

ORIGINAL

Prompt increases in retinol-binding protein 4 and endothelial progenitor cells during acute exercise load in diabetic subjects

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Abstract. The present study was undertaken to determine whether acute exercise load alters serum retinol-binding protein 4 (RBP4) and numbers of endothelial progenitor cells (EPC) in diabetic subjects. Sixty-two subjects with type 2 diabetes mellitus were enrolled in the present study. They were 50 males and 12 females with the ages of 65.1 ± 8.1 (mean \pm SD) years. Cardio-pulmonary exercise stress test (CPX) was carried out, and the numbers of EPC and serum RBP4 levels before and after the CPX were measured. RBP4 is a cytokine synthesized in hepatocytes, white adipose tissues and skeletal muscles, and serum RBP4 was determined by ELISA. EPC was determined as CD34⁺/133⁺ cells by FACS. The subjects were subgrouped into two groups with or without nephropathy. Serum RBP4 levels promptly increased from 48.2 ± 4.3 (mean \pm SEM) to 54.3 ± 4.2 μ g/mL after the CPX (mean exercise time of 8 min) in the diabetic subjects without nephropathy ($p=0.0006$), but did not in those with nephropathy. There was a positive correlation between changes in serum RBP4 during the exercise and estimated glomerular filtration rate ($r=0.30$, $p=0.018$). Also, an acute exercise load promptly increased the number of EPCs in the diabetic subjects with and without nephropathy. These findings suggest that a prompt increase in exercise-induced RBP4 is retarded by progression of nephropathy, and that an exercise-induced mobilization of EPCs could maintain endothelial cells in diabetic subjects.

Key words: Diabetes mellitus, Retinol-binding protein 4, Endothelial progenitor cells

RETINOL-BINDING PROTEIN 4 (RBP4) is a 21KDa protein, which is synthesized mainly in hepatocytes. Also, RBP4 is expressed in skeletal muscles and white adipose tissues, that are sensitive to insulin [1]. It is known that plasma RBP4 levels are increased in subjects with obesity, impaired glucose tolerance and diabetes mellitus, in particular, in diabetic nephropathy [2-5]. RBP4 increases insulin resistance by inhibiting insulin signaling in muscles and increasing hepatic glucose output [6]. It binds to the large transthyretin homotetramer, and alterations in RBP4-transthyretin binding contribute to elevated serum RBP4 levels in insulin-resistant states [7]. However, there are controversial reports regarding insulin resistance [8].

Recently Kahn and her associates [9] evaluated muscular RBP4 during exercise load in the rats. In their study muscular RBP4 mRNA expression was elevated in diabetic rats compared with controls. Exercise mimics muscular RBP4 mRNA expression. Similarly, we found that exercise load promptly increases the number of bone marrow-derived vascular endothelial progenitor cells (EPCs), determined as CD34⁺/133⁺ cells, in diabetic subjects and ischemic heart disease, and EPCs may participate in maintaining vascular endothelial cells against arteriosclerotic change [10, 11]. Therefore, there is a possibility that acute exercise may promptly affect vascular biology in some pathological state.

In the present study we determined whether acute exercise load alters serum RBP4 levels and the numbers of CD34⁺/133⁺ cells in diabetic subjects. The acute exercise test is the standard tolerance test for producing anaerobic condition [12]. Furthermore, whether progression of diabetic nephropathy alters exercise-induced RBP4 and EPCs was also examined.

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Methods

Sixty-two subjects with type 2 diabetes mellitus were enrolled in the present study between March, 2006 and December, 2010. They were collected from the outpatient clinic of Jichi Medical University Saitama Medical Center. They were 50 males and 12 females with the ages of 65.1 ± 8.1 years (mean \pm SD) ranging from 47 to 79 years. Type 2 diabetes mellitus was diagnosed by The Japan Diabetes Society (JDS) criteria. Hemoglobin A1c (HbA1c; NGSP) was 7.6 ± 1.1 % and duration of diabetes mellitus was 14.9 ± 8.5 years. Twenty-three subjects had diabetic retinopathy, 28 had diabetic neuropathy and 32 had diabetic nephropathy. Thirty-two

subjects had hypertension, 38 had dyslipidemia and 18 had obesity. Twenty-four subjects were current smokers. The following subjects including hemodialysis treatment, maintenance medication of nitroglycerin, infectious diseases, malignancy and the past history of intrapelvic system surgery were excluded from the present study. According to the progression of diabetic nephropathy by Research Committee of the Japanese Ministry of Health, Labour and Welfare for Disorders of Diabetes Mellitus (The Research Committee of Diabetic Nephropathy, 2001), we divided the diabetic subjects into stages 1-4. The numbers of subjects taking medication for diabetes mellitus, hypertension and dyslipidemia are summarized in Table 1. Risk factors for

Table 1 Clinical characteristics of the diabetic subjects with and without nephropathy

Clinical stages	Stage 1	Stages 2-4	<i>p</i> value
Subjects (male/female)	30(24/6)	32(26/6)	
Age (years)	64.4 ± 1.5	65.8 ± 1.4	0.515
Height (cm)	163.3 ± 1.5	162.2 ± 1.2	0.564
Weight (kg)	65.4 ± 1.9	62.3 ± 2.1	0.281
BMI	24.4 ± 0.6	23.5 ± 0.6	0.277
Duration of diabetes mellitus (years)	13.5 ± 1.6	16.1 ± 1.5	0.236
Systolic blood pressure (mmHg)	130.8 ± 2.4	129.4 ± 2.3	0.684
Diastolic blood pressure (mmHg)	75.9 ± 1.2	72.8 ± 1.5	0.124
HbA1c (NGSP) (%)	7.5 ± 0.2	7.69 ± 0.2	0.610
Total cholesterol (mg/dL)	189 ± 5.3	196 ± 6.8	0.457
Triglyceride (mg/dL)	113 ± 10.6	124 ± 10.2	0.432
HDL-Cholesterol (mg/dL)	53.5 ± 2.5	52.0 ± 2.7	0.689
LDL-Cholesterol (mg/dL)	112 ± 4.2	117 ± 5.9	0.511
BUN (mg/dL)	14.2 ± 0.7	16.5 ± 0.8	0.031
Creatinine (mg/dL)	0.76 ± 0.03	0.85 ± 0.03	0.033
Uric acid	5.22 ± 0.2	4.98 ± 0.2	0.417
eGFR (mL/min/1.73m ²)	77.8 ± 2.7	69.3 ± 3.3	0.052
Albuminuria (mg/g creatinine)	13.1 ± 1.7	440 ± 145	0.0001
Adiponectin (μ g/mL)	7.77 ± 0.9	9.62 ± 1.0	0.173
RBP4 (μ g/mL)	48.2 ± 4.3	53.5 ± 3.6	0.349
EPCs (cells/100 μ L)	88.9 ± 18.6	63.2 ± 13.4	0.259
Smoking, <i>n</i> (%)	12 (40)	12 (38)	0.847
Dyslipidemia, <i>n</i> (%)	18 (60)	20 (63)	0.840
Hypertension, <i>n</i> (%)	14 (47)	18 (56)	0.316
Obesity, <i>n</i> (%)	10 (33)	8 (25)	0.657
Retinopathy, <i>n</i> (%)	10 (33)	13 (40)	0.497
Neuropathy, <i>n</i> (%)	12 (40)	16 (50)	0.429
Medication, <i>n</i> (%)			
ACEI	5 (17)	6 (19)	0.830
ARB	6 (20)	13 (41)	0.078
Calcium channel blockers	5 (17)	8 (25)	0.421
Diuretics	1 (3)	0 (0)	0.298
Statins	13 (43)	11 (34)	0.469

Values are mean \pm SE. Values are analyzed by Student's *t*-test or chi-square for independence test. GFR, glomerular filtration rate; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker

atherosclerosis were defined as follows: Hypertension was defined as systolic blood pressure of greater than 140 mmHg, diastolic blood pressure of greater than 90 mmHg, or the subject's having taken antihypertensive agents. Dyslipidemia was defined as a total cholesterol level of greater than 220 mg/dL, a high-density lipoprotein cholesterol level of less than 40 mg/dL, and a triglyceride level of greater than 150 mg/dL, or the subject's having taken either statins or fibrates. Obesity was defined as BMI of greater than 25.

Blood samples were collected from the subjects in the sitting position to determine HbA1c, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, blood urea nitrogen (BUN), and creatinine at the outpatient clinic. Urine samples were collected in the morning to measure urinary excretion of albumin and creatinine. In all the subjects endothelial function tests of flow-mediated dilatation (FMD) and nitroglycerin-mediated dilatation (NMD) were performed. And cardio-pulmonary exercise stress test (CPX) was carried out, and the number of bone-marrow derived CD34⁺/133⁺ cells in the peripheral blood and serum RBP4 levels before and after the CPX were determined. The present study was approved by the ethical committee of Jichi Medical University for human studies. We obtained informed consent from the subjects who joined the present protocol.

Measurements

Blood samples were collected into tubes and centrifuged at 3,000 rpm at 4 °C for 15 minutes. The supernatants were decanted and frozen at - 80 °C until assayed. Serum RBP4 was measured by the methods of ELISA using Human RBP4 ELISA kits (AdipoGen, Seoul, Korea). Urinary excretion of albumin was determined by latex agglutination immunoassay (Eiken, Tokyo). Serum adiponectin was measured using Human adiponectin ELISA kits (Otsuka Pharmaceutical Co, Tokyo, Japan). Renal function was calculated as the estimated glomerular filtration rate (eGFR) by the Modification of Diet in Renal Disease equation (MDRD) revised for Japanese by Japanese Society of Nephrology (Matsuo *et al.*, 2009). The measurement of the number of endothelial progenitor cells (EPCs) were defined as CD34⁺/133⁺ cells by fluorescence-activated cell sorting (FACS) (FACS CaliburTM; BD Biosciences, San Jose, CA, USA). The value for HbA1c (%) is estimated as National Glycohemoglobin Standardization Program

(NGSP) equivalent value (%), which is calculated by the formula $\text{HbA1c (\%)} = \text{HbA1c (JDS) (\%)} + 0.4\%$. This equation is derived from the relation between HbA1c (JDS) measured by the previous Japanese standard substance and measurement of HbA1c (NGSP), according to the committee of Japan Diabetes Society on the diagnostic criteria of diabetes mellitus from Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus [13].

Flow-mediated dilatation (FMD)

Endothelial function was evaluated by flow-mediated dilatation (FMD). FMD indicates an arterial response to reactive hyperemia, causing endothelial dependent dilatation [14, 15]. According to the procedure previously described, FMD of right brachial artery was determined by one investigator, the first author, between 16:00 and 18:00 after 15 minutes rest using 12-MHz ultrasound equipment (UNEXEF18G[®], UNEX Corporation, Nagoya, Japan). The brachial artery in longitudinal section just above the antecubital fossa was imaged, and internal diameters from anterior to posterior intimal interfaces were measured at end-diastole using B-mode imaging as baseline. A pneumatic cuff was inflated on the forearm at a pressure of over 50 mmHg higher than systolic blood pressure for 5 minutes. The intravascular blood flow velocities and vessel diameter were measured from 20 to 120s after cuff deflation in the same manner as that as baseline. FMD was calculated as the percent increase in vessel diameter following reactive hyperemia ((Maximum diameter following reactive hyperemia-baseline diameter)/ baseline diameter × 100). Fifteen minutes after FMD examination, nitroglycerin-mediated dilatation (NMD), which is endothelium-independent dilatation, was performed after administering sublingual nitroglycerin 0.3-mg-spray. The maximum vessel diameter by nitroglycerin was measured 4 minutes after the administration, and then NMD was calculated.

Exercise capacity

Cardio-pulmonary exercise stress test was performed between 14:00 and 15:00 on the separate day to measure oxygen consumption at the anaerobic threshold (AT) and peak oxygen consumption (peak VO₂) using an electronically braked cycle ergometer (Ergometer 232C[®], Minato Medical Science, Osaka, Japan) at a constant rate of 60 rpm. The work rate was increased using a 15 W/min ramp until leg fatigue, shortness of

breath, significant ST depression or maximum heart rate (220 - age) arrival. Expired air gas was analyzed continuously using a metabolic cart (AE-300S®, Minato Medical Science). The anaerobic threshold was determined by the V-slope method by the starting point of the non-linear increase in carbon dioxide output [16]. The mean exercise time of subjects during the procedure of CPX was approximately 8 min.

Statistical analysis

All values are expressed as mean \pm SEM. The values were analyzed by Student's *t*-test to compare the differences between the groups. Corresponding data were analyzed by Student's paired *t*-test. Categorical data were analyzed by chi-square test. Simple regression analysis was performed to evaluate correlation between the parameters. The statistical package of SPSS Statistics® 16.0 (IBM, Tokyo, Japan) was employed for the present analysis. A *p* value less than 0.05 was considered significant.

Results

We compared clinical features in the two groups of diabetic subjects with and without nephropathy (Table 1). The stage of diabetic nephropathy was determined according to the Research Committee of Diabetic Nephropathy [17]. BUN and serum creatinine were significantly increased, but eGFR was reduced, in the subjects with nephropathy than those without nephropathy (BUN; *p* = 0.031, serum creatinine; *p* = 0.033, eGFR; *p* = 0.052). Otherwise, there was no difference in any parameter between the two groups of subjects.

Fig. 1 shows changes in serum RBP4 levels after the cardio-pulmonary exercise test in the diabetic subjects. The maneuver of cardio-pulmonary exercise increased maximally oxygen consumption at the anaerobic threshold and peak oxygen consumption to 12.7 ± 3.0 mL/kg/min and 20.7 ± 4.6 mL/kg/min in the diabetic subjects, respectively. These two parameters were not different between two groups of diabetic subjects with and without nephropathy (13.1 ± 0.5 vs. 12.4 ± 0.6 mL/kg/min, *p* = 0.369; and 21.9 ± 0.9 vs. 20.6 ± 0.9 mL/kg/min, *p* = 0.297). Serum RBP4 levels promptly increased from 48.2 ± 4.3 to 54.3 ± 4.2 μ g/mL in response to the exercise load when getting to peak VO₂ in the subjects without nephropathy (stage 1) (*p* = 0.0006) (Fig. 1a). However, serum RBP4 levels remained unchanged under the acute exercise load in the subjects with nephropathy (53.5 ± 3.6 to 52.2 ± 2.9 μ g/mL, Fig. 1b). The relation of changes in serum RBP4 levels (Δ RBP4) during the exercise with eGFR in all the diabetic subjects is shown in Fig. 2. There was a positive correlation between Δ RBP4 and eGFR (*r* = 0.30, *p* = 0.018).

The numbers of EPC under basal condition were 88.9 ± 18.6 and 63.0 ± 13.4 cells/100 μ L in the diabetic subjects without and with nephropathy, respectively. There was no difference in basal numbers of EPC between the two groups of subjects (*p* = 0.315). They were significantly increased in response to an acute exercise load in both the subjects with and without nephropathy (Fig. 3). An increase in the numbers of EPC seemed likely to be less in the subjects with nephropathy, but it was not significant.

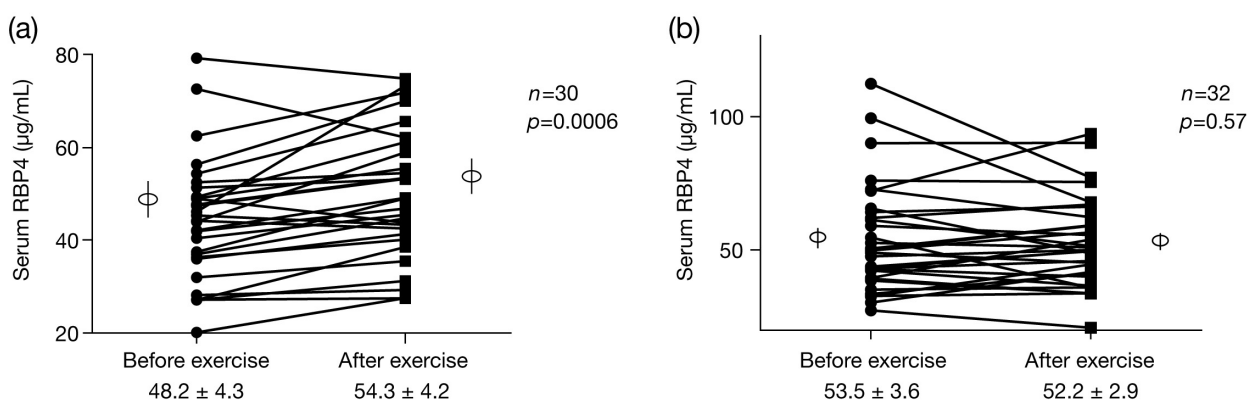


Fig. 1 Serum RBP4 levels in response to an acute exercise load in diabetic subjects with nephropathy (a) the subjects with stage 1 (*n* = 30), (b) the subjects with stages 2-4 (*n* = 32). Open circles show means \pm SE.

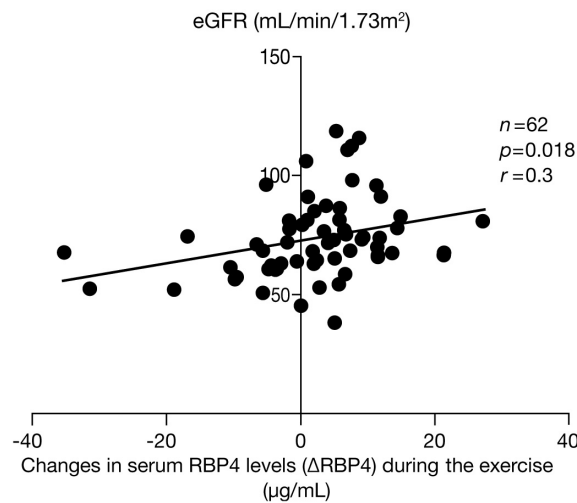


Fig. 2 The relationship of changes in serum RBP4 levels (Δ RBP4) during the acute exercise load with estimated GFR in the diabetic subjects ($n=62$)

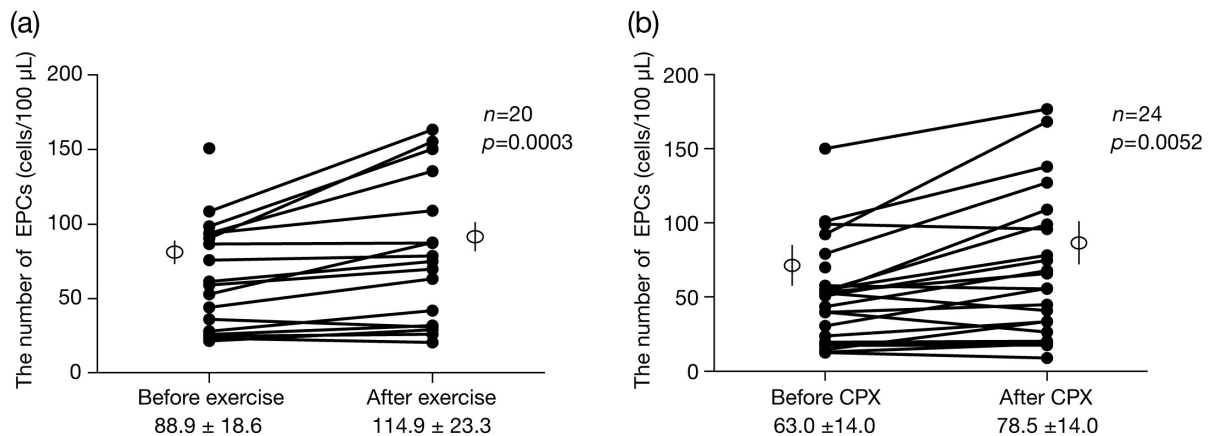


Fig. 3 The number of endothelial progenitor cells (EPCs) in response to the acute exercise load in diabetic subjects with nephropathy (a) the subjects with stage 1 ($n=20$), (b) the subjects with stages 2-4 ($n=24$). Open circles show means \pm SE.

Discussion

Serum RBP4 levels are finely altered in association with varying disorders. In our previous studies we have demonstrated that an elevation in serum RBP4 levels is found in diabetes mellitus and atherosclerotic disorders such as cerebral infarction [18, 19]. Particularly, in diabetic subjects serum RBP4 levels gradually increased according to the progression of nephropathy [3, 5, 18]. Therefore, there is a negative correlation between eGFR and serum RBP4 levels. Also, the subjects with newly onset or past history of cerebral infarction have an elevation of serum RBP4 levels, and concomitantly

have a reduction in serum adiponectin levels [19]. The elevated circulatory RBP4 increases insulin resistance by inhibiting insulin signaling in muscular tissues and increasing hepatic glucose output [6, 20]. Persistent insulin resistance may develop arteriosclerotic disorders, including ischemic heart disease, cerebrovascular disease and peripheral arterial disease (PAD) [21-23]. The prevalence of diabetes mellitus, hypertension and dyslipidemia could be underlied in the subjects with cerebral infarction. Therefore, an increase in serum RBP4 levels is closely associated with underlying disorders and atherosclerosis, independently of the primary carrier for vitamin A (retinol) in plasma.

RBP4 is the primary carrier for retinol in plasma, and synthesized by liver. Also, RBP4 expression is resided in extrahepatic tissues including white adipose tissues and skeletal muscles [1]. Furthermore, kidney has a role in maintenance of whole body retinol homeostasis. Our studies have demonstrated that serum RBP4 levels are gradually increased according to the progression of diabetic nephropathy [18], and there was a negative correlation between serum RBP4 and eGFR in the diabetic subjects. Though clearance study was not carried out, a decrease in GFR could affect RBP4 accumulation in the systemic circulation. In addition, protein complex of RBP4 and transthyretin homotetramer in the systemic circulation may reduce renal clearance of RBP4.

The present study demonstrated that serum RBP4 levels were promptly increased in response to an acute exercise load in the diabetic subjects without nephropathy, but not in those with nephropathy (stages 2-4). Its response was obtained during only 8 min (mean) exercise, and an exercise load is clinically the standard formula for producing anaerobic condition [12]. Furthermore, changes in serum RBP4 levels had a positive correlation with eGFR in all the diabetic subjects. Exercise load could stimulate synthesis and/or release of RBP4 in the subjects without nephropathy. RBP4 is synthesized in skeletal muscles and white adipose tissues as well as in hepatocytes [1, 20]. Kahn *et al.* [9] reported that RBP4 mRNA was verified in rat skeletal muscles. Its expression was 4-times greater in the diabetic rats than the controls. Acute exercise promptly increased muscular RBP4 mRNA in the rats. This observation might suggest the prompt increase in serum RBP4 following acute exercise load could be derived from muscular tissues in the present study. The phenomenon disappeared in the progression of diabetic nephropathy. Basal levels of serum RBP4 increased according to diabetic nephropathy, and the response of RBP4 release to the exercise load could be impaired in the diabetic subjects with progression of nephropathy. As the exercise study was performed for less than 10 min, the involvement of renal clearance of RBP4 may be excluded in the present study. However, the response of RBP4 to acute exercise may not directly associate with the progression of diabetic nephropathy. Instead, we suppose that the undetermined arteriosclerotic change may in parallel underlie on the progression of nephropathy, which could be related to the alteration in serum RBP4 in the present study. Despite of no direct

evidence in the present study, there might be association of prompt change in serum RBP4 with muscular production of RBP4 in diabetic subjects. In addition, as aforementioned the present study is the first observation to depict the phenomenon that RBP4 promptly increased during an acute exercise, and thus its physiological or pathological role remained obscure. Further study will be necessary to elucidate the involvement of muscular tissues in RBP4 release and pathophysiological role of RBP4 during the acute exercise.

Endothelial progenitor cells are determined as CD34⁺/133⁺ cells by FACS [24]. There was no difference in the numbers of EPC among any group of stage 1 through stage 4 of diabetic subjects. In our previous studies basal counts of EPC gradually reduced according to the progression of either diabetic nephropathy or neuropathy, but it was not significant [25]. Acute exercise load significantly increased the amounts of EPC in the diabetic subjects without microvascular complication and with moderate progression of nephropathy. However, the response of EPC counts to an exercise load disappeared in the subjects with advanced diabetic complications [25]. The findings of prompt increases in serum RBP4 levels and EPC counts after the acute exercise load were the same direction in the present study. Though the mechanism for exercise-promoted EPCs is not evident, acute exercise promotes endothelial repair by inducing bone marrow-derived EPCs. Such an exercise-induced mobilization by EPCs is maintained even if the subjects have intermediate progression of nephropathy.

In summary, the present study demonstrated that acute exercise load promptly increased serum RBP4 in the diabetic subjects without nephropathy, but did not in those with nephropathy. The response of EPC counts to an acute exercise was obtained in both the subjects with and without nephropathy. These findings suggest that a prompt increase in exercise-induced RBP4 is retarded by progression of nephropathy, and that an exercise-induced mobilization of EPCs could maintain endothelial cells in diabetic subjects.

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