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Exogenous Glutathione Attenuates Stunning Following Intermittent Hypoxia in Isolated Rat Hearts

K.S. SEILER[1], J.P. KEHRER[2], and J.W. STARNES[1,2]

[1]*Department of Kinesiology, College of Education;* [2]*Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, TX 78712*

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An isolated rat heart model of intermittent hypoxia was used to investigate the impact of exogenous supplementation of glutathione and two thiol delivery vehicles on functional recovery during reoxygenation and whether efficacy was dependent on enhanced intracellular thiol concentration. Hearts from F344 rats were perfused in the Langendorff mode and exposed to three, 5 minute bouts of global, substrate free, normothermic hypoxia separated by 5 minute reoxygenation periods. Changes in coronary flow, heart rate, systolic and diastolic pressure, and rate pressure product were evaluated throughout in control hearts and compared with hearts in which one of the following was provided during the hypoxic periods: reduced glutathione (GSH, 1 or 10 mM), 10 mM GSH mono-ethyl ester (GSHMEE), or 1 mM L-2-oxothiozolidine-4-carboxylate (OZT). After three hypoxic periods plus reoxygenation, rate pressure product in control hearts was ~ 60% of pre-hypoxic values. Exposing hearts to 1 or 10 mM GSH, 10 mM GSHMEE, or 1 mM OZT significantly ($p < 0.05$) enhanced post-hypoxic recovery of rate pressure product and attenuated the rise in diastolic pressure during hypoxia. This improvement in function was not associated with an elevated intracellular thiol concentration in treated hearts. Cumulative oxidative changes may occur during intermittent hypoxia via a mechanism localized on or near the sarcolemmal membrane. These changes appear to precede the appearance of significant intracellular oxidative stress and may be due to alterations in the reduced status of critical membrane bound proteins. Exogenously administered thiols attenuate protein alterations via a localized increase in thiol availability without an increase in gross measures of intracellular thiol or glutathione content.

Key words: free radicals, ischemia, reperfusion, oxidative stress, antioxidants

INTRODUCTION

Transient and reversible myocardial dysfunction following brief ischemia has been termed myocardial stunning.[1] A mechanism proposed for stunning that has substantial experimental support is oxidative stress from the generation of free radicals.[2,3] Among the numerous cellular antioxidant defenses present in the myocardium, glutathione (GSH) may be one of the most important due to both its high cytosolic concentration (millimolar range) and the diverse mechanisms by which it can function to reduce oxidative stress.[4]

Address for correspondence: J.W. Starnes, Department of Kinesiology, 222 Bellmont, University of Texas at Austin, Austin, TX 78712, USA, phone: (512) 471–8589, FAX: (512) 471–0946

Several studies employing extended periods of myocardial ischemia or hypoxia have reported a decrease in the intracellular content of GSH at the termination of the insult[5,6] and after subsequent reperfusion.[7,8] The results of Curello *et al.*,[5] and Park *et al.*[6] suggest that oxidative stress is not limited to the free radical burst associated with tissue reoxygenation.[9,10] In the Park *et al.* study, both total GSH and mitochondrial GSH were significantly depleted during hypoxia, but did not undergo further depletion upon reoxygenation. Additionally, a marked depression occurred in Ca^{++}-ATPase activities of sarcoplasmic reticulum and sarcolemmel preparations isolated at the end of hypoxia, consistent with oxidative stress.[6] Blaustein *et al.*[11] and Singh *et al.*[12] have reported a positive relationship between intracellular GSH content and recovery of mechanical function following ischemic or hypoxic insult. These two studies compared functional recovery among hearts that had been 1) untreated, 2) chemically depleted of GSH, or 3) perfused with GSH supplemented buffer. These results suggest that pharmacological strategies to successfully maintain intracellular GSH status during an ischemic or hypoxic stress could have clinical benefit. In contrast, Tani found no positive impact of glutathione, cysteine, N-acetyl-L-cysteine, or dithiothreitol on functional recovery of isolated rat hearts subjected to 30 minutes of normothermic, zero-flow ischemia.[13] Based on these contrasting results, the potential of exogenous GSH as a cardioprotectant remains uncertain.

In the present study, brief, intermittent hypoxia was employed to further evaluate the efficacy of GSH as a cardioprotectant. This model was employed for three reasons. First brief periods of ischemia or hypoxia produce a degree of transient, reversible dysfunction consistent with clinical scenarios. In contrast, the relatively long ischemic bout employed in the study of Tani may have failed to reveal a cardioprotective effect of GSH due to overwhelming irreversible injury from severe calcium accumulation and myocyte contracture. Second, intermittent oxygen deprivation provides multiple reoxygenation periods which are considered to be critical events in free radical production. Finally, substrate free hypoxia was chosen rather than ischemia in order to allow GSH presentation during the period of greatest intracellular thiol depletion. In addition to GSH, other thiol compounds were also employed in order to maximize the delivery of GSH across the sarcolemmal membrane. We hypothesized that the efficacy of GSH as a cardioprotectant is dependent upon its access to the cytosol and maintenance and/or elevation of the intracellular thiol pool.

MATERIALS AND METHODS

Perfusion Procedures

All experiments were performed on hearts from male and female Fischer 344 rats weighing 150–225 grams. Preliminary studies demonstrated no gender difference in response to the hypoxic stress employed. After anesthetization with sodium pentobarbital (50 mg/kg, i.p.) and heparinization (100 IU) via the hepatic vein, hearts were excised and immersed in ice-cold 0.9% NaCl. The arrested heart was then cannulated and perfused at 37°C at a constant pressure of 80 cm H_2O.

The perfusion medium was a modified Krebs-Henseleit bicarbonate solution containing in mM: NaCl 118.5, KCl, 4.7, $CaCl_2$ 3.0, KH_2PO_4 1.2, $MgSO_4$ 1.0, Na_2EDTA 0.5, $NaHCO_3$ 24.7, insulin 12 IU/l. The pH was between 7.35 and 7.4 in all preparations. The final free calcium concentration of the buffer was determined to be 2.1 mM via a calcium sensitive electrode. The normoxic buffer was gassed with a 19:1 mixture of O_2–CO_2 and supplemented with 10 mM glucose. The hypoxic buffer was gassed with 19:1 N_2–CO_2 and glucose was replaced with 10 mM mannitol to maintain osmolarity. Preliminary experiments indicated that adding mannitol (a proposed •OH radical quencher) did not affect the functional response to intermittent hypoxia compared to hearts perfused with mannitol-free buffer during hypoxia.

Heart rate and intraventricular systolic and diastolic pressures were monitored via a 20 gauge needle introduced through the left ventricular apex and attached via a fluid filled, 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). Ventricular work (rate pressure product) was taken as the product of heart rate and intraventricular developed pressure (peak systolic – diastolic pressure). Coronary flow was determined by weighing timed collections and normalized for heart weight.

Intermittent Hypoxia

The hypoxic buffer was unsupplemented (CON), or contained 10 mM GSH (GSH10), 1 mM GSH (GSH1), 10 mM GSH monoethyl ester (GSHMEE10), or 1 mM L2-oxothiazolidine-4-carboxylic acid (OTZ). GSH was obtained from Sigma Chemical Co., St. Louis and GSHMEE and OTZ were generous gifts from Clintec Nutrition Co., Deerfield, IL. All hearts were initially perfused for 30 minutes under normoxic conditions prior to the determination of baseline flow and contractile measurements. Hearts were then subjected to three, 5 minute periods of hypoxia separated by 5 minute reoxygenation periods. Heart rate, systolic pressure, diastolic pressure, and coronary flow measurements were made at one, three, and five minutes of each period. Coronary flow was determined using a 20 second collection period starting 10 seconds before and ending 10 seconds after each minute time point. Contractile measurements were acquired in the same manner over a 10 second period. After assessing functional changes following 3 complete hypoxia-reoxygenation cycles, hearts were freeze clamped using aluminum clamps cooled in liquid nitrogen for subsequent determination of intracellular thiol content.

Extended Hypoxia

To directly compare the oxidative impact of the above protocol with that of a more prolonged hypoxic stress, six hearts was exposed to 30 minutes of continuous hypoxia and immediately freeze clamped for the measurement of intracellular thiol concentration.

Intermittent Hydrogen Peroxide Exposure

In order to provide additional comparative information relevant to the intermittant hypoxic protocol employed here, the impact on function and intracellular thiol status of an intermittent oxidative stress without hypoxia was determined in an additional group of hearts. These hearts were subjected to three, 5 minute exposures of 250 μM H_2O_2 (Sigma) under normoxic perfusion conditions. Peroxide exposures were separated by 5 minute peroxide-free perfusion periods with or without 5 mM GSH in the buffer. After the third five minute peroxide-free recovery period, hearts were rinsed with GSH-free buffer to rinse out extracellular GSH and then freeze clamped for measurement of intracellular thiols.

Intracellular Thiol Measurement

Intracellular non-protein thiol (primarily GSH) concentrations were determined by a modification of the colorimetric method of Akerboom and Sies.[14] Identification of the measured thiols as up to 90% reduced glutathione was confirmed in a subset of hearts by comparing results of the non specific colormetric thiol determination with the GSH specific enzymatic recycling method of Tietze.[15]

Statistical Analysis

One way ANOVA was used to compare coronary flow, diastolic pressure, and rate pressure product, at each time point throughout hypoxia and reoxygenation among the various groups, as well as the intracellular thiol status following three hypoxia-reoxygenation cycles. For points where one-way ANOVA detected significant differences, Fishers' LSD test was employed to perform multiple comparisons. Differences were considered significant at the 0.05 level.

TABLE 1 Baseline Functional Characteristics of Intermittent Hypoxia Treatment Groups.

Group	*Heart Rate*	*LVDP*	*Rate Pressure Product*	*Coronary Flow*
Control (n = 16)	268	69	18 490	8.6
	±6	±1	±640	±0.3
10 mM GSH	271	70	18 970	9.0
(n = 7)	±8	±2	±840	±0.4
1 mM GSH	273	66	18 020	9.4
(n = 9)	±7	±1	±500	±0.3
10 mM GSHMEE	260	66	17 200	8.6
(n = 8)	±6	±1	±340	±0.4
1 mM OTZ	264	67	17 690	9.2
(n = 5)	±12	±1	±760	±0.4

Values are Mean ± SEM. LVDP = Left ventricular developed pressure

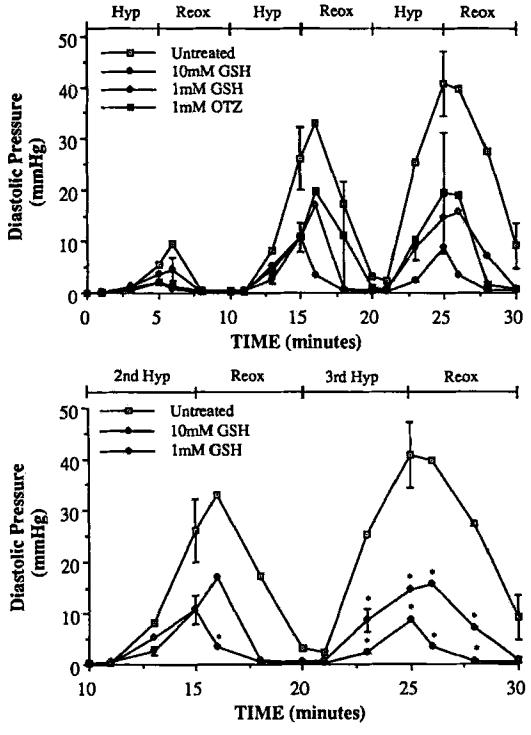

FIGURE 1 Upper panel: Changes in end-diastolic pressure throughout three cycles of hypoxia-reoxygenation in 1 mM GSH (n = 9), 10 mM GSH (n = 7), 1 mM OTZ (n = 5), and Untreated (n = 16) hearts. The impact of OTZ on diastolic pressure was variable and not significantly different from Untreated. Diastolic pressure responses to GSHMEE were omitted for clarity. Representative error bars are included in the figure. Lower panel: Expanded portion of the same graph depicted in the upper panel, with OTZ group omitted. Exogenous GSH administered during hypoxia significantly attenuated the rise in end-diastolic pressure observed with intermittent hypoxia. * = $P < 0.05$ vs Untreated at same time point.

RESULTS

Baseline Function

Normoxic baseline values for heart rate, left ventricular developed pressure, and their product (rate pressure product) for each of the five experimental groups subjected to intermittent hypoxia are presented in Table 1. No significant differences in baseline normoxic function were observed among the groups. Subsequent data depicting changes in rate pressure product (Figure 2) are compared as a percentage of the normoxic baseline condition for each individual group.

Functional Recovery after Intermittent Hypoxia

The first five minute bout of substrate-free hypoxia was well tolerated by all hearts. Diastolic pressure was only slightly elevated at the end of the first hypoxic bout and rapidly returned to baseline in all groups after five minutes of reoxygenation (Figure 1, upper panel). Rate pressure product declined similarly (40–60%) in all groups and recovered similarly to 80–95% of original normoxic values after reoxygenation (Figure 2, upper panel). Furthermore, the coronary flow response did not differ significantly among the groups after the first cycle of hypoxia and reoxygenation (Figure 3).

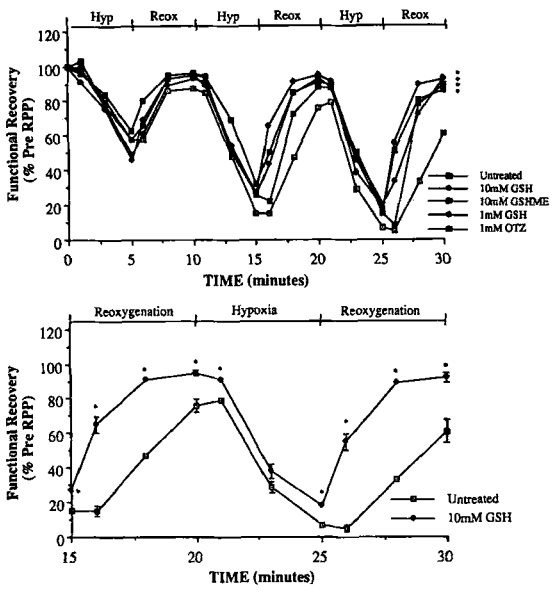

FIGURE 2 Upper panel: Changes in heart rate × developed pressure (RPP) throughout three cycles of hypoxia-reoxygenation in GSH, GSHMEE (n = 9), OTZ and Untreated. Recovery values are expressed as a percentage of rate pressure product determined during normoxia. Error bars have been omitted for clarity. Functional recovery in all treated groups was similarly and significantly ($p < 0.05$) enhanced compared to untreated hearts. Lower panel: Expanded portion of the same graph depicted differences in functional recovery between untreated hearts and hearts exposed to 10 mM GSH during each 5 minute hypoxic period. Representative error bars are included. * = $P < 0.05$ vs Untreated at same time point.

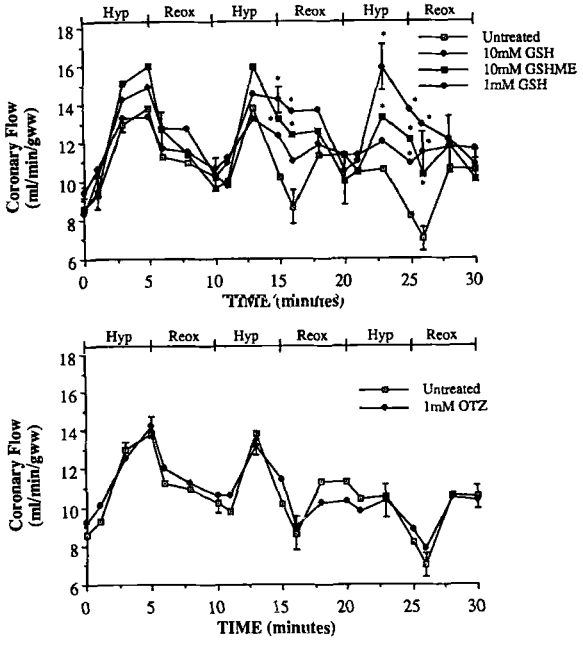

FIGURE 3 Upper panel: Changes on coronary flow throughout three cycles of hypoxia-reoxygenation. Exogenous GSH or GSHMEE significantly enhanced the hyperemic response to intermittent hypoxia compared to Untreated. Representative error bars are included in figure. * = $P < 0.05$ vs Untreated at same time point. Lower panel: Exogenous 1 mM OTZ did not alter the coronary flow response to intermittent hypoxia.

The impact of the second and third 5 minute bouts of hypoxia was more severe in all hearts; however, these effects were significantly attenuated in hearts treated with GSH. The presence of either 10 mM or 1 mM GSH during hypoxia significantly attenuated the hypoxia-induced rise in diastolic pressure compared to control hearts (Figure 1). Administration of 1 mM OTZ also tended to attenuate the rise in diastolic pressure; however, this response was highly variable and did not reach statistical significance (data not shown). In the second and third hypoxic period, GSH treatment significantly preserved the coronary hyperemic response compared to control hearts (Figure 3, upper panel). In contrast, OTZ did not alter the coronary flow response (Figure 3, lower panel). Overall, the coronary flow response to the various drug treatments inversely tracked the diastolic pressure responses suggesting that a major reason for the enhanced coronary flow was a reduction in vascular compression.

The impact of the various thiol treatments on mechanical recovery during the hypoxia-reoxygenation transitions is depicted in Figure 2. All thiol donor groups were similarly effective in providing greater mechanical recovery than untreated hearts (~ 60% RPP recovery vs 90% RPP after 3 hypoxia/reoxygenation cycles, lower panel). Coronary flow during recovery did not appear to be improved by any of the thiol donor compounds.

Intracellular Thiol Status

Total soluble thiol concentrations are displayed in Figure 4. In untreated hearts perfused norm-

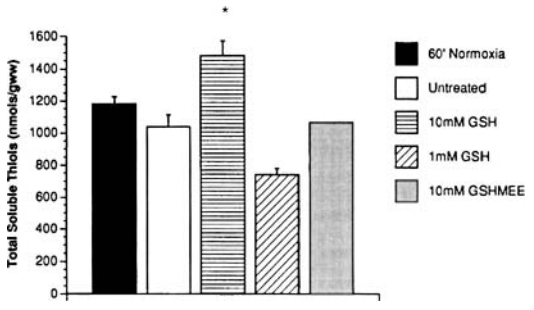

FIGURE 4 Total soluble thiols were measured in tissue frozen immediately after the third hypoxia-reoxygenation cycle. Total perfusion time for all hearts was 60 minutes. Total soluble thiols in hearts frozen after 60 minutes of normoxic perfusion are depicted by the solid black bar. * = $P < 0.05$ vs Untreated.

oxically for 30 minutes followed by 3 hypoxia-reoxygenation cycles (total perfusion time 60 minutes) total soluble thiols were not different at the 95% confidence level compared to hearts perfused under normaoxic conditions for 60 minutes. In comparison, total soluble thiols were decreased over 40% in hearts frozen immediately after a single 30 minute bout of hypoxia. The impact of exogenous GSH supplementation during hypoxia on total soluble thiols in hearts exposed to three complete cycles of intermittent hypoxia and reoxygenation is depicted in Figure 4. Only the 10 mM GSH group was found to have significantly elevated intracellular GSH content. Interestingly, hearts perfused with 1 mM GSH, whose functional recovery was significantly improved, had a significantly lower intracellular thiol content compared to controls ($P < 0.05$). Furthermore, no change in GSH content was found with 10 mM GSHMEE, suggesting that it was not a more effective GSH delivery agent than GSH alone over the brief time of exposure observed. In conjunction with the functional results already described, these data suggest that the beneficial effects of GSH augmentation were not dependent on an increase in intramyocyte glutathione.

Intermittent Hydrogen Peroxide Exposure

Mechanical function of control hearts was almost completely abolished after being subjected to three five minute exposures of 250 μM hydrogen peroxide during normoxic perfusion, each followed by a five minute recovery period (RPP = 11 ± 5% of pre-exposure value five minutes after third exposure). Intracellular thiol concentration in these hearts was reduced to approximately ⅓ of normal values (394 ± 33 nmols/g wet wt., n = 4). The addition of 5 mM GSH to the perfusate during each of the three, five minute peroxide-free recovery periods significantly improved both mechanical function and intracellular thiol content (57 ± 9% and 891 ± 178 nmol/g wet wt., $P < 0.05$).

DISCUSSION

The intermittent hypoxia model used in the present study did not cause a significant decline in intracellular glutathione content, yet produced measureable cardiac dysfunction that was greatly attenuated by exogenous GSH. This observation runs counter to our hypothesized mechanism for the efficacy of GSH. Furthermore, the results of the present study differ from previous reports which demonstrated a positive relationship between post ischemic/hypoxic myocardial functional recovery and residual intracellular glutathione content[11,12,16] by demonstrating that exogenous glutathione can be cardioprotective independent of a change in bulk intramyocyte thiol status. However, the results of this study do not contradict previous findings. Instead, they suggest that in the time course of hypoxia/reoxygenation cell dysfunction, reactive oxygen species generated externally or near the sarcolemmal membrane may overwhelm the limited thiol defenses in these regions before a marked depletion in GSH occurs in the cytosolic and mitochondrial regions. As the duration of insult is extended, these interior compartments, which house most of the intracellular GSH, also become progressively depleted.

The most thorough examination of the efficacy of thiol based exogenous protection to date was carried out by Tani.[13] The results of that study suggested that exogenous thiols in the form of

GSH, cysteine, dithiothreitol, or N-acetyl-cysteine provided no protection from myocardial ischemia reperfusion injury in the isolated heart. Hearts were perfused with the thiol compounds for 20 minutes before and for thirty minutes after ischemia. Thirty minutes of ischemia resulted in similar and severe myocardial injury in non-thiol controls and all thiol perfused groups; after 30 minutes of reperfusion, recovery of rate pressure product was only about 25% in control hearts and associated with severe calcium uptake. A possible explanation for the negative results reported by Tani may be that the ischemic insult was so severe that factors in addition to oxidative stress contributed to much of the perfusion damage. Taken together, the contrasting results of Tani and those reported herein suggest that the efficacy of exogenous GSH is strongly dependent upon insult duration and/or timing of presentation.

Consistent with earlier studies, we found that 30 continuous minutes of substrate-free hypoxia, or three five minute intermittent H_2O_2 exposures was sufficient to reduce intracellular thiol content by approximately 50% and 67%, respectively, corresponding to severe mechanical dysfunction. Furthermore, in H_2O_2 exposed hearts, exogenous GSH significantly attenuated both the decline in intracellular thiols and mechanical function. These data confirm the vulnerability of the intracellular thiol pool to a non-physiological level exogenous oxidative stress or moderately long hypoxia. In contrast, the mild intermittent hypoxia model utilized in this study did not appreciably deplete the intracellular thiol pool.

The nearly compete resistance of the myocardium to intracellular GSH augmentation was surprising but not unexplainable. Studies that have demonstrated significant uptake of GSH or a GSH transport form such as GSH monoethyl ester, have investigated uptake in pharmacologically depleted tissue.[17–19] We also observed uptake of glutathione in hearts severely depleted of GSH by exposure to exogenous hydrogen peroxide. However, when levels remain near normal, glutathione concentration appears to be strongly feedback regulated in the cell.[20] Consequently, it is not unreasonable that the glutathione supplementation agents used in the present study during intermittent hypoxia did not significantly augment cytosolic GSH. The lone exception observed was 10 mM GSH. At this concentration, extracellular levels are higher than normal cytosolic levels (approximately 2.5–5 mM) and the concentration gradient may have helped drive GSH into the cell.

It is particularly interesting to note that 1 mM GSH provided as much protection as 10 mM GSH despite the fact that hearts treated with 1 mM GSH actually had lower intramyocyte thiol content versus control hearts (Figure 4) after the third hypoxia reoxygenation cycle. Why this was the case is unclear. However, the fact that the cardioprotective effect appears independent of total cellular GSH concentration suggests that GSH attenuates myocardial stunning via a membrane localized mechanism. There are several key myocardial membrane proteins involved in ion flux which contain functionally important sulfhydryl groups. Exogenous thiol donors have been reported to prevent, and in some cases reverse, oxidative modification of these membrane proteins.[21–23]

The structure of L-2-oxothiazolidine-4-carboxylate (OTZ) provides some insight into the mechanism of its cardioprotective effect a well as that of GSH. OTZ is a thiol delivery agent but not a thiol donor in its intact form due to the cyclic structure of thiozolidine. In normoxic buffer, the compound is sufficiently stable such that assay of 1 mM OTZ with DTNB reveals no thiol group binding (Data not shown). Consequently, it seems likely that the protective effect of OTZ depends on the breakdown of the molecule by the enzyme 5-oxoprolinase residing on or immediately inside the sarcolemmal membrane. The protection observed in OTZ hearts in the absence of increased cytosolic GSH suggests that its cardoprotection is independent of the GSH peroxidase pathway.

CONCLUSIONS

The present results add to the growing body of evidence suggesting that antioxidant protection against myocardial stunning can occur extracellularly. We have demonstrated that exogenous glutathione significantly enhances the rate of contractile recovery during the immediate transition period following brief hypoxia. This beneficial effect is independent of a change in intracellular thiol or GSH status and is consistent with a mechanism involving the protection of oxidizable thiol groups on membrane proteins involved in ion flux.

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