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Short communication

Determination of plasma activities of purine nucleoside phosphorylase by high-performance liquid chromatography: estimates of nonparenchymal cell injury after porcine liver transplantation

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Abstract

An assay is described for measurement of purine nucleoside phosphorylase (PNP) in plasma by high-performance liquid chromatography (HPLC). A plasma sample was incubated with hypoxanthine and ribose-1-phosphate in phosphate-free medium at pH 7.4 to catalyse the production of inosine by plasmatic PNP. The reaction was stopped by addition of perchloric acid to inactivate the enzyme and to precipitate plasma proteins. After centrifugation and neutralization of the supernatant with NaOH the increase in the substrate inosine was determined by HPLC.

Plasma activities of PNP averaged 5.0 mU/ml before and 12.3 mU/ml ($p < 0.001$), 5 min after porcine liver transplantation. At the same time points, the plasma activities of the frequently used liver enzymes lactate dehydrogenase or alanine aminotransferase remained virtually unchanged. Thus, plasmatic activities of PNP may be a suitable and early indicator of ischemic alterations to the graft in vivo.

1. Introduction

Reperfusion of ischemic organs is often compromised by an impaired vascular conductivity [1]. A growing body of evidence has emerged to suggest that injury to the nonparenchymal cell population, e.g. endothelial cells, represents an early event during organ preservation and reperfusion [2–4], likely to play a pivotal role with regard to the loss of organ viability after transplantation [4,5].

Purine nucleoside phosphorylase (PNP) is an enzyme active in the catabolism of adenine nucleotides catalysing the reversible conversion of inosine to ribose-1-phosphate and hypoxanthine in the presence of inorganic phosphate.

The localisation of PNP is rather specific, showing merely high activities in nonparenchymal cells like the vascular endothelium and Kupffer cells, while only little or no activities are found in the parenchyma of liver, kidney or heart muscle [6]. Thus, PNP has been proposed as a marker of oxidative injury to liver endothelial cells [3], and recently interest has focussed on the

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measurement of PNP activities in the eluate prior to reperfusion as an early marker of organ viability after warm ischemia [3] or ischemic storage [7].

The enzymatic determination of PNP is usually based on the production of hypoxanthine, which is further catabolised to uric acid in presence of an excess of xanthine oxidase [8,9]. Uric acid production could be followed spectrophotometrically by an increase in absorbance at 293 nm.

However, since many physiological proteins show relevant absorption at this wavelength, the method requires time-consuming purification of the sample material prior to analysis and is thus poorly applicable for the measurement of plasmatic enzyme activities.

Therefore, coupled assays have been developed, in which the reaction products are further metabolized by catalase and a NAD-dependent aldehyde dehydrogenase, and the conversion of NAD to NADH followed spectrophotometrically at 334 nm [10,11].

High-performance liquid chromatography has also proven suitable for the determination of PNP, providing an easy separation of substrate and products after incubation of the sample. This technique has already been applied to isolated, washed erythrocytes [12]. However, the release of PNP from the nonparenchymal cell population of ischemically injured organs can only be followed in the erythrocyte-free plasma of experimental animals or humans.

The present study describes an assay for the determination of PNP in plasma samples, based on the reverse reaction, which catalyses the formation of inosine and inorganic phosphate from ribose-1-phosphate and hypoxanthine. One major advantage of this principle is the fact, that the reverse reaction may not be influenced by contaminations of the sample material as adenosine, hypoxanthine, uric acid or uricase.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade.

Hypoxanthine and perchloric acid were obtained from Merck Chemicals (Darmstadt, Germany), all other reagents from Sigma Chemicals (St. Louis, MO, USA).

2.2. Procedures

Orthotopic liver transplantations were performed in 18 pigs. The livers were flushed and cold stored in University of Wisconsin organ preservation solution for 6 h at 4°C prior to implantation into the recipient. Plasma samples were obtained from central venous blood of the recipient prior to reperfusion of the graft (baseline values) as well as 5 and 60 min after restoration of liver blood flow. Care has to be taken to avoid hemolysis, since high concentrations of PNP are found in erythrocytes. The samples were stored frozen below −40°C until the day of analysis.

2.3. Assay technique

Aliquots of 100 μl EDTA plasma were pipetted into 75 × 12 mm tubes, containing 100 μl of 8 m*M* ribose-1-phosphate in phosphate-free buffer at pH 7.4 0.05M (3-[N-morpholino]propanesulfonic acid (MOPS). After addition of 800 μl 1 m*M* hypoxanthine in 0.05 *M* MOPS-buffer of pH 7.4, the mixture was incubated at 25°C in a shaking water bath (SW-U3, Julabo Labortechnik, Seelbach, Germany) for 20 min. The reaction was stopped by addition of 0.25 ml of 10% perchloric acid. The samples were then centrifuged in the cold (4°C) for 10 min at 9500 *g* to precipitate the denaturated enzymes along with contaminating plasma proteins.

From the clear supernatant 0.5 ml were transferred to clean glass vials, neutralized with 0.1 ml 1 *M* NaOH and kept at 4°C in the autosampler platform of the HPLC system.

A 25-μl aliquot of the neutralized supernatant was injected into a high-performance liquid chromatography system (Merck-Hitachi D6000). The mobile phase was composed of 50 m*M* potassium phosphate buffer and 1 m*M* tetrabutylammonium phosphate (pH 5.0). The sample was pumped with the eluent through LiChrocart RP 18 column (125 × 4.0 mm I.D., 10 μm particle size, Merck) at a rate of 2.0 ml/min.

The concentrations of hypoxanthine and inosine in the sample were calculated from external standards following the absorbance at 254 nm using a Merck-Hitachi detector type L 4250. The total time required for the run of one sample was 5 min.

The activity of PNP was calculated from the increase in product, based on the assumption that 1 unit of PNP metabolizes 1 μmol hypoxanthine to inosine per min at 25°C. A sample without ribose-1-phosphate was run simultaneously and served as a blank.

3. Results and discussion

Preliminary investigations on the reverse reaction of PNP have previously been performed on erythrocytes by Halfpenny and Brown [12] by stopping the reaction by heat inactivation of the enzymes. However, when applied to plasma samples, heating resulted in a turbidity of the samples, which were not cleared after centrifugation. Therefore we used perchloric acid to terminate the reaction and to precipitate plasma proteins. Care must be taken to keep the samples in the cold after addition of $HClO_4$, to avoid nonenzymatic decomposition of inosine, which might occur in acidic solution at higher temperatures.

The linearity of the assay was tested over a range up to 200 mU/ml. Different amounts from 0.125 to 1.0 μg of a commercialized nucleoside phosphorylase preparation were added to a plasma sample and plotted against the activities calculated from the recovery of inosine in the chromatogram. We found a linear correlation between the amount of protein added and the measured activity of PNP up to 200 mU/ml with a correlation coefficient of $r^2 = 0.995$.

Original chromatograms from assays of porcine plasma with and without addition of PNP are shown in Fig. 1.

The formation of inosine was also found to be linearly correlated with the time of incubation. Fig. 2 shows the linear increase of inosine with time of incubation up to 40 min. A reaction time of 20 min was chosen.

The stability of the samples after centrifugation and transfer of the supernatant into the HPLC vials was confirmed for at least 2 h.

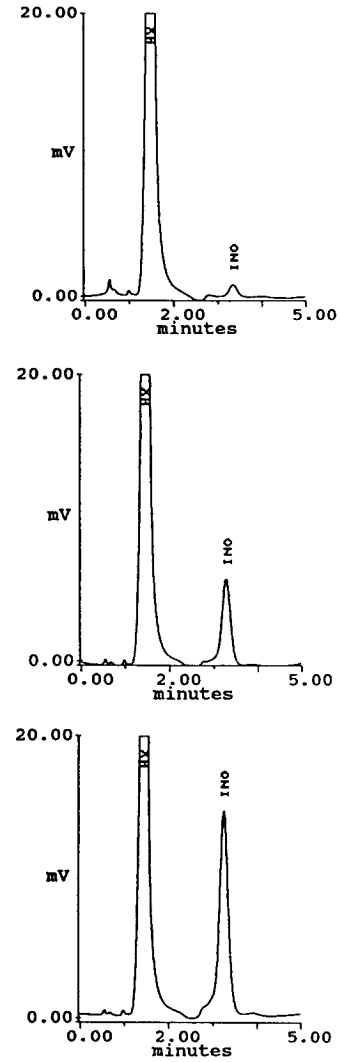

Fig. 1. Original chromatograms of native plasma (containing 2 mU/ml of PNP) and after addition of 1.25 μg/ml or 2.50 μg/ml of PNP to the plasma prior to the incubation (from top to bottom). Peaks are identified as hypoxanthine (HX) and inosine (INO).

Nine measurements of a given amount of nucleoside phosphorylase gave a mean activity of 82.0 mU/ml with a relative standard deviation of 3.0%, confirming sufficient reliability of the procedure.

The mean plasma activities of purine nucleoside phosphorylase before and after orthotopic liver transplantation corresponded well to

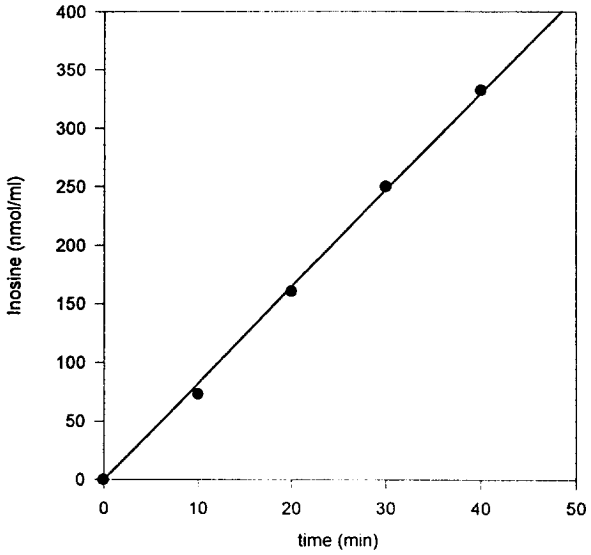

Fig. 2. The effect of incubation time on the production of inosine.

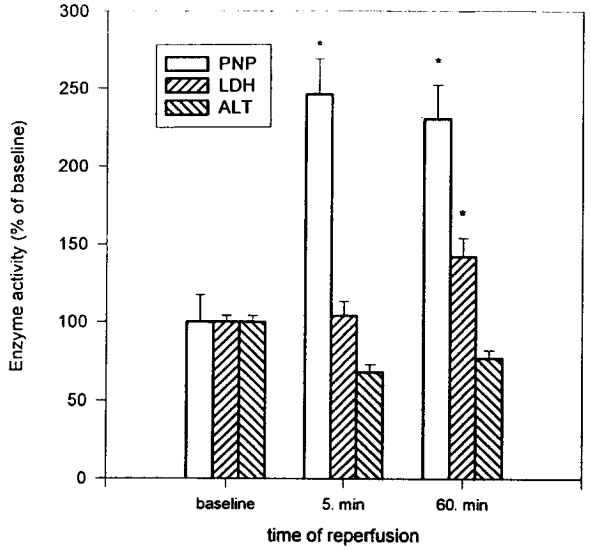

Fig. 3. Relative changes from baseline values of different plasma enzyme activities after porcine liver transplantation. LDH and ALT were measured by standard enzymatic procedures; values are represented as mean ± S.E.M. of $n = 18$ experiments; * = $p < 0.001$ vs baseline values; ANOVA and Tukey–Kramer test for multiple comparisons.

the experimental trauma. Only little activity was detectable in porcine plasma under baseline conditions (5.0 ± 0.86 mU/ml, mean ± standard error of the mean), when the integrity of the vascular endothelium is presumed to be intact. However, a significant increase in systemic plasma activity of PNP up to 12.3 ± 1.17 mU/ml was observed as early as 5 min after onset of postischemic liver reperfusion with virtually unchanged values after 60 min (11.5 ± 1.08 mU/ml).

These results are in good agreement with the data previously reported from Rao et al. [3], who used an isolated liver preparation after warmischemic insult, and confirm the value of PNP as an early and sensitive marker of liver injury after ischemic alteration in an in vivo model.

For comparison, the plasma activities of the commonly used parenchymal liver enzymes lactate dehydrogenase (LDH) or alanine aminotransferase (ALT) were measured using standard enzymatic procedures.

Under the described experimental conditions there were no discriminative changes for both enzymes as early as 5 min after onset of reperfusion and only a 42% increase in plasma activity of LDH after 1 h of reperfusion (cf. Fig. 3).

4. Conclusion

The present data indicate that measurement of PNP in plasma can be easily performed by the HPLC technique with the use of a time-effective autosampler device after termination of the reaction with perchloric acid.

The plasmatic activities of PNP after experimental liver transplantation in animals seem to be an early and more sensitive parameter than parenchymal liver enzymes such as LDH or ALT, for the detection of tissue alteration upon reperfusion of the liver. The measurement of plasmatic activities of PNP thus may become interesting also in the postoperative diagnostic follow up in human liver transplantation.

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