenous GSH did not maintain intracellular thiol concentration after 20 and 30 min of hypoxia and subsequent reoxygenation (Figure 5), which is consistent with other studies that were unsuccessful in significantly raising intracellular thiol concentration through the administration of exogenous GSH.[11,21] The measured increase in thiol levels in hearts subjected to prolonged hypoxia was likely due to contamination of intracellular thiol determinations with extracellular GSH trapped in the interstitium and vasculature within non-reperfused (no-reflow) regions, which were revealed by dye-injection experiments. Furthermore, total thiol content in hearts perfused with 5 mM GSH and freez-clamped without rinsing with GSH-free buffer is more than doubled (data not shown), indicating that the total volume of vascular and interstitial space remaining ischemic due to no-reflow is sufficient to generate this artifact. Therefore, we conclude that the apparent increase in thiol concentration in the more severely injured hypoxic hearts was an artifact of "no-reflow" and not due to exogenous GSH traversing the sarcolemmal membrane to an extent sufficient to prevent cytosolic declines.

GSH-induced attenuation in reoxygenation contractile dysfunction after 20 and 30 min of hypoxia did not appear to be due to a specific protective effect on electrical conduction pathways. PR interval, QRS duration and total conduction time were not altered in any hearts after

these durations of hypoxia, suggesting that conducting tissue is more resistant to damage or dysfunction than other components of the myocardium. Electrical conduction velocity was not decreased until the duration of hypoxia was extended to the point of severe contractile dysfunction and the development of persistent no-reflow. Though exogenous GSH tended to attenuate this deterioration, an associated improvement in contractile recovery was not observed, suggesting that these were independent phenomena. After 50 min of hypoxia, overall myocardial function was severely compromised upon reoxygenation – contractile recovery was virtually absent, coronary flow did not rebound, and electrical activity was extremely disrupted or absent.

The data implicate the sarcolemma, which is critical to both electrical and mechanical function, as the functionally relevant target of exogenous GSH protection. This conclusion is based on the findings that GSH protects when administered only during hypoxia (Figure 7) when mitochondria are not producing free radicals and that the presence of 5 mM exogenous GSH does not elevate intracellular GSH or attenuate its decline (Figure 5). In addition, there is evidence that the myocyte is highly resistant to taking up the intact GSH tripeptide.[11,22] Considerable evidence has emerged demonstrating the susceptibility of sarcolemmal membrane proteins to oxidative insult,[5,23–25] which would be expected to have severe consequences manifested as rhythm disturbances and decreased contractile function. The position of the sarcolemma within the landscape of the intact heart makes it a potential target for free radicals produced within the cytosol (from mitochondria), within the lipid membrane itself, or external to the myocyte. Univalent reduction of oxygen within mitochondria is a well established source of reactive oxygen species,[14,26] but an unlikely one during the hypoxic period when GSH administration was effective. Other identified sources of reactive oxygen species include autooxidation of endogenous catecholamines released during ischemia[27–29] and production of free radicals by endothelial cells of the coronary vasculature.[26,30,31] Recently, spin trapping agents have directly identified hydroxyl radical production localized within the vascular compartment of cat hearts subjected to brief regional ischemia.[32] This source of free radicals could cause protein dysfunction in the adjacent sarcolemma by directly oxidizing regulatory sulfhydryl groups on proteins or indirectly altering protein function through lipid peroxide-induced changes in membrane fluidity.

Although the myocyte sarcolemma is highly resistant to GSH uptake, the compound is known to be taken up by endothelial cells.[33,34] Therefore, it is possible that endothelial GSH concentration was augmented by exogenous GSH. Such an increase in endothelial thiol concentration would likely go undetected in whole heart measurements of GSH because of the much larger volume of the myocyte. Exogenous superoxide dismutase and catalase have been shown to be cardioprotective in spite of similar inaccessibility to the myocyte cytosol.[14] Given the general effectiveness observed with SOD + catalase treatment against myocardial stunning, the data of Mickle *et al.*[35] are of interest. They examined the relative susceptibility to oxidative injury of cultured endothelial cells, myocytes and fibroblasts from human hearts. The effectiveness of SOD + catalase, ascorbic acid, and Trolox (water soluble analog of alpha-tocopherol) in protecting against a free radical generating system (hypoxanthine–xanthine oxidase) was compared in the isolated cell types. Furthermore, SOD + catalase protected endothelial cells against oxidative insult, but not myocytes. In contrast, ascorbic acid and Trolox were protective of myocytes, but not endothelial cells. We have previously observed that the cysteine thiol carrier L-2-oxothiozolidine-4-carboxylate (OTZ), which becomes a thiol donor only when cleaved by 5-oxoprolinase located intracellularly, is cardioprotective in a manner similar to GSH.[11] Because OTZ did not increase intramyocyte thiol status, its likely target of protection was endothelial cells. In support of this

possibility, OTZ has been reported to increase intracellular GSH in endothelial cells and protect against oxidative injury.[36] Because exogenous GSH fails to traverse the sarcolemmal membrane over the experimental duration observed, the results are consistent with protection of the sarcolemmal membrane from free radical production by the vascular endothelium.

In summary, it appears that exogenous GSH protects against myocardial dysfunction associated with oxygen deprivation and the extent of protection is dependent on the duration of hypoxia and the functional parameter being evaluated. The cardioprotection was not due to enhancement of intracellular GSH levels since intracellular thiols were decreased similarly in Control and GSH-supplemented hearts. However, the mechanism may involve protection against oxygen radical production since their production has been reported to be considerably higher after moderate periods of oxygen deprivation than after longer periods[15] which corresponds to when GSH-related cardioprotection was and was not observed, respectively. The observations herein are consistent with a proposed mechanism in which GSH enters vascular endothelial cells and protects against thiol oxidation of adjacent sarcolemmal membrane proteins involved in intracellular ion homeostasis.

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