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Urinary Excretion of Ceruloplasmin Is Elevated in the Subjects with "Borderline Glucose Tolerance Test"

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MURATA, M., NARITA, T., KOSHIMURA, J. and ITO, S. *Urinary Excretion of Ceruloplasmin Is Elevated in the Subjects with "Borderline Glucose Tolerance Test"*. Tohoku J. Exp. Med., 1999, **188** (1), 1-10 —— To examine whether or not there are any renal alterations in subjects with borderline glucose tolerance and in patients with non-insulin dependent diabetes mellitus (NIDDM) classified by the criteria of Japan Diabetic Association, urinary excretions of plasma proteins including albumin, ceruloplasmin (Cerulo) and IgG were measured in timed overnight urine samples. Eighty middle-aged, non-obese, normotensive, untreated men with urinary albumin excretion rates below 20 μg/minutes, β_2-microglobulin excretion rates below 140 μg/minutes and creatinine clearance values exceeding 80 ml $\cdot$ min^{-1} $\cdot$ (1.73 m^2)$^{-1}$ were included in this study. Three groups were defined according to the results of 75 g oral glucose tolerance test (OGTT) as follows: D group, 10 subjects with NIDDM; B group, 40 subjects with "borderline glucose tolerance test" and N group, 30 subjects with normal glucose tolerance. The fractional clearance (Θ) of Cerulo, but not albumin and IgG, was elevated in 37.5% of the B group compared with the upper limit of that of the N group. Furthermore, Θ-Cerulo and Θ-IgG increased in the D group compared with those of the N and the B groups. Recently, we found that Θ-Cerulo and Θ-IgG increased in healthy volunteers when GFR was elevated by acute protein loading and that increase in Θ-Cerulo is remarkable than increase in Θ-IgG. The present result, taken together with our recent finding mentioned above, suggests that increases in Θ-Cerulo and Θ-IgG may not be due to an impairment of charge selectivity in the glomerular basement membrane, but due to an increase of intraglomerular hydraulic pressure. ———— ceruloplasmin; borderline diabetics; diabetic nephropathy

Proteinuria, including albuminuria, is involved in the onset and progression of diabetic nephropathy (Parving et al. 1996). Proteinuria in diabetic nephropathy has been reported to be due to increased intraglomerular hydraulic pressure (Hostetter et al. 1982; Viberti and Keen 1984; Zatz et al. 1985; Anderson and Brenner 1988), loss of charge selectivity (Deckert et al. 1984; Nakamura and

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Myers 1988; Gausch et al. 1993), loss of pore size selectivity in the glomerular basement membrane (Myers et al. 1982; Winetz et al. 1982) or combination of these factors. Although loss of pore size selectivity seems to be present in advanced diabetic nephropathy of patients with insulin dependent diabetes mellitus (IDDM) (Friedman et al. 1983; Tomlanovich et al. 1987; Deckert et al. 1993), controversy remains regarding which of these three factors precedes the other two and which factor plays an important role in the onset of diabetic nephropathy in non-insulin dependent diabetes mellitus (NIDDM). Furthermore, it is unclear whether or not these three factors occur in non-diabetic subjects with slight hyperglycemia.

To examine whether or not there is any renal involvement in non-diabetics with slight hyperglycemia, subjects with "borderline glucose tolerance test (borderline GTT)" as classified according to the criteria of the Japan Diabetes Association (Japan Diabetes Association [JDA] Committee 1982), were selected from a population given 75 g oral glucose tolerance test (OGTT). The urinary excretions of albumin, IgG and ceruloplasmin (Cerulo) in timed overnight urine samples from these subjects were compared with those from subjects with normal glucose tolerance and from non-insulin dependent diabetic patients with normoalbuminuria.

Materials and Methods

Subjects

Male subjects between the ages of 35 and 65 years who had undergone the two-day screening examination at Akita Red-Cross Hospital were recruited into this study. Careful examination of the subject's medical histories showed that they had no previous renal, cerebrovascular or peripheral vascular diseases and had not received any medication. They were also free from coronary artery disease, according to the result of a questionnaire on symptoms of angina pectoris and electrocardiogram's finding. Systolic and diastolic blood pressure was measured in the sitting position three times over two days. Mean blood pressure (mBP) ($1/3 \times$pulse pressure$+$mean diastolic pressure) and body mass index (BMI) (kg/m^2) were calculated.

Samples

Blood was drawn for the measurement of serum total cholesterol (TC), serum high-density lipoprotein cholesterol (HDL-C), serum triglycerides (TG), serum albumin (s-Alb) and fasting plasma glucose (PG 0) levels before the 75 g OGTT. Blood was also drawn at 60 and 120 minutes for the measurement of plasma glucose concentrations (PG 60, PG 120). Spot urine was collected in the morning to evaluate urinary sediment. The subjects were asked to collect their overnight urine samples. They were instructed to empty their bladders before going to bed, and to void into plastic containers the next morning or during the night (the timed

overnight urine samples). The time of each voiding was noted. Serum was taken in the morning after the timed overnight urine collection. The creatinine levels in the timed overnight urine and serum samples were measured by the modified Jaffe method (Koopman et al. 1987), and creatinine clearances (C-Cr) ($ml \cdot min^{-1} \cdot [1.73\ m^2]^{-1}$) were calculated. The β_2-microglobulin levels in the timed overnight urine samples were measured by sandwich enzyme immunoassay (Koopman et al. 1987), and the albumin levels were measured by radioimmunoassay (Koopman et al. 1987). The excretion rates of β_2-microglobulin (β_2-MGER) and albumin (AER) were then calculated.

Criteria for choosing subjects

The following subjects were excluded from the study: Subjects with urinary tract infection, acute or chronic infection such as positive rheumatoid factor or C-reactive protein, positive surface antigen for hepatitis B virus, BMI $\geqq$ 27 kg/m^2, systolic BP $\geqq$ 140 mmHg, diastolic BP $\geqq$ 90 mmHg, C-Cr $\leqq$ 80 $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$, β_2MGER $\geqq$ 140 $\mu g/min$ or AER $\geqq$ 20 $\mu g/min$. The remaining 80 subjects were divided into three groups as follows, according to the result of 75 g OGTT: N group, 30 subjects with normal glucose tolerance according to the criteria of the JDA (PG 0 $<$ 6.1 mmol/liter, PG 60 $<$ 8.9 mmol/liter, PG 120 $<$ 6.7 mmol/liter), who had no history of "borderline GTT"; B group, 40 subjects with borderline GTT according to the criteria of the JDA (6.1 mmol/liter $\leqq$ PG 0 $<$ 7.8 mmol/liter, PG 60 $\geqq$ 8.9 mmol/liter or 6.7 mmol/liter $\leqq$ PG 120 $<$ 11.1 mmol/liter), who had no history of diabetes; D group, 10 subjects diagnosed as having NIDDM by World Health Organization (WHO) and JDA criteria.

Assay procedure

Levels of serum and urinary Cerulo were measured by immunoradiometric assay (IRMA) according to the previously reported method (Yamazaki et al. 1995). Human Cerulo was purchased from Cosmo Bio Co., Ltd. (Tokyo). Two kinds of anti-human ceruloplasmin antisera were purchased from INCSTAR Co. (Stillwater, MN, USA) (raised in goat) and Dako Co., Ltd. (Denmark) (raised in rabbit). Anti-human ceruloplasmin antisera raised in rabbit was affinity-purified using Sepharose 4B-Cerulo conjugates that was prepared from 1 g of CNBr activated Sepharose 4B and 1 mg of human Cerulo, as recommended by the manufacturer (Pharmacia Fine Chemistry, Uppsala, Sweden). Radioiodinated purified anti-human Cerulo antibody was prepared by the chloramine T method, using 20 μg of antibody, 10 μg of chloramine T and 500 μCi of NaI-125. After being labeled, the radioiodinated antibody was separated from free iodine by a Sephadex G-10 (Pharmacia Fine Chemistry) column in which 0.1 mol/liter phosphate buffer (pH 7.4) containing 0.05% Tween 20 and 0.3% bovine serum albumin (BSA) (washing buffer) was used as an elution buffer.

To the assay tubes (Nunctubes, Roskilde, Denmark), 0.3 ml of aliquots of

anti-human Cerulo antisera raised in goat (coating antisera, 1 : 1000 dilution) were added and the reaction was allowed to proceed at room temperature for 24 hours. After three washes with washing buffer, 0.1 ml of 0.1 mol/liter phosphate buffer pH 7.4 containing 0.15% Tween 20, 0.9% BSA and 0.2 ml of either standard or samples, were added to the tubes. After 24 hours of incubation, the tubes were washed three times. Then 0.3 ml of radioiodinated antibody was added to the tubes, which were incubated for 24 hours. After three washes, radioactivity was measured using a gamma counter.

Levels of serum and urinary IgG were measured by IRMA using rabbit anti-human IgG purchased from Dako Co., Ltd. (coating antisera) and radioiodinated affinity-purified rabbit anti-human IgG from MBL Co., Ltd. (Watertown, MS, USA). In all assays, the intra- and interassay coefficients of variations were below 5% and 10%. In the assay method, the detection limits of Cerulo and IgG were below 100 pg/ml and 1 ng/ml, respectively.

Renal Fractional protein clearance (Θ) was calculated as renal clearance of protein/creatinine clearance. All results were corrected for variation in body surface area.

Statistical analysis

Data are presented as mean $\pm$ S.D. or median with range. When the data were normally distributed, statistical analysis among the three groups was performed by one-way analysis of variance (ANOVA) and multiple comparison test by Fisher's PLSD method. When the data were not normally distributed, analysis was performed by Kruskal-Wallis test and a multiple comparison test using a Stat View Software package.

Results

Table 1 shows the clinical characteristics of the subjects. Age was higher in the D group than in the B group. BMI, mean blood pressure (mBP), TC, TG, HDL-C, C-Cr and β_2-MGER did not differ among the three groups.

Table 2 and Fig. 1 shows the fractional clearance of albumin (Fig. 1A), Cerulo (Fig. 1B) and IgG (Fig. 1C). Θ-Alb was similar in the three groups. Θ-Cerulo in the B and D groups was higher than that of the N group ($p<0.01$ and 0.001, respectively). Θ-Cerulo in the D groups was elevated as compared with that of the B group ($p<0.05$). Θ-IgG was higher in the D group than in the N group, but did not differ between the N and the B groups.

Fig. 2 shows the relationship between Θ-Cerulo and PG 120 in the study subjects. Θ-Cerulo in 37.5% (15/40) of the subjects in the B group (shown by closed circle) was higher than the mean + 1 S.D. value in the N group. As shown in Table 3, PG 0, PG 60, PG 120, age BMI, mBP, TC, TG, C-Cr and β_2-MGER were similar in the 37.5% of the B group who showed elevated Θ-Cerulo and the other 62.5%. Only the value of HDL-C in the former subgroup was decreased

TABLE 1. *Clinical characteristics of subjects*

	N group	B group	D group
Number	30	40	10
Age (years)	49.2±7.1	50.0±7.5	55.5±6.7[a,b]
PG0 (mmol/liter)	5.42±0.31	5.64±0.46[a]	6.46±0.71[c,d]
PG60 (mmol/liter)	6.53±1.28	10.33±1.59[c]	13.33±2.13[c,d]
PG120 (mmol/liter)	5.26±0.94	7.04±1.59[c]	13.44±1.69[c,d]
BMI (kg/m^2)	23.6±2.1	23.7±2.4	24.0±2.6
mBP (mmHg)	79.8±6.6	82.5±7.9	82.6±6.0
TC (mg/100 ml)	203.1±29.9	193.0±37.3	205.9±47.2
TG (mg/100 ml)	131.8±77.2	137.0±103.6	125.2±44.7
HDLC (mg/100 ml)	54.9±15.6	50.0±11.2	50.0±13.2
C-Cr (ml/min/1.73 m^2)	131.9±30.1	136.2±31.3	134.2±26.4
β2MGER (mg/min)	51.9±29.0	53.01±32.19	67.6±26.0

Data are presented as mean±S.D.

a, significantly different from the N group at $p<0.05$; b, significantly different from the B group at $p<0.05$; c, significantly different from the N group at $p<0.001$; d, significantly different from the B group at $p<0.001$.

TABLE 2. *Serum levels and renal fractional clearances of albumin, ceruloplasmin and IgG*

	N group	B group	D group
s-Alb (g/100 ml)	4.55 (4.04–5.08)	4.53 (4.14–5.09)	4.38[a] (4.14–4.80)
Θ-Alb (10^{-8})	62.2 (39.1–217.6)	74.7 (27.4–207.9)	82.7 (53.3–169.8)
s-Cerulo (μg/ml)	262.3 (188.6–402.0)	281.3 (185.2–382.2)	296.3 (228.7–354.9)
Θ-Cerulo (10^{-8})	52.9 (15.8–152.0)	80.9[c] (23.2–320.2)	162.6[b,d] (42.1–369.4)
s-IgG (mg/100 ml)	1178 (716–2509)	1123 (662–2215)	1412 (628–2746)
Θ-IgG (10^{-6})	0.96 (0.44–2.00)	1.24 (0.52–3.80)	2.05[a] (0.77–3.28)

Data are presented as median (range).

a, significantly different from the N group at $p<0.05$; b, significantly different from the B group at $p<0.05$; c, significantly different from the N group at $p<0.01$; d, significantly different from the B group at $p<0.001$.

compared with the latter subgroup.

DISCUSSION

The present study showed that urinary excretion of Cerulo was elevated in the subjects with borderline GTT as compared with non-diabetic subjects with

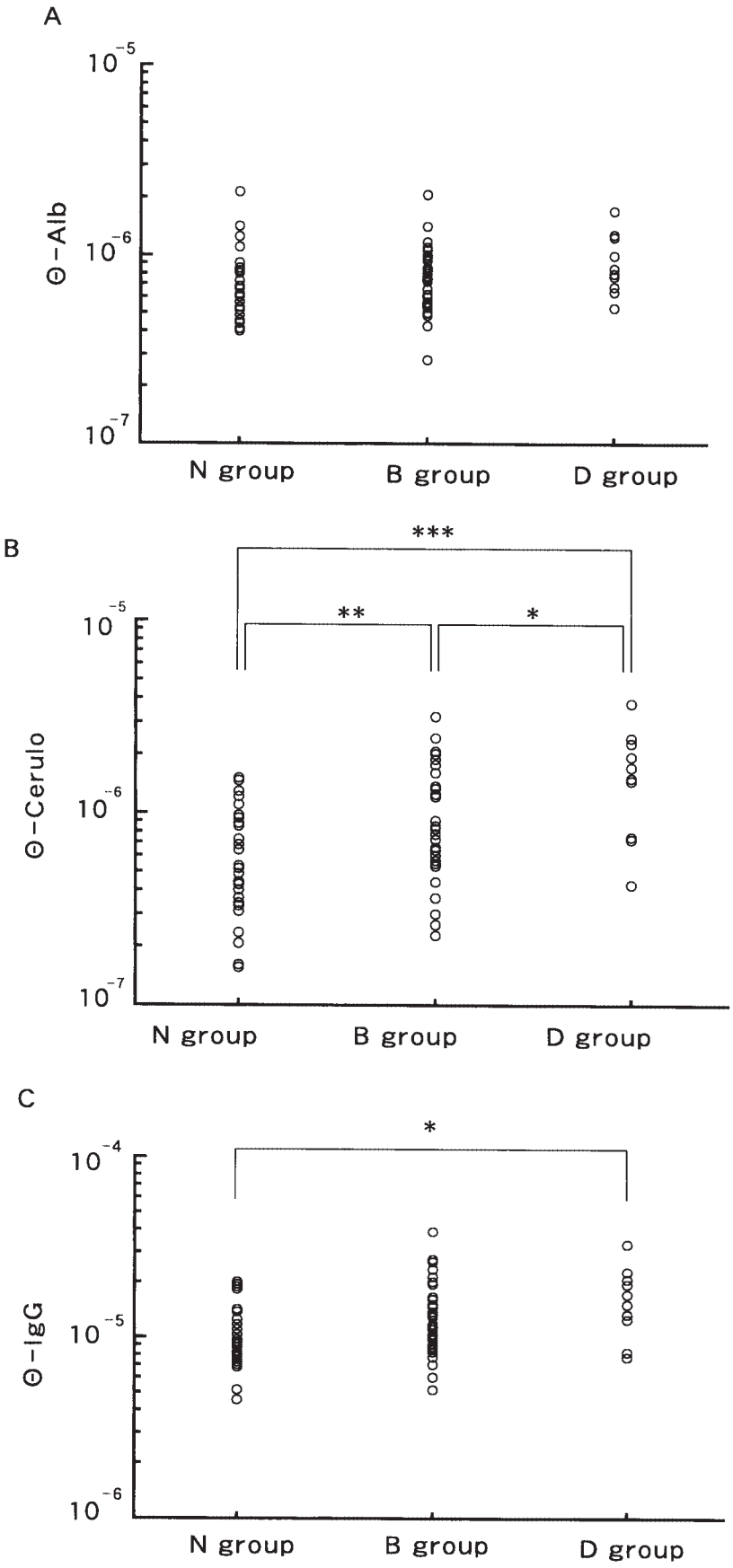

Fig. 1. Renal fractional clearance of albumin (A), ceruloplasmin (B) and IgG (C). Values are presented as open circles. Y axis is logarithmically transformed. Abbreviations: *, ** and ***; significantly different by non parametric test and multiple comparison test ($p<0.05$, $p<0.01$ and $p<0.001$, respectively).

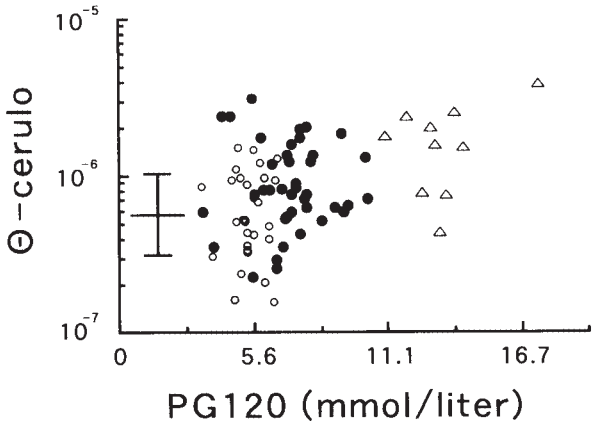

Fig. 2. Relationship between the renal fractional clearance of ceruloplasmin and the 120-minute values of plasma glucose during 75 g OGTT (PG 120). Y axis is logarithmically transformed. Upper, middle and lower horizontal bars at the left side of the figure represent the values of +1 S.D., mean and −1 S.D. of the N group, respectively. ○, N group; ●, B group; △, D group.

TABLE 3. *Comparison of clinical charasteristics between subgroups of subjects with borderline GTT who have higher and lower renal fractional clearance of ceruloplasmin (Θ-Cerulo)*

Θ-Cerulo	Higher group	Lower group
Number	15	25
PG0 (mmol/liter)	5.63±0.39	5.64±0.51
PG60 (mmol/liter)	10.57±1.12	10.19±1.83
PG120 (mmol/liter)	7.04±1.59	7.05±1.56
Age (years)	52.2±7.9	48.6±7.1
BMI (kg/m²)	24.5±2.2	23.3±2.4
MBP (mmHg)	82.6±8.2	82.4±7.9
TH (mg/100 ml)	200.5±46.6	188.4±30.7
TG (mg/100 ml)	160.9±157.3	122.6±49.2
HDL-C (mg/100 ml)	44.5±7.2	52.6±12.2[e]
C-Cr (ml/min/1.73 m²)	134.3±33.1	137.4±30.8
β2MGER (ng/min)	60.2±32.8	48.7±31.7

Data are presented as mean±S.D.

"Higher group" means the subjects whose renal fractional clearances of ceruloplasmin were higher than the mean +1 S.D. value in the subjects with normal GTT (N group) and "Lower group" means the remainders of borderline GTT.

e, significantly different by *t*-test ($p<0.05$).

normal glucose torelance, though urinary excretions of Alb and IgG were similar in the two groups mentioned above. Furthermore, urinary levels of β_2-microglobulin, which is a marker for tubular reabsorption of proteins, were similar in these two groups. These finding, together with those of Yamazaki et al. (1995), in which urinary excretion of Cerulo increased in parallel with enhanced urinary excretion of Alb in diabetic patients, led us to conclude that the urinary Cerulo

measured in the present study is of glomerular origin.

Urinary excretion of Cerulo (isoelectric point [pI] = 4.4) was elevated in the subjects with borderline GTT as compared to controls and it was greater in normoalbuminuric diabetic patients than in subjects with borderline GTT. These findings suggest that an impairment of glomerular charge selectivity may exist in the subjects with borderline GTT and may be emphasized in normoalbuminuric diabetic patients. However, enhanced urinary excretion of IgG in normoalbuminuric diabetic patients can not be explained by an impairment of glomerular charge selectivity, because pI of IgG is 7.4. Therefore, increased urinary excretion of Cerulo and IgG may be induced by another mechanism other than an impairment of charge selectivity. The idea was supported by another finding that increased urinary excretion of Alb was not found in diabetic patients who had enhanced urinary IgG excretion, despite of the fact that pI of Alb is more acidic than that of IgG. Furthermore, in the present study, urinary β_2-microglobulin excretion, which is a marker for tubular function, was not different among three groups. Thus, difference of urinary proteins excretions among three groups was not thought to be due to an alteration of renal tubular function.

Recently, Narita et al. (1999) measured urinary excretion rates of Alb, Cerulo, IgG and α_2-macroglobulin in healthy volunteers after acute protein loading. They found that urinary excretions of Cerulo and IgG increased after acute protein loading, though those of Alb and α_2-macroglobulin did not change. Furthermore, increased urinary excretion of Cerulo was more remarkable than that of IgG. As it is well known that acute protein loading causes increased GFR, in other words, increased intraglomerular hydraulic pressure (Bosch et al. 1983; Hostetter 1986), it seems reasonable to consider that increased urinary excretions of Cerulo and IgG may be induced by enhanced intraglomerular hydraulic pressure. Based on these findings, the subjects with borderline GTT may have increased intraglomerular hydraulic pressure and this enhanced intraglomerular hydraulic pressure may be emphasized in the diabetic patients who had increased urinary excretions of Cerulo and IgG.

To find out whether an impairment of charge selectivity in the glomerular basement membrane (Deckert et al. 1988) was present in diabetic patients or not, measurement of urinary excretions of plasma proteins with different pI and similar molecular radius has been introduced (Deckert et al. 1993). However, in view of the report (Narita et al. 1999) that enhanced GFR may cause urinary excretions of various plasma proteins with similar molecular radius, irrespective of their pI, development of a new method seems to be necessary to elucidate an impairment of charge selectivity in the glomerular basement membrane.

Although enhanced urinary Cerulo excretion was found in the subjects with borderline GTT as compared with controls, it was not demonstrated in all of these subjects. Approximately 40% of the subjects with borderline GTT had a higher Θ-Cerulo than the upper limit of that in the subjects with normal glucose toler-

ance, and the other 60% had similar level to Θ-Cerulo levels in the control subjects (Fig. 2). Clinical characteristics were compared between these two subgroups. No significant differences were found among the levels of glucose tolerance, aging, obesity, blood pressure and renal function (Table 3). Serum HDL-C levels were significantly lower in the subjects with borderline GTT with elevated Θ-Cerulo than in the other subgroup. However, the pathophysiological significance of the finding remains unclear.

In conclusion, the present cross-sectional study indicated that the subjects with borderline GTT may have enhanced intraglomerular hydraulic pressure. This changes, which occurred in approximately 40% of the subjects with borderline GTT in this study, may initiate or predict incipient nephropathy in NIDDM. Prospective study of renal function in these subjects is necessary to elucidate this hypothesis.

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