THE USE OF LYSOSOMAL ENZYMURIA IN THE EARLY DETECTION AND MONITORING OF THE PROGRESSION OF DIABETIC NEPHROPATHY

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ABSTRACT

Recent acquisitions on the early detection and monitoring of the progression of diabetic complications (nephropathy) using the techniques of enzymology (lysosomal enzymes) are reviewed. It appears that the kidney is the principal source of urinary lysosomal enzymes. Urinary samples for lysosomal enzyme determination can be either 24-hour or spot-collection. The use of synthetic substrates (4-methylumbelliferyl substrates) provides an easy, inexpensive, sensitive and highly reproducible method of lysosomal enzyme assay. It is recommended that more than one enzyme be assayed in the process. The use of fractional enzyme excretion (FEE) ratios is further recommended. The urinary lysosomal glycosidases investigated and found to be of particular diagnostic value in the early detection of diabetic nephropathy include N-acetyl- β -D-glucosaminidase (β -hexosaminidase, NAG), β -glucuronidase and β -galactosidase, with NAG being the most useful indicator. Urinary NAG can be used in monitoring the progression of diabetic nephropathy. The fluorimetric assay of lysosomal glycosidases is particularly recommended in developing countries since it is simple, sensitive and inexpensive.

KEY WORDS

Lysosomal enzymuria, diabetic nephropathy, N-acetyl- β -D-glucosaminidase.

INTRODUCTION

Diabetes mellitus is defined as a state of chronic hyperglycaemia, i.e. an excessive concentration of glucose in the blood (1). There are two forms of human diabetes mellitus: non-insulin-dependent diabetes (NIDDM), also referred to as type II, maturity onset, or non-ketotic diabetes; and insulin-dependent diabetes mellitus (IDDM), also referred to as type I, juvenile onset, ketosis-prone diabetes (2). Diabetic nephropathy is the most common cause of end-stage renal failure in both IDDM and NIDDM (3). In the early stages of diabetic nephropathy, there are no clinical signs or symptoms of renal disease. Glomerular changes can be identified only by renal biopsy, and electron microscopy (4,5). The first clinical evidence of nephropathy is microalbuminuria (i.e. the

appearance of low, but abnormal, albumin levels in the urine, >30 mg/24h). Without specific interventions, approximately 80% of type 1 diabetics have their urinary albumin excretion increase at a rate of 10-20%/year to the stage of overt nephropathy or macroalbuminuria (>300 mg/24h; clinical albuminuria) over 10-15 years, developing hypertension along the way. Approximately 30% of individuals with type 2 diabetes are found to have microalbuminuria or macroalbuminuria (overt nephropathy) shortly after the diagnosis of their illness, because diabetes is actually present for many years previously (6). Once overt diabetic nephropathy is established, renal function declines about 10% per year, ending in end-stage renal failure (7).

The choice of this topic was motivated by the increasing prevalence of this disease in third-world countries in general and in Cameroon in particular. Mortality and morbidity rates are high due to a lack of proper management and treatment of accompanying complications. Renal involvement (diabetic nephropathy) is perhaps the most serious complication, and is known to increase morbidity and mortality rates in diabetic patients. We believe that these rates could be lowered by the

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early detection and monitoring of the progression of diabetic complications using the techniques of enzymology, which are simple and inexpensive.

The major factor known to be responsible for changes in serum levels (8,9) and urinary levels (10) of intracellular enzymes is injury to organs or tissues rich in such enzymes. Lysosomal enzymes are found in the kidneys, in most other tissues and in circulating blood cells. These hydrolases catalyse the hydrolysis of glycoproteins, glycolipids and glycosaminoglycans (3), and may act extracellularly to breakdown endothelial membrane glycoconjugates (11). The use of lysosomal enzymes excreted in urine as non-invasive tests for renal damage has been appreciated for years (12).

Urinary Lysosomal Enzyme Assays.

There are several problems one must be aware of when collecting urine samples for lysosomal enzyme analysis (13,14).

1 Urine sample collection and some practical considerations.

Urine samples should be free of contaminants, mid-stream samples are preferable in man. Collection vessels should always be maintained at 4°C to avoid loss of enzyme activity. Inhibitors should be removed prior to assay by short-term dialysis or filtration through sephadex or ultrafiltration, but when fluorimetric techniques are used, dilution is sufficient (15).

Since diurnal variations of urinary enzymes, including lysosomal enzymes, are known to occur, 24-hour urine samples are best (3), or samples should be collected over a carefully timed period, such as between 06:00hrs and 09:00hrs (15). However, in most places in Africa the continuous supply of electricity may not always be reliable, therefore the practical problem of refrigerating specimens between the times of collection and assay, and the inherent instability of some urinary enzymes, may make unfeasible a 24-hour urine sample collection. Hence, it is preferable to perform enzyme assays on a spot urine and report results on a per mg creatinine basis (16). Studies have shown that this is a valid way to estimate and express lysosomal enzymuria (10,12,17).

2. Measurement of Lysosomal Enzyme Activities.

Historically, two general types of assays have been used to estimate lysosomal enzymes, namely colorimetric and

fluorimetric methods. A common substrate used in the colorimetric assay of lysosomal acid phosphatase, for example, is p-nitrophenyl phosphate. However, the p-nitrophenyl glycoside substrates used in the colorimetric method to estimate glycosidase activities (18) have the disadvantage of low sensitivity which requires that large volumes of urine be used. The use of large volumes of urine is undesirable, because this increases the problems caused by inhibitory substances that may be contained in the urine sample.

Nowadays, the fluorimetric assay methods which use 4-methylumbelliferyl glycoside substrates are much more popular than the colorimetric procedures. The fluorimetric glycosidase assays are simple and inexpensive and allow for the sensitive and reproducible assay of lysosomal glycosidases (3). The fluorimetric method is about 20 times more sensitive than the spectrophotometric procedure (19). The basic fluorimetric assay is exemplified by the following procedure that is used for the assay of N-acetyl- β -D-glucosaminidase (NAG; β -hexosaminidase), β -glucuronidase, β -galactosidase, and acid phosphatase. The incubation medium for β -hexosaminidase contains 2.0mM 4-methylumbelliferyl- β -D-glucosaminide in 0.1M sodium citrate buffer (pH 4.6) with bovine serum albumin (1mg/ml). The β -glucuronidase incubation medium contains 10mM 4-methylumbelliferyl- β -D-glucuronide in 1M sodium acetate buffer (pH 4.8). The β -galactosidase incubation medium contains 3.33mM 4-methylumbelliferyl- β -D-galactoside in citrate-phosphate buffer with 0.2M sodium chloride (pH 4.4). The incubation medium for acid phosphatase contains 10mM 4-methylumbelliferyl-phosphate in 1M sodium acetate buffer (pH 4.5) (10,20,21,22,23).

The reaction is initiated by adding 0.01ml of urine to 0.09ml of incubation mixture for each enzyme assay. All assays are performed at 37°C; the incubation time being 30 min for β -hexosaminidase, β -glucuronidase and β -galactosidase, and 15 min for acid phosphatase. The reaction is terminated by adding 2.9ml of 0.3M glycine/ammonia buffer (pH 10.0), to make a final volume of 3.0ml. The amount of fluorescent 4-methylumbelliferone product, released from the appropriate substrate, is determined using a Turner model III fluorimeter with settings of 360nm for excitation and 520-580nm for emission against a reaction blank. The fluorimetric assays are highly reproducible, usually exhibiting a standard error of 4% or less (3,10,12,16,20,21,22,23). Urinary enzyme activities are expressed as units/mg creatinine. One unit of enzyme activity equals 1nmol of substrate hydrolysed per hour at 37°C. Berty *et al* (24) introduced the use of fractional enzyme

excretion (FEE) ratio to compare the urinary excretion of lysosomal enzymes under a variety of physiological conditions. The FEE value is the ratio of enzyme clearance to creatinine clearance and is calculated from the following equation:

$$\text{FEE} = \frac{(\text{U/P}) \text{ Enzyme activity (Units/ml)}}{(\text{U/P}) \text{ creatinine (mg / dl)}} \times 100$$

Where U = urine and P = plasma, U/P = urine to plasma ratio.

The greatest advantage of the FEE ratio is that it takes into consideration the blood level of a particular enzyme, as well as the enzyme's concentration in the urine. The value of this ratio has been demonstrated in several recent studies (10,12). For example, VenderJagt *et al* (16) used the ratio to compare the urinary excretion of some lysosomal hydrolases in healthy elderly and young adults to evaluate changes in renal function with age.

Significance of Lysosomal Enzymuria as a marker of the preclinical stage of diabetic nephropathy.

The kidney readily compensates when damaged and since it also has an excess of functional capacity, classical tests of renal function such as the measurement of glomerular filtration rate (GFR) and tubular reabsorption maxima lack sensitivity (15).

Urinary lysosomal enzymes have been used in nephropathy studies in humans and animals in 2 ways. Firstly, they provide a sensitive non-invasive test for renal damage and with careful selection of enzymes they can be used to determine the primary site of damage in the nephron (25). For example, b-hexosaminidase, the most studied urinary lysosomal enzyme, b-galactosidase and b-glucuronidase are all located in the proximal tubule of the nephron (3,26), while the specific activity of acid phosphatase is enriched in the glomeruli. Secondly, because the excretion of urinary enzymes varies with the activity of renal disease, they can be used to monitor the rate of recovery (27). In diabetic patients, urinary excretion of lysosomal enzymes appears before endogenous creatinine clearance, blood urea nitrogen or urinary osmolality values are altered (28).

Urinary excretion of smaller molecular weight proteins such as N-acetyl-β-D-glucosaminidase (NAG) and retinol binding protein (RBP) indicate proximal tubular dysfunction, and may identify diabetic patients at risk of developing diabetic

nephropathy (29). In a trial to assess renal tubular function, Salema *et al* (29) found that diabetic patients with microalbuminuria had concomitant renal tubular disorder indicated by high urinary NAG and RBP. Meanwhile, patients without microalbuminuria had both tubular markers excreted in urine in significantly higher amounts than controls.

β-Hexosaminidase, with a molecular weight of about 140,000 da, is probably excreted in urine not as a result of glomerular leakage or tissue damage (28). This assertion is supported by the serum concentration of the enzyme which is too low to account for the urinary rate of excretion, by the differences of serum and kidney isoenzymes of the enzyme and by the fact that other non-lysosomal kidney enzymes are not increased in diabetic urine (30). In the study by Bomback *et al* (31) it was found that urinary excretion of β-galactosidase was significantly higher in both diabetic female and male patients compared with controls, whereas the change in the serum level of activity was not statistically significant.

To identify early markers of the preclinical stage of diabetic nephropathy, a study was made of the activities of the specific tubular enzymes, such as N-acetyl-β-D-glucosaminidase (NAG), β-glucuronidase, γ-glutamyl transferase, alkaline phosphatase, lactate dehydrogenase (32) and β-galactosidase (31,33), and albumin, total protein and β-2-microglobulin (34) in urine and/or serum, in various stages of diabetic nephropathy. Patients without the clinical signs of diabetic nephropathy (i.e. proteinuria) manifested a significant rise of excretion of lysosomal enzymes of the proximal tubules (NAG, β-glucuronidase and β-galactosidase), with NAG activity being the highest (34,35). Puncture biopsy of the kidneys was made in patients without proteinuria, and with concomitant elevation of the excretion of lysosomal enzymes, and electron microscopy revealed changes in the proximal tubules (32). The morphological changes in renal tissue, as shown by investigations of renal biopsies, confirm the diagnostic significance of lysosomal enzymuria as a marker of early tubular involvement, i.e. as a marker of the preclinical stage of diabetic nephropathy (36).

Increase in urinary activity of lysosomal enzymes appeared in diabetic patients prior to clinical proteinuria, and with appearance of proteinuria, urinary lysosomal enzymes (i.e. NAG, β-glucuronidase and β-galactosidase) more increased, indicating the severity of the nephropathy (34). They could therefore be used in the monitoring of the progression of diabetic nephropathy (3). This is supported by the study of Maisant *et al*. (37) on children suffering from diabetes mellitus,

which showed that the increasing level of urinary NAG might represent the increasing severity of nephrotic damage by diabetes.

Onset of Diabetic Nephropathy

Renal involvement (nephropathy) is perhaps the most serious complication of diabetes mellitus, the hallmark being proteinuria (38). Alterations in the size and charge selectivity of the glomerular basement membrane (GBM) have been considered as possible mechanisms responsible for the development of proteinuria in a diabetic individual (39). In the glomerulus, receptors for insulin are found in both the epithelial and endothelial cells. Insulin is felt to be concerned mainly with the control of basement membrane synthesis which may help explain why insulin lack may be associated with disordered basement membrane synthesis (40). Heparan sulfate is the predominant glycosaminoglycan (GAG) proteoglycan in the GBM. It confers a negative charge on the GBM, and its loss has been related to the presence of albumin in the urine (41).

Genetics plays an important role in the development of diabetic nephropathy: Patients who have one or two deletions of the angiotensin-converting enzyme (ACE) gene, a defect in the sodium proton pump, or a family history of hypertension are at increased risk for progression to diabetic nephropathy (42). However, in such patients, nephropathy does not occur until type 1 diabetes develops; the worse and more prolonged the hyperglycaemia, the greater the risk of diabetic nephropathy (43).

Early diabetic glomerulopathy has been detected by electron microscopy in normoalbuminuric patients and found to be more advanced in those with microalbuminuria and proteinuria (4). Structural changes underlying diabetic nephropathy in Type 1 diabetes are predominant in the glomerulus. Glomerulopathy, characterized by thickening of the glomerular basement membrane (GBM) and mesangial expansion, is the most important renal structural change in type 1 diabetic patients with diabetic nephropathy. Morphological lesions develop concomitantly in the arterioles, tubules and interstitium. Mesangial fractional volume [V_v (mes/glom)], an estimate of mesangial expansion, is the structural parameter that best correlates with glomerular filtration rate (GFR) and it is also closely related to the presence of proteinuria and hypertension (44). Collagen IV deposition precedes the hyperfiltration and enlargement of glomeruli in early-stage diabetic nephropathy. Dedifferentiation of mesangial cells may be associated with collagen IV deposition (45).

The course of renal function is heterogeneous in type 2 diabetic patients and reflects heterogeneous patterns of renal lesions. A subset of patients with normoalbuminuria (5), microalbuminuria and proteinuria is characterized by the typical diabetic glomerulopathy usually observed in type 1 diabetic patients, with altered albumin excretion rate (AER), for example, glomerular basement membrane thickening and mesangial fractional volume expansion (38). Glomerular hypertrophy, one of the histological changes in diabetic nephropathy, is present in type 2 as well as in type 1 diabetic patients (5).

A proposed mechanism for development of nephropathy in patients with type 1 diabetes is elevation of growth hormone levels due to poor glycaemic control, resulting in hyperperfusion of the glomerulus and glomerular hypertension. Mesangial cells in the glomerulus respond to glomerular hypertension by producing growth factors, especially tumor necrosis factor- α , which results in increased glomerular permeability, proliferation of glomerular epithelial cells, and excessive production of basement membrane and collagen tissue. These effects eventually lead to glomerular scarring and renal failure (46).

Renal tubular damage may already exist in early-stage of diabetic nephropathy in both type-1 and type-2 diabetic patients. Maeda *et al* (47) reported renal hypertrophy, glomerular injury and structural alteration of the proximal straight tubules during the acute phase of diabetes in nonobese diabetic mice. The renal lesions in type-2 diabetic patients may be caused by cardiovascular diseases such as arteriosclerosis and hypertension (48). Microalbuminuria (MA), i.e. slightly elevated albumin excretion in the urine, is now considered to be an atherosclerotic risk factor. MA predicts future cardiovascular disease risk in diabetic patients, in elderly patients, as well as in the general population. It has been implicated as an independent risk factor for cardiovascular disease and premature cardiovascular mortality for patients with type 1 and type 2 diabetes mellitus, as well as for patients with essential hypertension. Although microalbuminuria is associated with a certain degree of sub-clinical atherosclerotic damage, it is not known how early in the atherosclerotic process microalbuminuria appears (49).

Oxidative stress has been considered to be a common pathogenetic factor of diabetic nephropathy (50,51). But the reason why renal cells are susceptible to oxidative injury in diabetes is not clear. Vitamin C plays a central role in the antioxidant defense system and exists in two major forms. The charged form, ascorbate, is taken up into cells via

sodium-dependent facilitated transport. The uncharged form, dehydroascorbate, enters cells via glucose transporter and is then converted back to ascorbate within these cells (50). Because dehydroascorbate and glucose compete for glucose transporters, hyperglycemia will exclude vitamin C from the cell and resulted in a decreased antioxidant capacity in some cell type that is dehydroascorbate dependent. As such, we hypothesized that some renal cells were dehydroascorbate dependent and the susceptibility of renal cells to glucose-induced injury was mediated by hyperglycemic exclusion of dehydroascorbate uptake through competing for glucose transporter. Tubular epithelial cells are dehydroascorbate dependent. Vitamin C exclusion from tubular epithelial cells through competition of glucose and dehydroascorbate for common transport mechanism in diabetes will deprive the cells of antioxidant ability and could lead to reactive oxygen species accumulation (50). Dicarbonyl also may play important roles in the development of diabetes complications, and its response to hyperglycemia could determine individual susceptibility to diabetic nephropathy. Progression of diabetic nephropathy is significantly related to elevated dicarbonyl stress (51).

CONCLUSIONS

1. Renal function can be impaired in both type-1 and type-2 diabetic patients. Renal tubular damage may already exist in early-stage of diabetic nephropathy.
2. The kidney is the principal source of urinary lysosomal enzymes. Urinary samples for lysosomal enzyme determination can be either 24-hour or spot-collection.
3. The use of synthetic substrates (4-methylumbelliferyl substrates) provides an easy, inexpensive, sensitive and highly reproducible method of lysosomal enzyme assay. It is recommended that more than one enzyme be assayed in the process. The use of fractional enzyme excretion (FEE) ratios is further recommended.
4. The urinary lysosomal glycosidases investigated and found to be of particular diagnostic value in the early detection of diabetic nephropathy include N-acetyl- β -D-glucosaminidase (β -hexosaminidase, NAG), β -glucuronidase and β -galactosidase, with NAG being the most useful indicator.
5. Urinary NAG can be used in the monitoring of the progression of diabetic nephropathy.
6. The fluorimetric assay of lysosomal glycosidases is particularly recommended in developing countries since it is simple, sensitive and inexpensive.

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