Molecular and Cellular Biochemistry **144:** 85–93, 1995.

Glutathione disulfide as an index of oxidative stress during postischemic reperfusion in isolated rat hearts

Robert J.A.M. Verbunt, Willem G. van Dockum, E.M. Lars Bastiaanse, Janneke M. Egas and Arnoud van der Laarse

Department of Cardiology, University Hospital Leiden, Leiden, The Netherlands

Received 1 September 1994; accepted 21 November 1994

Abstract

The objectives of this study were to determine 1) whether reactive oxygen species generated upon postischemic reperfusion lead to oxidative stress in rat hearts, and 2) whether an exogenous prooxidant present in the early phase of reperfusion causes additional injury. Isolated buffer-perfused rat hearts were subjected to 30 min of hypothermic no-flow ischemia followed by 30 min of reperfusion. Increased myocardial content of glutathione disulfide (GSSG) and increased active transport of GSSG were used as indices of oxidative stress. To impose a prooxidant load, cumene hydroperoxide (20 μM) was administered during the first 10 min of reperfusion to a separate group of postischemic hearts. Reperfusion after 30 min of hypothermic ischemia resulted in a recovery of myocardial ATP from 28% at end-ischemia to 50–60%, a release of 5% of total myocardial LDH, and an almost complete recovery of both coronary flow rate and left ventricular developed pressure. After 5 and 30 min of reperfusion, neither myocardial content of GSSG nor active transport of GSSG were increased. These indices were increased, however, if cumene hydroperoxide was administered during early reperfusion. After stopping the administration of cumene hydroperoxide, myocardial GSSG content returned to control values and GSH content increased, indicating an unimpaired glutathione reductase reaction. Despite the induction of oxidative stress, reperfusion with cumene hydroperoxide did not cause additional metabolic, structural, or functional injury when compared to reperfusion without cumene hydroperoxide. We conclude that reactive oxygen species generated upon postischemic reperfusion did not lead to oxidative stress in isolated rat hearts. Moreover, even a superimposed prooxidant load during early reperfusion did not cause additional injury. (Mol Cell Biochem **144:** 85–93, 1995)

Key words: rat hearts, ischemia-reperfusion, oxidative stress, glutathione disulfide, glutathione reductase, reactive oxygen species

Introduction

It is widely believed that reperfusion of ischemic myocardium is not entirely beneficial, since reperfusion may cause injury additional to the injury induced by ischemia (for recent reviews [1–3]). In the pathogenesis of reperfusion-induced injury, reactive oxygen species such as hydrogen peroxide, the superoxide radical, and the hydroxyl radical have been implicated. Investigators using spin-trapping techniques have reported that a burst of reactive oxygen species is generated during the initial minutes of reperfusion [4–6]. If the quantity of reactive oxygen species exceeds the capacity of the antioxidant defense mechanisms, oxidative stress occurs. Oxidative stress is defined as a disturbance in the prooxidant/antioxidant balance in favor of the former, leading to potential damage [7]. Both ischemia and reperfusion may contribute to oxidative stress, as ischemia impairs the antioxidant defense mechanisms [8, 9], and reperfusion is associated with generation of reactive oxygen species which form a prooxidant challenge [1–3].

Address for offprints: R.J.A.M. Verbunt, University Hospital Leiden, Department of Cardiology, Building 1, D4-P19, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands

A major antioxidant defense mechanism against reactive oxygen species is the glutathione redox cycle. This redox cycle has a protective function in maintaining thiol groups of enzymes and other proteins in their reduced state, and in preventing peroxidation of membrane lipids [10]. The glutathione redox cycle detoxifies reactive oxygen species by reaction with reduced glutathione (GSH). The superoxide radical either reacts nonenzymatically with GSH [10], or is dismutated to hydrogen peroxide. The reaction of hydrogen peroxide with GSH is catalyzed by glutathione peroxidase. The glutathione redox cycle thereby prevents the formation of the highly toxic hydroxyl radical, since the superoxide radical and hydrogen peroxide are the precursors of the hydroxyl radical. The reaction of GSH with reactive oxygen species leads to the formation of glutathione disulfide (GSSG). The majority of GSSG is reduced back to GSH by glutathione reductase, but part of GSSG is actively transported out of the cell [11]. If the glutathione redox cycle is overwhelmed by reactive oxygen species, the glutathione reductase reaction becomes rate-limiting. This leads to intracellular accumulation of GSSG and increased transport of GSSG out of the cell. Increased tissue content of GSSG and increased active transport of GSSG are both considered to be sensitive indices of oxidative stress [2, 11, 12].

In isolated rat hearts, it was reported that myocardial content of GSSG was increased during postischemic reperfusion [13], but not during reoxygenation of hypoxic hearts [14–17] when compared to control levels. In preliminary studies we failed to find an increase in myocardial content of GSSG during postischemic reperfusion of isolated rat hearts. Therefore, we decided to study the occurrence of oxidative stress in isolated perfused rat hearts in more detail. Isolated buffer-perfused rat hearts were subjected to 30 min of hypothermic no-flow ischemia, and myocardial content of GSSG and release of GSSG from postischemic hearts were measured as indices of oxidative stress. We selected this model, since hypothermic ischemia, in contrast to normothermic ischemia, prevented the development of no-reflow areas during reperfusion. Furthermore, we tested whether postischemic hearts could withstand a superimposed prooxidant load during early reperfusion. To this end, we administered an exogenous prooxidant (cumene hydroperoxide) upon reperfusion to a separate group of postischemic hearts, and examined whether this superimposed prooxidant load during reperfusion caused additional injury.

Materials and methods

Isolated heart preparation

Male Wistar rats weighing 285–330 g were anesthetized with diethyl ether. After intravenous injection of 300 IU of sodium heparin, the hearts were excised, placed in ice-cold heparinized perfusion buffer, and trimmed of extraneous tissue. The hearts were mounted on a non-recirculating Langendorff perfusion apparatus, and perfused retrogradely with Krebs-Henseleit bicarbonate buffer at 37°C. The perfusion buffer contained (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11, and was gassed continuously with 95% O_2 + 5% CO_2. The hearts were perfused at a perfusion pressure of 65 mmHg which was maintained by a pump (Watson-Marlow 101 U). The perfusion pressure was measured with a pressure transducer (Statham P23Db) via a side arm of the aortic cannula. The perfusion pressure signal was coupled back to the pump by an electronic feedback system to keep the perfusion pressure constant. Throughout the experiments, heart rate was kept at 300–330 beats/min by right ventricular pacing. Myocardial temperature decreased from 37°C to 30–31°C during ischemia (hypothermic ischemia), although the temperature in the glass chamber enclosing the heart was maintained at 36.5 ± 1°C during ischemia. In separate experiments, myocardial temperature was maintained at 37°C during ischemia by immersing the heart in perfusion buffer (normothermic ischemia). Left ventricular pressure was measured by inserting a 23 gauge needle into the left ventricle through the apex. The needle was connected via a fluid-filled polyethylene catheter to a pressure transducer (Statham P23Db). Left ventricular developed pressure was calculated as the difference between peak systolic and end-diastolic pressure. Coronary flow rate was measured electromagnetically (Skalar) and by timed collection of coronary effluent. Perfusion pressure, left ventricular pressure, and coronary flow rate were recorded on a chart recorder (Gould 2400). The experiments have been approved by the Institutional Committee on Animal Experiments.

Experimental protocol

First the hearts were allowed to stabilize for 30 min. Then the hearts were subjected to 30 min of hypothermic no-flow ischemia, followed by either 5 or 30 min of reperfusion. The 5 min time point was chosen as a precaution not to miss a transient increase in myocardial GSSG content due to reactive oxygen species generated during the initial minutes of reperfusion. The control group was subjected to 30 min of non-ischemic perfusion. To test whether postischemic hearts could withstand a superimposed prooxidant load during early reperfusion, we administered cumene hydroperoxide to two separate groups of postischemic hearts at the onset of reperfusion either for 5 min (this group was reperfused for only 5 min), or during the first 10 min of the 30 min reperfusion period. Cumene hydroperoxide (0.3 μmol/min) was

administered to the perfusion buffer by means of an infusion pump. The average concentration of cumene hydroperoxide in the perfusion buffer during the first 10 min of reperfusion ranged among the hearts from 19–24 μM depending on the recovery of coronary flow rate. To exclude a potential effect of hypothermia on the occurrence of oxidative stress during reperfusion, two additional groups of hearts were subjected to 30 min of normothermic ischemia followed by either 5 or 30 min of reperfusion. At the end of each experiment, the hearts were freeze-clamped for tissue analysis using Wollenberger tongs precooled in liquid nitrogen.

Evans Blue perfusion

Hearts were examined for the development of no-reflow areas following 30 min of either hypothermic or normothermic ischemia. At various time points during reperfusion Evans Blue was administered to the perfusion buffer (final concentration of 0.2 g/l) for 1 min by means of an infusion pump. After Evans Blue perfusion the hearts were frozen in isopentane precooled in liquid nitrogen and 2–5 transverse sections (30 μm) were cut. These sections were viewed with a microscope, subsequently digitized using a video frame grabber, and analyzed with standard software equipment as described [18]. The no-reflow areas, i.e. areas devoid of Evans Blue, are expressed as a percentage of total myocardial area.

Biochemical assays

Myocardial GSSG and GSH

Heart tissue was processed as described by Akerboom and Sies [19]. Briefly, the freeze-clamped hearts were pulverized in an aluminum mortar at liquid nitrogen temperature. For determination of total glutathione (GSH+GSSG), frozen heart powder was homogenized in ice-cold 1 M perchloric acid containing 2 mM EDTA, whereas for selective determination of GSSG, 50 mM N-ethylmaleimide (NEM) was added to the perchloric acid/EDTA solution. NEM traps GSH and prevents oxidation of GSH to GSSG. The homogenates were stored at –70°C for not longer than 9 days. GSH+GSSG and GSSG were subsequently assayed by an enzymatic recycling method described by Tietze [20] using NADPH, glutathione reductase, and 5′,5-dithiobis(2-nitrobenzoic acid). Excess NEM was removed by chromatographic separation on a QAE-25 Sephadex column [19]. GSH was calculated by subtracting GSSG from GSH+GSSG. Myocardial contents of GSSG and GSH are expressed as GSH equivalents/mg protein (i.e. 1 mol GSSG equals 2 mol GSH-eq).

Myocardial adenine nucleotides

ATP, ADP and AMP were assayed in freeze-dried heart powder. A weighed aliquot was extracted in 3 M perchloric acid. The extracts were neutralized with $KHCO_3$. After centrifugation, the supernatants were analyzed by reversed-phase high performance liquid chromatography [21]. Myocardial contents of adenine nucleotides are expressed as μmol/g dry weight.

Myocardial protein

Protein content was measured according to the method of Lowry *et al.* [22] using bovine serum albumin as a standard.

Release of GSSG and GSH

Samples of coronary effluent were collected at the end of the stabilization period (pre-ischemia), and at 1, 3, 5, 10, 20, 30 min after the onset of reperfusion and stored on ice. For determination of GSH+GSSG release, the samples were assayed immediately after the perfusion experiment. For determination of GSSG release, aliquots of approximately 2 ml of coronary effluent were collected in preweighed vials containing 0.5 ml of ice-cold 50 mM NEM and 10 mM EDTA, and immediately stirred and weighed. The samples were extracted 8 times with equal volumes of diethyl ether at 0–4°C to remove excess NEM. After diethyl ether extraction, recovery of GSSG at concentrations of 50 and 100 nM in the NEM/EDTA solution was 95 ± 6% (mean ± SD, n = 8). GSH+GSSG and GSSG were assayed according to Tietze [20]. Release of GSSG and GSH is expressed as GSH-eq/min/g.

Release of lactate dehydrogenase (LDH)

The coronary effluent was collected in 5 min fractions on ice for determination of LDH release. LDH activity was spectrophotometrically assayed using a test kit (Boehringer Mannheim).

Release of GSH, GSSG, and LDH into the effluent is expressed per g ventricular wet weight. Since the hearts become progressively edematous during the course of the perfusion experiments, ventricular weight (VW) at the start of the perfusion experiment was estimated on the basis of body weight (BW). From a separate group of 29 rats (BW ranging from 250–350 g) the hearts were excised and the atria removed. The ventricles were rinsed, blotted, and weighed. VW (in g) correlated with BW (in g) according to: $VW = 2.48*10^{-3}*BW+0.163$, r=0.84.

Chemicals

GSSG, glutathione reductase, and NADPH were purchased from Boehringer Mannheim, NEM from ICN Biomedicals, 5′5′-dithiobis(2-nitrobenzoic acid) from Janssen Chimica, QAE Sephadex A25 from Pharmacia, and cumene hydroperoxide from Fluka AG. All other chemicals were of analytical grade.

Analysis of data

Data are expressed as means ± SD. The unpaired Student's *t* test was used to compare two group means, and one-way ANOVA was used to compare more than two groups. Repeated-measures ANOVA was used to compare within groups data obtained at pre-ischemia with data obtained during reperfusion. If a significant value of F was obtained, the Student-Newman-Keuls test was used to make pairwise comparisons. A difference was considered statistically significant if $p < 0.05$.

Results

No-reflow areas

The no-reflow phenomenon prevents reperfusion of part of the ischemic myocardium upon restoration of arterial supply [23]. Hearts subjected to 30 min of hypothermic ischemia showed 29 ± 4% no-reflow areas after 1 min of reperfusion (n = 4), but these areas were completely dissolved as soon as arrhythmias had stopped and paced contractions were reestablished (usually between 2 and 5 min) (n = 4). On the other hand, hearts (n = 4) subjected to 30 min of normothermic ischemia showed 34 ± 3% no-reflow areas still after 30 min of reperfusion, despite the finding that arrhythmias in this group had also stopped within 5 min.

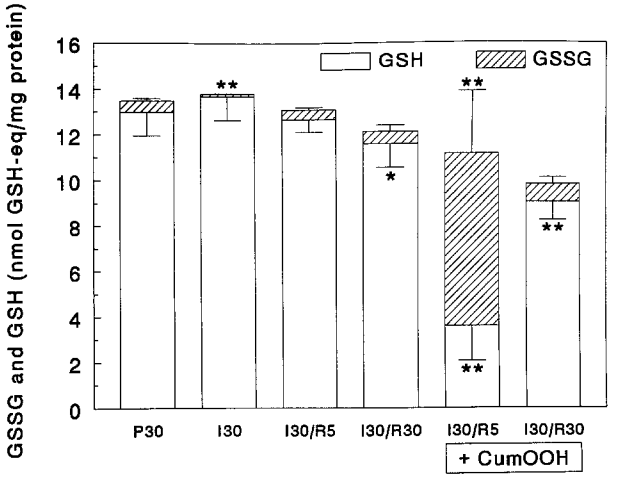

Fig. 1. Myocardial contents of GSSG and GSH during postischemic reperfusion with or without administration of cumene hydroperoxide. Isolated rat hearts were subjected to 30 min of non-ischemic control perfusion (P30), to 30 min of hypothermic no-flow ischemia (I30), and to 30 min of hypothermic ischemia followed by 5 or 30 min of reperfusion (I30/R5 and I30/R30, respectively). Cumene hydroperoxide (CumOOH, 20 μM) was administered at the onset of reperfusion either for 5 min (these hearts were reperfused for only 5 min) (I30/R5+CumOOH), or during the first 10 min of 30 min of reperfusion (I30/R30+CumOOH). Data represent means ± SD for 5–7 hearts. *p<0.05, **p<0.01 vs. P30.

Metabolic data

Myocardial GSSG and GSH

Myocardial contents of GSSG and GSH after 30 min of non-ischemic control perfusion, after 30 min of hypothermic ischemia, and after 5 and 30 min of reperfusion are shown in Fig. 1. Myocardial GSSG content after 30 min of ischemia was decreased compared to the control value, which may be due to less generation of reactive oxygen species during ischemia. Myocardial GSSG content after 5 and 30 min of reperfusion was not different from that after control perfusion. Myocardial GSH content was unchanged after 30 min of ischemia and after 5 min of reperfusion, but was decreased by 10% after 30 min of reperfusion, when compared to GSH content after control perfusion.

Myocardial content of GSSG was also not increased during reperfusion following either 15 or 45 min of hypothermic no-flow ischemia, or 30 min of normothermic no-flow ischemia. After 5 and 30 min of reperfusion, these values were, respectively, following 15 min of hypothermic ischemia 0.47 (n = 3) and 0.58 nmol GSH-eq/mg protein (n = 3), following 45 min of hypothermic ischemia 0.33 (n = 6) and 0.35 nmol GSH-eq/mg protein (n = 7), and following 30 min of normothermic ischemia 0.30 (n = 3) and 0.23 nmol GSH-eq/mg protein (n = 4). These results suggest that the absence of an increase in myocardial GSSG content during reperfusion can be explained neither by the duration of the preceding ischemic period nor by the hypothermia.

Administration of cumene hydroperoxide (20 μM) at the onset of reperfusion for 5 min resulted in a marked increase in myocardial GSSG content and decrease in GSH content (Fig. 1). If the administration of cumene hydroperoxide was stopped after 10 min of reperfusion, GSSG content decreased to control values and GSH content increased again during the subsequent 20 min of reperfusion without cumene hydroperoxide (Fig. 1). These results indicate that neither the formation of GSSG catalyzed by glutathione peroxidase, nor the reduction of GSSG to GSH catalyzed by glutathione reductase were impaired during postischemic reperfusion.

Release of GSSG and GSH

Release of GSSG and GSH into the effluent at the end of the stabilization period (i.e. pre-ischemic value) and during reperfusion after 30 min of hypothermic ischemia is shown in Fig. 2. The rate of GSSG release increased from 0.16 nmol GSH-eq/min/g at pre-ischemia to 0.29 and 0.30 nmol GSH-eq/min/g after 5 and 10 min of reperfusion, and returned to the pre-ischemic value after 20 min of reperfusion (Fig. 2A). These increases in GSSG release after 5 and 10 min of reperfusion were accompanied by a corresponding increase in GSH release (Fig. 2B). The ratios of GSSG to GSH in the effluent after 5 and 10 min of reperfusion were 0.019 ± 0.010 and 0.031 ± 0.013, respectively. These values are not greater

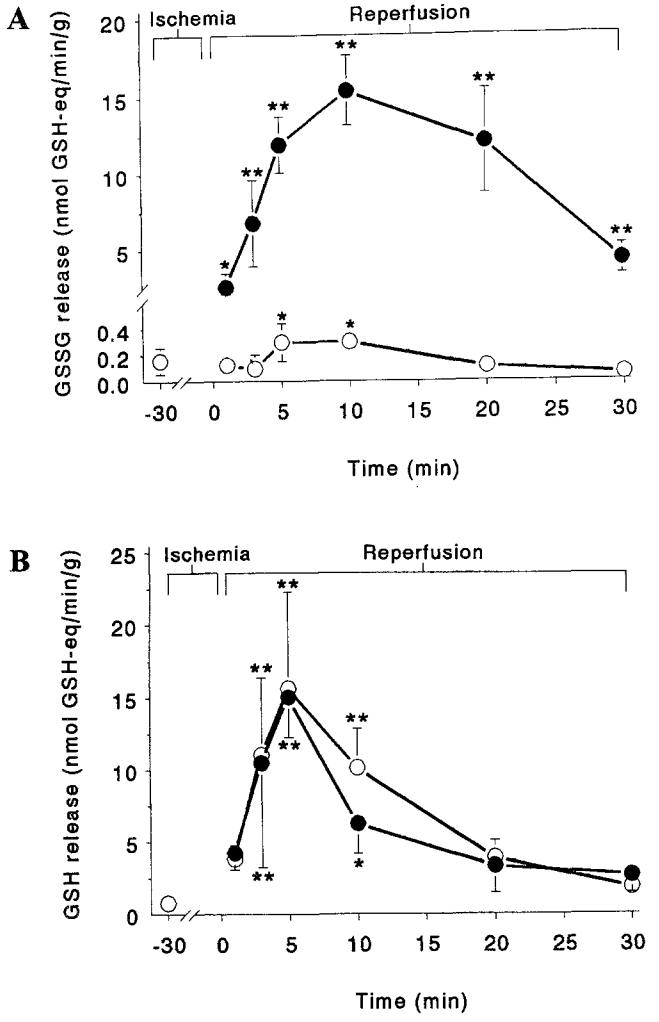

Fig. 2. Time courses of release of GSSG (A) and GSH (B) into the effluent during postischemic reperfusion with or without administration of cumene hydroperoxide. Isolated rat hearts were subjected to 30 min of hypothermic no-flow ischemia followed by 30 min of reperfusion (○), or followed by 30 min of reperfusion in the presence of 20 μM cumene hydroperoxide during the first 10 min (●). Data represent means ± SD for 5–6 hearts. *P<0.05, **P<0.01 vs. pre-ischemic values (–30 min).

than the ratio of GSSG to GSH in myocardial tissue after 30 min of control perfusion (0.038 ± 0.008). Therefore, it is unlikely that the increased release of GSSG after 5 and 10 min of reperfusion is due to oxidative stress. Namely, reperfusion-induced oxidative stress leads to intracellular accumulation of GSSG and to selective transport of GSSG out of the cell. The latter should increase the ratio of GSSG to GSH in the effluent. Our results can be explained by aspecific release of GSSG and GSH from severely injured cells, since aspecific release does not increase the ratio of GSSG to GSH in the effluent compared to the ratio in tissue. The cumulative release of GSSG during 30 min of reperfusion was 5.1 ± 1.6 nmol GSH-eq/30 min/g, and the cumulative release of GSH was 209 ± 52 nmol GSH-eq/30 min/g, resulting in a ratio of GSSG to GSH of 0.026 ± 0.011. This ratio was also not greater than the ratio of GSSG to GSH in myocardial tissue after control perfusion (0.038 ± 0.008). The cumulative release of GSH+GSSG during 30 min of reperfusion accounted fully for the 10% decrease in myocardial content of GSH+GSSG after 30 min of reperfusion.

Active transport of GSSG due to reperfusion-induced oxidative stress was also not increased during reperfusion following 15 or 45 min of hypothermic ischemia, or 30 min of normothermic ischemia (2–5 hearts; data not shown).

Release of GSSG into the effluent was markedly increased during reperfusion when cumene hydroperoxide (20 μM) was administered at the onset of reperfusion for 10 min (Fig. 2A), indicating the presence of oxidative stress. Cumulative GSSG release during reperfusion was 314 ± 60 nmol GSH-eq/30 min/g in the presence of cumene hydroperoxide. Reperfusion with cumene hydroperoxide did not affect GSH release, compared to reperfusion without cumene hydroperoxide (Fig. 2B): cumulative release of GSH during reperfusion was 176 ± 53 nmol GSH-eq/30 min/g in the presence, and 209 ± 52 nmol GSH-eq/30 min/g in the absence of cumene hydroperoxide (P = NS). The cumulative release of GSH+GSSG during 30 min of reperfusion accounted fully for the 27% decrease in myocardial content of GSH+GSSG after 30 min of reperfusion, when cumene hydroperoxide was administered during early reperfusion.

Release of LDH

The cumulative release of LDH activity during 30 min of non-ischemic control perfusion was 0.7 ± 0.1 U/g. During 30 min of reperfusion after 30 min of hypothermic ischemia a total of 17.8 ± 3.6 U/g of LDH activity was released into the effluent, which corresponds to 5% of total myocardial LDH content [24]. The cumulative release of LDH activity was not increased when 20 μM cumene hydroperoxide was administered during the first 10 min of reperfusion (20.6 ± 5.6 U/g, P = NS), indicating that cumene hydroperoxide did not cause additional structural cell damage. Although the hearts subjected to normothermic ischemia sustained probably more severe structural injury than the hearts subjected to hypothermic ischemia, they did not release more LDH activity (19.4 ± 3.3 U/g), most likely due to trapped LDH in no-reflow areas.

Myocardial adenine nucleotides

Myocardial ATP content was decreased to 28% after 30 min of hypothermic ischemia, and recovered to 50–60% during reperfusion, when compared to control perfusion (Fig. 3). ADP content was halved after 30 min of ischemia, and remained unchanged during subsequent reperfusion. Myocardial AMP content was increased 6-fold at the end of the ischemic period, and returned to control values after 5 min of reperfusion. Administration of 20 μM cumene hydro-

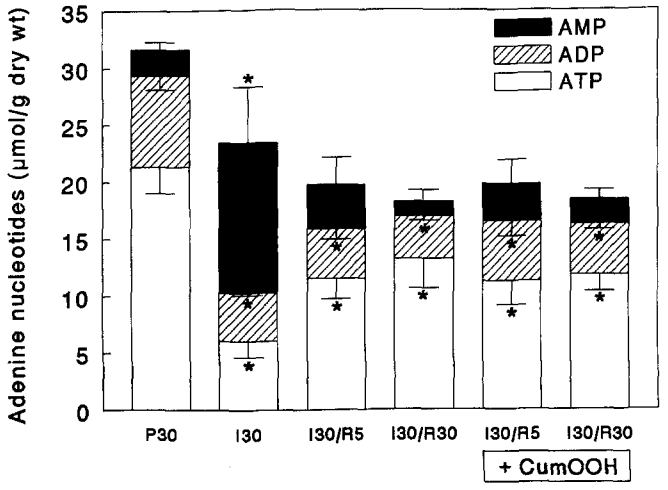

Fig. 3. Myocardial contents of adenine nucleotides during postischemic reperfusion with or without administration of cumene hydroperoxide. Isolated rat hearts were subjected to 30 min of non-ischemic control perfusion (P30), to 30 min of hypothermic no-flow ischemia (I30), and to 30 min of hypothermic ischemia followed by 5 or 30 min of reperfusion (I30/R5 and I30/R30, respectively). Cumene hydroperoxide (CumOOH, 20 µM) was administered at the onset of reperfusion either for 5 min (these hearts were reperfused for only 5 min) (I30/R5+CumOOH), or during the first 10 min of 30 min of reperfusion (I30/R30+CumOOH). Data represent means ± SD for 3–6 hearts. *p<0.01 vs. P30.

peroxide during early reperfusion did not affect the recovery of adenine nucleotides during reperfusion (Fig. 3). At the end of 30 min of normothermic ischemia, ATP content was decreased to 5% only, and recovered to 20% during reperfusion. One should realize that this latter value reflects the ATP content of myocardium that for approximately two thirds is reperfused, the remainder being ischemic after 30 min of reperfusion due to no-reflow.

Functional data

Coronary flow rate

The pre-ischemic coronary flow rate was 11.3 ± 0.7 ml/min. An hyperemic response was observed during reperfusion after 30 min of hypothermic ischemia (Fig. 4A). The coronary flow rate peaked after 10 min of reperfusion (14.7 ± 1.7 ml/min) and declined gradually to the pre-ischemic value after 30 min of reperfusion. Administration of 20 µM cumene hydroperoxide during the first 10 min of reperfusion had no significant effect on the recovery of coronary flow rate (Fig. 4A). Following normothermic ischemia, however, coronary flow recovered within 2 min to only 67% and remained constant for the rest of the reperfusion period.

Left ventricular developed pressure

All hearts showed ventricular fibrillation within 30 s after the

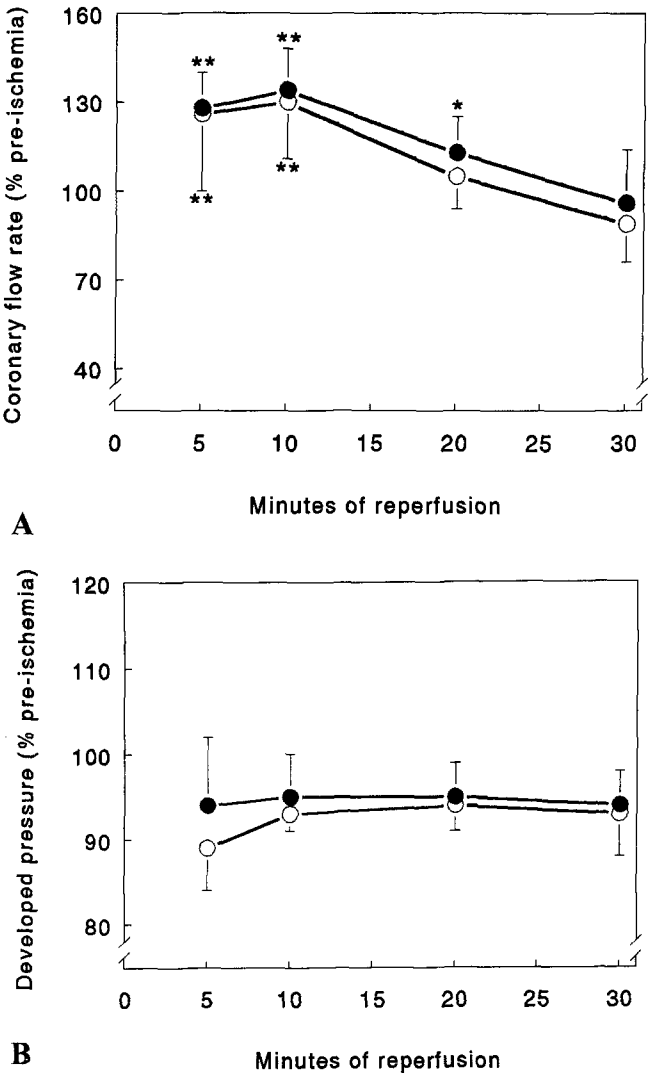

Fig. 4. Recoveries of coronary flow rate (A) and left ventricular developed pressure (B) during postischemic reperfusion with or without administration of cumene hydroperoxide. Isolated rat hearts were subjected to 30 min of hypothermic no-flow ischemia followed by 30 min of reperfusion (○), or followed by 30 min of reperfusion in the presence of 20 µM cumene hydroperoxide during the first 10 min (●). Data represent means ± SD for 5–7 hearts. *p<0.05, **p<0.01 vs. pre-ischemic values.

onset of reperfusion, but nearly all converted spontaneously to paced rhythm within 5 min. In addition, the hearts were prone to brief periods of arrhythmias during the remaining reperfusion period. Hearts which showed arrhythmias at the selected time points (at 5, 10, 20, 30 min after the onset of reperfusion) were excluded from analysis of developed pressure at that time point. The pre-ischemic left ventricular developed pressure was 70.2 ± 5.3 mmHg. The developed pressure tended to be lower during reperfusion (P = NS) (Fig. 4B). Administration of cumene hydroperoxide during reperfusion had no significant effect on the recovery of developed

pressure (Fig. 4B). Following normothermic ischemia, developed pressure recovered only partially to 10, 30, and 38% after 10, 20 and 30 min of reperfusion, respectively.

Discussion

The present study demonstrates that postischemic reperfusion of isolated buffer-perfused rat hearts did not lead to oxidative stress, since neither myocardial content of GSSG nor active transport of GSSG were increased during reperfusion. These results indicate that the quantity of reactive oxygen species generated upon reperfusion was insufficient to exceed the antioxidant capacity of the glutathione redox cycle. The antioxidant capacity of the glutathione redox cycle is closely related to the functioning of the glutathione reductase reaction, which is critically dependent on the availability of reducing equivalents such as NADPH. In our experiments, the glutathione reductase reaction was not impaired during reperfusion, as myocardial content of GSSG rapidly decreased and that of GSH rapidly increased after the administration of cumene hydroperoxide had been stopped. Kehrer *et al.* showed that even in severely energy depleted heart tissue (ATP and phosphocreatine <4%) the availability of NADPH was sufficient to reduce GSSG [25]. As in our experiments the quantity of reactive oxygen species generated upon reperfusion was insufficient to disturb the prooxidant/antioxidant balance, it is unlikely that the quantity was sufficient to cause injury additional to ischemia-induced injury. This hypothesis is supported by our finding that even if the prooxidant/antioxidant balance was disturbed by the administration of cumene hydroperoxide, this superimposed prooxidant load during reperfusion did not cause additional structural, functional or metabolic injury, when compared to reperfusion without cumene hydroperoxide.

In our isolated rat heart model, the hearts were subjected to 30 min of hypothermic ischemia. We selected this model, since the hypothermia prevented the development of no-reflow areas during reperfusion, which were 34 ± 3% of total myocardial area after 30 min of reperfusion following 30 min of normothermic ischemia. The presence of no-reflow areas (i.e. permanently ischemic areas) next to reperfused areas disturbs an accurate assessment of both oxidative stress and metabolic, functional, and structural injury during reperfusion. The hearts subjected to hypothermic ischemia sustained less metabolic and functional injury than the hearts subjected to normothermic ischemia. They sustained probably also less structural injury, although LDH release was not different between both groups, which is most likely due to trapped LDH in the no-reflow areas of the hearts subjected to normothermic ischemia. Less structural injury results in less aspecific release, which facilitates the detection of active transport of GSSG under conditions of oxidative stress. It has been suggested that the occurrence of oxidative stress during reperfusion is correlated with the severity of ischemic injury [1, 2]. However, we found that oxidative stress was absent following ischemic periods causing both relatively mild injury (15 and 30 min of hypothermic ischemia) and severe injury (30 min of normothermic and 45 min of hypothermic ischemia).

Using increased myocardial content of GSSG and increased release of GSSG as indices of oxidative stress, the occurrence of oxidative stress during postischemic reperfusion has been studied in various species. In agreement with our results, absence of these indices of oxidative stress during reperfusion has been reported in studies in canine hearts *in vivo* [26–28], and in one study in isolated rabbit hearts [29]. We interpret the increased GSSG release in the study reported by Lesnefsky *et al.* [27] to be due to aspecific release of GSSG from severely injured cells, as the increase in GSSG release was accompanied by a corresponding increase in GSH release. This interpretation is further supported by their finding that myocardial content of GSSG was not increased at various time points during reperfusion. Our results are also in agreement with results from hypoxia-reoxygenation studies in isolated rat hearts, since myocardial content of GSSG was not increased during reoxygenation of hypoxic hearts when compared to control levels [14–17]. Isolated buffer-perfused hearts are not exposed to neutrophils, which may be an important source of reactive oxygen species during reperfusion [1, 2]. However, oxidative stress was also absent in postischemic blood-perfused hearts [26–28].

In contrast to our results, oxidative stress did occur during postischemic reperfusion of isolated rabbit hearts in studies by Ferrari and colleagues [9, 30, 31], and during reperfusion of isolated rat hearts in one study [13]. In these latter four studies, myocardial content of GSSG was increased after 30 min [9, 13, 30] or 60 min of reperfusion [31]. Three of these studies [9, 13, 31] reported an unaffected activity of glutathione reductase during reperfusion, as determined after isolation of the enzyme from postischemic hearts. The increased GSSG content in these studies can therefore only be explained by an insufficient supply of NADPH during reperfusion to reduce GSSG. However, this explanation is supported neither by our finding of an unimpaired glutathione reductase reaction during reperfusion, as determined in intact tissue, nor by the finding that even in severely energy depleted hearts the supply of NADPH was sufficient to reduce GSSG [25].

The cumulative release of GSSG was also increased from isolated rat hearts subjected to ischemia followed by *recirculating* perfusion [8, 32]. We consider recirculating perfusion unsuitable to investigate oxidative stress, since GSH in perfusion medium equilibrated with 95% O_2 + 5% CO_2 at 37°C is prone to rapid autooxidation to GSSG [11]. Auto-

oxidation of GSH in perfusion medium yields false-positive indices for oxidative stress. Notably, myocardial content of GSSG was not increased during reperfusion in both studies [8, 32].

Also in the human heart the occurrence of oxidative stress during reperfusion after aortacoronary bypass grafting has been suggested [33]. This study reported increases in release of GSSG during reperfusion, which were more pronounced following ischemic periods of longer duration. Prolonged ischemia induces more cell death, however, and thus leads to more aspecific release of GSSG. In our opinion, solely aspecific release should be ruled out to avoid that increases in release of GSSG are falsely interpreted as an index of oxidative stress.

In summary, postischemic reperfusion of isolated rat hearts did not lead to oxidative stress, which may be due to an unimpaired glutathione reductase reaction during reperfusion. These results suggest that the quantity of reactive oxygen species generated upon reperfusion was insufficient to cause injury additional to ischemia-induced injury, which is supported by our finding that even a superimposed prooxidant load during the early phase of reperfusion failed to cause additional injury.

Acknowledgements

This investigation was supported by grant D91.015 from the Netherlands Heart Foundation. The authors wish to thank D.E. Atsma for critical review of the manuscript.

References

1. Opie LH: Reperfusion injury and its pharmacologic modification. Circulation 80: 1049–1062, 1989
2. Ferrari R, Ceconi C, Curello S, Cargnoni A, Pasini E, Visioli O: The occurrence of oxidative stress during reperfusion in experimental animals and men. Cardiovasc Drugs Ther 5: 277–288, 1991
3. Hearse DJ, Bolli R: Reperfusion induced injury: Manifestations, mechanisms, and clinical relevance. Cardiovasc Res 26: 101–108, 1992
4. Garlick PB, Davies MJ, Hearse DJ, Slater TF: Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. Circ Res 61: 757–760, 1987
5. Zweier JL, Kuppusamy P, Williams R, Rayburn BK, Smith D, Weisfeldt ML, Flaherty JT: Measurement and characterization of postischemic free radical generation in the isolated perfused heart. J Biol Chem 264: 18890–18895, 1989
6. Sun JZ, Kaur H, Halliwell B, Li XY, Bolli R: Use of aromatic hydroxylation of phenylalanine to measure production of hydroxyl radicals after myocardial ischemia *in vivo*: Direct evidence for a pathogenetic role of the hydroxyl radical in myocardial stunning. Circ Res 73: 534–549, 1993
7. Sies H: Oxidative stress: Oxidants and Antioxidants. Academic Press, London, pp xv–xxii, 1991
8. Julicher RHM, Tijburg LBM, Sterrenberg L, Bast A, Koomen JM, Noordhoek J: Decreased defence against free radicals in rat heart during normal reperfusion after hypoxic, ischemic and calcium-free perfusion. Life Sci 35: 1281–1288, 1984
9. Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Visioli O: Oxygen-mediated myocardial damage during ischaemia and reperfusion: Role of the cellular defences against oxygen toxicity. J Mol Cell Cardiol 17: 937–945, 1985
10. Meister A, Anderson ME: Glutathione. Annu Rev Biochem 52: 711–760, 1983
11. Ishikawa T, Sies H: Cardiac transport of glutathione disulfide and S-conjugate: Studies with isolated perfused rat heart during hydroperoxide metabolism. J Biol Chem 259: 3838–3843, 1984
12. Jaeschke H, Mitchell JR: Use of isolated perfused organs in hypoxia and ischemia/reperfusion oxidant stress. Methods Enzymol 186: 752–759, 1990
13. Arduini A, Mezzetti A, Porreca E, Lapenna D, DeJulia J, Marzio L, Polidoro G, Cuccurullo F: Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat heart. Biochim Biophys Acta 970: 113–121, 1988
14. Guarnieri C, Flamigni F, Caldarera CM: Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J Mol Cell Cardiol 12: 797–808, 1980
15. Brasch H, Schoenberg MH, Younes M: No evidence for an increased lipid peroxidation during reoxygenation in Langendorff hearts and isolated atria of rats. J Mol Cell Cardiol 21: 697–707, 1989
16. Darley-Usmar VM, O'Leary V, Stone D: The glutathione status of perfused rat hearts subjected to hypoxia and reoxygenation: The oxygen paradox. Free Radic Res Commun 6: 261–267, 1989
17. Park Y, Kanekal S, Kehrer JP: Oxidative changes in hypoxic rat heart tissue. Am J Physiol 260: H1395–H1405, 1991
18. Holman ER, Van Dijkman PRM, Van der Wall EE, Matheijssen NAA, Van der Meer P, Van Echteld CJA, Ruigrok TJC, De Roos A, Van der Laarse A: Assessment of myocardial perfusion during ischemia and reperfusion in isolated rat hearts using gadopentetic acid-enhanced magnetic resonance imaging. Coron Artery Dis 2: 789–797, 1991
19. Akerboom TPM, Sies H: Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol 77: 373–382, 1981
20. Tietze F: Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Anal Biochem 27: 502–522, 1969
21. Wynants J, Van Belle H: Single-run high-performance liquid chromatography of nucleotides, nucleosides, and major purine bases and its application to different tissue extracts. Anal Biochem 144: 258–266, 1985
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275, 1951
23. Sheppard AJ, Gavin JB: The transmural progression of the no-reflow phenomenon in globally ischemic hearts. Basic Res Cardiol 83: 611–617, 1988
24. Mohanlal RW, Mauve I, Zoet ACM, Van der Laarse A: Reperfusion-induced enzyme release: Washout effect or manifestation of reperfusion damage? Cardiovasc Res 22: 603–610, 1988
25. Kehrer JP, Lund LG: Cellular reducing equivalents and oxidative stress. Free Radic Biol Med 17: 65–75, 1994
26. Lenz ML, Michael LH, Smith CV, Hughes H, Shappell SB, Taylor AA, Entman ML, Mitchell JR: Glutathione disulfide formation and lipid peroxidation during cardiac ischemia and reflow in the dog *in vivo*. Biochem Biophys Res Commun 164: 722–727, 1989
27. Lesnefsky EJ, Dauber IM, Horwitz LD: Myocardial sulfhydryl pool alterations occur during reperfusion after brief and prolonged

myocardial ischemia *in vivo*. Circ Res 68: 605–613, 1991
28. Ondrejickova O, Ziegelhoeffer A, Gabauer I, Sotnikova R, Styk J, Gibala P, Sedlak J, Horakova L: Evaluation of ischemia-reperfusion injury by malondialdehyde, glutathione and gamma-glutamyl transpeptidase: Lack of specific local effects in diverse parts of the dog heart following acute coronary occlusion. Cardioscience 4: 225–230, 1993
29. Lesnefsky EJ, Ye J: Exogenous intracellular, but not extracellular, iron augments myocardial reperfusion injury. Am J Physiol 266: H384–H392, 1994
30. Ceconi C, Curello S, Cargnoni A, Ferrari R, Albertini A, Visioli O: The role of glutathione status in the protection against ischaemic and reperfusion damage: Effects of N-acetyl cysteine. J Mol Cell Cardiol 20: 5–13, 1988
31. Qiu Y, Galiñanes M, Ferrari R, Cargnoni A, Ezrin A, Hearse DJ: PEG-SOD improves postischemic functional recovery and antioxidant status in blood-perfused rabbit hearts. Am J Physiol 263: H1243–H1249, 1992
32. Janssen M, Koster JF, Bos E, De Jong JW: Malondialdehyde and glutathione production in isolated perfused human and rat hearts. Circ Res 73: 681–688, 1993
33. Ferrari R, Alfieri O, Curello S, Ceconi C, Cargnoni A, Marzollo P, Pardini A, Caradonna E, Visioli O: Occurrence of oxidative stress during reperfusion of the human heart. Circulation 81: 201–211, 1990