Energy-linked cardiac transport system for glutathione disulfide

Toshihisa Ishikawa, Maria Zimmer and Helmut Sies

Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstrasse 5, D-4000 Düsseldorf, FRG

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The relationship between the rate of glutathione disulfide (GSSG) export and the energy state was studied in isolated perfused rat heart. The intracellular GSSG level was maintained at saturation for transport (7.5 nmol GSSG · min $^{-1}$ · g heart $^{-1}$) by continuous perfusion with 20 μM *t*-butyl hydroperoxide. GSSG release was substantially restricted upon the addition of inhibitors of mitochondrial respiration such as KCN, antimycin A or rotenone. In contrast, no effect was observed on GSSG release during potassium-induced cardiac arrest, although changes in oxygen consumption and coronary flow were similar to those observed with KCN. The dependence of the GSSG transport rate on the cytosolic free ATP/ADP ratio reveals that GSSG transport is half-maximal at $(ATP/ADP)_{free} \approx 10$. The capacity of GSSG transport was unchanged by infusion of epinephrine, norepinephrine or dibutyryl cyclic AMP.

Glutathione disulfide *Membrane transport* *(Isolated perfused rat heart)* *Energy metabolism* *ATP/ADP ratio* *^{31}P-NMR*

1. INTRODUCTION

Glutathione disulfide (GSSG) and glutathione *S*-conjugates, generated from glutathione peroxidase and glutathione transferase reactions, are eliminated from cardiac cells [1,2]. The elimination is an important step in detoxication as well as in interorgan glutathione turnover and processing to mercapturic acids in the kidney. Work with isolated perfused heart showed the existence of a common transport system for GSSG and glutathione *S*-conjugates, and an activation energy of 12 kcal·mol^{-1} was estimated for GSSG transport [1].

This study addresses the question of dependence of cardiac GSSG transport on the cytosolic free ATP/ADP ratio, $(ATP/ADP)_{free}$. The investigation of GSSG transport in isolated perfused heart has several advantages as compared to other organs, for example: (i) saturation in the rate of GSSG release with respect to the intracellular GSSG level can be achieved and maintained over a sufficient experimental period; (ii) the energy state can be changed by the inhibition of mitochondrial respiration to decrease $(ATP/ADP)_{free}$ or by a potassium-induced cardiac arrest to increase $(ATP/ADP)_{free}$; (iii) the level of $(ATP/ADP)_{free}$ can be assessed by creatine phosphate/creatine; (iv) the intracellular pH as well as creatine phosphate and ATP levels are readily monitored with ^{31}P-NMR methods.

Here we present evidence that the cardiac transport of GSSG is an active transport, and show that the rate of transport is a function of cytosolic $(ATP/ADP)_{free}$.

2. MATERIALS AND METHODS

2.1. *Biochemicals and enzymes*

NADPH, NADH, ATP, glycerate 3-phosphate, phospho*enol*pyruvate, dibutyryl cyclic AMP, pyruvate kinase, myokinase and 3-phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase (Boehringer, Mannheim), antimycin A, rotenone, epinephrine, norepinephrine and creatine kinase (Sigma), *N*-ethylmaleimide (Merck)

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and quaternary aminoethyl (QAE)-Sephadex A-25 (Pharmacia) were obtained from the commercial sources indicated. All other chemicals were of analytical grade.

2.2. *Perfusion of rat heart*

Hearts of male Wistar rats of 190–220 g body wt, fed on stock diet (Altromin), were perfused as described [1], and the performance of cardiac functions was monitored according to [1,2]. The oxygen concentration in effluent perfusate was measured with a Clark-type oxygen electrode. For measurement, a Tygon tube with a diameter of 1 mm was inserted into the pulmonary artery and a portion of the effluent perfusate was pumped through the electrode chamber at a constant rate of 0.8 ml/min.

2.3. *Assay of metabolites in heart*

The perfused hearts were freeze-stopped using aluminium tongs cooled with liquid N_2, and were pulverized in a percussion mortar at liquid N_2 temperature. 200–250 mg of powder was successively homogenized in 1.2 ml of 1 M perchloric acid containing 2 mM EDTA (for the assay of adenine nucleotides, creatine and creatine phosphate) or in 1.2 ml of 1 M perchloric acid containing 2 mM EDTA and 50 mM *N*-ethylmaleimide (for GSSG assay). GSSG was measured according to [1]. ATP, ADP, AMP, creatine and creatine phosphate were determined by conventional enzymatic analyses [3,4].

2.4. *Assay of GSSG in effluent perfusate*

This was carried out as described [1].

2.5. *Measurement of intracellular pH with ^{31}P-NMR*

^{31}P-NMR spectra of isolated perfused hearts were recorded with a Bruker WH 360 NMR spectrometer operated in the Fourier transform mode. The heart was placed in a 15-mm NMR sample tube and inserted into a probe which was seated in the bore of a superconducting magnet (8.5 T). Spectra were taken by 80 scans of a 60° broadband pulse with a 0.6 s interpulse delay. The intracellular pH was determined from the chemical shift of inorganic phosphate signal as described in [5,6].

3. RESULTS AND DISCUSSION

3.1. *Effect of inhibition of mitochondrial respiration on GSSG release*

When the heart was perfused with 20 μM *t*-butyl hydroperoxide, the level of intracellular GSSG was increased, and GSSG release from the heart reached a maximal rate of 7.5 nmol·min^{-1}·g heart^{-1} [1]. The rate of GSSG release remained constant during the perfusion, and no remarkable change was observed in cardiac functions such as heart beat rate, left ventricular pressure, coronary flow and oxygen consumption rate. This enabled us to assess the capacity of cardiac GSSG transport under various perfusion conditions by referring to the maximal rate of GSSG release.

Fig.1 shows the effect of the inhibition of mitochondrial respiration with KCN on the maximal rate of GSSG release. When KCN was infused at a concentration of 0.5 mM during the perfusion with *t*-butyl hydroperoxide, the oxygen consumption of the heart dropped from 5.1 to 1.2 μmol·min^{-1}·g heart^{-1}, and a remarkable decrease was observed in the rate of GSSG release, in spite of the increase of coronary flow rate. As shown in fig.1, changes were reversible. After termination of the KCN infusion the decreased GSSG release recovered to the initial rate. A similar change in GSSG release was observed with an anoxic perfusion. When antimycin A or rotenone was infused at 5 μM, a more remarkable decrease was seen in the GSSG release rate; however, the effect was irreversible (not shown).

3.2. *Effects of cardiac arrest and cardiac stimulation on GSSG release*

Fig.2 shows the effect of potassium-induced cardiac arrest on the rate of GSSG release. KCl was infused at 20 mM during perfusion with *t*-butyl hydroperoxide. Thereby, the heart beat was completely arrested, due to the depolarization of the resting membrane potential of the cardiac cells. Changes in the coronary flow and the oxygen consumption were very similar to those observed upon the addition of KCN (fig.1A). However, interestingly, the maximal rate of GSSG release was little affected. This result, therefore, presents a control for the effect of KCN on GSSG release rate shown in fig.1.

Likewise, the cardiac function was stimulated by

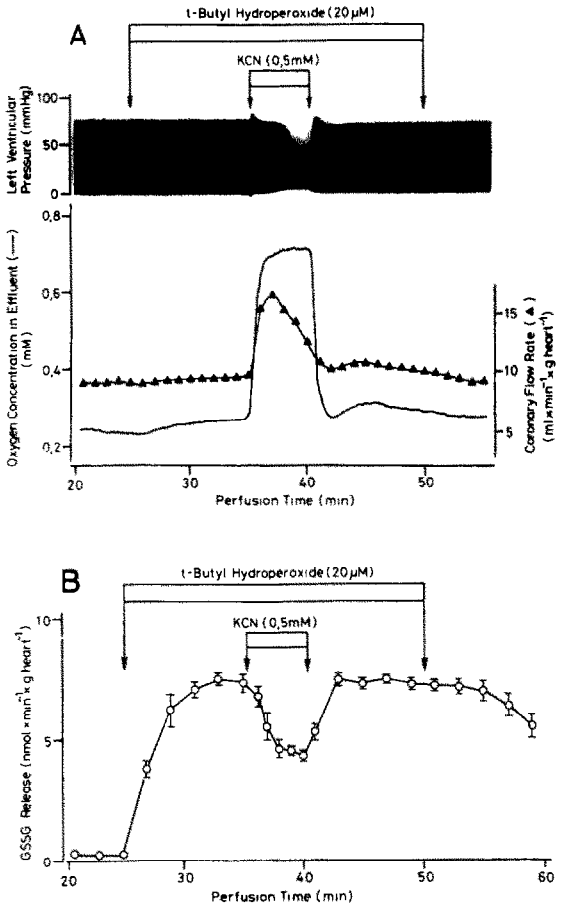

Fig.1. Effects of the inhibition of mitochondrial respiration by KCN on cardiac functions and on GSSG release. The heart was perfused with 20 μM *t*-butyl hydroperoxide for 25 min, and during the perfusion KCN was infused at 0.5 mM for 5 min. (A) Time courses of left ventricular pressure, oxygen concentration in effluent perfusate and of coronary flow rate. (B) GSSG release shown as mean ± SE of 3 different experiments.

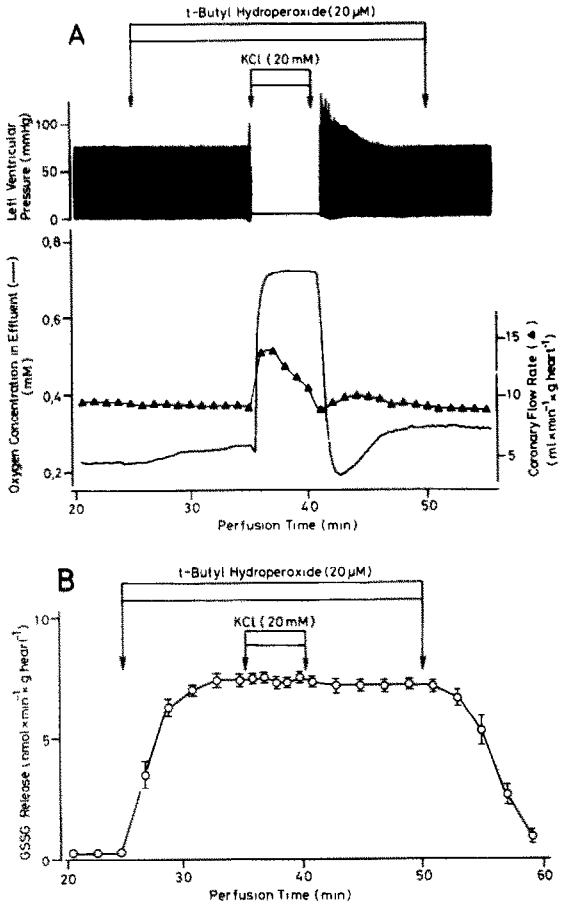

Fig.2. Effect of potassium-induced cardiac arrest on GSSG release. The heart was perfused with 20 μM *t*-butyl hydroperoxide for 25 min, and during the perfusion KCl was infused at a concentration of 20 mM for 5 min. (A) Time courses of left ventricular pressure, oxygen concentration in effluent perfusate and of coronary flow rate. (B) GSSG release shown as mean ± SE of 3 different experiments.

the addition of epinephrine (50 nM), norepinephrine (50 nM) and dibutyryl cyclic AMP (50 μM), and the maximal rate of GSSG release was not significantly affected (not shown). The results indicate that the GSSG release is a process independent of the membrane potential and of the hormonal regulation related with adrenergic hormones.

3.3. *GSSG transport and cardiac energy state*

Table 1 summarizes data on GSSG release rate, intracellular GSSG, ATP, ADP, AMP, creatine phosphate (CrP) and creatine (Cr) in hearts perfused under various conditions. Perfusion with 20 μM *t*-butyl hydroperoxide enhanced the intracellular GSSG level by 150 nmol·g heart^{-1} without any remarkable change in the energy parameters, as compared with the control. The addition of 20 mM KCl to induce cardiac arrest during perfusion with *t*-butyl hydroperoxide increased the ratio of creatine phosphate to creatine, whereas no significant change was observed in the GSSG

Table 1

Effects of KCl, KCN and antimycin A on GSSG release and on metabolites in the heart perfused with *t*-butyl hydroperoxide

	GSSG release (nmol·min^{-1}·g heart^{-1})	Intracellular GSSG (nmol·g heart^{-1})	μmol·g heart^{-1}				
			ATP	ADP	AMP	CrP	Cr
Control	0.10 ± 0.01	15 ± 1	3.72 ± 0.12	0.66 ± 0.01	0.13 ± 0.03	4.88 ± 0.13	4.26 ± 0.09
t-Butyl hydroperoxide (20 μM)	7.5 ± 0.3	161 ± 27	3.74 ± 0.10	0.65 ± 0.02	0.17 ± 0.01	4.49 ± 0.28	4.16 ± 0.27
+ KCl (20 mM)	7.6 ± 0.2	170 ± 6	3.65 ± 0.08	0.66 ± 0.01	0.17 ± 0.01	5.40 ± 0.27	3.55 ± 0.09
+ KCN (0.5 mM)	4.3 ± 0.5	252 ± 18	1.98 ± 0.12	0.93 ± 0.05	0.51 ± 0.07	0.50 ± 0.09	6.86 ± 0.30
+ antimycin A (5 μM)	2.9 ± 0.2	283 ± 10	1.39 ± 0.10	1.06 ± 0.05	1.33 ± 0.18	0.28 ± 0.02	7.27 ± 0.06

In the control experiment, hearts were perfused under standard perfusion conditions for 40 min and freeze-stopped. In the other experiments, hearts were perfused with 20 μM *t*-butyl hydroperoxide from 25 min perfusion time, and KCl, KCN or antimycin A was infused from 35 min as shown in figs 1 and 2. The perfusion was terminated by freeze-stop at 40 min. GSSG release rate was observed at 39–40 min perfusion time. Metabolites in the heart were assayed as described in section 2. Data are shown as means ± SE (n = 4)

release and intracellular GSSG level. It is noteworthy that the intracellular GSSG level was increased in hearts which were perfused with KCN or antimycin A, while the rate of GSSG release dropped strikingly (fig.1, table 1). Thus, the decline of the GSSG release rate shown in fig.1 is not ascribable to a decrease in intracellular GSSG level. In those hearts, ATP content was about half the control level and creatine phosphate content was conspicuously low (table 1).

In heart, adenine nucleotides are found in both mitochondrial and extramitochondrial spaces [7]. A substantial fraction of cytosolic ADP and a part of ATP are bound to contractile proteins [8]. Therefore, assuming near-equilibrium of the creatine kinase reaction, the cytosolic free ATP/ADP ratio, $(ATP/ADP)_{free}$, was estimated as a suitable parameter for the evaluation of the cytosolic energy state. Fig.3 shows the relationship between GSSG transport rate and the estimated $(ATP/ADP)_{free}$. Heart perfused under standard perfusion condition gave an $(ATP/ADP)_{free}$ of 180 ± 11 (n = 4), and 171 ± 10 (n = 4) was seen for heart perfused with 20 μM *t*-butyl hydroperoxide for 15 min. Potassium-induced cardiac arrest enhanced the ratio up to 253 ± 13 (n = 4). When mitochondrial respiration in heart was inhibited with KCN or antimycin A, the $(ATP/ADP)_{free}$ level dropped maximally by one order of magnitude. In this case, the GSSG transport was found to be closely linked with the lowered level of $(ATP/ADP)_{free}$. Under the corresponding conditions, intracellular pH assessed with ^{31}P-NMR shifted just from 7.05 to 6.80. Thus, the result shown in fig.3 suggests the direct dependence of the capacity of cardiac GSSG transport on the cytosolic energy state.

3.4. *Concluding remarks*

Although the phenomenon of GSSG release was observed in different organs and cell types [10], the detailed mechanism has not been fully understood. Only in erythrocytes, the ATP dependence of the GSSG release was proven by using inside-out membrane vesicles [11]. Recently, it has been reported that a plasma membrane preparation from rat hepatocytes contained ATPase activity which was activated by GSSG [12], and the implication of the ATPase activity with hepatic transport of GSSG is suggested. The present study demonstrates a close relationship between the rate of GSSG release and cytosolic free ATP/ADP ratio, proving the existence of an energy-dependent GSSG transport system in heart. This is the first report which demonstrates this in an intact perfused organ.

As exemplified by a competition between GSSG and glutathione *S*-conjugate releases, cardiac GSSG transport system is considered to take a role

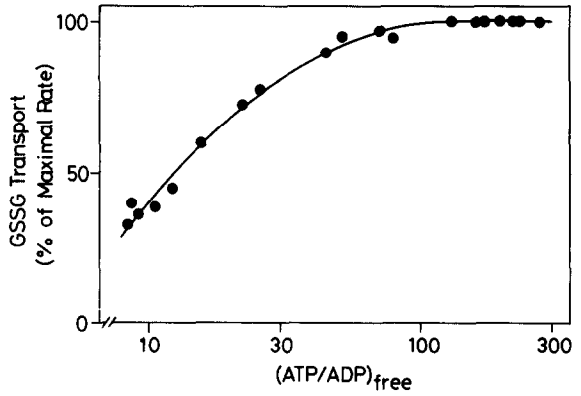

Fig.3. Relationship between rate of GSSG transport and $(ATP/ADP)_{free}$. Hearts were perfused with 20 μM *t*-butyl hydroperoxide and with KCl (20 mM), KCN (50, 100 or 500 μM) or antimycin A (5 μM) in the same manner as shown in figs 1 and 2, and were freeze-stopped at 40 min perfusion time. GSSG transport rate is shown as the ratio of the GSSG release rate at 39–40 min to the release rate at 34–35 min; 100% corresponds to 7.5 ± 0.2 nmol·min^{-1}·g heart^{-1} (n = 20). $(ATP/ADP)_{free}$ was estimated from the contents of creatine and creatine phosphate in the freeze-stopped heart, assuming near-equilibrium of creatine kinase reaction. The equilibrium constant of the reaction was:

$$K_{CK} = \frac{[ATP][Cr]}{[ADP][CrP][H^+]} = 1.66 \times 10^9\ M^{-1}\ [9].$$

$[H^+]$ was determined in a separate series of experiments with ^{31}P-NMR. pH = 7.05 for data of $(ATP/ADP)_{free} \geq 80$, pH = 6.80 for $(ATP/ADP)_{free} < 80$.

in the elimination of glutathione *S*-conjugates which have been generated from glutathione transferase reaction ([1,2]; see [13] and [14] for rat cardiac glutathione transferases). However, the energy dependence of glutathione *S*-conjugate transport is more difficult to study because the maximal rate of glutathione *S*-conjugate release cannot be maintained for a sufficient time.

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