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In Situ Microdialysis for Monitoring of Extracellular Glutathione Levels in Normal, Ischemic and Post-Ischemic Skeletal Muscle

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Microdialysis probes were inserted into the tibialis anterior muscle and into the femoral vein of anaesthetised Sprague-Dawley rats for monitoring of reduced (GSH) and oxidized (GSSG) extracellular glutathione. The dialysates were analysed using HPLC. The levels of GSH and GSSG were high immediately after implantation in the skeletal muscle and declined to steady state levels after 90 minutes into the same range as that found in the venous dialysate. Total ischemia was induced two hours after implantation of the dialysis probe after steady state levels had been reached. The extracellular levels of GSH increased during total ischemia and had doubled at the end of the ischemic period compared to preischemic values. During the following initial 30 minutes of reperfusion the levels increased further to four-fold the preischemic levels. The levels of GSSG also increased (100%) during the initial 30 minutes of reperfusion. The extracellular GSH levels remained elevated for 1 hour of reperfusion, but the GSSG levels returned to preischemic levels. The results indicate that intermittent hypoxia or anoxia in muscle tissue through hypoperfusion or ischemia decreases intracellular GSH stores by leakage, reducing the intracellular antioxidative capacity and increasing the risk for oxidative reperfusion injury upon final normalization of tissue blood supply.

Keywords: Skeletal muscle, Ischemia, Glutathione, Microdialysis

INTRODUCTION

Tissue ischemia is a major problem in many clinical situations. In the heart and the brain, the results of hypoxia are often immediate and catastrophic, but skeletal muscle is regarded as fairly resistant to long periods of ischemia. However, the development of microsurgical techniques for revascularization in replanted, severed limbs and free transplantation of vascularized skeletal muscle grafts will expose tissues to very long periods of ischemia before successful revascularization can be finally accomplished. Therefore, it is important to understand the mechanisms associated with ischemia-reperfusion injury to skeletal muscle and to explore ways to ameliorate their effects. The pathogenic mechanisms of the tissue damage

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occurring after ischemia and reperfusion have been the subject of much interest in recent years, but are still incompletely understood.

Oxygen radicals play an important role in the pathogenesis of ischemia-reperfusion injury in most organs including skeletal muscle,[1,2,3,4,5,6] as shown by the generation of lipid-peroxidation[2,7] and by the attenuation of the ischemia-reperfusion injury after administration of scavengers of reactive oxygen metabolites.[1,2,3,4,5,6]

Glutathione (GSH) is the major reducing agent in the cells protecting them from the toxic effects of hydrogen peroxide and other reactive oxygen species.[8,9] Thus GSH functions directly in the destruction of hydrogen peroxide and lipid peroxides by providing a substrate for the GSH peroxidase enzyme, which catalyses the formation of glutathione disulfide (GSSG), and water from two molecules of GSH and hydrogen peroxide.[8,9] GSSG can be reduced to GSH by the enzyme glutathione reductase which utilizes NADPH as a cofactor.[8,9] The intracellular GSSG/GSH ratio and the export of GSSG by cells have been suggested to be important indicators of oxidative stress.[1,9] Several studies have clearly illustrated the protective roll of GSH under conditions at which a peroxidative process is induced by chemicals.[10,12,13] However, the role of GSH in ischemia-reperfusion injury to the skeletal muscle has not been well established. In previous studies we and others have found that during reperfusion after severe ischemia there was a marked depletion of GSH in the skeletal muscle.[7,14,15]

In the present study we investigated this phenomenon further using microdialysis. Microdialysis allows for continuous monitoring of biochemical events in the extracellular tissue *in vivo*.[16]

MATERIAL AND METHODS

Chemicals

Oxidized glutathione (GSSG) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany) and reduced glutathione (GSH) from Fluka (Buchs, Switzerland). The derivatization reagent N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) was from the same company. Glutathione reductase, cysteine and NADPH were obtained from Sigma Chemical Co (St Louis, MO, USA). Methanesulphonic acid was from Fluka and acetonitrile (HPLC grade) was from Rathburn Chemicals (Walkerburn, Scotland). Phosphoric acid (85%), Na_2EDTA (Titriplex III), perchloric acid and Methyl Orange were from E. Merck (Darmstadt, Germany).

Rat Model

Male Sprague-Dawley rats (250–300g) were anaesthetized intraperitoneally before the tourniquet application using a mixture of ketamine (Ketalar®) and xylazine (Rompun®), given in doses of 50 mg and 5 mg per kg body weight respectively. Repeated doses were given to maintain anaesthesia throughout the course of the experiment. An elastic rubber band was applied around the proximal thigh in a standardized manner[17,18] After the ischemic period the tourniquet was released by cutting the rubber band. The ischemic leg was maintained at 37°C using a plastic pad with circulating water.

Experimental Set-up

In one group of rats (n = 3) a dialysis probe was implanted into the tibialis anterior muscle and another one was inserted into the femoral vein. In another group of rats (n = 7) a dialysis probe was implanted only into the tibialis anterior muscle and a tourniquet on the hindlimb was applied for two hours followed by reperfusion for one hour. Biopsies were taken at the end of the experiment for measurement of muscle tissue GSH concentrations.

Microdialysis

The microdialysis probes were 10 × 0.5 mm and their molecular cut-off was 20 000 Daltons. The

probe was perfused continuously at a flow rate of 2.0 μl/min with Ringer's solution via a microinjection pump. Fractions were collected every 30 minutes into tubes containing 60 μl 10 mM hydrochloric acid (a total of 120 μl/fraction) and immediately frozen in liquid nitrogen. The microdialysis probes and the pump were from CMA/Microdialysis, Sweden.

Preparation of Muscle Biopsies for GSH Measurements

The muscle biopsies were weighted and homogenized in 1 M perchloric acid containing 1 mM Na_2EDTA (4 ml/g muscle). The muscle was first minced into small pieces and then homogenized in a Polytron (2 × 30 seconds). The homogenate was then centrifuged for 5 minutes at 2,000 *g* to remove the protein precipitate. Perchlorate was then removed from 1 ml aliquots of supernatants in glass tubes, containing 5 μl of Methyl Orange (0.5 g/l) by adding a few drops of 5 M K_2CO_3 during mixing on a magnetic stirrer until there was a colour change. After 5 minutes, centrifugation was performed at 1,300 *g*.

Preparation of Muscle and Microdialysis Samples for Analysis of GSH

For the determination of the total amount of glutathione we added 10-μl aliquots of protein-free (from muscle or microdialysis) samples to tubes containing 340 μl of 0.1 M phosphate buffer, pH 7.0 and 10 μl of 0.1 mM NADPH, dissolved in the phosphate buffer. After mixing, 10 μl of glutathione reductase (6 U/ml) dissolved in the buffer was added, and the tubes were incubated for 5 minutes at room temperature. For the determination of reduced glutathione we omitted the reductase step and added the 10 μl aliquots of sample to 360 μl of phosphate buffer to obtain the same dilution of the sample as in the total glutathione method.

For derivatization of thiols we modified our earlier method[19] and added 1 ml of a solution containing 50 mM sodium carbonate and 2 mM Na_2 EDTA, pH 9.0 to the tubes, and then 100 μl of DACM, 10 μmol/L in acetone. The pH was adjusted to pH 9.0 by the addition of 13.5 μl of 1 M sodium hydroxide. After mixing, the tubes were placed in a water-bath at 37°C and derivatization was continued for 3 hours.

High-Performance Liquid Chromatography

The equipment used was a Constametric pump from LDC (Riviera Beach, FL, USA) and an automatic sample injector Model M 7110 from Magnus Scientific Instrumentation (Aylesbury, Buchs, UK), a Fluoromonitor III filter fluorimeter and a Model Cl-10 Integrator from LDC. The detector was operated with a mercury lamp, a 360 nm excitation filter and a 418–700 nm emission filter. Sample was injected by filling the 10 μl loop completely.

The analytical column was a Supelcosil LC-8 column (150 × 4.6 mm i.d., particle size 5 μm, Supelco, Bellefonte, PA, USA), which was thermostated at 28°C with a circulating water-bath. A Polygosil 60 C8 column (250 × 4.6 mm i.d., particle size 40–63 μm) from Machery-Nagel (Düren, Germany) was used as a silica saturation column.

The mobile phase was prepared from a solution containing 50 mM phosphoric acid and 6.3 mM methanesulphonic acid as the ion-pairing agent. The pH was adjusted to 2.2 with sodium hydroxide before the addition of acetonitrile (26%). The mobile phase was filtered through a 0.45 μm Durapore polyvinylidene difluoride filter, type HVLP (Millipore, Bedford, Mass., USA).

Statistical Analysis

The data are presented as mean ± S.E.M. A paired t-test was used to make statistical comparisons. A p value <0.05 was considered statistically significant.

RESULTS

Dialysate GSH (Fig. 1A) and GSSG (Fig. 1B) levels were high immediately after implantation of the probe and declined to steady state levels after 90 minutes. There were no major changes of either GSH (Fig. 1A) or GSSG (Fig. 1B) levels in the venous dialysate over time following the insertion of the probes. The levels of GSH and GSSG in the vein were in the same range as the steady state levels in the muscle (Fig. 1A and 1B).

In a second set of experiments we investigated the effect of ischemia and reperfusion on the levels of extracellular GSH and GSSG in the skeletal muscle. The ischemia was induced after steady state levels had been reached, two hours after implantation of the microdialysis probe. During the first hour of ischemia there were no changes in extracellular GSH and GSSG levels compared to preischemic levels (Figs. 2A and 2B). After 1.5 hours of ischemia, the GSH levels increased significantly compared to pre-ischemic values 4.60 ± 0.95 vs 2.18 ± 0.29 μM and were further enhanced after 2 hours of ischemia (Fig. 2A). There were no significant changes in the levels of GSSG during ischemia (Fig. 2B). During reperfusion the levels of GSH further increased compared to the preischemic period 8.8 ± 2.4 vs 2.18 ± 0.29 μM (Fig. 2A). The levels of GSSG were also enhanced during the first 30 minutes of reperfusion compared to preischemic values 0.35 ± 0.08 vs 0.14 ± 0.02 μM, but the levels of GSSG returned to preischemic levels after 60 minutes of reperfusion (Fig. 2B). The GSH levels remained elevated after 1 hours of reperfusion (Fig. 2A).

The tissue levels of total GSH (GSH+GSSG) were significantly reduced in the postischemic muscle compared to the non-ischemic muscle contralateral to that exposed to tourniquet ischemia, 584 ± 15 vs 678 ± 38 nmol/gram wet weight muscle ($p < 0.05$).

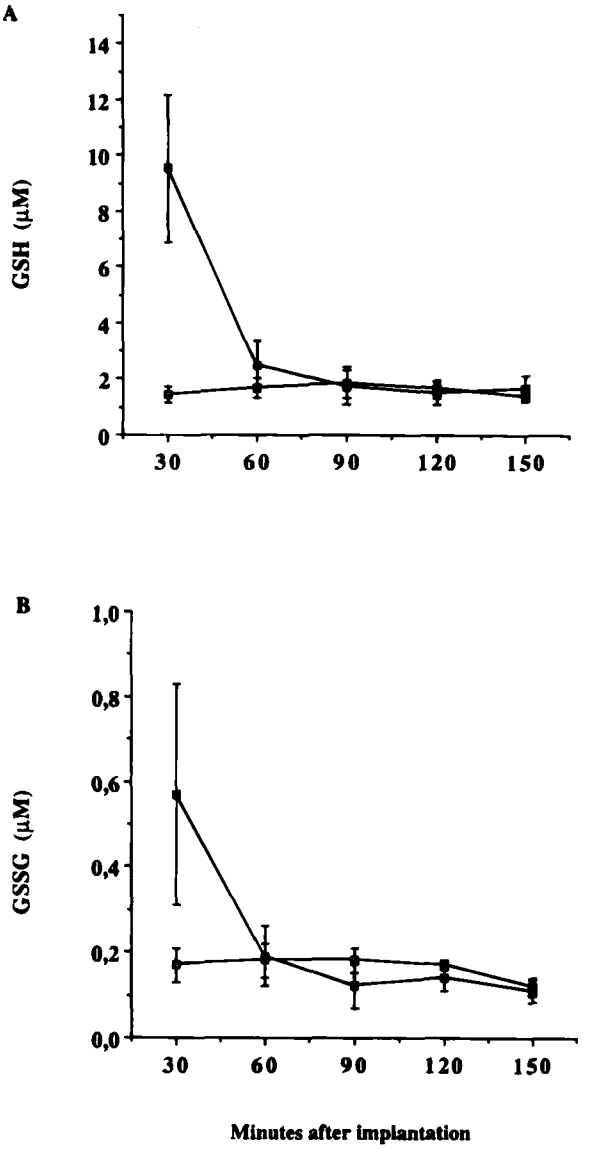

FIGURE 1 Time course of changes in extracellular (dialysate) concentrations of A: GSH and B: GSSG after implantation of the microdialysis probe into the muscle and the vein, n = 3. Data are plotted as mean ± S.E.M. –□– vein, –■– muscle.

DISCUSSION

The present study demonstrates the possibility to determine the extracellular level of GSH in muscle tissue and blood of anaesthetized rats by microdialysis, although the levels of GSH and

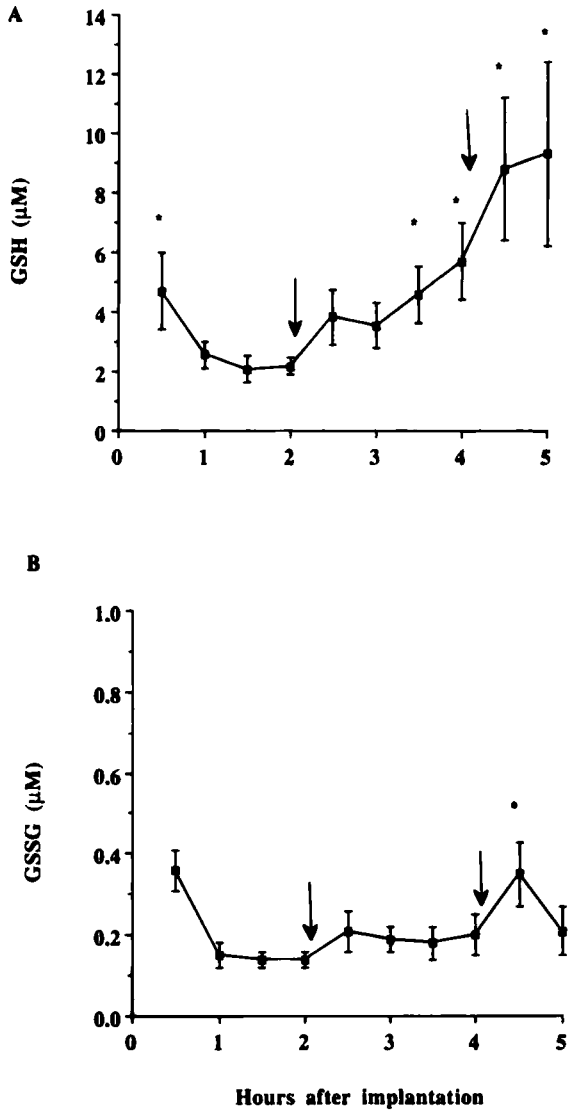

FIGURE 2 The extracellular levels of A: GSH and B: GSSG before and during 2 hours of ischemia followed by 1 hour of reperfusion. The arrows indicate the beginning and end of the ischemic period, n = 7. Mean ± S.E.M. p = <0.05 compared with steady state level = 2 hours after implantation of the microdialysis probe.

GSSG in the microdialysate are not the absolute extracellular values. Thus, the recovery of both GSH and GSSG are dependent on the properties of the membrane, most notably the molecular cut-off, its thickness and the perfusion speed.[16] In *in vitro* studies we have shown that the recovery of GSH and GSSG from standard solutions with the same microdialysis set-up as in this study were approximately 40% and 30%, respectively (data not shown), but the recoveries *in vivo* are unknown.

The present study shows that the plasma levels of GSH and GSSG are in the same range as those in the extracellular space in the muscle. It also shows that GSH is released during ischemia. The release of GSH was further enhanced during reperfusion and a small release of GSSG was also seen during the first 30 minutes of reperfusion. The trauma of insertion was also associated with a release of GSH and GSSG.

It has been established in several studies that upon oxidative stress glutathione is oxidized and exported from the tissue.[10,12,20] Eklöw *et al*[12] demonstrated a rapid and extensive release of GSSG from hepatocytes treated with t-BuOOH, with only a transient increase in GSSG inside the cells before efflux. Both during reperfusion and after the trauma of insertion of the microdialysis probe, there was an increased release of GSSG. Both trauma and reperfusion after ischemia have been shown to induce reactive hyperemia.[21,22] This reactive hyperemia, that increases the oxygen tension locally in the muscle, has been suggested to be a component for the generation of oxygen free radicals during reperfusion.[23,24,25,26] This may explain the increased levels of extracellular GSSG, both during reperfusion and immediately after implantation of the microdialysis probe. However, since there is a significant pool of intracellular GSSG,[7,14] the release of GSSG upon reperfusion may be a consequence of unspecific cell damage and not a consequence of increased generation of hydrogen peroxide or lipid peroxides.

The loss of GSH during reperfusion after severe ischemia in skeletal muscle has recently also been shown by Smith *et al*.[15] They suggested that this was due to increased oxidation of GSH. The apparent constant levels of GSSG shown previously in postischemic muscle[14]

and the results of the present study, suggest that the depletion of GSH is caused mainly by a release of GSH during ischemia and reperfusion and not by oxidation.

However, an experimentally induced marked depletion of glutathione in skeletal muscle has been shown to result in mitochondrial damage, where the histological changes observed were similar to those found after ischemia.[27,28] The skeletal muscle sensitivity to GSH depletion indicates that interventions aiming at increasing GSH levels during ischemia-reperfusion may be beneficial.

We conclude from the present study that reperfusion after ischemia is associated with a loss of total glutathione (GSH+GSSG). This seems to be caused by leakage of mainly GSH during both ischemia and reperfusion. This reduced intracellular anti-oxidative capacity will increase the risk for oxidative reperfusion injury upon final normalization of tissue blood perfusion. Furthermore, our results demonstrate that the microdialysis method will be useful in studies of GSH metabolism in normal and ischemic-reperfused skeletal muscle *in vivo* in intact animals.

Acknowledgments

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