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Impaired glutathione biosynthesis in the ischemic-reperfused rabbit myocardium

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Abstract Non-protein thiols (NP-SH) and the activities of the glutathione status-regulating enzymes γ-glutamylcysteine synthetase (G-GCS), γ-glutamyl transpeptidase (G-GT) and glutathione reductase (GR) were assessed in perfused rabbit hearts subjected to severe (60 min) or mild (7 min) total ischemia and 30 min reperfusion. Severe ischemia significantly decreased NP-SH, which were further depressed on reperfusion together with a significant decline in G-GCS activity; G-GT and GR activities were unchanged. Specific analytes were unaffected by mild ischemia-reperfusion. Thus, impaired enzymatic biosynthesis of GSH is operative in the reperfused rabbit myocardium after 60 min ischemia. This phenomenon may favour myocardial GSH depression and oxidative reperfusion injury after severe ischemia.

Key words: Glutathione; Glutathione-related enzyme; Ischemia-reperfusion; Perfused heart; Oxidative stress

1. Introduction

Experimental studies have proved that myocardial ischemia-reperfusion induces a decrease of tissue reduced glutathione (GSH) content, which has been attributed to oxidation and loss of the tripeptide from the injured myocytes [1,2]. Notably, cell GSH pool depression is relevant in conditioning myocardial oxidative injury [1–4]. Tissue levels of GSH are maintained via the activity of specific enzymes capable of favouring de novo biosynthetic processes and reconversion of GSH from its oxidized form, viz. γ-glutamylcysteine synthetase (G-GCS) and glutathione reductase (GR), respectively [5]. γ-Glutamyl transpeptidase (G-GT), which is a major GSH breakdown enzyme allowing tissue utilization of extracellular GSH, is also responsible for the regulation of GSH status in the cell environment [5].

In spite of the evidence of decreased GSH levels in the ischemic-reperfused myocardium, little is known concerning the activity of the aforementioned enzymes during ischemia-reperfusion of the mammalian heart. The present study, therefore, was designed to investigate glutathione status-regulating enzymes in the ischemic and reperfused rabbit heart.

2. Materials and methods

2.1. Experimental protocol

Hearts of New Zealand rabbits (body weight about 2.5 kg) were subjected to severe (60 min) or mild (7 min) ischemia followed or not by 30 min reperfusion. The duration of the mild ischemic period was selected to induce unambiguously no myocyte irreversible damage and necrosis [6,7]. Moreover, when extrapolated to the clinical setting, the first protocol could be assimilated to prolonged ischemia with myocardial infarction, and the second to transient reversible ischemia, i.e. angina pectoris [6,7].

As far as the severe ischemia-reperfusion protocol is concerned, in a first control group of 6 rabbits, the excised hearts were perfused aerobically for 110 min on a double-reservoir, Langendorff apparatus operating at 80 mmHg and kept at 37°C. The perfusion medium was a modified glucose-bicarbonate Krebs-Henseleit buffer, containing (in mM) 128 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose, and gassed with 95% O_2 and 5% CO_2 (pH 7.4). Heart rate was kept constant at 180 beats/min by right ventricular pacing. Contractile function was assessed via a fluid-filled latex balloon inserted into the left ventricular cavity through the mitral orifice and connected to a pressure transducer [8]. Developed pressure and end diastolic pressure were recorded on a Sensormedics Dynograph R 611 Recorder. Coronary flow was measured by 1 min perfusate collections [9]. When required, a rapid hypothermic arrest was obtained by cold buffer perfusion delivered by the second Langendorff reservoir.

A second group of 6 hearts was subjected to 60 min total ischemia, after a preliminary period of 50 min perfusion with normally oxygenated buffer.

A third group of 6 hearts was subjected to 60 min total ischemia, followed by 30 min reperfusion, after 20 min perfusion with normally oxygenated buffer.

Three groups of 5 rabbits were used in the mild ischemia-reperfusion protocol; in this regard, the control hearts were perfused aerobically for 110 min, while the ischemic and the ischemic-reperfused ones were subjected, respectively, to 7 min total ischemia after 103 min aerobic perfusion and to 7 min ischemia plus 30 min reperfusion after 73 min aerobic perfusion.

2.2. Biochemical analyses

Reagents were from Sigma Aldrich s.r.l., Milano, Italy, unless otherwise indicated. Heart samples were homogenized (1:8 w/v) in ice-cold 0.1 M Tris-HCl buffer, pH 7.7, plus 3 mM EDTA for enzymatic study, or in 4% sulfosalicylic acid for the assay of non-protein thiol compounds (NP-SH). Centrifugations at $400\times g$ for 10 min and $4500\times g$ for 20 min were performed to assess, respectively, G-GT activity and NP-SH. Further centrifugations at $3500\times g$ for 20 min and $105\,000\times g$ for 60 min were performed to measure the activities of G-GCS and GR, respectively.

The activity of G-GCS, the key enzyme catalyzing the first step in GSH biosynthesis [5,10], was assayed as described by Sekura and Meister [10], with slight modifications. Reaction mixtures contained 10 mM L-glutamic acid, 20 mM DL-α-aminobutyric acid, 5 mM Na_2ATP and a suitable supernatant homogenate aliquot, in 80 mM Tris-HCl buffer, pH 8.2, containing 4 mM EDTA, 10 μg bovine serum albumin and 0.04 M $MgCl_2$. The inorganic phosphate (P_i) formed by the specific enzymatic activity was detected spectrophotometrically at 405 nm using a commercially available kit (Boehringer Mannheim, Germany). Results are expressed as μg P_i/min per mg protein.

G-GT activity was determined using a commercial kit (Boehringer Mannheim, Germany), after 4 h incubation of the relative homogenate at 4°C in 0.1 M Tris-HCl buffer, pH 8.2, containing 6 mM EDTA and 1% sodium deoxycholate [11]. Enzyme-mediated 5-amino-2-nitrobenzoate (ANB) formation was followed spectrophotomet-

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rically at 405 nm. Results are expressed as mU (nmol ANB released/min) per mg protein.

For GR activity assay, appropriate amounts of cytosol were added to 1.0 ml reaction mixtures containing 0.1 M potassium phosphate buffer, pH 7.4, 1.0 mM EDTA, 0.16 mM NADPH and 1.0 mM GSSG [12]. Blanks were without GSSG, and NADPH disappearance was followed spectrophotometrically at 340 nm. Results are expressed as mU (nmol NADPH oxidized/min) per mg protein (NADPH extinction coefficient: 6220 M^{-1} cm^{-1}).

NP-SH, of which GSH represents virtually the only pool in the rodent myocardium [13], were measured spectrophotometrically at 412 nm [12], after reaction of suitable aliquots of the relative supernatant homogenate with 0.6 mM 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent) in 0.2 M potassium phosphate buffer, pH 8.5, plus 1.0 mM EDTA. Values were calculated as nmol NP-SH/mg protein, using an extinction coefficient of 13 600 M^{-1} cm^{-1} [12]. Protein concentrations were assayed according to Bradford [14].

2.3. Statistics

Results were calculated as means ± SD. Hemodynamic values obtained before and after ischemia-reperfusion were analyzed by paired Student's *t*-test [15]. Biochemical differences among the control, ischemic and ischemic-reperfused hearts were computed by the one-way analysis of variance plus Bonferroni's test [15]. $P < 0.05$ was regarded as statistically significant.

3. Results

In agreement with previous reports [1,2], severe ischemia induced a significant decrement of myocardial NP-SH levels (Table 1). Although G-GCS activity was decreased by about 13% at the end of 60 min ischemia, the level of statistical significance was not reached; G-GT and GR activities were not changed by the ischemic period (Table 1). Reperfusion after severe ischemia was associated with a further decrement of myocardial NP-SH ($P < 0.05$), and with a significant depression of G-GCS activity ($P < 0.05$; Table 1); G-GT and GR activities were also unaffected by reperfusion (Table 1).

Regarding the mild ischemia-reperfusion protocol, NP-SH levels were similar in the control, ischemic and ischemic-reperfused hearts (8.65 ± 2.3, 8.4 ± 1.7 and 8.5 ± 1.6 nmol/mg protein, respectively, P = NS), as were also the activities of G-GCS (9.1 ± 1.6, 8.9 ± 2.1 and 8.8 ± 1.8 μg P_i/min per mg protein, P = NS), G-GT (0.7 ± 0.25, 0.68 ± 0.2 and 0.73 ± 0.3 mU/mg protein, P = NS) and GR (32.7 ± 6.4, 33.2 ± 7.6 and 31.6 ± 6.7 mU/mg protein, P = NS).

Finally, in our experimental models, 60 min ischemia followed by 30 min reperfusion induced a significant decrease in developed pressure (from 91.5 ± 6.5 to 24 ± 4 mmHg, $P < 0.0001$) and coronary flow values (from 4.6 ± 0.4 to 2.2 ± 0.35 ml/min per g wet tissue, $P < 0.0001$), as well as an end-diastolic pressure rise (62 ± 5.5 mmHg), whereas the mild ischemia-reperfusion protocol did not affect significantly myocardial haemodynamics (not shown).

4. Discussion

The present study shows that severe, but not mild, ischemia-reperfusion can significantly lower NP-SH (namely, GSH) levels and GSH enzymatic biosynthesis in the rabbit myocardium. In particular, severe ischemia, per se, results in a decreased myocardial GSH content, with a further decrement after reperfusion. Ischemia-induced GSH decrease is conceivably due to enhanced tripeptide oxidation and/or consumption [1,2], as well as to impaired energy-dependent GSH formation [5]. The further GSH decrement observed after reperfusion is in agreement with previous studies carried out in perfused rabbit hearts subjected to severe ischemia-reperfusion [1,2]; this phenomenon may reflect GSH oxidation and loss from damaged myocytes [1,2]. It has been reported, however, that the net amount of GSH released during 30 min reperfusion does not quantitatively account for the cell GSH depression induced by severe ischemia-reperfusion in the perfused rabbit heart [2], and that in such a model mean cardiac GSSG levels rise of only 0.3 nmol/mg protein after 30 min reperfusion [1,2]. Thus, the impaired GSH enzymatic biosynthesis of the reperfused myocardium, which represents a previously unrecognized finding, may contribute to tissue GSH depression after severe ischemia. In this context, studies performed with the G-GCS inhibitor buthionine sulfoximine (BSO) have shown that GSH concentrations are 40% lower in the perfused hearts of BSO-treated animals than in those of controls after ischemia-reperfusion [3]. Hence, considering a substantial (approx. 70%) inhibition of G-GCS by BSO treatment in vivo [3] and the decrement of about 27% of G-GCS activity observed in our severe ischemia-reperfusion model, it may be calculated that such a decrement could have conditioned a 15% decline in heart GSH content. A similar G-GCS-related decline, although relatively small, might not be insignificant for the myocardium subjected to ischemia-reperfusion and oxidative stress [1]. Indeed, a decrement of only 30% of the cell GSH content induced right by G-GCS inhibition has been shown to cause a significant cell viability loss under oxidative stress conditions [16]. On the other hand, GSH is relevant in preventing mitochondrial dysfunction and derangement of cell calcium homeostasis [5,17], which are involved in the pathophysiology of irreversible ischemia-reperfusion damage [7].

Reperfusion induces a burst of myocardial radical generation and oxidant burden [8,18–20]. It is possible that radical species and lipid peroxidation by-products produced predominantly at reperfusion after more prolonged cardiac ischemia [8,18] could interact with G-GCS at the thiol group level of the active site [5], resulting in some enzyme inactivation. Antioxidant enzymes may indeed be inactivated by radical species [21]. A significant myocardial loss of G-GCS appears unlikely, considering that G-GT and GR activities are unaffected by ischemia and reperfusion, and that the molecular weight of these two latter enzymes is, respectively, lower than and very similar to that of G-GCS [5].

Therefore, reperfusion after severe ischemia induces a sig-

Table 1
Glutathione status-regulating enzymes and non-protein thiols in perfused rabbit hearts subjected to 60 min total ischemia and 30 min reperfusion

	Control	Ischemia	Ischemia-reperfusion
G-GCS	9.3 ± 1.7	8.1 ± 1.5	6.8 ± 1.3[a]
G-GT	0.65 ± 0.2	0.63 ± 0.15	0.69 ± 0.23
GR	31.5 ± 5.7	30 ± 7.5	29 ± 6.3
NP-SH	8.8 ± 1.7	5.9 ± 1.4[a]	3.7 ± 0.9[a,b]

G-GCS, γ-glutamylcysteine synthetase activity (μg P_i/min per mg protein); G-GT, γ-glutamyl transpeptidase activity (mU/mg protein); GR, glutathione reductase activity (mU/mg protein); NP-SH, non-protein thiols (nmol/mg protein). Means ± S.D. of 6 hearts in each group.

[a] $P < 0.05$ vs control (i.e., aerobic perfused) group.

[b] $P < 0.05$ vs ischemia group (one-way analysis of variance followed by Bonferroni's test). See Section 2 for further explanations.

nificant depression of heart G-GCS activity, which may negatively influence cardiac GSH status and favour oxidative reperfusion damage. Consistently, GSH and G-GCS have been stressed in myocardial cell protection against oxidant injury [22]. When extrapolated to the clinical setting, our data suggest that only reperfusion after severe ischemia, e.g. myocardial infarction with spontaneous and/or thrombolysis-induced reperfusion or prolonged cardiac arrest followed by reperfusion during heart surgery procedures, might be associated with a decline in myocardial GSH content and tripeptide biosynthesis. Administration of GSH precursors without inhibitory effects on G-GCS, such as γ-glutamylcysteine [17], could so be considered as cardioprotective agents in the clinical setting. Notably, the beneficial effect of γ-glutamylcysteine ethyl ester, as a result of cell GSH preservation, has been proved in the canine myocardium subjected to severe ischemia and reperfusion [23].

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