Proteins Released from Liver after Ischaemia Induced an Elevation of Heart Resistance Against Ischaemia-Reperfusion Injury: 1. Beneficial Effect of Protein Fraction Isolated from Perfusate after Ischaemia and Reperfusion of Liver

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Abstract. *Objectives.* Numerous mechanisms have been proposed to participate in adaptation of heart to ischaemia by ischaemic preconditioning. We have described previously a release of cardio-protective protein fraction during ischaemic preconditioning of dog heart. In the current study the effect of high soluble protein fraction (HS fraction) released from isolated perfused rat liver after ischaemia and reperfusion was examined on isolated perfused rat heart during ischaemia-reperfusion injury.

Methods. Livers were subjected to 30 or 60 min ischaemia followed with 120 min reperfusion. HS fraction was isolated using ammonium sulphate precipitation and dissolved in perfusion solution before Langendorf perfusion of isolated rat hearts. The protein pattern of HS fraction was detected with SDS-PAGE and western blot with ConA and anti ConA antibody. Hearts were then subjected to 20 min ischaemia followed by 20 min reperfusion. During reperfusion, the haemodynamic parameters of hearts were measured. Heart levels of adenine nucleotide were measured in $HClO_4$ extracts using HPLC on C_{18} column.

Results. Liver ischaemia induced changes in protein pattern of HS fraction released from the liver during reperfusion period. Particularly, we registered an increase in amount of several low-molecular weight proteins and decreased amount of high-molecular weight proteins. Proteins in this fraction isolated from perfusate after liver ischaemia interact with ConA with lower intensity as proteins isolated from perfusate after control non-ischaemic condition. HS fraction isolated from perfusate after ischaemia and reperfusion of liver had beneficial effect on heart

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function during 20 min ischaemia and subsequent 20 min reperfusion, documented by: i) decrease of arrhythmia score from 2 to 1 in 5 min of reperfusion and from 2 to 0 in 10 min of reperfusion; ii) improved heart contractility monitored as stabilised $[dP/dt]_{max}$ and increased Q parameter; iii) increased coronary flow. Proteins isolated from liver perfused under control non-ischaemic condition did not induce similar effects. The stabilisation of heart haemodynamics, observed after administration of HS proteins isolated from perfusate after ischaemia and reperfusion was associated with slight increase in ATP and ADP levels as well as decrease in AMP level.

Conclusion. HS fraction released from liver after 60 min global ischaemia induced an elevation of heart resistance against ischaemia-reperfusion injury.

Key words: Ischaemic preconditioning — Ischaemia reperfusion injury — Cardio-protective proteins — Heart ischaemia — Liver ischaemia

Introduction

A short period of ischaemia followed by reperfusion is known to protect the heart during subsequent ischaemia-reperfusion injury (Murry et al. 1986; Reimer et al. 1990). This protection was called ischaemic preconditioning. Numerous mechanisms have been reported to be involved in the phenomenon of preconditioning. They include: i) inhibition of mitochondrial ATPase (Bosetti et al. 2000); ii) release of endogenous mediators such as adenosine (Liu et al. 1991; Mullane 1992) and catecholamines coupled with changes in G-proteins (Ravingerová et al. 1995, 1996, 2002); iii) formation of nitric oxide (Végh et al. 1992; Takano et al. 1998; Suematsu et al. 2001; Laude et al. 2003) and cyclooxygenase products, most likely prostacyclin (Végh et al. 1990); iv) expression of proto-oncogenes (Brand et al. 1992); v) activation of ATP-sensitive K^+-channels (Gross and Auchampach 1992; Sanada et al. 2001; Munch-Ellingsen et al. 2000; Pain et al. 2000; Yue et al. 2002; Ravingerová et al. 2002); vi) release of reactive oxygen species (Ambrosio et al. 1994; Das et al. 1999) and bradykinin (Végh et al. 1993; Kositprapa et al. 2001); vii) role of several kinases including MAP kinases (Vogt et al. 1994; Maulik et al. 1996, 1998; Nakano et al. 2000; Barančík et al. 1999, 2000; Strohm et al. 2000; Schulz et al. 2002) and many others.

It has also been reported that release of some cardio-protective proteins such as heat stress proteins (Marber et al. 1993; Sakamoto et al. 2000; Zhou et al. 2001) might participate in preconditioning phenomenon. Ziegelhöffer et. al. (1995) showed that a high soluble protein fraction (HS fraction) released from the dog myocardium during ischaemic preconditioning was found to be cardio-protective against ischaemia similarly as ischaemic preconditioning itself. These results indicate that during ischaemia some proteins that exert cardio-protective effect are released from the heart.

The aim of the current study was to examine whether any cardio-protective peptides are released during ischaemia also from other tissues than heart itself,

e.g., from the liver. In the present study we examined the changes of protein pattern released from isolated perfused rat liver after global ischaemia and subsequent reperfusion. We also tested the effect of HS fraction released from isolated perfused rat liver after ischaemia on the function of isolated perfused rat heart during ischaemia-reperfusion injury.

Materials and Methods

Treatment of animals

The male Wistar rats with body weight of 200–250 g kept on standard pellet diet and tap water ad libidum were used in all experiments. The animals were anaesthetized with sodium pentobarbitone (40 mg/kg) i.p. and heparinized (500 IU) before each experiment.

Ischaemia of isolated rat livers and isolation of HS fraction from perfusate

The surgical procedure for the preparation of the rat liver for perfusion was the same as in our previous works (Bezek et al. 1990; Kukan et al. 1990), whereas the perfusion system was modified according to Ballet et al. (1988). During surgery, the livers were perfused using a single-pass mode at 37 ℃ with approx. 400 ml of haemoglobin-free Krebs–Henseleit bicarbonate buffer at pH 7.4, fortified with glucose (10 mmol/l) and saturated with 95 % oxygen and 5 % carbon dioxide. After surgery the liver was transferred to a heated porcelain chamber drained into a reservoir. Ischaemia was induced by interrupting the perfusate flow for 30 s in the control livers. In the other two groups, the livers were subjected to ischaemia for either 30 min or 60 min by stopping the flow and by transferring the livers from the perfusion circuit to a water bath, where they were incubated at 37 ℃ in a Krebs-Henseleit bicarbonate buffer. All three groups of livers were then reperfused for 120 min in the recirculating system with the same perfusion medium as given above at constant pressure of 12 cm H_2O, which was maintained by an overflow mechanism (see Fig. 1 of Ballet et al. (1988) for details). The volume of the perfusion medium was 200 ml.

After reperfusion the perfusates were lyophilised, dissolved in bi-distilled water, dialysed against bi-distilled water and finally HS fractions were isolated by ammonium sulphate precipitation. Briefly: Proteins were precipitated overnight with 2.128 mol/l ammonium sulphate at 4 ℃ and the precipitates were centrifuged at 3000 × *g*. Supernatants were additionally precipitated with ammonium sulphate (added 200 mg of solid ammonium sulphate into each 1 ml of supernatant) for 3 h at 4 ℃, pH was then adjusted to 4.3 by H_3PO_4 precipitated overnight and centrifuged at 3000 × *g*. Pellets were dissolved in bi-distilled water and dialysed overnight against water. All dialysed samples were freeze-dried and stored for further application or analysis. The protein patterns of all fractions were determined by means of SDS-PAGE electrophoresis in 6–18 % gradient gel using Laemmli procedure. Protein bands were visualised by Coomassie Blue or were blotted to nitrocellulose, and

glycoproteins were visualised by ConA, mouse anti ConA antibody and secondary anti mouse antibody linked with horseradish peroxidase using procedure described by Ziegelhöffer et al. (1995). Finally, the effect of HS fraction on isolated perfused rat heart during ischaemia – reperfusion injury was tested.

Ischaemia of isolated rat hearts

Hearts from male Wistar rats were divided into two experimental groups, control group and protein group ($n = 4$ in each group). Hearts from both groups were cannulated through the aorta and perfused in a non-recirculating mode according to Langendorff at a constant perfusion pressure equivalent to 75 mm Hg at 37℃. As a perfusion medium the modified Krebs–Henseleit buffer (pH 7.4) was used. Medium was gassed with 95 % O_2 and 5 % CO_2 and contained (in mmol/l): NaCl 118.0, $NaHCO_3$ 27.2, $MgSO_4$ 1.2, KH_2PO_4 1.0, $CaCl_2$ 1.59, KCl 3.0 and glucose 11.1. The solution was filtered through a 5μm porosity filter to remove contaminants. After 20 min stabilisation perfusion in absence (control group) or presence of HS fraction (0.1 mg/ml) the hearts were exposed to 20 min global ischaemia followed by 20 min reperfusion. During reperfusion the haemodynamic parameters of each heart were monitored. An epicardial electrogram (EG) was registered by means of two stainless steel electrodes attached to the apex of the heart and the aortic cannula and continuously recorded (Mingograph ELEMA-Siemens, Solna, Sweden). Heart rates were calculated from the EG. Coronary flow was measured by a time collection of coronary effluents. Left ventricular pressure was measured by means of a latex water-filled balloon inserted into the left ventricle *via* the left atrium (adjusted to obtain end-diastolic pressure of 7–10 mm Hg) and connected to a pressure transducer (P23 Db Pressure Transducer, Gould Statham Instruments Inc., USA). Maximum rates of pressure development ($[dP/dt]_{max}$) and parameter Q (represents the ratio of maximum rates of pressure falls and developments, $[dP/dt]_{min}/[dP/dt]_{max}$) were used as measures for hearts relaxation and contraction properties. Incidence of arrhythmias was evaluated using arrhythmia score according to the Lambeth conventions (Walker et al. 1988)

To examine the effect of HS fraction this fraction was added to the perfusion solution and applied during stabilisation period before ischaemia in the dose of 0.1 mg/ml. All other details were described elsewhere (Ravingerová et al. 2002)

Evaluation of levels of adenine nucleotides using HPLC method

Heart tissues were freezed according to Wollenberger (1958). Nucleotides were extracted with 10 % $HClO_4$ (Boehringer), 10 ml $HClO_4$ *per* one g of tissue. Extracts were centrifuged for 10 min at 3000 × *g*. Supernatants were diluted in bi-distilled water (1 : 1) and neutralized with K_2CO_3 (Lachema). Samples were then filtered to remove precipitated $KClO_4$ and filtrates were directly injected into the HPLC column (C18, 250 × 4.6 mm, 4 μm particles). Solution of 150 mmol/l KH_2PO_4, 150 mmol/l KCl, pH = 5.5 was used as mobile phase in which linear gradient of acetonitrile (0–15 % *per* 1 h) was used for nucleotides elution from the column.

Results

The protein pattern of HS fraction after liver ischaemia is presented in Fig. 1. Global ischaemia (30 or 60 min) induced visible changes in the amount and spectrum of proteins in HS fraction that was released from liver to the perfusion medium (Fig. 1A). Particularly, ischaemia induced gradual elevation of the levels of two low molecular weight proteins (bands 2 and 3) and a decrease in the level of one low molecular weight protein (band 1, Fig. 1A). Other changes in protein pattern in HS fraction as well as in fraction precipitated by 2.128 mol/l ammonium sulphate were not so pronounced. Ischaemia of liver induced a decrease in the proportion of proteins that are able to interact with ConA, i.e., proportion of glycosylated proteins, in both protein fractions (Fig. 1B).

When the HS fraction isolated from perfusion medium after 60 min global ischaemia of isolated perfused liver was administered to the perfusion medium for heart perfusion, it induced a beneficial effect on heart function during postischaemic reperfusion after 20 min global ischaemia of isolated rat heart. This cardioprotective effect is indicated by: i) decreased incidence of arrhythmias from the level of arrhythmia score 2, e.g., ventricular arrhythmias lasting over 30 s, bigeminia and trigeminia occurred in the control group at the 5^{th} min and the 10^{th} min of reper-

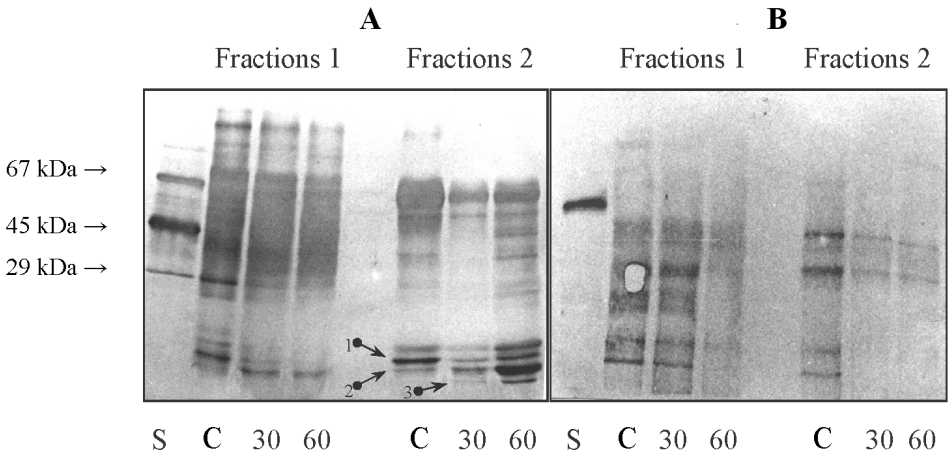

Figure 1. Characterisation of proteins released from the liver to perfusate after 30 s (C), 30 min (30), and 60 min (60) of global ischaemia. S, low molecular standards. Protein fractions: Fraction 1 – precipitated by 2.128 mol/l ammonium sulphate, and Fraction 2 – high soluble protein fraction (HS fraction). Fractions were isolated as described in Materials and Methods. **Panel A:** Proteins were separated on 6–18 % gradient gel and visualised by Coomassie Blue. **Panel B:** After separation on the same system proteins were electrotransferred to nitrocellulose membrane and stained by ConA, mouse anti ConA antibody and anti-mouse antibody linked with horseradish peroxidase. Similar results were obtained for four independent experiments.

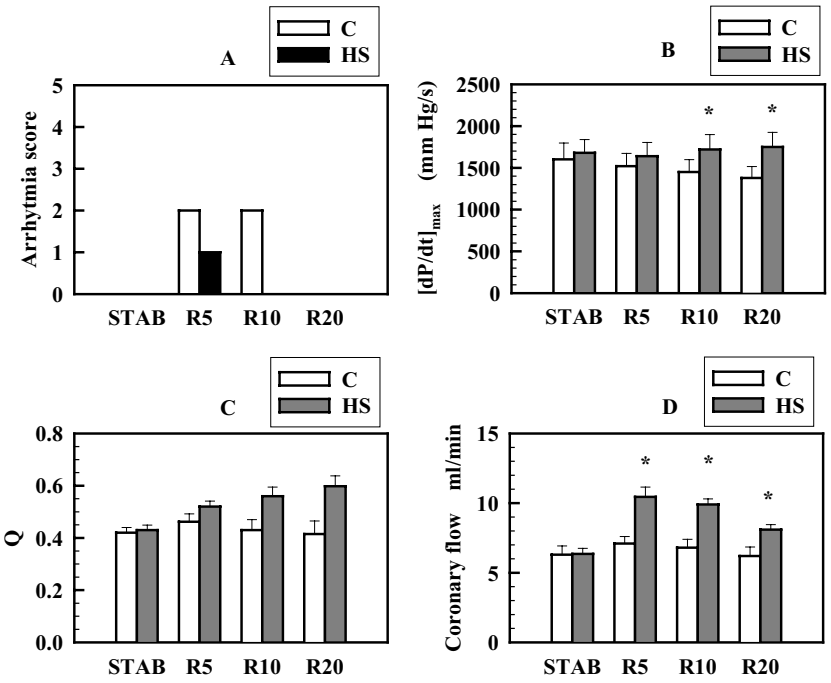

Figure 2. Protective effect of HS fraction (0.1 mg/ml) on heart against ischaemia and reperfusion injury. Hearts were perfused without HS fraction in control experiment (C). **Panel A:** effect of HS fraction on arrhythmia score registered during reperfusion after 20 min of ischaemia. **Panel B:** effect of HS fraction on contraction index (maximum rate of pressure development $[\mathrm{d}P/\mathrm{d}t]_{\max}$) measured during reperfusion after 20 min of ischaemia. **Panel C:** effect of HS fraction on parameter of contractility Q (ratio of maximum rate of pressure falls and maximum rate of pressure development $[\mathrm{d}P/\mathrm{d}t]_{\min}/[\mathrm{d}P/\mathrm{d}t]_{\max}$) measured during reperfusion after 20 min of ischaemia. **Panel D:** effect of HS fraction on coronary flow registered during reperfusion after 20 min of ischaemia. Results on the panels B–D represent mean value ± S.E.M. from four independent experiments. * level of significance $p < 0.05$ computed using Student's t-test; STAB, stabilisation perfusion; R5 –, R10 – and R20 – fifth, tenth and twentieth min of reperfusion.

fusion to the level 1, e.g., ventricular arrhythmias under 30 s occurred at the 5^{th} min and level 0, e.g., no arrhythmias occurred at the 10^{th} min of reperfusion in the group perfused with HS fraction in medium before ischaemia (Fig. 2A); ii) increased level of coronary flow from the interval 6.2 ± 1.5–7.1 ± 1.3 ml/min, observed for control group, to the interval 8.9 ± 1.0–10.45 ± 1.5 ml/min observed for the group with administrated HS fraction (Fig. 2D); iii) improved contractility of myocardium manifested by both stabilisation of $[\mathrm{d}P/\mathrm{d}t]_{\max}$ values and increased Q values with time in the group with administrated HS fraction (Fig. 2B and C). The index of contraction $[\mathrm{d}P/\mathrm{d}t]_{\max}$ was found to exceed the level 1700 mm Hg/s while

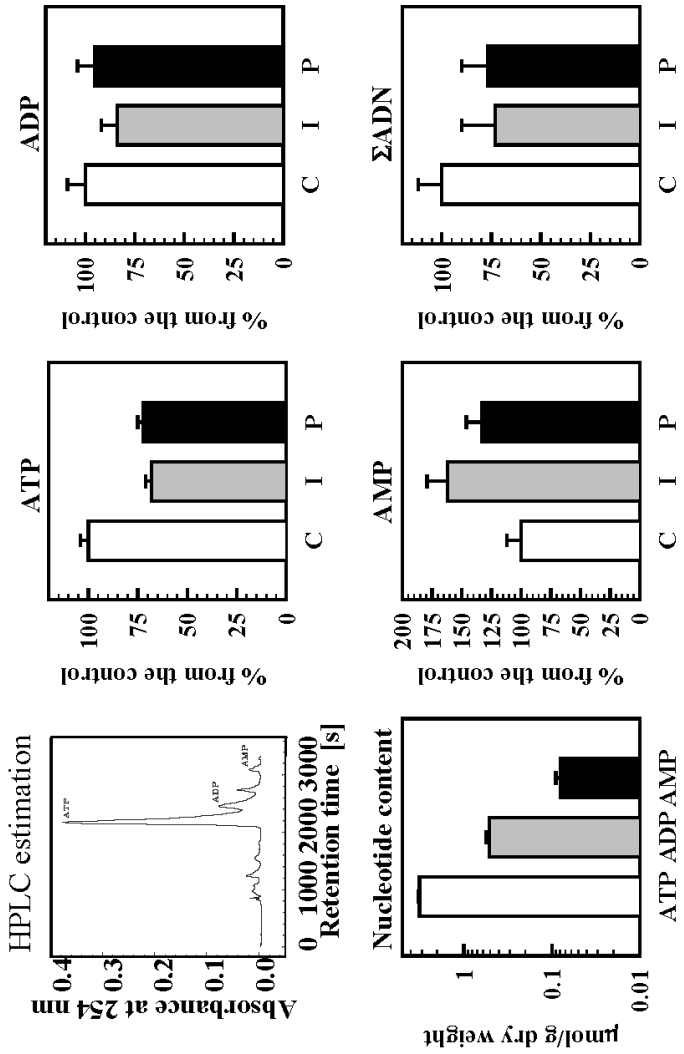

Figure 3. Levels of ATP, ADP AMP and sum of all three nucleotides (ΣADN) in cardiac tissue in control (C) and after 20 min of ischaemia followed by 20 minutes reperfusion in the absence (I) or presence (P) of HS fraction (0.1 mg/ml). Left upper panel represent typical HPLC registration of ATP, ADP and AMP. Left lower panel documented nucleotide content in control rat hearts. The results represent mean value ± S.E.M. from four independent experiments.

in control this value has decreased from 1602 to 1379 mm Hg/s during 5–20 min of reperfusion (Fig. 2B). Parameter Q was increased with time during reperfusion in the HS group while changeless in the control group (Fig. 2C). Thus, $[\mathrm{d}P/\mathrm{d}t]_{\min}$ has to be increased with time in both groups as it could be deduced from Fig. 2B and C. The heart rates of both groups were in the interval 186–212 min^{-1} in both groups and there were no significant differences of this parameter between both groups during the whole experiment.

Effect of HS fraction on adenine nucleotide levels of heart tissue is documented in Fig. 3. The levels of ATP, ADP and ΣADN (whole amount of adenine nucleotides) after ischaemia-reperfusion injury significantly decreased, the level of

AMP significantly increased. In the protein group the level of ATP non-significantly increased against control group but was still significantly decreased against the level before ischaemia-reperfusion. The level of ADP in the protein group significantly increased against control group and was not significantly different from the level before ischaemia. The level of AMP in the protein group non-significantly decreased against control group but was still significantly increased against the level before ischaemia. The level of ADN in the protein group non-significantly increased but was still significantly decreased against the level before ischaemia, similarly as the levels of ATP.

Discussion

It is known that some stress proteins are released from the heart tissue during preconditioning (Marber et al. 1993; Sakamoto et al. 2000; Zhou 2001) and it can be assumed that some proteins can be involved in the preconditioning protection. Consitent with this hypothesis are the findings of Ziegelhöffer et. al. (1995) that proteins released from the dog myocardium during ischaemic preconditioning are cardio-protective against ischaemia similarly as ischaemic preconditioning itself. Our results indicate that during liver ischaemia several proteins that protect myocardium against ischaemia-reperfusion injury were released into the perfusion medium.

We have examined that the HS fraction released from isolated rat liver after 60 min ischaemia had a protective effect on isolated rat heart during ischaemia-reperfusion injury. This beneficial effect of HS fraction is documented by decreased number of arrhythmias and improved contractility of hearts. Presence of HS fraction in the perfusion medium before ischaemia also induced an enhancement in the level of coronary flow during postischaemic reperfusion. Increase of this parameter may indicate an improvement in uptake of oxygen and metabolic substrates into the heart tissue during reperfusion.

The SDS-PAGE electrophoresis of HS fractions of both groups showed a visibly changed protein pattern of this fraction after liver ischaemia in comparison with the control group: the amounts of several low-molecular weight proteins increased, and the amounts of high-molecular weight proteins decreased. Even if biochemical analysis indicated protein degradation, the HS fraction seemed to be cardio-protective. These findings indicate that cardio-protective proteins may be also degradation products of some high-molecular weight proteins and are formed and released during ischaemia in an enhanced amount.

Our results showed that HS fractions have not a considerable influence on energetic metabolism of heart cells during ischaemia and reperfusion, because only a non-significant decrease of dephosphorylation of adenine nucleotides could be registered. However, significantly increased level of ADP after HS fraction administration was measured, indicating that stimulation of adenylate kinase reaction ($ATP + AMP \rightarrow 2ADP$) occurred. Stimulation of this reaction can also be an

adaptive mechanism that protects the energetic stage of cardiomyocytes during ischaemia-reperfusion injury (Pucar et al. 2002).

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