

Myocardial Glutathione Depletion Impairs Recovery of Isolated Blood-perfused Hearts After Global Ischaemia

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(Received 10 February 1992, accepted in revised form 13 May 1992)

S. W. WERNs, J. C. FANTONE, A. VENTURA, B. R. LUCCHESI. Myocardial Glutathione Depletion Impairs Recovery of Isolated Blood-Perfused Hearts After Global Ischaemia. *Journal of Molecular and Cellular Cardiology* (1992) **24**, 1215-1220. This study was performed to determine whether depletion of myocardial glutathione would impair recovery of left ventricular function of blood-perfused, isolated hearts after reversible ischaemic injury. Cats were treated with either vehicle or buthionine sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of glutathione. The feline isolated hearts were perfused with the blood of normal donor cats before and after 40 min of global myocardial ischaemia. The myocardial concentration of glutathione of the BSO group, 178 ± 38 ng/mg tissue, was significantly less than that of the control group, 292 ± 38 ng/mg tissue ($P < 0.05$). The peak left ventricular developed pressure (LVDP) 1 h after reperfusion, expressed as a fraction of the peak LVDP before ischaemia, was 0.87 ± 0.10 for the control group and 0.64 ± 0.08 for the BSO group ($P = 0.05$ vs. control). The peak left ventricular dP/dt after reperfusion, expressed as a fraction of the peak dP/dt before ischaemia, was 1.08 ± 0.14 for the control group and 0.78 ± 0.09 for the BSO group ($P = 0.05$ vs. control). The myocardial creatine kinase activity of the BSO group, 1046 ± 46 U/g tissue, was not significantly different from that of the control group, 1038 ± 17 U/g tissue ($P = 0.87$). Thus, depletion of myocardial glutathione resulted in impaired post-ischaemic contractile function that cannot be attributed to a greater extent of irreversible cell injury.

KEY WORDS: Glutathione; Buthionine sulfoximine; Reperfusion injury.

Introduction

Myocardial stunning refers to the delayed recovery of contractile function after either regional or global myocardial ischaemia which results in reversible myocardial injury [1]. Numerous studies have supported the hypothesis that the pathogenesis of myocardial stunning involves the formation of oxygen metabolites, such as superoxide anion, hydrogen peroxide and hydroxyl radical [1]. Reduced glutathione (GSH) and glutathione peroxidase constitute the major intracellular defenses against damage by hydrogen peroxide and lipid hydroperoxides [2, 3]. This study was designed to evaluate the importance of myocardial glutathione by determining the effect of myocardial glutathione depletion on myocardial stunning after global myocardial ischaemia. Depletion of glutathione was achieved by treatment with buthionine sul-

foximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of glutathione [4].

Methods

Preparation of blood-donor cat

The blood-perfused, feline isolated heart preparation was developed by Vogel and Lucchesi [5] and has been used to study the effects of a variety of pharmacological interventions, including β -adrenergic blockade [6], calcium channel blockade [7], and SOD and catalase [8], on myocardial injury due to global ischaemia and reperfusion. Hearts from mongrel cats of either sex were isolated and perfused via the aorta with blood drawn from a blood-donor cat. Blood-donor cats (3-5 kg) were anesthetized with 0.7 ml/kg iv of a dial-ureth-

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ane solution containing: allobarbitol, 100 g/l; urethane, 400 g/l; monoethylurea, 400 g/l. The cats were anticoagulated with 300 U/kg iv sodium heparin. A femoral vein was isolated, cannulated and connected through a roller pump to a venous reservoir. The reservoir level was adjusted to maintain the diastolic blood pressure of the blood-donor cat above 75 mmHg. One femoral artery was isolated, cannulated and connected to a second roller pump for withdrawal of arterial blood. The other femoral artery was cannulated to monitor systemic blood pressure. The trachea was cannulated for positive-pressure ventilation. Tidal volume was adjusted to maintain arterial pH, P_{O_2} , and P_{CO_2} within normal limits.

Preparation of isolated heart

Buthionine sulfoximine was obtained from Sigma Chemical, St. Louis, MI. After preparation of the donor cat as described above, the control or BSO-treated cats were anesthetized with 35 mg/kg iv sodium pentobarbital and anticoagulated with 300 U/kg iv sodium heparin. The heart was excised quickly and mounted on the perfusion apparatus. Arterial blood, drawn by the roller pump from the blood-donor cat, was maintained at 37°C by a heat exchanger and monitored continuously with a thermistor probe. The perfusion pressure was recorded with a pressure transducer connected to a side arm of the aortic cannula, and was maintained at 75 mmHg by manually varying the flow rate of the perfusion pump. The isolated hearts were suspended in a water-jacketed vessel maintained at 37°C. During ischaemic arrest the hearts were immersed in 0.9% sodium chloride solution. Otherwise the hearts were kept moist by periodically applying 0.9% sodium chloride to the surface.

The vena cava and pulmonary veins were ligated and the pulmonary artery was cannulated to collect the coronary venous effluent, which was returned to the jugular vein of the blood-donor cat via the venous reservoir and roller pump. A left ventricular drainage cannula was secured at the apex of the heart with purse string sutures. Blood collected through the drainage cannula (<1 ml/min) was returned to the venous reservoir. The hearts

were paced electrically from the right atrial appendage at a rate of 150 beats/min. An electrocardiogram was recorded from the isolated heart with one electrode attached to the aortic cannula and another to the apex of the heart.

Left ventricular volume was controlled with a water-filled latex balloon placed in the ventricle through the left atrial appendage. The balloon was connected by a short length of stiff polyethylene tubing to a syringe and a pressure transducer. Left ventricular pressure and its electronically derived first derivative (dP/dt) were recorded through this system. Ventricular function curves were inscribed by increasing the volume of the balloon by 0.2 ml increments so that ventricular diastolic pressure varied between 0 and 35 mmHg.

After stabilization for 60 min, the left ventricular pressure and dP/dt were recorded for left ventricular end-diastolic pressures varying from 0 to 35 mmHg by adjusting the balloon volume. The balloon volume was adjusted to the value that produced a left ventricular diastolic pressure of 15 mmHg. The hearts were subjected to zero-flow ischaemia for 40 min, followed by reperfusion. Fifty per cent of the hearts from each group developed ventricular fibrillation after reperfusion and were defibrillated with injections of 100 μ l of saturated KCl solution into the perfusion cannula. The perfusate was not allowed to recirculate. After reperfusion for 60 min the hemodynamic measurements were repeated.

Tissue creatine kinase and glutathione

Samples of blood were obtained from the pulmonary artery cannula 10 min after reperfusion to measure oxidized glutathione as described below. After reperfusion for 60 min and recording of the post-ischaemic left ventricular function curves, the hearts were perfused with 100 ml of Krebs's buffer to wash out the blood in the vascular space. The hearts were then freeze-clamped with tongs precooled in liquid nitrogen.

Creatine kinase

Previous studies have shown that the myocardial CK activity of isolated, blood-perfused cat hearts was unchanged after 40 min of

ischaemia, suggesting reversible cell injury [5]. The myocardial CK activities were determined using a commercial spectrophotometric assay (Sigma Chemical, St. Louis, MI). Phosphocreatine formed in the CK-catalyzed reaction between ATP and creatine is hydrolyzed to yield inorganic phosphorus, which is quantitated by the Fiske and SubbaRow method. Thus, the myocardium was homogenized in a phosphate-free buffer of 250 mM sucrose, 1 mM EDTA, 0.1 mM mercaptoethanol [9]. The CK activities of tissue samples was extrapolated from a standard curve of phosphate and CK standards.

Glutathione

The myocardial concentration of total glutathione was measured as described by Griffith and Meister [10]. The assay for GSH is an enzymatic recycling assay in which GSH is sequentially oxidized by 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and reduced by glutathione reductase and NADPH [11]. The rate of 5-thio-2-nitrobenzoic acid formation is monitored at 412 nm, and is proportional to the sum of GSH and 2X GSSG. The glutathione concentration of the tissue sample was extrapolated from a standard curve.

The concentration of oxidized glutathione in pulmonary artery plasma was measured as described by Anderson [11]. Fifty microliters of 500 mM EDTA was added to 5 ml of blood. Aliquots of 400 μ l were centrifuged at 10 000 g for 90 seconds. One hundred μ l of 10% sulfosalicylic acid was added to 200 μ l aliquots of plasma and centrifuged at 10 000 g for 5 min. The supernatants were stored overnight at 4°C. On the following day 4 μ l of 2-vinylpyridine were added to 200 μ l aliquots of supernatant. The samples were mixed with 6 μ l of triethanolamine and incubated for 60 min at room temperature. The concentration of oxidized glutathione was extrapolated from a standard curve.

Data analysis

All data are expressed as means \pm S.E.M. Differences were considered significant for $P < 0.05$. An unpaired *t* test was used to compare the control and BSO groups.

Results

The first objective was to identify a schedule of treatment with buthionine sulfoximine (BSO) that significantly reduces the concentration of glutathione in the cat heart. The myocardial concentration of glutathione was 998 ± 79 ng/mg tissue ($n = 2$) in non-ischaemic cat hearts, a value similar to that reported for the mouse heart [10]. Four hours after the injection of BSO 1.6 g/kg ip, the myocardial concentration of glutathione was 790 ng/mg ($n = 2$). The concentration of glutathione was 617 ng/mg ($n = 1$) 18 h after BSO 1.6 g/kg ip. After treatment with BSO 1.6 g/kg ip for 2 days, the myocardial concentration of glutathione was 272 ng/mg ($n = 2$). Therefore, the latter schedule of administration was selected for subsequent experiments.

Experiments were performed to examine the correlation between the post-ischaemic recovery of left ventricular function and the myocardial concentration of glutathione. Cats were assigned randomly to treatment with either vehicle ($n = 6$) or BSO ($n = 6$) 1.6 g/kg ip 42 and 18 h before the experiments. The peak left ventricular developed pressure (LVDP) for the control group was 142 ± 4 before ischaemia and 121 ± 12 after reperfusion ($P = 0.11$; see Fig. 1). The peak LVDP for the BSO group was 165 ± 14 before ischaemia and 106 ± 16 after reperfusion ($P = 0.005$). The peak LVDP after reperfusion, expressed as a fraction of the peak LVDP before ischaemia, was 0.87 ± 0.10 for the control group and 0.64 ± 0.08 for the BSO group ($P = 0.05$ vs. control). Similarly, the peak left ventricular dP/dt after reperfusion, expressed as a fraction of the peak dP/dt before ischaemia, was 1.08 ± 0.14 for the control group and 0.78 ± 0.09 for the BSO group ($P = 0.05$ vs. control; see Figure 1).

After reperfusion for 10 min, the concentration of oxidized glutathione in the pulmonary artery plasma was 6.3 ± 0.2 μ M for the controls ($n = 3$) compared to 3.3 ± 0.4 μ M for the BSO group ($n = 3$; $P < 0.01$ vs. controls). The myocardial concentration of glutathione was 292 ± 38 ng/mg tissue for the control group ($n = 6$), compared to 998 ± 79 ng/mg for the non-ischaemic hearts ($n = 2$). Thus, ischaemia and reperfusion resulted in a substantial decline in the myocardial concentra-

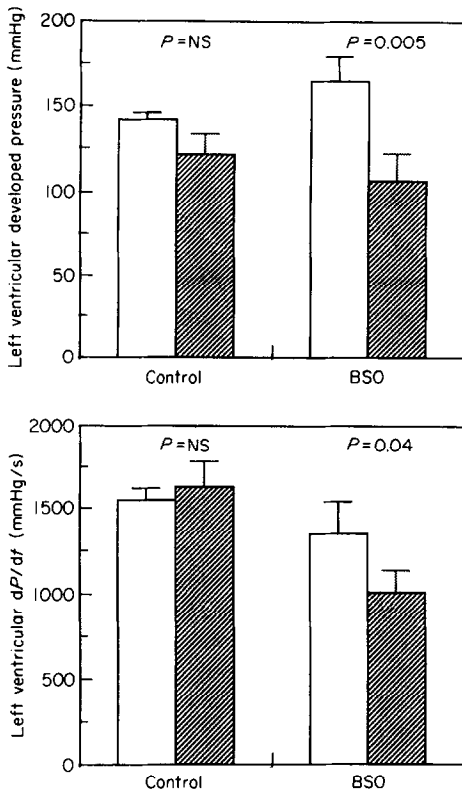


FIGURE 1. Left ventricular developed pressure and dP/dt before ischaemia and 1 h after reperfusion of the control group ($n=6$) and BSO group ($n=6$). □ = Pre-ischaemia, ▨ = post-reperfusion.

tion of glutathione. The myocardial concentration of glutathione of the BSO group, 178 ± 38 ng/mg ($n=5$), was significantly less than that of the control group ($P < 0.05$ vs. control). There was a significant correlation between the myocardial concentration of glutathione and the peak LVDP after reperfusion expressed as a percentage of the peak LVDP before ischaemia ($R = 0.64$, $P = 0.03$).

The myocardial creatine kinase activity (CK) of the BSO group, 1046 ± 46 U/g tissue ($n=5$), was not significantly different from that of the control group, 1038 ± 17 U/g tissue ($n=6$; $P = 0.87$). Therefore, the depletion of glutathione appears to have caused an impairment of post-ischaemic contractile function that cannot be attributed to a greater extent of irreversible cell injury.

Discussion

The results demonstrate that (1) there is a significant reduction of the total glutathione concentration of cat hearts subjected to global ischaemia for 40 min followed by reperfusion for 60 min, (2) the myocardial concentration of glutathione is significantly reduced in cats treated with BSO and (3) inhibition of glutathione synthesis by treatment with BSO results in significantly less recovery of left ventricular developed pressure and dP/dt after global ischaemia and reperfusion. The results support the hypothesis that myocardial glutathione protects the heart against injury caused by ischaemia and reperfusion.

Although myocardial glutathione depletion has been studied previously, a unique aspect of this study is the glutathione-depleted hearts were perfused with blood from a normal donor animal. Previous studies have examined either isolated crystalloid-perfused hearts [12], or the *in-situ* hearts of animals subjected to systemic depletion of glutathione [13]. Normal erythrocytes contain catalase, glutathione peroxidase, and glutathione, and may serve as a major defense against the effects of hydrogen peroxide [14–17]. Toth *et al.* [14] reported that addition of human erythrocytes decreased damage to cultured endothelial cells and isolated rat lungs which were exposed to hydrogen peroxide. Winterbourn *et al.* [15] demonstrated that human erythrocytes scavenge extracellular hydrogen peroxide, inhibiting the formation of hypochlorous acid and hydroxyl radicals. Several studies have concluded that the protection from hydrogen peroxide by erythrocytes is mediated primarily by catalase [15, 17]. Other investigators, however, have provided evidence that both erythrocyte catalase and glutathione can decrease reperfusion injury [16]. Brown *et al.* [16] added human erythrocytes to the perfusate during reperfusion of isolated rat hearts after global ischaemia. Intact erythrocytes decreased the myocardial concentration of hydrogen peroxide and improved the recovery of ventricular function. The beneficial effect of the erythrocytes was reduced by treatment of the erythrocytes with either aminotriazole, which inactivates catalase, or N-ethylmaleimide, which both alkylates GSH and inhibits regeneration of GSH by GSH reductase [18].

The current study is the first to demonstrate the importance of myocardial glutathione in the presence of intact erythrocytes.

Blaustein *et al.* [12] treated rats with diethylmaleate (DEM), which reduced the cardiac glutathione concentration to 35% of control hearts. The rat hearts were subjected to crystalloid perfusion and global ischaemia for 20 min. The glutathione-depleted hearts exhibited impaired recovery of systolic function after reperfusion. DEM depletes glutathione via the formation of a thioether adduct, but it also inhibits protein synthesis, which might influence the results of the latter study [19]. BSO, however, does not inhibit protein synthesis, and reduces the concentration of glutathione by inhibiting gamma-glutamylcysteine synthetase, the rate-limiting enzyme in the synthesis of glutathione [10, 19, 20]. Only one previous study has employed BSO to inhibit myocardial glutathione synthesis [13]. The experimental preparation employed in the latter study, however, consisted of coronary artery occlusion *in vivo*, which resulted in myocardial infarction, and in addition to the heart, circulating erythrocytes, leukocytes and plasma were subjected to glutathione depletion. The experimental preparation utilized in the current study featured untreated erythrocytes and leukocytes, and normal plasma, and the duration of ischaemia produces reversible myocardial injury rather than myocardial infarction. Previous work from this laboratory demonstrated a pattern of hemodynamic and biochemical changes consistent with reversible injury, i.e. myocardial stunning, after isolated, blood-perfused cat hearts were subjected to 40 min of ischaemia: impaired recovery of systolic function without the accumulation of calcium and loss of creatine kinase exhibited by infarcted tissue [4].

Additional studies have attempted to determine whether an increased cellular concentration of GSH would ameliorate post-ischaemic myocardial injury. Enrichment of the cardiac perfusate with GSH was reported to improve the recovery of left ventricular function of isolated rat hearts subjected to global ischaemia and reperfusion [12, 21]. The improvement in function, however, is probably not due to an increased intracellular pool of GSH. Glutathione itself is not effectively transported into cells [22], suggesting that the GSH

in the cardiac perfusate may have exerted unrecognized effects. Employing a model of hypoxic injury to renal tubules, Weinberg *et al.* [23] concluded that the cytoprotective effect of exogenous GSH may be due to a protective effect of the glycine derived from GSH. The exogenous GSH added to isolated renal proximal tubules was degraded rapidly to its component amino acids, glutamate, cysteine and glycine. Treatment with cysteine, but not glycine, increased the intracellular concentration of GSH, but only glycine attenuated hypoxic injury to the renal tubules.

N-acetylcysteine has been shown to increase intracellular concentrations of GSH by increasing the availability of cysteine [22]. Administration of N-acetylcysteine to dogs did not limit the extent of myocardial necrosis after coronary artery occlusion, although the post-ischaemic recovery of left ventricular function was improved [24]. Compared to control hearts, isolated rabbit hearts treated continuously with N-acetylcysteine before, during and after global ischaemia exhibited less efflux of creatine kinase and better left ventricular function after reperfusion [25]. The interpretation of the latter study is confounded by the fact that pretreatment with N-acetylcysteine exerted only a modest effect on the post-ischaemic myocardial concentration of GSH (8.3 nmol/mg protein *vs.* 6.5 nmol/mg protein in controls), and the addition of N-acetylcysteine to the perfusate throughout ischaemia and reperfusion would have exerted an antioxidant action.

Ferrari *et al.* [26] measured the release of GSH and oxidized glutathione from human hearts subjected to global myocardial ischaemia during coronary artery bypass grafting, and found indirect evidence of "oxidative stress" during reperfusion. Thus, both clinical and experimental studies suggest that myocardial glutathione stores may be an important component of the heart's susceptibility to reperfusion injury.

Acknowledgements

The authors would like to thank Caren M. Stalburg, Beatrice Manzor, Sean Pfeffer, and GuIm Kwon for their technical assistance. Supported by grant HL-19782-14 from the National Institutes of Health, Heart, Lung and Blood Institute.

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