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Urinary Fructose-1,6-Bisphosphatase (FBP-1,6) and N-Acetyl-β-Hexosaminidase (HEX) in Monitoring Kidney Transplantation - Literature Review

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1. Introduction

Kidney grafts begin normal function immediately after transplantation, after several, or after dozen or so days delay. **A**cute **K**idney **I**nsufficience (AKI), **N**ephrotic **S**yndrome (NS) and **C**hronic **A**llograft **N**ephropathy (CAN), sometimes defined as "**I**nterstitial **F**ibrosis / **T**ubular **A**trophy (IF/TA) without any specific etiology" (Reuter et al., 2010), are the main types of complications connected with kidney transplantation.

In the diagnosis of the function of transplanted renal allografts, activities of enzymes excreted into urine by particular fragments of nephron are used. Increase of enzymuria precedes some clinical symptoms and is a more sensitive diagnostic marker than tubular proteinuria. Increase of enzymuria may also be useful for determination the time since renal tubules damage took place. Fructose-1,6 – bisphosphatase (FBP-1,6)- the principal enzyme of gluconeogenesis and N-acetyl-β-hexosaminidase (HEX)- the most active of the lysosomal exoglycosidases, taking part in the degradation of glycoconjugates (glycoproteins, glycolipids, proteoglycans), are markers used for monitoring kidney allograft function both in early and late post transplant periods, because of localization in the proximal contorted and straight tubules of nephron.

2. Urinary fructose-1,6-bisphosphatase (FBP-1,6) key enzyme of gluconogenesis

2.1 Renal glucose production

Kidneys require a continuous supply of glucose as a metabolic fuel. In the absence of dietary intake of carbohydrates, glycogen stores (mostly liver's) are able to supply glucose for only ten to eighteen hours. Fortunately liver and kidneys are able to produce glucose via the gluconeogenesis pathway (Champe et al., 2008) [Fig. 1.].
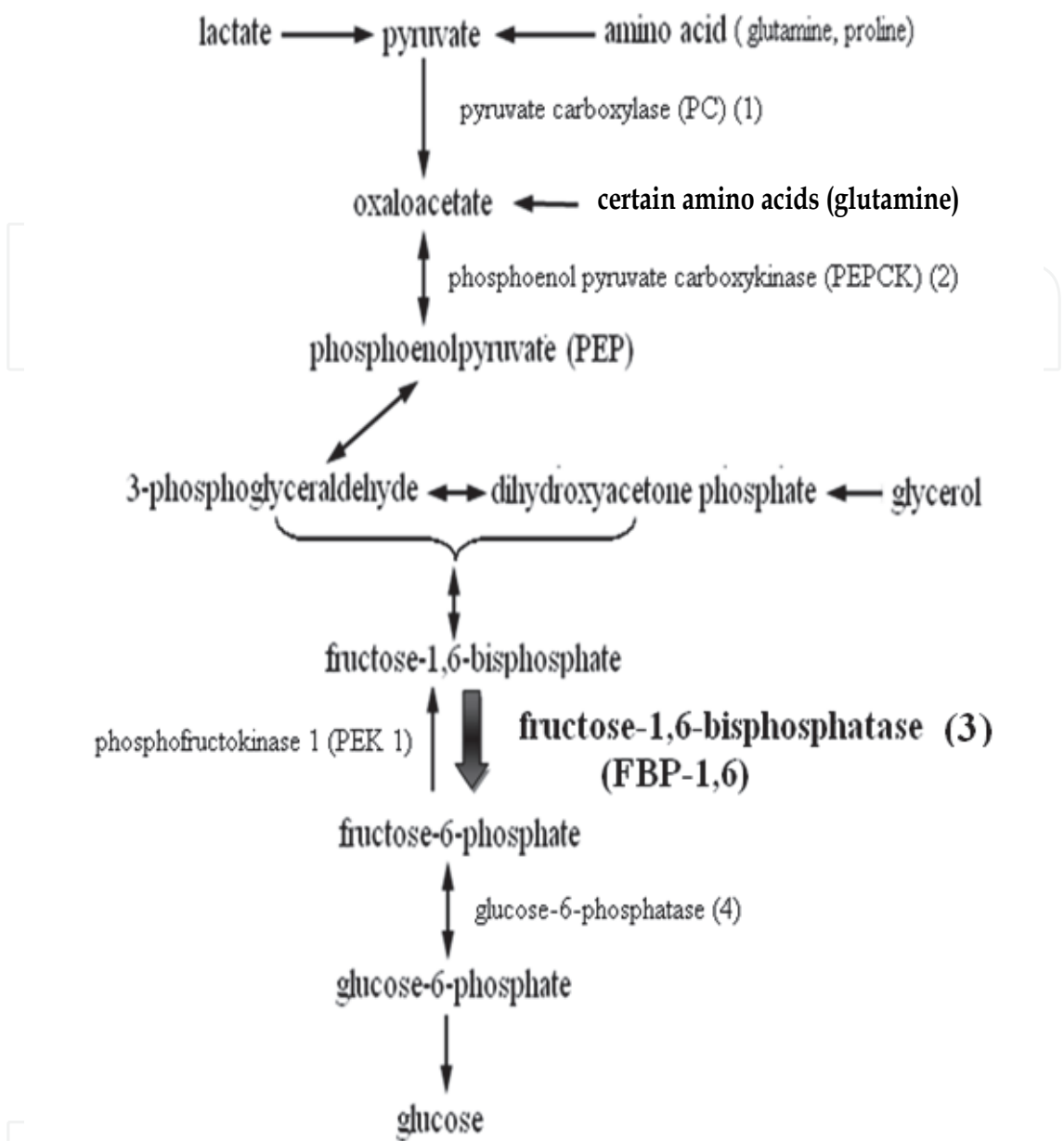
lactate ⟶ pyruvate ⟵ amino acid (glutamine, proline)

pyruvate carboxylase (PC) (1)

oxaloacetate ⟵ **certain amino acids (glutamine)**

phosphoenol pyruvate carboxykinase (PEPCK) (2)

phosphoenolpyruvate (PEP)

3-phosphoglyceraldehyde ⟷ dihydroxyacetone phosphate ⟵ glycerol

fructose-1,6-bisphosphate

phosphofructokinase 1 (PEK 1)   **fructose-1,6-bisphosphatase (3)**
(FBP-1,6)

fructose-6-phosphate

glucose-6-phosphatase (4)

glucose-6-phosphate

glucose

Fig. 1. Renal gluconeogenesis. The key enzymes of gluconeogenesis: (1) **P**yruvate **C**arboxylase (PC), (2) **P**hospho**E**nol **P**yruvate **C**arboxy**K**inase (PEPCK), (3) **F**ructose-**1,6-B**is**P**hosphatase (FBP-1,6), (4) glucose-6-phosphatase. The main renal gluconeogenic substrates are: glutamine, lactate, glycerol.

Renal glucose production accounts for between 5% to 25% of the total glucose formed by gluconeogenesis, in the body (Conjard et al., 2001; Meyer et al., 2003). The proximal renal tubules are capable of synthesizing glucose from a variety of precursors, including lactate, pyruvate, glutamine (the main substrate of renal gluconeogenesis), glutamate, and glycerol. The three segments of the renal proximal tubules PCT1, PCT2 and PST [Fig. 2.] can synthesize glucose at different rates from lactate. PCT2 and PST segments synthesize more glucose from lactate than the PCT1 segment (Yáñez et al., 2003; Yáñez et al., 2005). The PCT1 segment is specialized in the reabsorption of lactate in kidney (Yáñez et al., 2005). The segments PCT1, PCT2 and PST synthesize glucose from glutamine with about the same

capacity (Conjard et al., 2001), which indicates that glutamine is the main gluconeogenic substrate for the kidney (Stumvoll et al., 1997). Also fructose, propionate and proline are potential candidates for renal glucose precursors (Conjard et al., 2001; Meyer et al., 2003; Stumvoll et al., 1997). Fructose-1,6-bisphosphatase (FBP-1,6. EC 3.1.3.11) [Fig. 1.] is a key regulatory enzyme for renal gluconeogenesis.
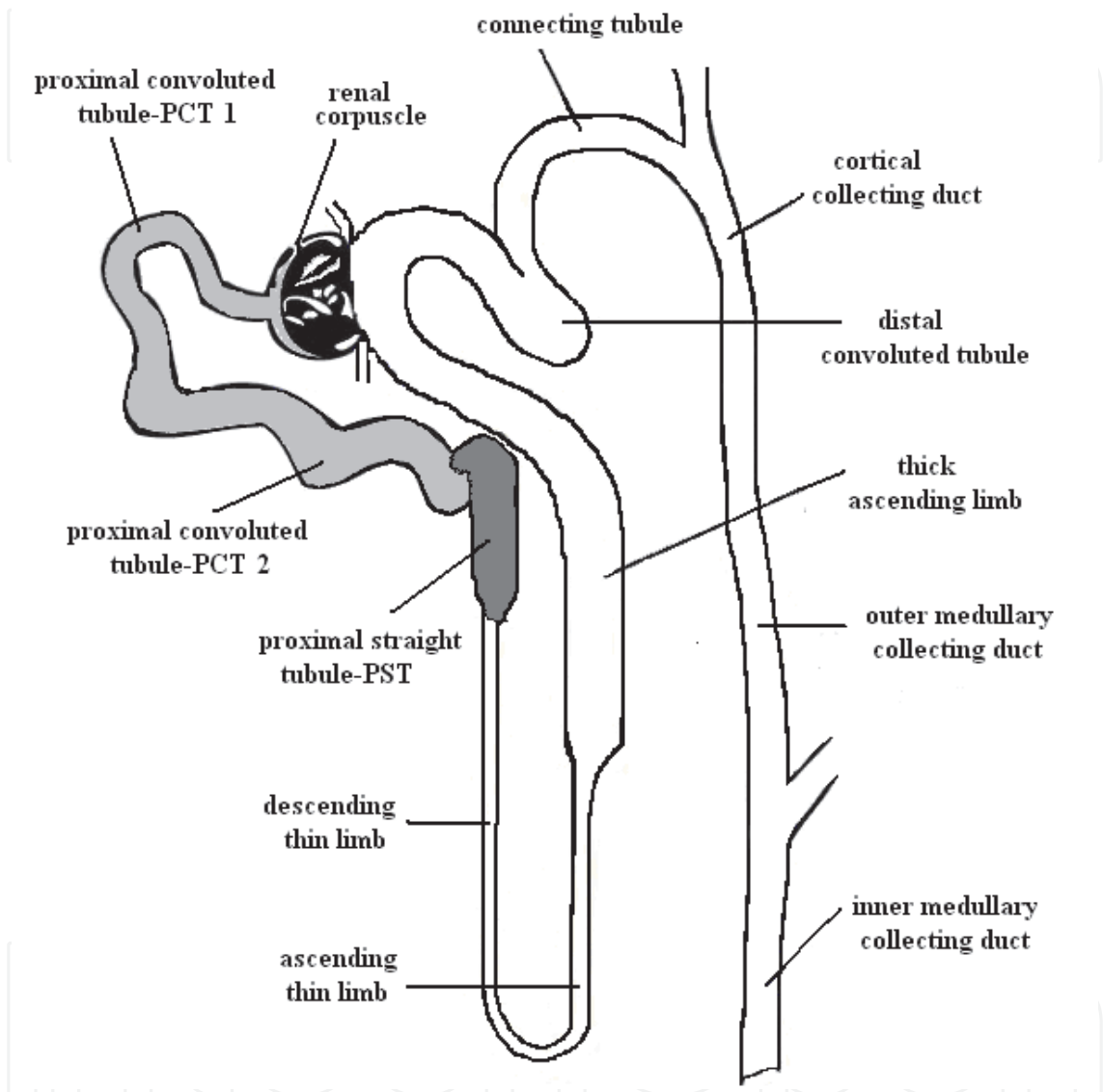
Fig. 2. Localization of FBP-1,6 and HEX in the nephron. FBP -1,6 is confined to the **P**roximal **C**onvoluted **T**ubule –PCT1, PCT2 and **P**roximal **S**traight **T**ubule –PST. Lysosomal N-acetyl-β-D-hexosaminidase (HEX) is distributed along the nephron with highest activity in the proximal convoluted tubule PCT1 and PCT2.

2.2 Structure and action of kidney FBP-1,6

FBP-1,6 catalyzes the irreversible conversion of fructose-1,6-bisphosphate (Fru-1,6-P_2) to fructose-6-phosphate and inorganic phosphate in the reaction:

Fructose 1,6-bisphosphate + H_2O = Fructose-6-phosphate + phosphate
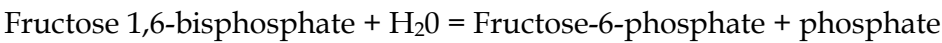
The mammalian kidney FBP-1,6 is a tetramer composed of four identical subunits with molecular weight ranging from 36 kDa to 41 kDa per subunit (e.g. each subunit of the pig kidney enzyme consists of 337 amino acids), organized as a dimer of dimers [Fig. 3.].
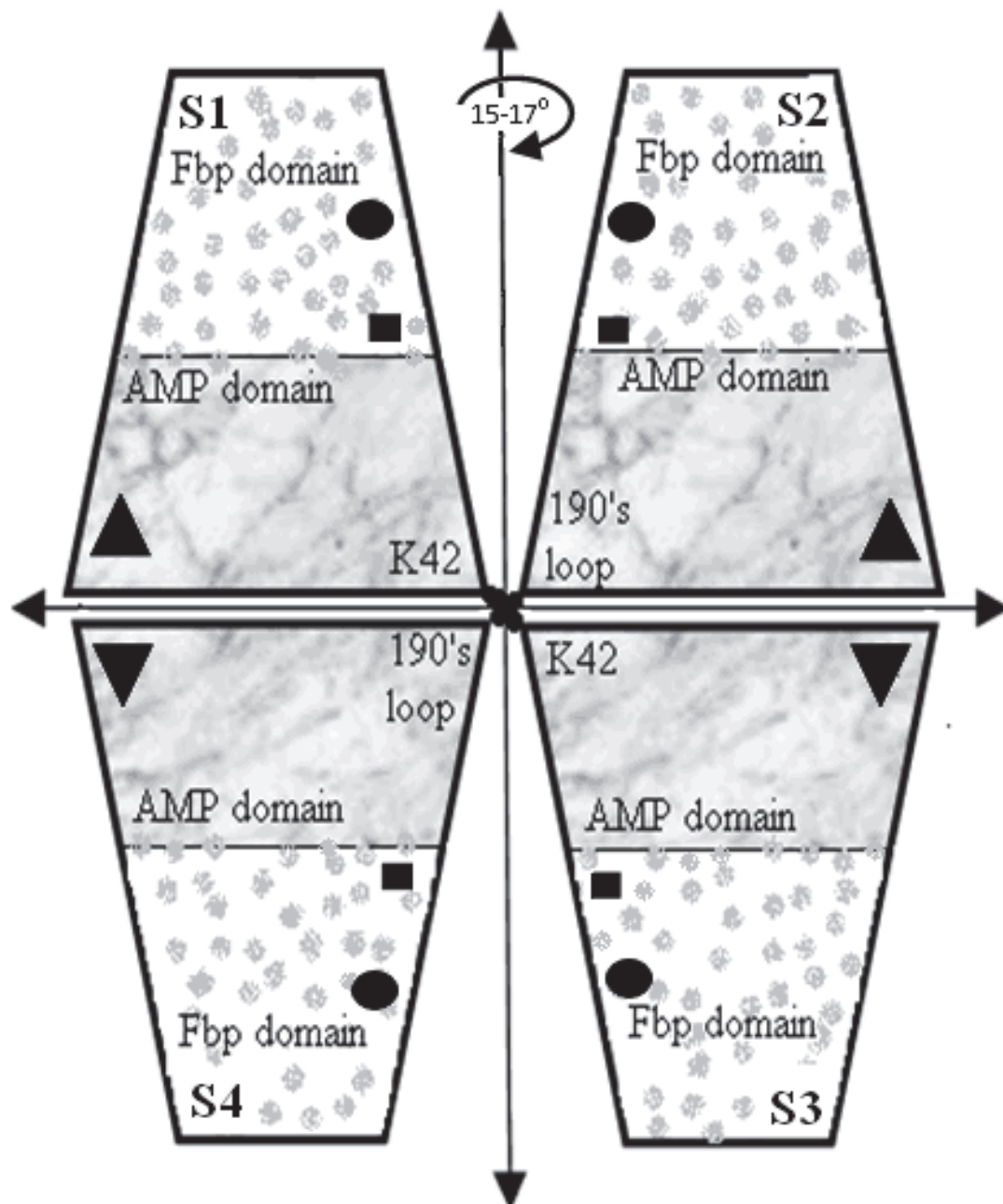

Fig. 3. The quaternary structure of fructose-1,6-bisphosphatase (R-state). The four subunits of FBP-1,6 are designated S1,S2,S3,S4 and are labeled clockwise. The S1 and S2 subunits correspond to the upper dimer, and the S3, S4 subunits correspond to the lower dimer. Each subunit is composed of two folding domains. The AMP domain has the AMP binding site (▲), while the Fru-1,6-P$_2$ (Fbp) domain contains the active site (●). The divalent metal sites (■) are located between the AMP and Fbp domains. Residues of the 190's loop make critical contacts with Lys-42 (K42). The R→T transition of FBP-1,6 involves a 15°-17° clockwise rotation of the S1:S2 dimer in respect to the S3:S4 dimer.

The tetramer of FBP-1,6 requires divalent cations: magnesium, manganese, zinc or cobalt for its catalytic activity (Dżugaj, 2006; Sáez et al., 1996; Yáñez et al., 2003). In each of four (S1, S2, S3 and S4) subunits, X-ray crystallographic studies have identified that the AMP and FBP domains [Fig. 3.]. Fru-1,6-P_2 (substrate) binding site (●) is located in each of four FBP domains. The AMP-binding sites (▲) are located at AMP domains approximately 30Å from the active site. One divalent metal-binding site (■) is located at each of the FBP domain between the Fru-1,6 P_2 and AMP binding domains of each subunit [Fig. 3.]. Pig kidney FBP-1,6 exists in at least two quaternary conformations called **R** (relaxed)-active, and **T** (tense)-inactive state, depending on the relative concentrations of the FBP-1,6 effectors. The allosteric transition R→ T of kidney FBP-1,6 involves a 15^0-17^0 clock-wise rotation around the vertical axis of the S1:S2 (upper) dimer, with respect to the S3:S4 (lower) dimer (Kelly et al., 1996; Lu et al., 1996; Zhang et al., 1994) [Fig. 3.]. Localization the 190's loops and Lys-42 near the horizontal and vertical molecular symmetry axes is critical for the R→T transition. AMP (reflecting shortage of energy) inhibits the enzyme, shifting FBP-1,6 from the R to the T conformation [Fig. 3.]. In the absence of AMP the FBP-1,6 exists in the active R state. Fructose-1,6-bisphosphate, fructose-6-phosphate and ATP (reflecting excess of energy), as well as metal cations, stabilize the R (active) state (Kelly-Loughane & Kantrowitz, 2001; McIninch & Kantrowitz, 2001). Mammalian kidney FBP-1,6 is activated by high glucagon/insulin ratio, which elevate cAMP level and increase activity of protein kinase A. Increased activity of protein kinase A favors the phosphorylation of bifunctional complex 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Phosphorylated phosphofructokinse-2 is inactive, which decreases the level of fructose 2,6-P_2 (inhibitor of FBP-1,6) (Champe et al., 2008). FBP-1,6 has two synergistic natural inhibitors: mentioned earlier adenosine-5'-monophosphate (AMP), and fru-2,6-P_2, a product of the bifunctional phosphofructokinase-2/ fructose bisphosphatase-2 (Hines et al., 2007; Sáez et al., 1996; Wu et al., 2006). Excess of FBP-1,6 substrate i.e Fru-1,6-P_2, acts as an inhibitor on FBP-1,6. Are novel inhibitors of fructose-1,6-bisphosphatase such as achyrofuran, anilinoquinazoline (also an inhibitor of tyrosine kinase) and 3-(2-carboxyethyl)-4,6-dichloro-1H-indole-2-carboxylic acid (MDL-29951)- also an antagonist of the glycine site of the NMDA receptor (Wright et al., 2001; Wright et al., 2003).

2.3 Ultrastructural localization of the FBP-1,6 in kidney

FBP-1,6 immunoreactivity was detected in kidney cortex cells, but not in the medulla. In the cortex all epithelial cells the PCT1, PCT2, and PST segments, forming the proximal convoluted tubules were FBP-1,6 positive (Yáñez et al., 2003). Aldolase B, but not aldolase A, colocalizes and forms a complex with FBP-1,6 (Yáñez et al., 2005).

Expression FBP-1,6 activity in cytoplasm and apical cells membranes PCT1, PCT2 and PST segments of the proximal tubules was demonstrated (Yáñez et al., 2003) [Fig. 2.]. Expression of FBP-1,6 was also observed in the basolateral membrane region of the PCT1 segment of the proximal tubule and colocalized with the **M**ono**C**arboxylate **T**ransporter 1 (MCT1) (Yáñez et al., 2003; Yáñez et al., 2005). MCT1 contributes to the transport of monocarboxylic acids in many tissues, which suggest that the PCT1 segment is specialized in the reabsorpion of lactate in kidney. The actual level of lactate transporter expression in the PCT1 segment might be the reason for the difference in the rates of glucose neosynthesis from lactate in the human renal proximal tubules (Yáñez et al., 2003).

The subcellular location of kidney FBP-1,6 is similar to liver FBP-1,6. The histochemical analysis revealed that the anti-FBP-1,6 antibody detected two single bands with a molecular

weight of 36kDa and 40 kDa, in kidney and liver, respectively (Yáñez et al., 2003). The immunofluorescence and confocal analysis revealed that FBP-1,6 was present in the cytosol and inside the nucleus of hepatocytes and proximal cells of the nephron (Sáez et al., 1996). Nuclear FBP-1,6 function is similar to the function of the cytosolic form. FBP-1,6 can be translocated inside the nuclei of proximal tubule cells (Yáñez et al., 2003; Yáñez et al., 2005).

The peroxidase reaction in light microscopy, yielded a strong signal FBP-1,6 in the proximal straight and convoluted tubules located at the cortex, but not in cells of the kidney medulla. The FBP-1,6 reaction is concentrated in the apical membrane region and is also observed in the basolateral membrane region (Yáñez et al., 2005). No immunoreactivity was observed in glomeruli, ascending and descending loops of Henle, collecting tubules, distal tubules and in cell of the kidney medulla (Sáez et al., 1996; Yáñez et al., 2003).

Determination of the fructose-1,6-bisphosphatase activities in the fractions of kidney and liver (assayed spectrophotometrically at 30^0C, by following the rate of NADH formation) revealed that FBP-1,6 activity is present in both tissues in the initial homogenate, the cytoplasmic extract and the high speed supernatant. No activity of FBP-1,6 was observed in the nuclear fraction, light or the heavy mitochondrial and microsomal fraction (Sáez et al., 1996).

2.4 Genetics and localization of the FBP-1,6 isozymes in human tissues

Human FBP-1,6 is coded in 2 distinct genes, *FBP1* and *FBP2* (Matsuura et al., 2002). *FBP1* consist of seven exons and spans over 31 kilobases at chromosome 9q22.2-q22.3 and expresses a 362-amino acid (el-Maghrabi et al., 1995). Messenger RNA (mRNA) from liver, kidney and monocytes is identical (Kikawa et al., 1994). *FBP2* which was isolated from muscle, codes 339 amino acids, and shares 77% amino acid sequences identity with *FBP1* (Tillmann & Eschrich, 1998).

FBP-1,6 is expressed in variety of organs such as: liver, kidney, muscle, brain, lung, small intestine, retina (Cloix et al., 1997; Mamczur et al., 2010; Nomura et al., 1994; Velásquez et al., 2010; Yáñez et al., 2003; Yáñez et al., 2005). Existence of four isozymes: muscle, liver, brain and intestine in tissues of vertebrates has been postulated (Dżugaj, 2006). In mammals two isoforms of FBP-1,6 exist, which are encoded by separate genes. In humans L-FBP-1,6 which is expressed mainly in liver and kidney, and M-FBP-1,6 is expressed mainly in muscle. Both isforms (L-FBP-1,6 and M-FBP-1,6) were found in several organs such as lung and brain (Bigl et al., 2008).

3. FBP-1,6 in kidney transplantation

After damage of the epithelial cell membranes, FBP-1,6 is released into urine. Therefore urinary FBP-1,6 activity was recommended for diagnoses of complications in the early post-transplantation period. Urinary FBP-1,6 was assayed in the second morning midstream urine portion in patients after transplantation. Cold ischemia time was 20.6±8.4h (range 3-32h), with a median of 22 h. Urinary FBP-1,6 excretion was significantly lower in grafts stored for a shorter time than 22 hours (0.69±0.42 U/g creatinine) as compared to FBP-1,6 urinary excretion in grafts stored > 22 hours (1.13±0.56 p=0.035 U/g creatinine). Multiple regression analysis demonstrated that the cold ischemia time was the only pre-transplant factor, which significantly correlated with early post-transplant urinary FBP-1,6 excretion (multiple R=0.65, F=14.6, p<0.001). After transplantation the patients were given cyclosporine A (CsA), but CsA levels did not correlate with urinary FBP-1,6 activities during

the first four postoperative days. The duration of the cold ischemia time is significantly correlated with the degree of urinary excretion of FBP-1,6 during the first four postoperative days (Kotanko et al., 1997).

Sharp increase in the activities of lactate dehydrogenase (LDH), glucose-6-phosphatase (G6Pase), malic enzyme (ME) and fructose-1,6-bisphosphatase (FBP-1,6) was observed in homogenates of renal cortex and medulla, after 5 min of warm ischemia induced by occlusion of rat renal left artery. Warm ischemia lasting 15-60 min resulted in a progressive decrease in the activity of the above enzymes. Reperfusion of rat kidneys for 60 min after prolonged warm ischemia caused complete recovery of the activity of the above enzymes (Khundmiri et al., 2004).

Five to ten day after uncomplicated renal transplantation enzymuria reaches a constant value, which is much higher than the average in healthy humans (Jung et al., 1992). Activity of urinary FBP-1,6 reached a lower level on the 4th day after transplantation, and remained on a stable low rate. During the four first days after transplantation the activity of urinary FBP-1,6 amounted to 0.9±0.5 U/g (0.1±0.06 U/mmol urinary creatinine); range 0.2-2.1 U/g (0.02-0.24 U/mmol)] (Kotanko et al., 1997). However in complicated transplantations urinary excretion of FBP-1,6 significantly increases, contrary to urinary creatinine concentration, which change slowly (Jung et al., 1992).

During experimental transplantation renal grafts taken from non-heart beating donors, fructose- 1,6-bisphosphate was used, for protection graft microcirculation against ischemic damage. Microdialysis probes in the renal cortex of pig kidney were used to monitor pyruvate concentration during hypotermic machine perfusion with a perfusate containing 29.4 mM exogenous fructose-1,6-diphosphate (FDP). During glycolysis fructose-1,6-diphosphate (FDP) is generated from glucose and is transformated into pyruvate. In the absence of oxygen, pyruvate is transformed into lactate rather than acetyl-CoA and is accumulated in the cell. The decrease of pH inhibits glucose conversion to fructose-1,6-diphosphate. Significant increases in pyruvate production ($p<0.05$) were observed after 12h of perfusion in the interstitial fluid of FDP-treated kidneys, as compared to control kidneys. After 24 h of graft perfusion the concentration of pyruvate was 149.1±58.4 µM in the FDP group and 55.6±17.9µM in the control group. The addition of FDP to the fluid during ischemic kidney perfusion, resulted in enhanced pyruvate production in the extracellular space. FDP has rheologic and antioxidant effects in addition to its ability to promote glycolysis and improve red blood cell deformability (Baicu et al., 2004).

FDP prevents the decline of renal cortical Na-K-ATPase activity induced by ischemia end gentamicin toxicity. Lu and Lin (Lu & Lin, 1989) have shown that fructose-1,6-diphosphate (FDP) has the ability to restrain the oxygen-radical related reperfusion injury. They observed reduction in renal Na-K-ATPase activity by 18.6% after 30 min of renal pedicle clamping and 60 min of reflow as well as a 32.5% reduction in renal Na-K-ATPase activity after 90 min of injection 100mg/kg gentamicin. Application of 4g/kg fructose-1,6-diphosphate (FDP) and 60 min infusion after reflow in the ischemic group, and 90 min infusion after injection of the drug in gentamicine-treated group, increased renal Na-K-ATPase activity in the FDP-treated group in comparison to the control kidney (Lu & Lin, 1989).

Expression of urinary FBP-1,6 protein is utilized to evaluate the degree of chronic post transplant nephropathy. Increased expression key renal enzymes of the citrate circle i.e. NAD$^+$-dependent **I**socitrate **DeH**ydrogenase (IDH) and **I**ron-**R**esponsive **E**lement-**B**inding **P**rotein-**1**/aconitase (IREBP1), as well as mitochondrial CK, creatine kinase B-type, ornithine

aminotransferase and FBP-1,6 were observed in IF/TA. FBP-1,6 protein was expressed at a significantly lower level in aTX(allogenic graft) than in sTX (syngenic graft) (Reuter et al., 2010). The above data is supported by analysis of gene expression profiles of renal transplants, which revealed reduced FBP-1,6 mRNA level in graft with IF/TA (Scherer et al., 2003).

Determination of urinary FBP-1,6 activity was not only used for evaluation of transplanted kidneys, but also to estimate the degree of damage to renal tubules of diabetic patients (Eid et al., 2006), in idiopatic nephrotic syndrome (Kępka et al., 2008) acute pyelonephritis (Zoch-Zwierz et al., 2004), heavy metal (e.g.$HgCl_2$) or, maleic acid intoxication (Kotanko et al., 1984), after trichloroethylene (TCE) administration (Khan et al., 2009). The urinary FBP-1,6 activity significantly increases after nephrotoxic drugs (ifosfamide, cisplatinum) (Pfaller et al., 1994) prednisolone, and azathioprine (Kotanko et al., 1986), cyclosporine, sirolimus and tacrolimus (Klawitter et al.,2010).

4. HEX - most active exoglycosidase in catabolism of glycoconjugates

All renal glycoconjugates (glycoproteins and glycolipids, present in renal cell membranes, and participating in inter cellular communication and interactions, as well as glycosaminoglycans of the renal cell surfaces, and extra cellular matrix), contain oligosaccharide chains (Gabius, 2009).Glycoconjugates are subject to constant synthesis and degradation. The protein cores of the glycoconjugates are synthesized in the cytoplasm, but glycosylation occurs in endoplasmatic reticulum and Golgi Apparatus (Zwierz et al., 1999). Lysosomes are the principal sites of intracellular digestion of sugar chains of glycoconjugates (Cuervo, 2004). Exoglycosidases which successively release individual sugars from non reducing ends of oligosaccharide chains are localized in lysosomes (Strecker et al., 1988; Winchester, 2005) [Fig. 4].

N–acetyl-β-hexosaminidase (HEX) (EC 3.2.1.52) is the most active of lysosomal exoglycosidases (Popko et al., 2008), taking part in the catabolism of oligosaccharide chains of all types of glycoconjugates (Zwierz et al., 1999). The fact that HEX has the highest activity of amongst exoglycosidases may be explained by the fact that aminohexoses (N-acetyl-glucosamine and N-acetyl-galactosamine) are components of all types of glycoconjugates: glycoproteins, glycolipids and glycosaminoglycans of proteoglycans (Chojnowska et al., 2011). HEX releases monomers of N-acetyl-glucosamine and N-acetyl-galactosamine from the non reducing ends of oligosaccharide chains of glycoconjugates (Zwierz et al., 1999). Isoenzymes of HEX are composed of two polypeptide chains (α and β) which are products of gene duplication (Pennybacker et al., 1996). Of the heat labile isoenzymes S (αα) and A (α,β), heat stable isoenzyme B (ββ) (Pérez & Tutor, 1998) and P (ββ) (Arciuch et al., 1999), HEX A and HEX B are the most common. In the majority of tissues HEX A (MW 96-110 kDa) and HEX B (MW 100-110 kDa) have similar contribution to the total HEX activity, because in normal conditions HEX S has only trace activity (Zwierz et al., 1999), and HEX P appears only in several situations e.g. pregnancy (Arciuch et al., 1999).

Kidneys are a rich source of HEX (Borzym-Kluczyk et al., 2005). HEX activity in the nephron is highest in proximal convoluted tubules. In proximal convoluted tubules HEX activity is three times higher than in renal corpuscles (Jung et al., 1992). HEX A and HEX B with the distinct dominance of HEX A are present in renal medulla and renal cortex (Tassi et al., 2000). In the urine of healthy humans the activity of HEX is low, mainly due to HEX A

derived from naturally exfoliated proximal convoluted tubule cells. Urinary excretion of HEX depends on age, sex, race and blood pressure (Agirbasli et al., 1996). Although HEX is present in variety of tissues and body fluids, urinary HEX activity is the most significant (Ostrowska et al., 1993).
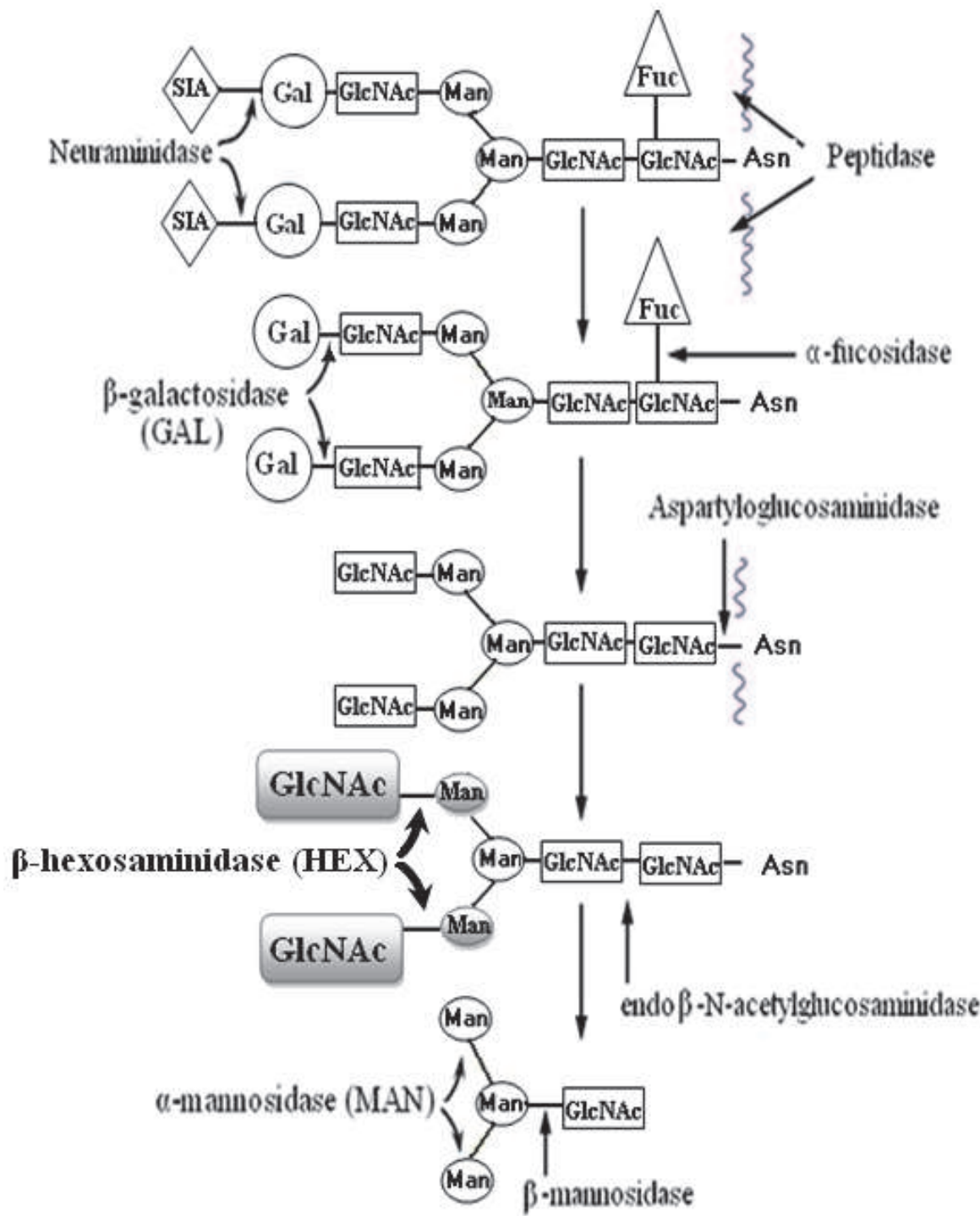
Fig. 4. Catabolism of the sugar chains of N-linked glycoproteins.
Asn- asparagine,Gal-galactose, GlcNAc-N-acetylglucosamine,Fuc-fucose, Man- mannose, SIA – Sialic acid (N-acetylneuraminic acid).

Urinary HEX activity is independent of diuria, diurial variations, low pH, or presence of the morphotic elements of blood (Costigan et al., 1996). Urinary HEX activity increases from several to dozens of times after damage to the epithelium of proximal convoluted tubules cells (Rustom et al., 1998). As cells of renal proximal convoluted tubules are very sensitive to hypoxia, all pathological states occurring with hypoxia, lead to dysfunction of the epithelium of renal proximal convoluted tubules, and leakage of HEX into urine (Chujo et al., 2008). The kidneys are critical targets for toxicity induced by heavy metals e.g. cadmium (Jung et al., 1993), and HEX activity in urine is recommended as a simple, stable, and cheap, screening indicator for early detection of renal dysfunction caused by cadmium (Jung et al., 1993).

5. HEX in renal transplantation

HEX was used for evaluation of the condition of human kidney grafts before transplantation, condition of transplanted kidneys, and estimation of toxicity of drugs used during renal transplantation.

In evaluating the contribution of the Na^+/H^+ antiporter of kidney donors and recipients to post reperfusion injury and recovery after transplantation, HEX urinary excretion rate was treated as independent predictor. Positive association of donor antiport activity with total HEX excreted within the first three days after transplantation reflecting acute post perfusion injury, confirmed antiport active contribution to renal susceptibility to the ischemic insult. Urinary HEX excretion was treated as one of the independent predictors of short-term graft outcome, and the length of the recipient's stay at hospital (Matteucci et al., 1999). Excretion into urine of trimethylamine-N-oxide (TMAO) - derived from renal medullary osmolytes, and metabolites of glycolysis (lactate) or Krebs cycle (acetate, citrate) (reflecting energy supply to the cells) was used for the evaluation of the influence of retrieval conditions (cold storage and reperfusion) on renal medulla injury in an isolated perfused pig kidney model. Determination of the above substances by proton NMR spectroscopy as early markers of ischemia reperfusion injury during cold storage and reperfusion, is a proof that increase in TMAO and metabolites of glycolysis or Krebs cycle are relevant markers of graft damage during cold storage and reperfusion. In the model above HEX was not efficient in assessing tubular damage during cold storage and reperfusion, which may be explained by the fact that increase in TMAO and disregulation of glycolysis and Krebs cycle precede any increase in excretion of HEX connected with damage of cell, and leakage of lysosomal enzymes taking part in the degradation of damaged tissues (Hauet et al., 2000). Working on the same model it was stated that TMAO (trimethylamine-N-oxide), DMA (dimethylamine) and acetate are more efficient parameters for assessing the impact of preservation solutions on renal medulla injury than HEX (Hauet et al., 2000).

HEX activity is an established biomarker for assessing the condition of machine perfused kidneys for transplantation, and for predicting kidney transplant outcome. Out of six biomarkers evaluated (**L**actate **D**e**H**ydrogenase-LDH, **AS**partate **A**mino**T**ransferase-ASAT, **G**lutathione –**S**-**T**ransferase-GST, HEX, **H**eart type **F**atty **A**cid **B**inding **P**rotein-H-FABP), ROC analysis certifies that only three of them (GST-AUC 0.67, HEX-AUC 0.64, H-FABP-AUC 0.64) measured in machine perfusion perfusate at the end of machine perfusion, are independently associated with the risk of delayed graft failure (Moers et al., 2010). Therefore it was concluded that GST, HEX and H-FABP are independent predictors for delayed graft failure, but not the graft survival. Increased activity of GST, HEX and H-

FABP in the machine perfusate could be an additional factor to consider in adjusting postoperative recipient management, but not in the decision to transplant or discard kidneys donated after brain death (Moers et al., 2010). Urinary excretion of the HEX activity (determined by fluorometric method) was used for the evaluation of the influence of long-term blood pressure and renal function in kidney donors, and of renal tubular function after transplantation. Excretion of HEX was positively correlated with the excretion of total protein, albumin and β_2 – microglobulin (p= 0.000, p<0.007, and p= 0.002, respectively) (Talseth et al., 1986). Renal allograft function during the first 24 h after transplantation was compared with the function of remaining second kidney of the living donor in the first hour after transplantation. A 4-fold (significantly) higher level of HEX excretion in the recipients compared with the donors, falling to 2–fold (significantly) higher level after 6 hours, and only tendening to increase after 24 h after transplantation, was observed (Bugge et al., 1999). HEX is a specific indicator of ischemia/reperfusion injury of proximal tubules and attenuation of this injury by atrial natriuretic peptide (Chujo et al., 2008). During rejection in 92% of renal allografts, a significant increase in total HEX activity, and decrease in urinary activity of HEX A, with simultaneous increase in activity of HEX B, and intermediary forms of HEX, was observed (Whiting et al., 1983). Urines from transplant patients showed HEX isoenzyme patterns similar to the normal urine, but in episodes of rejection, dependent on the degree on severity of rejection, total HEX activity increased. Increase in total HEX activity was caused by intermediate forms which increased more than 10-fold, however with a simultaneous decrease in activity of HEX A and HEX B (Kind, 1982).

Two mathematical models: model A based on excretion of one, and the model B based on excretion of four enzymes were used for evaluation of the applicability of urinary excretion of **F**ructose-1,6-**Bis**Phosphatase (FBP-1,6), **G**lutathione **S-T**ransferase (GST), HEX and **P**yruvate **K**inase (PK), in renal allograft of patients after transplantation, who were given cyclosporin A (CyA group) or azathioprine and prednisolone (CON-group). In excretion of one enzyme (model A) the best results were obtained in the CON-group, using GST or FBP-1,6 urinary activities. Urinary HEX and PK were less efficient than GST and FBP-1,6. In the CyA group, FBP-1,6 or GST recognized 7 out of 9 graft rejections; PK diagnosed 5, but HEX only 4 rejections. In the CON-group, using model B, four of the above urinary activities were sufficient to detect all 12 graft rejections. FBP-1,6 was the best individual marker enzyme, followed by HEX. The combinations HEX/PK/GST and HEX/GST sufficed to detect all 12 rejections in the CON group, but only one in the CyA group (Kotanko et al., 1986).

Daily monitoring of urinary HEX excretion is a helpful adjunct method in diagnosis of renal damage caused by rejection and ischemia (Sandman et al., 1973), as determination of urinary HEX excretion is a reliable, rapid, sensitive and simple method. Excretion of urinary HEX may be a sign of impending rejection of kidney transplant, and daily measurement of HEX output may be useful in the early diagnosis of rejection (Koivula et al., 1978).

It is stressed that urinary HEX activities measured and reported every day are essential, if an early warning of kidney rejection is to be detected. However, during evaluation of increases in urinary HEX activity, causes of increase not connected with rejection (gentamycine toxicity, urinary tract infection, septicemia, pancreatitis, cardiac failure and thrombosis renal artery or vein) should also be considered (Corbett et al., 1978).

Increases in serum (Loko et al., 1991), and urinary total HEX activity in renal transplant patients (Keyser et al., 1976), caused by HEX intermediate forms, which resemble the

increase caused by oral contraceptive steroids, pregnancy, diabetes mellitus and liver diseases were reported. Chronic renal failure resulted in a decrease of serum HEX activity. In control serums and serum with elevated HEX activity HEX A was the dominant activity. Activity of HEX B was low or not detected. An intermediate serum HEX peak was present as a minor peak in the control sera, but intermediate forms accounted for about 40% of serum HEX activity in the renal transplant group. Urinary HEX provided a sensitive indicator of active renal disease (Thompson et al., 1977). Increased urinary HEX preceded the increase of serum creatinine, and was therefore the earliest detectable indication of rejection. Determinations the urinary HEX available to the clinician on the same day are possible without preservation of urine, and they are not subject to bacterial contaminations. There is agreement that the determination of HEX in urine will provide a slightly earlier results than with other methods, however HEX urinary activities may have a serious limitation in cases of bacterial or toxic complications (Keyser et al., 1976; Thompson et al., 1977).

During evaluation of HEX and **A**lanine **A**mino**P**eptidase (AAP) activities, **C**orrected to baseline **F**ractional **E**xcretion (CFE), and in differential diagnosis of **C**yclo**s**porine **A**cute **N**ephrotoxicity (CsAN) or in **A**cute **R**ejection (AR) crisis, after renal transplantation, CFE values above normal for both HEX and AAP, were more frequently found in episodes of acute rejection, than in cyclosporine acute nephrotoxicity episodes (76vs 0%; p<0.001). Consequently a rise in CFE levels for both HEX and AAP is strongly suggestive of acute rejection crisis (Bornstein et al., 1996). Using a spectrophotometric method for HEX, and separation of urinary inhibitors on Sephadex G-25, the CFE normal value for HEX was 4.26±3.23, and the abnormal was 10.76 + 2SD. Out of 21 AR episodes, 16 had abnormal CFE values for both HEX and AAP, 4 for HEX alone and 1 for AAP alone. Abnormal CFE values for both HEX and AAP were more frequently found in AR episodes than in CsAN episodes (76 vs 0%; p<0.001). The sensitivity, specificity, positive predictive value and diagnostic efficiency in regard to AR and CsAN is 0.84; 0.66; 0.7; 0.73 for HEX alone, and 0.63; 0.95; 0.93; 0.80, for HEX and AAP, respectively. On the basis of above results it was concluded that determination of both enzymes presents a rapid, reliable, cheap, noninvasive and easily available method to help assessing the postoperative period of patients undergoing renal transplantation (Bornstein et al., 1996). Evaluating HEX and AAP as indicators of graft function in renal transplant recipients taking cimetidine, urinary HEX and AAP, excretions were found to be normal throughout the period of treatment with cimetidine, which indicated that observed continuously high concentrations of creatinine in serum were not due to impairment of graft function (Krishna et al., 1986). Therefore urinary activities of HEX and AAP may be valuable as complementary renal-function tests in transplant recipients receiving cimetidine, in addition to being useful as early markers of rejection (Krishna et al., 1986).

Urinary HEX activity is a valuable indicator of drug nephrotoxicity. Very high and overlapping urinary excretion of HEX in about 35% of renal transplant patients treated with cyclosporin and prednisolne, or prednisolone with azathioprine, was reported (Tataranni et al., 1992). However, in the group of patients affected by autoimmune steroid-unsensitive uveitis treated with cyclosporin at progressively decreasing doses, urinary HEX activity decreased in parallel to decreasing cyclosporin doses. HEX release to the tissue culture medium was used as a specific marker of renal tubular cell injury for in vitro evaluation the nephrotoxicity of two immunosuppressive drugs: FK506 and cyclosporin A (Moutabarrik et al., 1992). HEX release to the medium from tubular cells

incubated with 0.1 and 1 µM FK506, as well as 1 µM cyclosporin A for 10, 24, and 48 hr, was not different from that of the control. In contrast to the above statement, 50 µM of FK506, and CsA induced a significant release of HEX to the medium from tubular cells treated for 10, 24, and 48hr (p<0.01) with these drugs, whereas 10 µM CsA significantly increases HEX release only after 48hr incubation. At the same concentrations, CsA seemed to induce a greater HEX release than FK506 (p<0.01) (Moutabarrik et al., 1992). Gentamicin nephrotoxicity accompanied by increased urinary excretion of HEX and β-galactosidase, appeared consistently within three days after the start of treatment, and fell towards pre-treatment levels a week after stopping the treatment (Wellwood et al., 1978). Other antimicrobial agents (ampicillin, ampicillin+cloxacilin, cephalexin, sulphonamides, nalidixic acid, nitrofurantoin, tetracycline) did not change urinary HEX excretion. It is worth of noting that increase in urinary HEX excretion after gentamicine treatment, preceded increase in serum creatinine and urinary proteins. Causes of impairment in renal function in patients with renal allografts are often difficult to determine, therefore rejection of the graft must always be taken into consideration. Graft nephrectomy in two patients and hemodialysis in another two were reported, after gentamycin treatment, and it is concluded that increase in urinary HEX should not be interpreted as an indicator of rejection of renal allograft when the patient is receiving gentamycin (Wellwood et al., 1978). During determination of HEX excretion 24 hr before and 24 hr after intravenous application of ioxaglate (contrast medium) in renal transplant patients, none of the patients receiving ioxaglate, experienced altered urinary HEX excretion, but only a tendency to increase, even in patients treated with cyclosporine, and patients with GFR <60ml/min. (Deray et al., 1995). The only objection to that results is the short time of determination of HEX urinary excretion after application of ioxaglate, as another researchers (Wellwood et al., 1978) observed increase in urinary HEX excretion three days after administration of gentamycine. However, it was found on the rat model, that ioxaglate induced a smaller increase in urinary HEX excretion than diatrizoate sodium meglumine (Deray et al., 1990). It was reported that **PenToXifylline** (PTX) seemed to be temporarily effective in reducing proteinuria and stabilizing renal graft function in more than half of patients at the end of 6 month follow-up in **C**hronic **A**llograft **N**ephropathy(CAN) (Shu et al., 2007). However in CAN, PTX increased urinary HEX excretion in the 3rd month of follow-up, but stabilized in the 6th month, although urinary HEX excretion was still significantly higher than before PTX treatment. Some researchers (Shu et al., 2007) believe that the failure to reduce urinary HEX by PTX implies that renal tubular damage is not modified by PTX, which decrease both proteinuria and urinary HEX excretion in Type 2 diabetic patients (Navarro et al., 2003).

6. Stabilization of FBP-1,6 and HEX activities in human urine

Excretion of enzymes into urine depends on circadian rhythm. The highest urinary excretion rates of arylsulphatase A, α-glucosidase, β-galactosidase and β-glucuronidase, during 24 hour collection, were found betwen 6^{00} – 9^{00} AM, and the lowest between 1^{00} -9^{00} PM. Therefore, collection of urine for 3-hours between 6^{00}- 9^{00} AM was recommended for determination urinary enzymes (Werner et al., 1970). Other authors (Jung et al., 1992) recommend 2-hour collection of urine, i.e. between 6^{00}-8^{00}AM. *Kotanko P.* and *Pfaller W.* (Kotanko et al., 1997; Pfaller et al., 1994) recommend collection of urine from second morning midstream, as an appropriate specimen for analysis of urinary enzymes. The first

portion of urine voided after night, should be definitely avoided, as storage of enzymes in urine during the night, may cause enzyme denaturation (Maruhn, 1983; Werner et al., 1970). Actually, urinary enzymes are commonly determined in urine of the second morning midstream collection, and calculated on gram or mole of excreted creatinine in this urine (Kotanko et al., 1997). Daily urinary creatinine excretion is stable and depends essentially on the muscle mass of the examined person.

During preparation of urine for analysis, epithelia and blood cells (Jung et al., 1992) should be removed by centrifugation in 3000-4500 rev/min by 5-10 min. Low molecular enzyme inhibitors e.g urea, should be removed by gel filtration on Sephadex G-25 or dialysis (Jung et al., 1992; Maruhn, 1979). Fresh urine stored in room temperature for several hours retain activity of the majority of enzymes. Freezing of fresh urine may decrease the activity of some enzymes. It was reported that freezing of urine after dialysis or gel filtration on Sephadex G-25 did not decrease the activity of the majority of urinary enzymes (Jung et al., 1992; Maruhn, 1979; Maruhn, 1983).

FBP-1,6 activity is comparatively stable in fresh urine stored at + 4°C. Therefore for preservation and determination of FBP-1,6, we (Kępka et al., 2009) recommend adjusting the pH of urine to pH 5.5-7.5, as FBP-1,6 activity decreased by 48% and 95%, respectively, in urine stored at pH 4.5, and 8.5. We (Kępka et al., 2009) do not recommend storage of unprotected urine, as the activity of FBP-1,6 stored for 6 days at a temperature of +4°C at pH 4.5 to 8.5, decreased significantly when compared to its initial activity. The pH 5.5-7.5 was optimal for determination and preservation the activity of FBP-1,6 in urine at a temperature of +4°C for 6 days with addition of protective substances. Positive effect of 2-mercaptoethanol (a two-fold increase in FBP-1,6 activity, with 2-mercaptoethanol added) was observed in the homogenate of the renal cortex (Burch et al., 1978). In our experience, the 2-mercaptoethanol (to protect –SH groups) added to urine may be used for successful protection of FBP-1,6 activity in urine stored for 7 days at +4°C (Kępka et al., 2009). Also adding 2-mercaptoethanol, and sodium azide (to prevent bacterial growth) into urine, had a statistically significant positive effect on FBP-1,6 activity during 7 days storage at +4°C. Our results suggest that 2-mercaptoethanol may be used for successful protection of FBP-1,6 activity in urine stored for 7 days at +4°C. We obtained the best protection of FBP-1,6 activity in urine stored for 7 days with 2-mercaptoethanol and sodium azide.

Addition of EDTA into urine had a statistically significant positive effect on FBP-1,6 stability during 4 days of storage at +4°C, but after storage longer than 5 days, significant decrease in FBP-1,6 activity was observed (Kępka et al., 2009). Our results are in agreement with the reports of Pfaller et al. (Pfaller et al., 1994) who, after storing urine for not longer than 5 days at a temperature of +4°C with the addition of EDTA, did not observed a significant decrease in FBP-1,6 activity.

There is the possibility of determining FBP-1,6 in urine stored with EDTA for 4 days at +4°C, because of an insignificant decrease in FBP-1,6 activity (by 4.6%).

HEX and majority of urinary enzymes were stable at pH 5.5-7.5 (Jung et al., 1983). The mean of total HEX activity in urine of normal males and females was 6.8 units (unit= μ mol of methylumbelliferone released from the substrate per hour per litre of urine) /mmol of creatinine, with CV within- batch of 3% and between-batch of 8%. Storage of urine samples at 4°C or at -20°C had little effect on the total HEX activity. Repeated analysis after urine storage for 2 weeks at 4°C showed a decrease which was newer greater than 5% of the total activity. During continuous flow analysis of urinary HEX isoenzymes separated on DEAE cellulose column in normal urine, a large HEX A and

smaller HEX B components were always present, usually in the ratio approximately 4:1. Slight traces of intermediate components, I_1 and I_2 were present in some normal urines, but contributed less than 2% of total activity. The reproducibility of isoenzyme profiles have CV 7.2%, and inclusion of 5% methiolate altered isoenzyme profile by less than 1%. Effect of dialysis on the activity of urinary HEX was minimal with CV of 5.6% (Kind, 1982). HEX is inactivated at urine pH higher than pH 8.0 (Islekel et al., 2007; Jung et al., 1982; Jung et al., 1983), but HEX- B isozyme is comparatively stable in alkaline urine (Islekel et al., 2007).

Urinary HEX activity is stable in fresh urine at room temperature for up to 3 hours, in temp. 4°C ≤ 50 days, and at -20°C for one year. HEX activity in crude urine conserved with 30% glycerol or ethylene glycol, was maintained for over 1 year in -20°C; in gel filtrate or dialyzate for 3 months in 4°C or -20°C (Jung et al., 1992). It was shown that using albumin solution for urine dilution is essential for stability of HEX in diluted urine (Sandman et al., 1973).

7. Conclusion

The literature review revealed that urinary FBP-1,6 and HEX are good markers for monitoring kidney to be transplanted, degree of the ischemic damage of kidney from the moment of resection to transplantation, as well as condition of the patient and transplanted kidney after transplantation. There were reports of cases where enzymuria precedes proteinuria and clinical symptoms of rejection. Determination of urinary FBP-1,6 is more complicated than HEX, because it requires more expensive reagents and strict conditions of urine handling and storage. FBP-1,6 and HEX present a rapid, reliable, cheap (particularly HEX), noninvasive and easily available methods to help assessing the postoperative period of patients undergoing renal transplantation. However, during evaluation of increase in urinary FBP-1,6 and HEX activities, causes of increase not connected with rejection, should also be considered.

8. Acknowledgment

9. References

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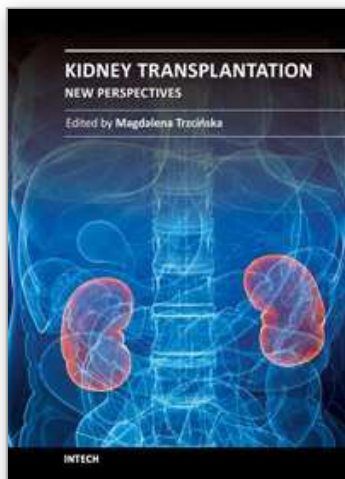
Kidney Transplantation - New Perspectives
Edited by Dr Magdalena Trzcinska

Although many years have passed since the first successful kidney transplantation, the method, although no longer considered a medical experiment, is still perceived as controversial and, as such, it triggers many emotions and that's why conscious educational efforts are still needed for kidney transplantation, for many people being the only chance for an active lifestyle and improved quality of life, to win common social acceptance and stop triggering negative connotations. Apart from transplantation controversies piling up over years transplantologists also have to face many other medical difficulties. The chapters selected for this book are of high level of content, and the fact that their authors come from many different countries, and sometimes even cultures, has facilitated a comprehensive and interesting approach to the problem of kidney transplantation. The authors cover a wide spectrum of transplant-related topics.

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