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MYOCARDIAL ANTIOXIDANT DEFENSE MECHANISMS: TIME RELATED CHANGES AFTER REPERFUSION OF THE ISCHEMIC RAT HEART

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It is well known that reperfusion damage of ischemic myocardium may be attributed to alterations in the antioxidant defense system against free radical aggression. In addition, the degree of myocardial damage may depend on the duration and severity of ischemia that precedes reperfusion. We carried out serial ischemic experiments (10, 30, 60 and 120 min) in ex-vivo rat hearts followed by 30 min reperfusion and we assayed the glutathione-dependent enzymatic activities (selenium-dependent glutathione-peroxidase: GSH-Px; selenium-independent glutathione peroxidase: GST-Px; glutathione-transferase: GST and glutathione-reductase: GS-SG-Red), Catalase activity (CAT) and non-proteic thiol compounds (NP-SH) at the end of reperfusion. We found a significant reduction of NP-SH, GSH-Px and CAT in ischemic/reperfused hearts from 30 min on, while GST activity was increased. In addition, we observed the appearance of a selenium-independent glutathione peroxidase activity (GST-Px) belonging to the GST system. In conclusion, we found the longer the duration of ischemia the greater the inbalance between the myocardial antioxidant system especially the GST activation, suggesting in particular for GST-Px, a role in the control of the damage against oxygen toxicity during ischemia/reperfusion.

KEY WORDS: Myocardial ischemia/reperfusion, antioxidant enzymes, glutathione transferase.

Abbreviations CAT, catalase; GSH-Px, selenium-dependent glutathione peroxidase; GS-SG Red, glutathione reductase; GST, glutathione transferase; GST-Px, selenium-independent glutathione peroxidase; NP-SH, non proteic thiol compounds.

INTRODUCTION

It is now accepted that reperfusion of the ischemic myocardium, depending on the severity and duration of the preceding blood flow deprivation, may be a potential source of accelerated and amplified myocardial injury.[1-4] In this context, it has been suggested that oxygen radical-mediated tissue damage may play a critical role.[5-11] Previous studies have shown that post-ischemic reperfusion is associated with a burst of free radical production[9,10] and increased accumulation of lipid peroxide.[12-13] Furthermore, the possibility that myocardial ischemia and reperfusion may alter the defense mechanisms against oxygen toxicity has also been

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emphasized.[14-18] In addition, a recent study has shown a susceptibility of the main cellular antioxidant enzymes to oxidant metabolites.[19] Thus, the ratio of free radical products and antioxidant enzymatic activities could be critical in the progression of tissue damage. The present study was aimed to evaluate the influence of different periods of global ischemia followed by a standard period of 30 min reperfusion on rat myocardial antioxidant activities. We tested the glutathione-dependent enzymatic activities (glutathione peroxidase: GSH-Px §, EC 1.11.1.9.; glutathione reductase: GS-SG Red, EC 1.6.4.2.; and glutathione S-transferase: GST, EC 2.5.1.18.), Catalase activity (CAT, EC 1.11.1.6.) and non proteic thiol compounds (NP-SH). We found a time-dependent loss of some protective intracellular antioxidants (i.e GSH-Px, NP-SH) together with activation of others (i.e. GST) and in addition, the appearance of new activity, selenium-independent glutathione peroxidase (GST-Px §), suggesting a change in the tissue expression of glutathione-dependent detoxifying enzymes, possibly related to metabolic changes which occur with ischemic-reperfusion progression in the myocardium.

MATERIALS AND METHODS

Heart Perfusion and Experiment Procedure

Male rats of the Sprague-Dowley strain weighing 250–300g were used. The rats were anesthetized with ether, the right femoral vein was exposed and 200 IU heparin was injected intravenously. Hearts were rapidily excised and chilled in a pre-cooled perfusion buffer until the contractions stopped. The hearts were perfused (70 mmHg) in retrograde fashion at the aortic root with a Krebs-Henseleit solution containing 118 mM NaCl, 25mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.4 mM $CaCl_2$, 11 mM glucose, pH 7.4, on a modified Langendorff apparatus. The chamber containing the hearts was thermostatically controlled at 37°C. The perfusion buffer was gassed with 95% O_2 and 5% CO_2 ($pO_2 > 600$ mmHg) and maintained at 37°C. The hearts were paced at a fixed rate of 240 beats/min (turned off during ischemia) using a square wave stimulator. Resting tension and developed tension were recorded by a force transducer (model 7004, Basile, MI, Italy) tied to the apex of the left ventricle and connected to a multichannel Sensormedics Dynograph R 611 recorder. A resting tension of 1 g was applied to the hearts on starting the perfusion. Total and global ischemia was induced by clamping the aortic perfusion line.

Thirty minutes of equilibration, during which time the hearts were aerobically perfused had undergone before any experiment.

The rats were randomly divided in four groups (n 6). After the stabilization period, the hearts were subjected to global and total ischemia for 10, 30, 60, 120, minutes, followed by 30 min of reperfusion. Control hearts (n 6 for each group) were perfused continuously in aerobic and normothermic conditions with Krebs-Henseleit buffer for the same period of ischemia plus reperfusion. At the end of the experiments the heart tissue was rapidly frozed using Wollemberg clamps precooled in liquid nitrogen and the organs were then stored in liquid nitrogen until analysis.

Homogenate and Supernatant Preparation

Rat heart tissues were weighted and homogenized (1:5 w/v) in ice cold 0.05 M potassium phosphate buffer (pH 7.4) with 30s bursts of a commercial homogenator

(Ultra Turrax, Tecmar Company, Cincinnati, OH). Non proteic thiol compounds (NP-SH) were determined in the supernatant obtained after centrifugation at 800 × g for 5 min at 4°C, to remove fibrous material.

CAT activity was assayed on the supernatant obtained from a second centrifugation at 2000 × g for 10 min at 4°C. GS-SG Red, GSH-Px, GST and GST-Px activities were measured on cytosol harvested after further centrifugation at 105000 × g for 60 min in a Beckman ultracentrifuge (model L3-50).

Biochemical Analyses

NP-SH (of which GSH represents the main biochemical pool) were measured by Ellman's method.[20] The NP-SH was extracted using an aliquot of the relative supernatant using sulfosalicylic acid at the final concentration of 4% and centrifuged for 15 min at 15000 × g to remove protein. Potassium phosphate buffer (2.0 ml, 0.1 M, pH 8.0) containing 1.0 mM EDTA and 0.1 ml of 0.3M Ellman reagent (5,5′-dithiobis-2-nitrobenzoic acid, Aldrich Europe, Belgium) was added to a sample of supernatant fraction (0.2-0.4 ml). No one reducing agent to generate SH groups from disulfides was used. The NP-SH values were measured spectrophotometrically at 412 nm in a Beckman spectrophotometer (model DB-G), and expressed as nmol NP-SH/G wet tissue.

CAT activity was assayed on the appropriate supernatant by spectrophotometric method, following the decrease in absorbence at 240 nm at 25°C, due to the hydrogen peroxide decomposition.[21] The supernatant was adjusted to 1% by adding Triton X-100 and incubated at room temperature for 30 min. The difference in adsorbance per unit time is a measure of catalase activity. The reaction was conducted in 3-ml cuvettes (1-cm light path). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0 (50 mM potassium dihydrogen phosphate and 50 mM disodium hydrogen phosphate), 14mM hydrogen peroxide (Perhydrol, Merk, F.R.G.) and 0.025-0.1 ml of appropiate supernatant sample which was added at the end to start the reaction. Specific activity was expressed as U/mg protein. One unit (U) is the amount of enzyme which decompose 1μmol H_2O_2 in 1 min at 25°C.

GS-SG Red activity was determined as previously reported.[22,23] The assay mixture (1 ml) contained 0.1 M potassium phosphate buffer pH 7, ImM GS-SG, ImM EDTA, 0.16 mM NADPH (Sigma Company, St. Louis, MO) and an appropiate amount of cytosol varying from 0.05-0.1 ml. The blank did not contain GS-SG. Enzyme activity was performed at 37°C by measuring the disappearance of NADPH at 340 nm and expressed as U/mg protein. One unit (U) is the amount of enzyme oxidizing 1 μmole of NADPH/min. A molar extinction coefficent for NADPH of 6.22×10^3 M^{-1} cm^{-1} was used.

GSH-Px activity was measured by the method of Paglia and Valentine[24] as modified by Di Illo *et al.*[25] The activity of selenium-independent glutathione peroxidase (GST-Px) was determined by measuring total glutathione peroxidase peroxidase activity using cumene hydroperoxide (1.2 mM) as the substrate and then subtracting from this the activity of selenium-dependent glutathione peroxidase measured with H_2O_2 (0.25 mM). The rate of reaction was recorded at 37°C by observing the decrease in absorbance at 340 nm of NADPH. GSH-Px activity was expressed as U/mg protein, each unit (U) representing the amount of enzyme oxidizing 1μmole

NADPH /min. GST activity was determined by following at 340 nm the rate of conjugation of glutathione with 1-chloro-2-4-dinitrobenzene (CDNB, Aldrich Europe, Belgium) according to Habig's method.[26] The standard assay mixture (final volume 2ml) contained 0.1 M potassium phosphate buffer with 1 mM EDTA, pH 6.5, 2 mM GSH and 1 mM CDNB. In the reference cuvette the enzyme source was omitted. The enzymatic assay was carried out at 37°C. Specific activity was expressed as U/mg protein. One unit (U) of enzymatic activity was defined as the amount of enzyme conjugating 1 μmole of substrate/min.

Protein concentrations were determined by Bradford's method,[27] using gamma-globulin as standard.

Statistical Analysis

Data were expressed as means ± SD. Biochemical data were analyzed by one way analysis of variance to test for any difference between the mean value of all groups. If differences were established, the Bonferroni's test was used to determine which means differed significantly. When comparisons involved control and ischemic/reperfused hearts, significance was determined by Student's t-test where P values < 0.05 were taken as significant.

RESULTS

Figures 1, 2 and 3 show the tissue biochemical parameters in isolated rat hearts perfused continuously in aerobic and normothermic conditions (Control) and subjected to ischemia for 10, 30, 60, and 120 min. and reperfused for 30 min. The Control hearts, continuously perfused for different times, did not display any significant modifications in their antioxidant defence system. After 10 min of ischemia followed by reperfusion (30 min), we observed a significant increase in GSH-Px and GST antioxidant activities respect to control (0.628 ± 14 vs 0.600 ± 0.020 U/mg protein, $P < 0.05$ Figure 1; 0.156 ± 0.013 vs 0.105 ± 0.010, U/mg protein, $P < 0.001$ Figure 2, respectively). However, more prolonged periods of ischemia revealed a different behaviour of GSH-Px and CAT respect to the GST system. In fact, while the GSH-Px and CAT show a progressively significant reduction in the enzymatic activity (Figure 1), GST shows higher activity respect to Controls at 30 and 60 min, with further significant increase at 120 min (Figure 2). In addition, the activity of selenium-independent glutathione peroxidase (GST-Px), which was not detectable in basal conditions nor after 10 minutes of ischemia, appeared in the ischemic reperfused tissue at 30 minutes, with stable values reaching 120 min of ischemia (Figure 3). NP-SH underwent a progressive and significant decrease at 30, 60 and 120 min. GS-SG Red activity resulted unmodified throughout the experimental times (Figure 3).

DISCUSSION

The salient biochemical findings of our study are: 1) a progressive and significant time-dependent decrease of non proteic thiol compounds (NP-SH), enzymatic activity for selenium-dependent GSH-Px and CAT as evaluated after 30 min of reperfusion following 30, 60 and 120 min of ischemia; 2) a concomitant stable increase of GST; and finally 3) the appearance of a "new" selenium-independent

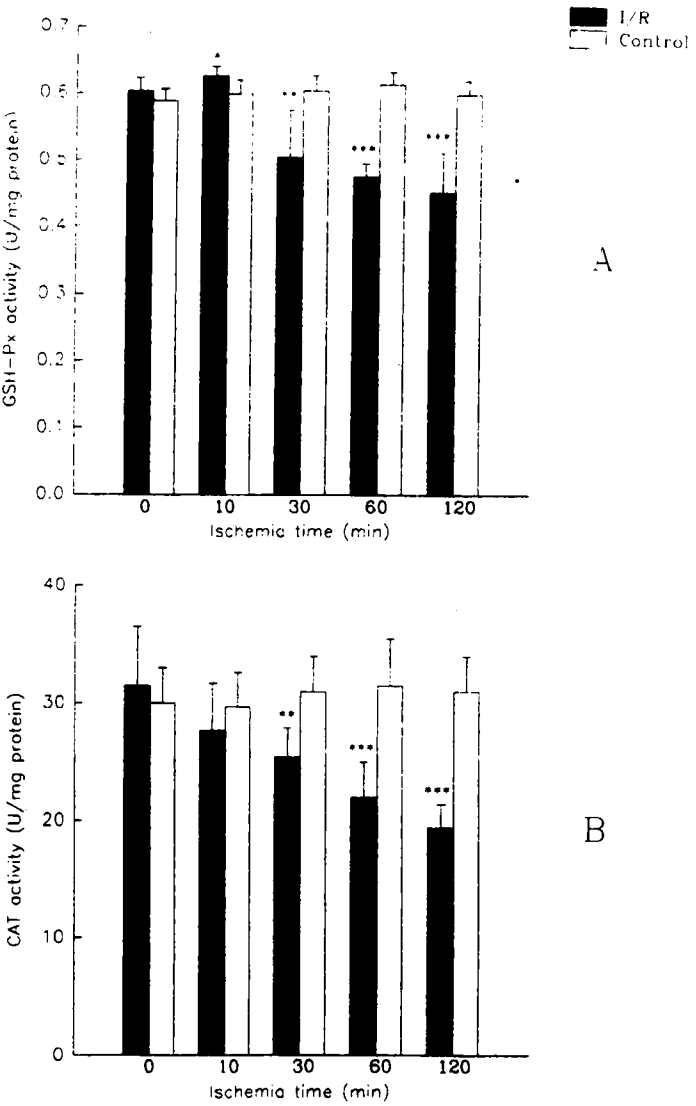

FIGURE 1 Time course of the effect of ischemia/reperfusion (I/R) of isolated rat heart on GSH-Px (Panel A) and CAT (Panel B) activities. Isolated rat hearts were perfused continuously in normothermic and aerobic conditions (Control) or reperfused for 30 min after 10, 30, 60, 120 min of ischemia. GSH-Px and CAT were measured as described in Materials and Methods. Values are presented as the means ± SD for six rats. *P < 0.05, **P < 0.01, ***P < 0.001, Control vs treated.

activity belonging to the glutathione transferase system (GST-Px). In agreement with other studies which show a decrease of NP-SH or GSH-Px after prolonged period of hypoxia-reoxygenation[14], or after ischemia-reperfusion,[15-18] we found that the "duration" of ischemia is important in determining the biochemical changes. In addition, these findings may suggest that a reduction of the defence mechanisms, observed during ischemia/reperfusion, could contribute to myocardial damage.

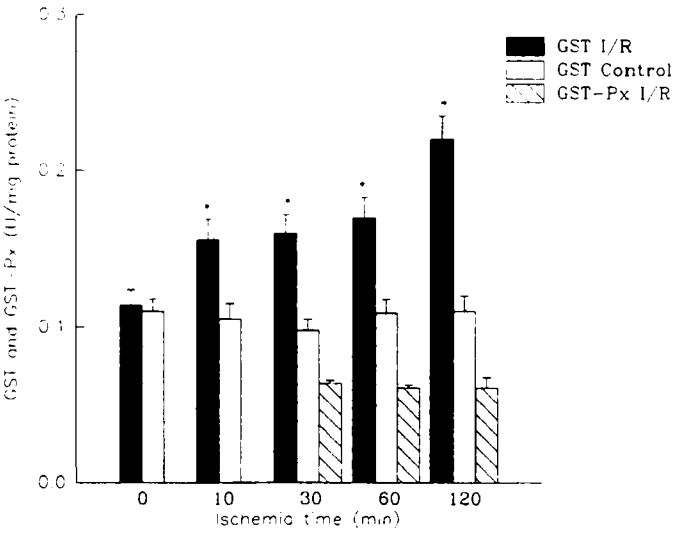

FIGURE 2 Time course of the effect of ischemia/reperfusion (I/R) of isolated rat heart on GST and GST-Px. Isolated rat hearts were perfused continuously or reperfused for 30 min after 10, 30, 60, 120 min of ischemia. Values are presented as the means ■ SD for six rats. *$P < 0.001$, Control vs treated.

Furthermore, we observed a significant increase in GSH-Px, GST and CAT after a short period of ischemia (10 min) followed by reperfusion, probably related to an initial non-specific response to the oxidative burst.[9-11,28] It is interesting to note that Pigeolet *et al.* have recently demonstrated an enzymatic antioxidant inactivation (i.e, GSH-Px and Catalase) by peroxide and oxygen free radicals.[19] Since an increased oxidative stress during reflow may be related to the duration of ischemia,[29-30] the possibility of a progressive secondary inactivation of the enzymatic system could be suggested in our experimental model. Simultaneous with reduction of GSH-Px activity, we observed an increased activity of cytosolic GST. To date, little attention has been given to GST as a system involved in the protection against myocardial oxidative stress.[31] Cytosolic GSTs of the rat heart have been studied in detail in control conditions, but not during ischemia and reperfusion.[32-33] In our study we show the behaviour of GST during myocardial ischemia/reperfusion of the rat. We found a divergent pattern of GST respect to GSH-PX, confirming a possible role against oxidative stress during ischemia and reperfusion. In this context, it is interesting to note that recent studies have emphasized an oxidative stress-induced activation of microsomal glutathione S-transferase in vitro[34] and in isolated rat liver reversed by treatment of microsomes with dithiolthreitol, a reducing agent.[35] In addition, we found the appearance of a selenium independent glutathione peroxidase activity belonging to the transferase system.[36]

It has been demonstrated that the glutathione peroxidase activity of GST (GST-Px) can reduce fatty acid hydroperoxides by catalysing the nucleophilic attack of GSH on electrophilic oxygen.[36] Since selenium-independent activity has been associated with alpha and mu GST family,[25] and rat hearts lack of isoenzymes of these classes,[32] the appearance of GST-Px (from 30 min of ischemia to 120 min) seems to reflect the adaptive isoenzymatic capacity of the heart to scavenge the cytotoxic

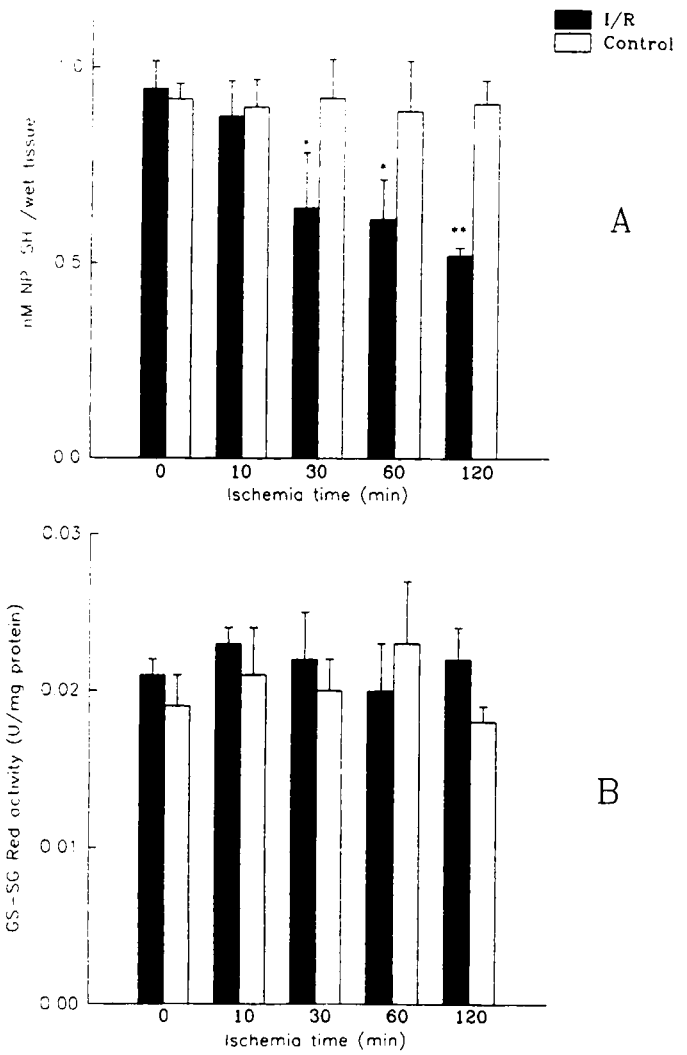

FIGURE 3 Time course of the effect of ischemia/reperfusion (I/R) of isolated rat heart on NP-SH (Panel A) and GS-SG Red (Panel B). Isolated rat hearts were perfused continuously or reperfused for 30 min after 10, 30, 60, 120 min of ischemia. Values are presented as the means ± SD for six rats. *P < 0.005, **P < 0.001, Control vs treated.

by-products of lipid peroxidation.[33] In addition, it has been suggested that GST-Px activity could represent an alternative pathway in the elimination of organic hydroperoxides when the selenium-dependent activity is critically depressed.[37-39] Finally, it is interesting to note that GST dependent reactions such as the attack of GSH on the epoxide of leukotriene A_4 have been observed in inflammatory processes[40] and a possible link between reperfusion injury and inflammation has been suggested.[41] In addition, a recent study has shown that isolated, buffer-perfused rat heart may

release vasocostrictive leukotrienes C_4, D_4 and E_4.[42] Thus, a further evaluation on the possible contribution of multifunctional GST to eicosanoid biosynthesis, particularly during ischemia and reperfusion could be suggested. In conclusion, our study suggests that the myocardial ischemia/reperfusion process is able to induce a biochemical inbalance between the myocardial antioxidant system parallel to the duration of the ischemic period. Furthermore, it should be proposed that the GST system, particularly the selenium-independent glutathione peroxidase activity, associated with this system, may play a role in the control of the damage against oxygen toxicity during ischemia/reperfusion.

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