Alteration of urinary sorbitol excretion in WBN-kob diabetic rats – treatment with an aldose reductase inhibitor

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Abstract

An accelerated polyol pathway in diabetes contributes to the development of diabetic complications. To elucidate diabetic nephropathy involving also renal tubular damage, we measured urinary sorbitol concentration concomitantly with urinary N-acetyl-D-glucosaminidase (NAG) excretion in WBN-kob diabetic rats.

Twenty-four-hour urinary sorbitol concentrations increased in the diabetic rats in parallel with whole blood sorbitol concentrations. An increase in 24-h urinary NAG excretion coincided with the elevated urinary sorbitol levels in the diabetic rats.

The administration of epalrestat, an aldose reductase inhibitor, reduced the increased whole blood and urinary sorbitol concentrations and urinary NAG excretion concomitantly with renal aldose reductase inhibition in the diabetic rats.

These results indicate that diabetic nephropathy involves distorted cell function of renal tubules, and that treatment with epalrestat may prevent at least the progress of the nephropathy.

Journal of Endocrinology (2004) **181**, 429–435

Introduction

A number of experimental and clinical studies suggest that, in addition to glycation, activation of the polyol pathway may play an important role in the development of diabetic complications (Gabbay 1973). The polyol pathway is an alternative route of glucose metabolism which is composed of two enzyme reactions, catalyzed by aldose reductase and sorbitol dehydrogenase. Aldose reductase, the rate-limiting enzyme in this pathway, catalyzes the reduction of glucose to sorbitol. Sorbitol is subsequently converted to fructose by sorbitol dehydrogenase. It is believed that acceleration of this pathway occurs in hyperglycemic metabolism, contributing to development of diabetic complications. To estimate the glucose metabolic state of the whole body and peripheral tissues, many investigators have determined sorbitol levels in various tissues, such as the erythrocytes, peripheral nerves, the lens, the retina and renal tissues, and analyzed the correlation between sorbitol levels and diabetic complications (Malone *et al.* 1980, Greene *et al.* 1985, Naeser & Brolin 1991, Stevens *et al.* 1995). Elevated levels of urinary sorbitol excretion measured by gas chromatography (Yoshii *et al.* 2001) or using the improved

method (Nakano *et al.* 2003) were also observed in the diabetic patient.

Recently, many aldose reductase inhibitors have been developed and have been shown to prevent or ameliorate diabetic complications (Kador *et al.* 1985); thus the importance of the polyol pathway in diabetes has attracted more attention.

On the other hand, it has been reported that N-acetyl-D-glucosaminidase (NAG) activity in urine increased during the development of the early stages of diabetic nephropathy, and is correlated with the increase of urinary microalbumin and other parameters relevant to diabetes (UK Prospective Diabetes Study 1993, Hsiao *et al.* 1996, Kato *et al.* 1997, Yamanouchi *et al.* 1998, Ishii *et al.* 2001). Although NAG is widely distributed in various organs, it is especially concentrated in the epithelial cells of the renal proximal tubules (Price 1992). Thus, the increase in urinary NAG activity suggests that the cell injury is in the renal proximal tubules.

Taken together, these findings prompted us to evaluate diabetic renal function, measuring urinary sorbitol levels, using our improved method, and NAG excretion in Wistar-Bonn/Kobori (WBN-kob) rats, a spontaneously

generated diabetic model, in response to treatment with an aldose reductase inhibitor.

Materials and Methods

Chemicals

Sorbitol, sodium hydroxide, zinc sulfate, ethylenediamine-N,N,N,N-tetraacetate (EDTA) and tris (hydroxymethyl) aminomethane (Tris) were of special grade and were purchased from Wako Pure Chemicals (Osaka, Japan). B-5 ion exchange resin was purchased from Katayama Chemical Co. (Nagoya, Japan). NAD^+ was obtained from Oriental Yeast Co. (Tokyo, Japan). Sheep liver sorbitol dehydrogenase (SDH) was from Roche Diagnostics GmbH (Mannheim, Germany) and *Pseudomonas* SDH was from Kikoman Co. (Tokyo, Japan).

Animals

Seventeen male 55-week-old WBN-kob rats weighing 330–370 g were purchased from Japan SLC Inc. Lab. (Hamamatsu, Japan). Rats were housed in a temperature-controlled room with lights on from 0700 to 1800 h and were allowed a standard rat chow diet and water available *ad libitum*. Two weeks after receiving the rats from the Japan SLC Inc. Lab., rats with blood glucose levels over 13.9 mmol/l were used as diabetic rats. The diabetic rats were randomly divided into two groups, i.e. diabetic rats and epalrestat-treated diabetic rats. Five male age-matched Wistar rats from Japan SLC Inc. Lab. were used as controls.

Epalrestat (an aldose reductase inhibitor, donated by Ono Pharmaceutical Co., Ltd, Osaka, Japan) was administered by oral gavage once daily at a dose of 50 mg/kg body weight for 49 days. Body weights of these rats were measured weekly and 24-h urinary specimens were collected before and every ten days after the onset of diabetes.

Whole blood specimens were obtained from cardiac paracentesis. The experiments conformed to the Guidelines on the Handling and Training of Laboratory Animals in our university.

Preparation of whole blood samples

One milliliter of whole blood obtained from each group of rats was diluted with distilled water to a final volume of 6.0 ml. After the addition of 1.0 ml of 0.475 mol/l NaOH, 1.0 ml of 0.293 mol/l ZnSO_4 was added. The mixture was stirred and centrifuged at $2000 \times g$ for 10 min, and the supernatant was used for whole blood samples.

Preparation of urine samples

Each group of rats was placed in individual metabolic cages to collect urine for a 24-h period for the measurement of

sorbitol concentrations. The rats were fed and given water during the collection of urine specimens, which were kept at -4°C prior to analysis.

Two milliliters of the urinary specimens were diluted with 1.0 ml distilled water and this diluted urine specimen was applied to an Amberlite MB-3 (ORGANO Corp., Tsukuba Plant, Japan) column (1×12.5 cm) to remove ionic substances which interfere with the assay system. Then, 20 ml distilled water were applied to the column to elute the urine specimen. After the addition of 1.0 ml of 0.475 mol/l NaOH to 5 ml of the eluate, 1.0 ml of 0.293 mol/l ZnSO_4 was added, and the mixture was stirred and centrifuged at $2000 \times g$ for 10 min. Then, the supernatant was used for urine samples.

Determination of sorbitol concentration

Sorbitol was assayed by a fluorometric and enzymatic method as described previously (Shinohara *et al.* 1998b). In brief, 2.0 ml whole blood or urine sample were mixed with 1.0 ml of 0.15 mol/l Tris-HCl buffer (pH 8.6) containing 4.5 mmol/l EDTA, 3.0 mmol/l NAD^+ and 2 U/ml SDH. This reaction mixture was incubated at 37°C for 30 min. Another 2.0 ml of the sample, mixed with 1.0 ml Tris-HCl buffer without SDH were also incubated as a blank. The relative fluorescence of NADH produced in the conversion of sorbitol to fructose catalyzed by SDH was measured using a JASCO spectrofluorometer FP-777 (JASCO, Tokyo, Japan), with an excitation wavelength of 366 nm and an emission wavelength of 452 nm. The inter- and intra-assay coefficients of variation were 4.4 and 4.5% respectively.

Measurement of urinary N-acetylglucosaminidase (NAG) activity

The urinary total NAG activity was determined with a spectrophotometric assay kit using 3,3-dichlorophenyl-sulfon-phthaleinyl-NAG as a substrate (Shionogi Pharmaceutical Co. Ltd, Osaka, Japan) (Noto *et al.* 1983).

The mean analytical recovery of NAG in human urine was 101%. The within- and between-assay coefficients of variation were about 5%.

Measurement of aldose reductase activity in rat kidney

Aldose reductase activity was assayed according to the method reported previously (Shinohara *et al.* 1998a).

Protein assay

The protein concentration in the sample was determined using the Bio-Rad protein assay kit (Bio-Rad Lab., Hercules, CA, USA).

Statistical analysis

ANOVA and a *post hoc* (Bonferroni/Dunn) test or Kruskal-Wallis test were used for comparing groups;

Table 1 Body weights and blood and urinary glucose levels in control, diabetic and Epalrestat-treated diabetic rats. Oral administration of Epalrestat at a dose of 50 mg/kg BW/day was started at 57 weeks of age (A=initial values at the beginning of the study) for 49 consecutive days (B=absolute values at the end of the study). Each value is the mean $\pm$ S.E.

Group	n	Body weight (g)		Blood glucose (mmol/dl)	Urinary glucose (mmol/mg:creatinine)
		A	B	B	B
Control	5	339 $\pm$ 9	634 $\pm$ 24	0.63 $\pm$ 0.03	0.007 $\pm$ 0.003
Diabetic	7	330 $\pm$ 8	312 $\pm$ 11*	1.78 $\pm$ 0.10*	7.809 $\pm$ 1.172*
Epalrestat-treated diabetic	10	347 $\pm$ 9	331 $\pm$ 11*	1.71 $\pm$ 0.11*	7.876 $\pm$ 0.944*

* $P < 0.05$, significantly different from the control group.

$P < 0.05$ was considered statistically significant. Linear relations between variables were evaluated by regression analysis.

Results

The changes in body weight and fasting blood glucose levels for all rat groups are shown in Table 1. In the diabetic rats, body weights were significantly decreased and fasting blood glucose levels were increased compared with control rats. The administration of epalrestat had no effect on the body weight or fasting blood glucose levels in the diabetic rats.

Table 2 shows whole blood sorbitol levels per gHb (Hb concentrations were similar in all groups) and urinary sorbitol and NAG excretion (similar results were obtained compared with those per day or per creatinine) in each group.

Diabetic rats revealed significantly higher levels of whole blood sorbitol than control rats. In epalrestat-treated diabetic rats, whole blood sorbitol concentrations tended to be suppressed compared with non-treated diabetic rats.

In diabetic rats, 24-h urinary sorbitol excretion, expressed as the amount of the increase at the end of the experiment, was significantly increased compared with

that of control rats. In epalrestat-treated diabetic rats, 24-h sorbitol excretion was significantly suppressed (0.824 $\pm$ 0.135 μ mol/mg creatinine) compared with diabetic rats.

Figure 1 shows a significant positive correlation between whole blood sorbitol concentration and 24-h urinary sorbitol excretion in control, diabetic and epalrestat-treated rats. Twenty-four-hour urinary sorbitol concentrations also correlated with fasting blood glucose and urinary glucose levels in control rats or in diabetic and epalrestat-treated rats (Figs 2 and 3).

To investigate whether 24-h urinary sorbitol excretion plays a role in diabetic nephropathy, we measured 24-h urinary NAG activity, which is a measure of diabetic renal function, and compared it with the change in 24-h urinary sorbitol excretion in these rats. Urinary NAG activity, expressed as the amount of the increase at the end of the experiment, is shown in Table 2. The diabetic rats showed a significant increase in urinary NAG activity in comparison with control rats. However, in epalrestat-treated diabetic rats, the increase in urinary NAG activity was significantly suppressed compared with that of non-treated diabetic rats.

Figure 4 shows a significant positive correlation between the 24-h urinary NAG activity and the 24-h sorbitol excretion in control rats or in diabetic and epalrestat-treated rats.

Table 2 Changes in whole blood sorbitol concentration (absolute values at the end of the study) and 24-h urinary sorbitol and NAG excretion (values are the difference between the initial value and that at the end of the study) in control, diabetic and Epalrestat-treated diabetic rats. Each value is the mean $\pm$ S.E.

Group	n	Whole blood sorbitol level (μ mol/gHb)	Urinary sorbitol level (μ mol/mg:creatinine)	Urinary NAG excretion (mU/mg:creatinine)
Control	5	0.55 $\pm$ 0.80	0.196 $\pm$ 0.031	10.8 $\pm$ 1.9
Diabetic	7	2.41 $\pm$ 0.41*	1.192 $\pm$ 0.072**	58.3 $\pm$ 7.8*
Epalrestat-treated diabetic	10	2.11 $\pm$ 0.18*	0.824 $\pm$ 0.135**†	35.8 $\pm$ 5.7*†

* $P < 0.001$, ** $P < 0.01$ compared with the control group; † $P < 0.05$ compared with the diabetic group.

Figure 1 Correlation between whole blood sorbitol and urinary sorbitol concentrations in control (●), diabetic (□) and epalrestat-treated diabetic (▲) rats. The solid line represents the combined control and diabetic rats. The dotted line represents the diabetic (epalrestat-treated and untreated) rats.

We also assessed the accelerated polyol pathway in the diabetic kidney by measuring aldose reductase (AR) activity and sorbitol content in all groups (Table 3).

In the diabetic state, AR activity and sorbitol content of the kidney were significantly increased compared

with those of control rats. In diabetic rats treated with epalrestat, the AR activity was significantly suppressed compared with that of none-treated diabetic rats. However, the sorbitol concentration tended to be reduced compared with that of diabetic rats, but was not statistically significant.

Figure 2 Correlation between urinary sorbitol excretion and fasting blood glucose levels in control (●), diabetic (□) and epalrestat-treated diabetic (▲) rats. The solid line represents the combined control and diabetic rats. The dotted line represents the diabetic (epalrestat-treated and untreated) rats.

Figure 3 Correlation between urinary sorbitol excretion and urinary glucose excretion in control (●), diabetic (□) and epalrestat-treated diabetic (▲) rats. The solid line represents the combined control and diabetic rats. The dotted line represents the diabetic (epalrestat-treated and untreated) rats.

Discussion

It is of crucial importance in diabetic treatment to prevent diabetic complications, such as nephropathy, as well as to assess long-term glycemic control. Although various factors are considered to be involved in the development of

diabetic complications, the activation of the polyol metabolic pathway caused by hyperglycemia has a critical role. Therefore, various investigators have evaluated the polyol pathway in various diabetic organs to predict the development of diabetic complications (Gabbay 1975). For example, sorbitol concentrations in erythrocytes, as an

Figure 4 Correlation between urinary sorbitol excretion and urinary NAG activities in control (●), diabetic (□) and epalrestat-treated diabetic (▲) rats. The solid line represents the combined control and diabetic rats. The dotted line represents the diabetic (epalrestat-treated and untreated) rats.

Table 3 Renal aldose reductase (AR) activity and sorbitol concentration in control, diabetic and Epalrestat-treated diabetic rats. Each value is the mean $\pm$ S.E. and is the absolute value at the end of the study

Group	<i>n</i>	AR activity (mU/mg protein)	Sorbitol concentration (nmol/mg protein)
Control	5	816 $\pm$ 8	2.98 $\pm$ 0.15
Diabetic	7	969 $\pm$ 10*	5.83 $\pm$ 0.55*
Epalrestat-treated diabetic	10	911 $\pm$ 9*†	5.09 $\pm$ 0.23*

* $P < 0.01$ compared with the control group; † $P < 0.01$ compared with the diabetic group.

intermediate metabolite of glucose metabolism, have been shown to correlate with the amount of sorbitol in nerve tissue and with retinopathy (Clements 1979, Greene 1983, Naeser & Brodin 1991). Several hypotheses explaining how the accelerated polyol pathway relates to the development of diabetic complications are proposed as follows: depletion of myo-inositol, resulting in suppression of phosphoinositide turnover and subsequent metabolic abnormalities (Greene & Lattimer 1983), decreased availability of NADPH (Lee *et al.* 1985), a cascade of metabolic imbalances initiated by an increased cytosolic ratio of free NADH/NAD⁺ resulting from increased oxidation of sorbitol to fructose (Williamson *et al.* 1993), and osmotic stress due to accumulation of intracellular sorbitol (Wick & Drury 1951). In addition to these hypotheses, accumulated evidence suggests that glycation and oxidative stress may have an interaction with the polyol pathway (Giugliano *et al.* 1996, Hamada *et al.* 1996).

We have previously reported an improved fluorometric and enzymatic method for measuring urinary sorbitol concentrations, which showed good sensitivity and reproducibility (Nakano *et al.* 2003) and confirmed that urinary sorbitol excretion and whole blood sorbitol levels in diabetic patients were significantly higher than those in nondiabetic controls, and that the difference was more significant in urine than in whole blood (Nakano *et al.* 2003). In the present study, we measured urinary sorbitol and NAG excretion as well as whole blood sorbitol concentration, and evaluated their correlation with AR activity in spontaneously generated diabetic WBN-kob rats. Both urinary sorbitol excretion and whole blood sorbitol levels increased in the diabetic state and were significantly correlated. Urinary sorbitol excretion also significantly correlated with fasting blood glucose and urinary glucose levels. Treatment with epalrestat significantly suppressed the increased urinary sorbitol excretion in the diabetic rats, in concordance with whole blood sorbitol concentration (Hotta 1995, Hayashi *et al.* 1998). Therefore, it is thought that urinary sorbitol excretion reflects the activated polyol pathway induced by hyperglycemia in various organs, consistent with our previous report using streptozotocin-induced diabetic rats

(Shinohara *et al.* 1998a) and as the polyol pathway exists in mesangial cells of rat glomeruli and may have some role in the development of mesangial cell dysfunction of streptozotocin-induced diabetic rats (Kikkawa *et al.* 1987), the measurement of urinary sorbitol levels may be a sensitive indicator of the control of the diabetic nephropathy.

We also demonstrated that there was good correlation between urinary sorbitol excretion and urinary NAG activity, which would be anticipated as an indicator of renal proximal tubular function. This glycolytic enzyme, NAG, which catalyzes the change of N-acetyl-D-glucosaminide into N-acetyl-D-glucosamine exists within the lysosome in the cell and consists of two major isozyme forms, A and B (Price 1992). Most of the urine from patients with renal glomerular and tubular lesions showed significant increases in values or percentages (20–30%) of NAG isozyme B (Numata *et al.* 1997). In diabetic patients total high urinary NAG activity might reflect lysosomal dysfunction of both glomerular and proximal tubular epithelial cells (Morita *et al.* 1991). The proportion of NAG isozyme B was elevated in the urine from diabetic patients when compared with normal individuals (Severini *et al.* 1988) and NAG isozyme B concentration in the spot urine specimens correlated well with total NAG activity (Itoh *et al.* 1994), probably because the molecular size of isozyme A normally prevents its passage through the glomerulus.

Although high NAG isozyme B levels were noted in urine collected from normal males who had ejaculated semen (Itoh *et al.* 1994), in our study each rat was kept separate in a cage to avoid copulation, and unexpectedly high values of NAG activity were excluded from the data.

Thus, the increase in urinary NAG activity in our diabetic rats may imply that diabetic nephropathy involves damage to the cells and cell function of renal tubules.

Moreover, these distorted metabolic parameters in the diabetic condition were improved by the administration of an AR inhibitor, suggesting that this treatment may be beneficial for preventing the progress of diabetic microangiopathy.

Acknowledgements

The authors thank Ms N Takekawa for preparing the manuscript.

Funding

This funding was supported by a grant from Fujita Health University.

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Received in final form 27 February 2004
Accepted 3 March 2004