Peroxidatic activity in heart effluent: a biochemical parameter for the assessment of experimental ischemia-reperfusion injury

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Abstract

Peroxidatic activity in heart effluent was defined as a new biochemical parameter for the experimental study of myocardial ischemia. The peroxidatic reaction was determined by dot blot analysis with 3,3'-diaminobenzidine as hydrogen donor. After ischemia, the level of peroxidatic activity in heart effluent was 2-3 times higher than before. The effects in experimental modulation of ischemia, such as nicorandil or aprikalim protection, and the reversibility of protection by glibenclamide, could accurately be noted using the level of peroxidatic activity in heart effluent as a biochemical parameter. The results were in good agreement with those obtained for other enzymes used as biochemical parameters in experimental heart ischemia-reperfusion studies.

Keywords: heart • Langendorff preparation • ischemia-reperfusion • nicorandil • aprikalim
glibenclamide • peroxidatic activity

Introduction

The phenomena induced by ischemia and reperfusion on myocardium are usually evaluated by physiological parameters, biochemistry, and morphology. The analysis of various enzymes released in heart effluent is currently used as a tool in the biochemical study of heart ischemia-reperfusion injury. On the other hand, the protective effects of some drugs against myocardial lesions due to ischemia and reperfusion are estimated by enzyme analysis in the heart perfusate. In a large variety of experimental models for ischemia and reperfusion on isolated heart, lactate dehydrogenase (LDH),

and creatine phosphokinase (CPK) were the main biochemical parameters used to describe the behavior of myocardium [1-11].

This paper defines the level of peroxidatic activity in heart effluent as an alternative biochemical parameter in the study of ischemia-reperfusion injury on myocardium. Some of our results using peroxidatic activity as a parameter in heart ischemia-reperfusion were previously reported [12].

Materials and methods

Samples

Hearts of 150-200 g male Wistar rats, in Langendorff preparation, were perfused with Krebs-Henseleit buffer,

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pH 7.2, gassed with 95% O₂, 5% CO₂, as below: (i) 30 min equilibration; (ii) 30 min global normothermic ischemia; (iii) 30 min reperfusion. Five to seven hearts were considered for every experimental model. In some experiments the perfusion buffer was supplemented, for the last 10 min of the equilibration step with protecting drugs, aprikalim (Apk) (1 uM), nicorandil (Nic) (0.2 uM, 0.5 uM, 1 uM), or Apk (1 uM) and glibenclamide (Glib) (4 uM), provided by Rhone-Poulenc Rorer (Nanterre, France). Samples were collected at 15 and 30 min during the equilibration step, and at 2, 5, 10, 15, 20, 25, and 30 min during reperfusion, respectively.

Hearts continuously perfused for 90 min were used as controls. Effluent aliquots were collected at times similar to those in every other experiment.

Analysis of peroxidatic activity in heart effluent

Aliquots of 20 ml heart effluent samples were blotted as dots on 0.2 mm nitrocellulose (NC) membrane (Sartorius, Goettingen, Germany), by repeatedly pipetting of 2 or 3 ml. The NC membrane was left to dry before a new aliquot pipetting, the diameter of the final spots being 5-6 mm. For other aliquots Bio Dot Apparatus (Bio-Rad, Richmond, Call USA) was successfully used for sample blotting on NC membrane. The peroxidatic reaction was developed in the dark, at room temperature for 2-24 hours, in 20 mM Tris-HCl buffer of various pH, supplemented with 0.025% 3,3'-diaminobenzidine (DAB) and 0.02% hydrogen peroxide [13]. The NC sheets were washed in 20 mM Tris-HCl buffer of the appropriate pH and dried in a filter paper sandwich.

Densitometry

The quantitative estimation of peroxidatic reaction was done on a Lucia S Image Analysis System (Nikon-

Optoteam, Vienna, Austria), using an algorithm for analyzing linear gel electrophoretograms [14].

CPK analysis

CPK was spectrophotometrically determined, at 340 nm, with kits for clinical use (Merck, Darmstadt, Germany).

Results

Influence of pH on peroxidatic reaction development

The development of peroxidatic reaction was done in 20 mM Tris-HCl buffer, in the pH range from 6.5 to 9.5. The optimal pH for peroxidatic reaction analysis was 7.0, as noted in Fig. 1, but buffers with pH between 7.0 and 7.3 could be used without any significant modification in quantitative data. Incubation buffer pH lower than 7.0 resulted in very high background on NC membrane, at short incubation time.

Time dependence of peroxidatic reaction development

At pH 7.0, a complete development of peroxidatic reaction occurred after 16 h incubation of NC membrane in reaction buffer (0.025% DAB, 0.02% H₂O₂ in 20 mM Tris-HCl), as shown in Fig. 2. The slow decrease in the values obtained by densitometrical analysis, at incubation times longer than 18 h, was due to the increase in the background level.

Fig. 1 Effect of pH on peroxidatic reaction development; reaction time was 16 hours.

Fig. 2 The development in time of peroxidatic reaction; pH of the reaction buffer was 7.0.

The time for peroxidatic reaction development was doubled when 10 mM sodium phosphate was used as reaction buffer (not shown).

The peroxidatic reaction in heart effluent as a biochemical parameter in the study of ischemia-reperfusion injury

In Fig. 3 are shown the results obtained by dot blot analysis for peroxidatic reaction in myocardial effluent before and after ischemia, in untreated and drug treated heart. Before ischemia, the heart effluent

showed low levels of peroxidatic reaction (see lanes A-C, preischemia, minutes 15 and 30). After ischemia, in the unprotected hearts the peroxidatic reaction level in the effluent was obviously increased (lane A, reperfusion, minutes 2 to 30). Heart pretreatment with Apk or Nic led to a decrease in peroxidatic reaction level in the effluent showing the protective effect of these drugs against ischemia-reperfusion injury (lanes B and C, reperfusion, minutes 2 to 30). Glib reversed this protective effect and the peroxidatic reaction in the myocardial effluent was increased, like in unprotected hearts.

Fig. 3 Image of the dot blot analysis results for peroxidatic reaction in the heart effluent obtained by various experimental schedule: lane A - results for heart injured by ischemia and reperfusion; lane B - effect of heart treatment by 1 mM Nic; lane C - effect of heart treatment by 1 mM Apk; lane D - reversal effect of Glib on protective effect of Apk (4 mM Glib + 1 mM Apk treated heart).

Fig. 4 Quantitative results using peroxidatic activity released in heart effluent as a biochemical parameter in the study of ischemia-reperfusion injury. **(a)** Lowering effects induced by Nic and Apk preischemic treatment on peroxidatic reaction level in heart effluent; **(b)** dose dependence of the protective effect due to Nic treatment.

Densitometrical analysis of the dots resulted in quantitative data susceptible for statistical evaluation. Fig. 4a shows the effects of Apk and Nic on myocardium during ischemia and reperfusion, in terms of peroxidatic reaction released in heart efflu-

ent. A significant decrease of peroxidatic reaction level was noted for drug treated hearts, as compared with control. The dose dependence of the protective effects of both drugs could be easily described using peroxidatic reaction as biochemical parameter, as is

Fig. 4 (cont'd.) (c) Similarity of the results obtained for CPK as compared with b; (d) reversal effect induced by Glib on the protection by Apk.

shown in Fig. 4b. The results were in good agreement with those obtained using CPK as biochemical parameter of heart injury (see Fig. 4c). A good correlation was also noted with the physiological behavior of heart, myocardial morphology and

ultrastructure (not shown). The ability of the new biochemical parameter to describe the effects of some modulation in experimental schedule is considered in Fig. 4d showing the reversal effect of Glib on myocardial protection by Apk.

Discussion

A large variety of compounds was analyzed in the heart effluent, during ischemia-reperfusion experiments, either in order to evaluate heart answer to experimental procedures, or to analyze the deepness of injury and the alterations in myocardial biochemistry. The most used biochemical parameter for controlling ischemia-reperfusion experiments were cytosolic enzymes released in heart effluent, such as LDH and CPK. Cardioprotective effect of drugs [12,15], biochemical mechanisms involved in ischemia-reperfusion injury [2,5-7] "stunned myocardium" [4,16], ischemic preconditioning [8-10], apoptosis in cardiomyocyte induced by hypoxia and reoxygenation [17], morphological studies [1], cell culture experiments [3] are some of the experimental approaches using this procedure. Other biomolecules, such as: membrane lipids and their metabolic derivatives [2,18], myosin subunits [19,20], troponin I [21], were identified and analyzed in heart effluent both to prove biochemical modifications induced by ischemia and reperfusion on myocardium, and to study the time-course of pathological events. There is a permanent interest to identify and to analyse other molecules in myocardial effluent after ischemia and reperfusion. Their study might complete our information about the effects of ischemia and reperfusion on the myocardium.

In this paper, the peroxidatic activity in the effluent was analyzed during heart ischemia-reperfusion experiments, and defined as a biochemical parameter describing the myocardial injury. The ability of this parameter to describe accurately and reproducibly the effects of various experimental models on heart behavior during ischemia-reperfusion was tested by preischemic treatment of myocardium with Nic or Apk. The previously reported protection of myocardium, against ischemia-reperfusion injury, by these drugs [22,23], and the reversal effect of Glib [22] were in a good manner evidenced by peroxidatic reaction analysis in heart effluent. Appropriate experiments are needed to identify the biochemical entities carrying peroxidatic activity in heart effluent. The reaction might be due to heme containing proteins like myoglobin, peroxisomal enzymes, or cytochromes. Electrophoretic pattern of proteins in heart effluent showed at least eight new bands between 14 and

94kD during postischemic reperfusion, as compared with preischemic effluent (not shown). The new protein bands in this molecular weight range could contain myoglobin, and/or both peroxisomal enzymes and various kinds of cytochromes. Samples analyzed after heart homogenisation and cell fractionation showed high level for peroxidatic activity in cytosolic fraction and practically no activity, detected by our procedure, for both mitochondrial and microsomal fractions (unpublished results). Therefore, the peroxidatic activity could be considered as a biochemical parameter in early evidence for cardiomyocyte injury by sarcolemmal damage.

In conclusion, the peroxidatic activity in heart perfusion buffer was defined as a biochemical parameter in the study of ischemia/reperfusion injury. The results obtained by using this parameter are similar to those described by other cytosolic enzymes released in myocardial effluent.

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