

Discussion

In this study we found that walking with a pedometer had a favorable effect on conventional metabolic parameters, such as blood pressure and serum triglycerides levels when the steps per day were greater than 8000. Meta-analysis of the effect of walking on serum lipids in 46074 subjects in 8 populations have shown that walking 6000 steps or more lowered TG by 10 mg/dL and increased HDL-C by 3 mg/dL compared to subjects walking less than 2000 steps per day²¹). In our study we did not find a significant effect of walking 6000 steps on lipid levels. Since our study subjects did not wear a pedometer before this study and thus we did not know the changes in the steps they walked between before and after this study, it was impossible to exactly explain the reason underlying this inconsistency between our study and their meta-analysis. This point might be a limitation of our study.

In our study, we measured several cytokines as well as conventional metabolic parameters. There was a significant reduction in plasma TNF- α levels after 50 days of walking, which could have a favorable effect on insulin sensitivities; however, there was no significant change in APN levels. There have been several studies conducted on the effects of exercise on plasma APN levels in obese or overweight subjects¹⁷⁻¹⁹). Yokoyama *et al.* reported that a 3-week intervention consisting of exercise, including walking and a bicycle ergometer, and diet therapy did not produce significant changes in APN levels in type 2 diabetic overweight individuals¹⁷), which was consistent with previous findings^{18, 19}). Our study differs from theirs in that we investigated the effect of walking with a pedometer. Also, we were able to compare the effects of walking on metabolic parameters between LT and MT groups. There is a report showing that considerable weight loss produces a significant increase in APN levels²²). Yang *et al.* showed that 21% BMI reduction by gastric partition produced a 46% increase in plasma APN levels²²). We presume that we did not observe significant changes in APN levels in our study because the weight loss produced by walking was too subtle.

In addition to the effect of walking on metabolic parameters, we also investigated the association between BMI, PBF and waist circumference versus various metabolic parameters. To our knowledge, there has been no previous study comparing the clinical significance of BMI, PBF and waist circumference versus metabolic parameters. Significant attention has been given to waist circumference, which is an important component for diagnosing metabolic syndrome²³). In our study, there was a stronger association with APN

levels than with BMI and PBF; thus, we presume that waist circumference could be a better predictor of adiponectin levels than BMI or PBF.

It is intriguing that there was a positive correlation of % changes in sBP with the number of steps walked per day, indicating how important it is to walk with a pedometer to lower systolic blood pressure.

For the accuracy and reliability of PBF by BIA, which was applied in this study, Tsui *et al.*²⁴) showed a high correlation between PBF by BIA and that by DXA ($r=0.89$, $p<0.0001$) in 96 (48 men and 48 women) type 2 diabetic subjects. This is in line with the data previously shown in healthy individuals²⁵).

In conclusion, we have reported that walking with a pedometer considerably improved blood pressure and TG levels but had no effect on serum APN levels in Japanese middle-aged men.

Acknowledgments

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References

- 1) Despres JP and Lamarche B: Low-intensity endurance exercise training, plasma lipoproteins and the risk of coronary heart disease. *J Intern Med*, 1994; 236:7-22.
- 2) Okada K, Hayashi T, Tsumura K, Suematsu C, Endo G, and Fujii S: Leisure-time physical activity at weekends and the risk of Type 2 diabetes mellitus in Japanese men: the Osaka Health Survey. *Diabet Med*, 2000; 17:53-58.
- 3) Hayashi T, Tsumura K, Suematsu C, Okada K, Fujii S, and Endo G: Walking to work and the risk for hypertension in men: the Osaka Health Survey. *Ann Intern Med*, 1999; 131:21-26.
- 4) Hu E, Liang P, and Spiegelman BM: AdipoQ is a novel adipocyte-specific gene dysregulated in obesity. *J Biol Chem*, 1996; 271:10697-10703.
- 5) Scherer PE, Williams S, Fogliano M, Baldini G, and Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem*, 1995; 270:26746-26749.
- 6) Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, and Matsubara K: cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (Adiponectin). *Mol Cell Biochem*, 1996; 157:175-183.
- 7) Pose Moist abundant Gene transcript 1). *Biochem Biophys Res Commun*, 1996; 221:286-289.
- 7) Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, and Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med*, 2002; 8:1288-1295.

- 8) Goldfine AB and Kahn CR: Adiponectin: linking the fat cell to insulin sensitivity. *Lancet*, 2003; 362:1431-1432.
- 9) Jansson PA, Pellme F, Hammarstedt A, Sandqvist M, Brekke H, Caidahl K, Forsberg M, Volkmann R, Carvalho E, Funahashi T, Matsuzawa Y, Wiklund O, Yang X, Taskinen MR, and Smith U: A novel cellular marker of insulin resistance and early atherosclerosis in humans is related to impaired fat cell differentiation and low adiponectin. *FASEB J*, 2003; 17:1434-1440.
- 10) Katsuki A, Sumida Y, Urakawa H, Gabazza EC, Murashima S, Matsumoto K, Nakatani K, Yano Y, and Adachi Y: Plasma levels of adiponectin are associated with insulin resistance and serum levels of triglyceride in Japanese metabolically obese, normal-weight men with normal glucose tolerance. *Diabetes Care*, 2003; 26:2964-2965.
- 11) Kern PA, Di Gregorio GB, Lu T, Rassouli N, and Ranganathan G: Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. *Diabetes*, 2003; 52:1779-1785.
- 12) Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, and Kahn SE: Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia*, 2003; 46:459-469.
- 13) Ezenwaka CE, Kalloo R, Uhlig M, and Eckel J: Relationship between adiponectin and metabolic variables in Caribbean offspring of patients with type 2 diabetes mellitus. *Horm Metab Res*, 2004; 36:238-242.
- 14) Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, and Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol*, 2000; 20:1595-1599.
- 15) Pellme F, Smith U, Funahashi T, Matsuzawa Y, Brekke H, Wiklund O, Taskinen MR, and Jansson PA: Circulating adiponectin levels are reduced in nonobese but insulin-resistant first-degree relatives of type 2 diabetic patients. *Diabetes*, 2003; 52:1182-1186.
- 16) Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, and Matsuzawa Y: Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation*, 1999; 100:2473-2476.
- 17) Yokoyama H, Emoto M, Araki T, Fujiwara S, Motoyama K, Morioka T, Koyama H, Shoji T, Okuno Y, and Nishizawa Y: Effect of aerobic exercise on plasma adiponectin levels and insulin resistance in type 2 diabetes. *Diabetes Care*, 2004; 27:1756-1758.
- 18) Hulver MW, Zheng D, Tanner CJ, Houmard JA, Kraus WE, Slentz CA, Sinha MK, Pories WJ, MacDonald KG, and Dohm GL: Adiponectin is not altered with exercise training despite enhanced insulin action. *Am J Physiol Endocrinol Metab*, 2002; 283:E861-865.
- 19) Ryan AS, Nicklas BJ, Berman DM, and Elahi D: Adiponectin levels do not change with moderate dietary induced weight loss and exercise in obese postmenopausal women. *Int J Obes Relat Metab Disord*, 2003; 27:1066-1071.
- 20) Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, and Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*, 1985; 28:412-419.
- 21) Hata Y and Nakajima K: Life-style and serum lipids and lipoproteins. *J Atheroscler Thromb*, 2000; 7:177-197.
- 22) Yang WS, Lee WJ, Funahashi T, Tanaka S, Matsuzawa Y, Chao CL, Chen CL, Tai TY, and Chuang LM: Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. *J Clin Endocrinol Metab*, 2001; 86:3815-3819.
- 23) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA*, 2001; 285:2486-2497.
- 24) Tsui EY, Gao XJ, and Zinman B: Bioelectrical impedance analysis (BIA) using bipolar foot electrodes in the assessment of body composition in Type 2 diabetes mellitus. *Diabet Med*, 1998; 15:125-128.
- 25) Sakamoto Y, Miura J, Yamaguchi Y, Ohno M, and Ikeda Y: Usefulness of bioelectrical impedance analysis for measurement of body fat. *Int J Obesity*, 1994; 18(suppl2):501.

Original Article

Association of Genetic Variation of the Adiponectin gene with Body Fat Distribution and Carotid Atherosclerosis in Japanese Obese Subjects

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Aim: The aim of this study was to investigate the effect of SNP45 of the adiponectin gene on body fat distribution and carotid atherosclerosis in Japanese obese subjects.

Methods: A total of 64 obese subjects were investigated. Genotypes of SNP45 were assayed by polymerase chain reaction-restriction fragment length polymorphism. Visceral fat area (VFA) and subcutaneous fat area (SFA) were measured using computed tomography. The progression of atherosclerosis was evaluated by plaque score (PS) of carotid artery using B-mode ultrasonography.

Results: Men carrying the G allele of SNP45 showed higher VFA (172.8 ± 50.8 vs. 147.1 ± 58.7 , $p=0.005$), lower SFA (209.9 ± 101.8 vs. 273.4 ± 142.2 , $p=0.007$), higher VFA/SFA (V/S) ratio (1.00 ± 0.46 vs. 0.60 ± 0.26 , $p<0.001$) and higher PS (9.5 ± 3.7 vs. 6.8 ± 4.2 , $p=0.012$) than those with TT genotype. Multivariate analysis showed that SNP45 was an independent determinant of V/S ratio and PS in men. In subgroup analysis, PS tended to be associated with V/S ratio only in the carrier of 45G allele.

Conclusion: These results suggest that the G allele could be a risk factor of metabolic syndrome and the development of atherosclerosis in Japanese obese subjects.

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Key words; Visceral obesity, Plaque score, Polymorphism, PCR-RFLP

Introduction

Adiponectin is a 244-amino acid protein synthesized and secreted exclusively by adipose tissue^{1,2)} and plays an important role in the regulation of energy homeostasis and insulin sensitivity³⁻⁵⁾. Adiponectin also has anti-atherogenic effects. This protein has been shown to suppress the expression of class A scavenger receptors in macrophages, affect the nuclear factor (NF)- κ B pathway and inhibit monocyte adhesion to aortic endothelial cells⁶⁻⁸⁾.

Genetic variations in the human adiponectin gene, especially two single nucleotide polymorphisms (SNPs) (+45T>G and +276G>T), have been reported to be associated with obesity, insulin resistance⁹⁾, type 2 diabetes^{10, 11)}, and coronary artery disease¹²⁾. Hara *et al.* reported that these two SNPs were associated with insulin resistance, indicating the pathogenesis of type 2 diabetes¹¹⁾. The mechanism underlying insulin resistance in type 2 diabetes is not fully understood, but many studies in nondiabetic populations have addressed the importance of upper body fat distribution. However, the association between these SNPs and body fat distribution has not been investigated. Based on these previous findings, it has recently been reported that the G allele of SNP45 was associated with susceptibility to coronary artery disease independent of conventional risk factors¹²⁾. Although, the mechanism is not clear, we hypothesized that SNP45 could modify body

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fat distribution and lead to more accumulation of visceral adipose tissue, resulting in metabolic abnormalities and the development of atherosclerosis in the process of increasing adipose tissue. To determine the validity of this hypothesis, we investigated the association of SNP45 with (1) various clinical and metabolic parameters, (2) body fat distribution, and (3) the progression of atherosclerosis using the plaque score of the carotid artery and maximum IMT in a group of Japanese obese patients.

Material and Methods

Subjects

Sixty-four Japanese obese subjects (40 men and 24 women, aged 54.2 ± 16.6 years, BMI 30.3 ± 5.3 kg/m²), receiving medical checkups in our institute from 2002 to 2004, were recruited for this study. These included 49 patients with type 2 diabetes, among whom 24 were treated with oral hypoglycemic agents, 13 with insulin, and 12 with diet alone. Subjects with other endocrine diseases or significant renal or hepatic disease were excluded.

Obesity was defined as a body mass index (BMI) ≥ 25 kg/m², based on the criteria of the Japan Society for the Study of Obesity¹³⁾. Diabetes mellitus was diagnosed according to World Health Organization criteria¹⁴⁾ and/or receiving treatment for diabetes mellitus. Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of Kanazawa University.

Screening of Mutations in the Adiponectin gene

Genomic DNA was extracted from peripheral blood leukocytes using the standard procedure. Genotypes were determined at position 45 relative to the translation start site (corresponding to GenBank AB012163S1, 2, 3) by PCR, followed by allele-specific hybridization.

DNA fragments containing SNP45 (372 bp) were amplified by PCR from genomic DNA using primers 5'-GCAGCTCCTAGAAGTAGACTCTGCTG-3' and 5'-GGAGGTCTGTGATGAAAGAGGCC-3'. PCR products were incubated at 25°C for 2 hours using *Sma*I (New England BioLabs Inc. UK). Digested products were separated by size on 3% agarose gel with ethidium bromide staining. The DNA segment from the G/G homozygote of SNP45 was digested into 209 and 163 bp fragments.

Laboratory Measurements

BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumfer-

ence at the umbilical level was measured in the exhalation phase of respiration while standing.

Venous blood samples were obtained after a 12-hour overnight fast. Serum total cholesterol (TC) and triglyceride (TG) were determined by enzymatic methods, and high-density lipoprotein cholesterol (HDL-C) levels were measured by a polyanion-polymer/detergent method. Serum immunoreactive insulin (IRI) was measured by enzyme-linked immunosorbent assay, blood glucose with the glucose oxidase method, and HbA_{1c} by high-pressure liquid chromatography. The insulin resistance index was calculated based on homeostasis model assessment (HOMA) [fasting glucose (mmol/L) \times fasting insulin (μ U/mL)/22.5]¹⁵⁾. Plasma adiponectin levels were measured with an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co., Tokushima, Japan), and leptin was measured by radioimmunoassay.

Body Fat Distribution

All subjects underwent computed tomography (CT) at the umbilical level to measure the cross-sectional abdominal subcutaneous fat area (SFA) and visceral fat area (VFA) using Fat Scan (N2 System Corp, Osaka, Japan)¹⁶⁾. The VFA/SFA ratio was calculated as visceral fat area divided by subcutaneous fat area.

Determination of Plaque Score and Max IMT

A high resolution B-mode ultrasonography unit (SS-A 370A; Toshiba; Tokyo) with a 7.5 MHz transducer was used to determine the plaque score of the carotid artery¹⁷⁾. Carotid Intima-Media Thickness (IMT) was measured at each common carotid, carotid bulb, and internal carotid artery.

The maximum IMT (Max-IMT) was defined as the highest IMT value at any location in the near and far walls of the carotid arteries, including atheromatous plaques on both sides. We defined a plaque, focal IMT thickening, as an area where IMT ≥ 1.1 mm, and calculated the plaque score by totaling the maximum thickness of all plaques on the near and far walls of vessels in the scanned area¹⁷⁾.

Statistical Analysis

All data are shown as the mean \pm SD. A chi-square test was used to confirm that the genotype frequency was in Hardy-Weinberg equilibrium and to compare differences. Continuous variables were compared by ANOVA after being adjusted for age, BMI, and sex. Univariate and stepwise regression analyses were employed to examine the association between the plaque score and clinical parameters. All statistical analyses were conducted with StatView 5.0 for Macintosh

Table 1. Genotype distribution and allele frequencies for the adiponectin gene SNP45

	SNP45 genotypes			Allele frequency	
	T/T	T/G	G/G	T	G
n (%)	34 (53.1)	25 (39.1)	5 (7.8)	0.72	0.28
male (n = 40)	23 (57.5)	13 (32.5)	4 (10.0)	0.74	0.26
female (n = 24)	11 (45.8)	12 (50.0)	1 (4.2)	0.71	0.29

Table 2. Clinical characteristics according to adiponectin genotypes at position 45

	T/T	T/G + G/G	P
n (%)	34 (53.1%)	30 (46.9%)	
M/F	23/11	17/13	0.36
Age (years)	53 ± 15	56 ± 18	0.39
Type 2 diabetes (%)	73.5	73.3	0.98
BMI (kg/m ²)	30.7 ± 6.3	29.4 ± 3.6	0.56
Waist (cm)	103.3 ± 14.2	103.2 ± 13.0	0.37
HOMA-R	3.6 ± 2.3	3.3 ± 1.9	0.80
HbA _{1c} (%)	7.1 ± 1.9	6.7 ± 1.6	0.32
Total cholesterol (mg/dL)	211 ± 35	204 ± 43	0.44
Triglycerides (mg/dL)	152 ± 104	151 ± 82	0.80
HDL cholesterol (mg/dL)	46 ± 10	45 ± 13	0.35
Adiponectin (μg/mL)	5.5 ± 2.3	6.8 ± 4.5	0.26
Leptin (ng/mL)	10.8 ± 6.4	14.1 ± 12.3	0.07
Systolic blood pressure (mmHg)	131 ± 19	135 ± 20	0.34
Diastolic blood pressure (mmHg)	79 ± 11	79 ± 14	0.65
Subcutaneous fat area (cm ²)	275.4 ± 127.5	246.5 ± 94.6	0.32
Visceral fat area (cm ²)	140.5 ± 56.6	151.1 ± 51.0	0.06
V/S ratio	0.56 ± 0.24	0.76 ± 0.45	0.009
Max IMT	1.89 ± 0.81	2.21 ± 0.91	0.27
Plaque score	6.1 ± 4.1	9.7 ± 3.9	<0.001

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation. Abbreviations: BMI=body mass index, HOMA-R=homeostasis model assessment of insulin resistance

*P values adjusted for age, sex, and body mass index.

(Abacus Concepts, Berkeley, CA). A *P* value of less than 0.05 was considered statistically significant. In step-wise analysis, an *F* value greater than 4 was significant.

Results

Genotypes and Allele Distribution of SNP45 of the Adiponectin gene

The genotype and allele frequencies of study subjects are shown in **Table 1**. Genotype distributions were in Hardy-Weinberg equilibrium at both loci, with T being the major allele. The frequency of the T allele of SNP45 was 72%, and the frequencies of the T/T genotype, T/G genotype, and G/G genotype were 53.1%, 39.1%, and 7.8%, respectively.

Clinical and Metabolic Characteristics of this Study According to SNP45 of the Adiponectin gene

Table 2 shows a comparison of clinical characteristics and body composition according to adiponectin genotypes. Subjects were divided into 45T/T homozygote and those carrying the G allele (45T/G and 45G/G).

No differences in sex, age, or the proportion with diabetes were observed between any groups. Plasma leptin levels tended to be higher in carriers of the 45G allele (10.8 ± 6.4 vs. 14.1 ± 12.3, *P*=0.07). Other variables (HbA_{1c}, plasma lipid, and plasma adiponectin levels) did not differ between these genotypes.

Table 3. Clinical characteristics according to gender

	men	women	<i>P</i>
n	40	24	
Age (years)	51 ± 16	60 ± 14	0.02
BMI (kg/m ²)	30.7 ± 6.3	29.4 ± 3.6	0.56
Waist (cm)	102.7 ± 13.7	104.2 ± 13.5	0.03
HOMA-R	3.6 ± 2.4	3.1 ± 1.3	0.76
HbA _{1c} (%)	6.7 ± 1.9	7.4 ± 1.5	0.14
Total cholesterol (mg/dL)	204 ± 40	216 ± 36	0.15
Triglycerides (mg/dL)	166 ± 108	132 ± 59	0.37
HDL cholesterol (mg/dL)	42 ± 9	53 ± 12	0.02
Adiponectin (μg/mL)	5.2 ± 2.5	7.6 ± 4.4	0.03
Leptin (ng/mL)	9.6 ± 9.2	16.8 ± 9.2	0.03
Systolic blood pressure (mmHg)	132 ± 19	135 ± 19	0.27
Diastolic blood pressure (mmHg)	80 ± 13	77 ± 9	0.65
Subcutaneous fat area (cm ²)	246.4 ± 129.1	287.6 ± 76.3	<0.001
Visceral fat area (cm ²)	158.0 ± 56.3	124.5 ± 43.1	0.002
V/S ratio	0.77 ± 0.41	0.47 ± 0.17	<0.001
Max IMT	2.09 ± 1.00	1.96 ± 0.61	0.18
Plaque score	8.1 ± 4.1	7.9 ± 5.0	0.41

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation. Abbreviations: BMI=body mass index, HOMA-R=homeostasis model assessment of insulin resistance

**P* values adjusted for age and body mass index.

Table 4. Body fat distribution and PS according to adiponectin genotypes at position 45 in men and women

	men			women		
	T/T	T/G + G/G	<i>P</i>	T/T	T/G + G/G	<i>P</i>
VFA	147.1 ± 58.7	172.8 ± 50.8	0.005	126.7 ± 52.0	122.7 ± 36.1	0.763
SFA	273.4 ± 142.2	209.9 ± 101.8	0.007	279.8 ± 95.6	294.3 ± 58.8	0.091
V/S ratio	0.60 ± 0.26	1.00 ± 0.46	<0.001	0.49 ± 0.18	0.44 ± 0.16	0.262
PS	6.8 ± 4.2	9.5 ± 3.7	0.012	4.4 ± 4.0	10.8 ± 3.8	0.135

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation. Abbreviations: VFA=visceral fat area, SFA=subcutaneous fat area

Relationship between Genotypes and Body Fat Distribution

When we considered the VFA/SFA ratio as a marker of body fat distribution, it was significantly higher in carriers of the 45G allele (0.76 ± 0.45 vs. 0.56 ± 0.24 , $P=0.009$), whereas there were no associations between SFA and SNP45. VFA tended to be higher in carriers of the 45G allele than TT homozygote (151.1 ± 51.0 vs. 140.5 ± 56.6 cm², $P=0.06$). Neither BMI nor waist circumference significantly differed between the two groups. Since there were sex differences in body fat distribution in this study (Table 3: men vs. women; VFA: 158.0 ± 56.3 vs. 124.5 ± 43.1 , $P=0.002$; SFA: 246.4 ± 129.1 vs. 287.6 ± 76.3 , $P<0.001$; V/S ratio: $0.773 \pm$

0.410 vs. 0.470 ± 0.176 , $P<0.001$), we performed separate analyses of the association between SNP45 and body fat distribution by sex (Table 4, Fig. 1). In men, SNP45 was associated with the VFA, SFA, and V/S ratio, whereas in women SNP45 was not associated with body fat distribution.

Furthermore, to evaluate the contribution of SNP45 to the V/S ratio in men, stepwise regression analysis was used (Table 5). Selected variables were age, BMI, SNP45, and adiponectin. The data showed that age, SNP45, and plasma adiponectin levels were independent determinants of the V/S ratio in men ($R^2=0.588$, $P<0.0001$).

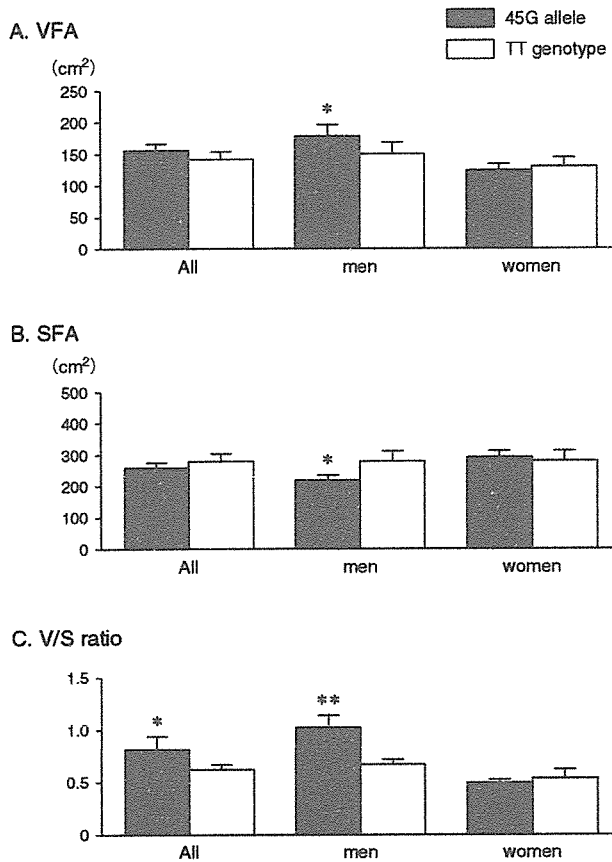


Fig. 1. Effect of SNP45 of the adiponectin gene on body fat distribution in all subjects, men and women

A. Effect of SNP45 on visceral fat area (VFA)
 B. Effect of SNP45 on subcutaneous fat area (SFA)
 C. Effect of SNP45 on VFA/SFA (V/F) ratio
 Data are the means \pm SE. * $P < 0.05$, ** $P < 0.001$

Relationship between Genotypes and Plaque Score of Carotid Artery

We investigated the effect of SNP45 on the plaque score and max IMT of carotid arteries. Carriers of the G allele had significantly greater PS than TT genotype after adjusting for age, sex, and BMI (10.0 ± 3.7 vs. 6.4 ± 4.2 , $P < 0.001$).

As shown in **Table 4**, SNP45 was associated with PS in men, and PS of the G allele tended to be higher than the TT genotype in women. To analyze the independent contribution of SNP45 to PS in men, stepwise regression analysis was applied (**Table 6**). Selected variables were age, BMI, V/S ratio, adiponectin, TC, HDL-C, systolic BP, and SNP45. The data showed that age and SNP45 were independent determinant of PS in men ($R^2 = 0.372$, $P = 0.0007$).

Table 5. Stepwise regression analysis for determinant of V/S ratio in men

Factor	β	F-value
Age	0.017	35.763
SNP45*	0.340	24.945
Adiponectin	-0.073	13.688

$R^2 = 0.588$

*TT genotype=0, TG genotype=1, GG genotype=2

Table 6. Stepwise regression analysis for determinant of PS in men

Factor	β	F-value
Age	0.143	13.362
SNP45*	2.509	7.563

$R^2 = 0.372$

*TT genotype=0, TG genotype=1, GG genotype=2

Table 7. Correlation of PS to V/S ratio according to the genotype in men and women

	equation	r	p
men			
all	$y = 4.05x + 4.71$	0.39	0.02
G allele	$y = 3.22x + 6.17$	0.38	0.14
TT genotype	$y = 2.91x + 4.93$	0.17	0.49
women			
all	$y = 4.67x + 5.85$	0.17	0.47
G allele	$y = 12.00x + 5.41$	0.60	0.06
TT genotype	$y = -2.57x + 5.59$	0.13	0.75

Relationship between V/S Ratio and Plaque Score of Carotid Artery

To examine the effect of the V/S ratio on PS, we performed univariate analysis (**Table 7**).

There was a significant positive correlation between the V/S ratio and PS in men ($r = 0.39$, $P = 0.02$), whereas in women that correlation were not statistically significant ($r = 0.17$, $P = 0.47$).

Next, to investigate the impact of SNP45 on the association between the V/S ratio and PS, we subdivided into two groups according to the genotype of SNP45 in men and women. The V/S ratio tended to be associated with PS in the G allele in both men and women (men: $r = 0.38$, $p = 0.14$; women: $r = 0.60$, $p = 0.06$, respectively). In contrast, in subjects with the TT genotype, there was no relationship between the V/S ratio and PS.

Discussion

Our study had three major findings in Japanese obese subjects. First, SNP45 in the adiponectin gene was associated with body fat distribution. Second, SNP45 was associated with the development of carotid atherosclerosis. Moreover, SNP45 had an impact on the effect of visceral obesity for the progression of atherosclerosis. Third, there was a gender difference in the effect of SNP45.

First, we demonstrated that the G allele had higher VFA, lower SFA, and a significantly higher V/S ratio compared to the TT genotype in men. Multivariate regression analysis showed that SNP45 was an independent determinant of the V/S ratio. These results indicated that the G allele of SNP45 is a risky genotype of visceral adiposity, resulting in metabolic syndrome. To our knowledge, this is the first study to demonstrate the association of SNP45 with body fat distribution. Some reports have shown that SNP45 contributes to obesity, insulin resistance, or dyslipidemia^{10, 18, 19}. In contrast, Ukkola *et al.* reported that SNP45 was found in equal frequency among obese and non-obese Swedish subjects²⁰. In French Caucasians, the 45G allele frequency was similar in morbidly obese adults and control subjects²¹. The inconsistency between these reports suggested that SNP45 could not be associated simply with weight or prevalence of obesity, but might contribute to body fat distribution in the process of becoming obese. Since visceral adipose tissue is widely believed to play a key role in the pathogenesis of metabolic abnormalities, the G allele of SNP45 could be an independent risk factor for metabolic syndrome.

Second, another important finding of the present study was the significant association between SNP45 and carotid artery PS in men. A similar trend was observed in women. Multivariate regression analysis showed that SNP45 was an independent determinant of PS. These findings suggest that SNP45 may affect the development of carotid atherosclerosis not only by modulating visceral obesity but also by other pathways.

To the best of our knowledge, PS tends to be associated with the V/S ratio only in the G allele in both men and women. In a previous study, we described a strong association between the V/S ratio and carotid artery PS in Japanese males with metabolic syndrome²², but patients with the TT genotype were protected from the atherogenic effect of visceral obesity. We hypothesized that visceral obesity might exaggerate the dysregulation of adiponectin properties of the G allele. The mechanism was unclear, but this hypothesis needs confirmation by expression studies.

Third, in this study the degree of the effect of SNP45 on body fat distribution and PS was different between men and women. Adipose tissue is sexually dimorphic in humans, with gender-specific differences in body fat distribution^{23, 24}. Gonadal steroids are the major mediator of sex dimorphism of body composition in adults^{25, 26}. Estrogen regulates both the metabolism and location of adipose tissue and plays a role in adipogenesis, adipose deposition, lipogenesis, lipolysis, and adipocyte proliferation²⁷. Furthermore, in recent studies, Clegg *et al.* reported that gonadal steroids mediate body fat distribution and interact with the integrated adiposity messages conveyed to the brain²⁸. Taken together with previous studies, our findings suggest that estrogen may interact with the adiponectin gene in adipocyte and modulate the effect of SNP45.

In addition, estrogen is known to have a cardio-protective effect. *In vivo* evidence suggests that the effect of estrogen on adhesion molecules is mediated by the inhibition of nuclear factor (NF)- κ B DNA binding^{29, 30}. As adiponectin has been shown to suppress the expression of class A scavenger receptors in macrophages, to affect the NF- κ B pathway and to inhibit monocyte adhesion to aortic endothelial cells⁶⁻⁸, atherogenic properties of the G allele may be suppressed by the effect of estrogen. Estrogen could interact with SNP45 and modulate the atherogenic function of adiponectin, but further large studies are needed to confirm the mechanism of gender-specific differences in the effect of SNP45.

The mechanistic relationship between SNP45 and both body fat distribution and the progression of atherosclerosis is unclear. SNP45 is located in exon 2 of the adiponectin gene and does not cause an amino acid change (GGT to GGG, Gly15Gly). One possibility is that SNP45 may have linkage disequilibrium with other undiscovered SNPs of the adiponectin gene having an effect on adiponectin expression, secretion, structure, or action. Another possibility is that SNP45 located in exon 2 is relatively close to the exon-intron boundary which may affect splicing machinery and effect adiponectin expression. The G allele of SNP45 may act through decreased adiponectin expression, which may cause increased visceral adipose tissue. Indeed, in Japanese type 2 diabetes, SNP45 is reported to be associated with reduced adiponectin levels¹¹. Similar findings have been shown in an other study³¹. Furthermore, recent studies have reported various adiponectin functions as an adipocyte differentiation factor, helping to maintain equilibrium adipocyte size, as an autocrine/paracrine factor in adipose tissue and as a participating factor in the regulation of adipocyte metabolism and adipose tissue mass. In 3T3-L1 preadipocytes,

adiponectin overexpression accelerates cell proliferation and differentiation, while in mature adipocytes, autocrine adiponectin increases glucose uptake and lipid accumulation³²⁾. Transgenic overexpression of adiponectin in the physiological range induced morbid obesity without insulin resistance in ob/ob mice²¹⁾. These reports indicated that hyperadiponectinemia may induce simple obesity with more subcutaneous fat accumulation, while decreased adiponectin levels may induce visceral obesity. Interestingly, the present study showed that hypoadiponectinemia was the third independent determinant of the V/S ratio. Due to these previous findings combined with our present study, the G allele might be genetically determined to have hypoadiponectinemia, contributing to the progression of visceral obesity. In contrast, the TT genotype might favor the accumulation of subcutaneous adipose tissue through hyperadiponectinemia, preventing insulin resistance, and eventually metabolic syndrome.

Adiponectin exists largely as low molecular weight (LMW) hexamers and high molecular weight (HMW) multimers^{32, 33)}. Recent article showed that the ratio of HMW to total adiponectin was responsible for metabolic effects³⁴⁾. Another study showed that HMW adiponectin was an important factor in metabolic syndrome³⁵⁾. Therefore, the alternative possibility of the atherogenic effect of SNP45 is that the proportion of HMW adiponectin might decrease in the G allele of SNP45, leading to atherosclerosis. As we measured total adiponectin and did not assess multimeric forms of adiponectin, further study is needed.

In conclusion, we demonstrated that SNP45 was associated with body fat distribution and PS of carotid arteries. The TT genotype is a protective genotype from metabolic syndrome and atherosclerosis progression in Japanese obese subjects. The mechanism by which SNP45 affects body fat distribution and the development of atherosclerosis has not been clarified at present. Further investigations will be needed to elucidate the functional mechanism of this polymorphism.

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References

- 1) Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, and Matsubara K: cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun*, 1996; 221:286-289
- 2) Scherer PE, Williams S, Fogliano M, Baldini G, and Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem*, 1995; 270:26746-26749
- 3) Combs TP, Berg AH, Obici S, Scherer PE, and Rossetti L: Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J Clin Invest*, 2001; 108:1875-1881
- 4) Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, and Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med*, 2002; 8:1288-1295
- 5) Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, and Matsuzawa Y: Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med*, 2002; 8:731-737
- 6) Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Ishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, and Matsuzawa Y: Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation*, 2001; 103:1057-1063
- 7) Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, and Matsuzawa Y: Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF- κ B signaling through a cAMP-dependent pathway. *Circulation*, 2000; 102:1296-1301
- 8) Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, and Matsuzawa Y: Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation*, 1999; 100:2473-2476
- 9) Fumeron F, Aubert R, Siddiq A, Betoulle D, Pean F, Hadjadj S, Tichet J, Wilpart E, Chesnier MC, Balkau B, Froguel P, and Marre M: Adiponectin gene polymorphisms and adiponectin levels are independently associated with the development of hyperglycemia during a 3-year period: the epidemiologic data on the insulin resistance syndrome prospective study. *Diabetes*, 2004; 53:1150-1157
- 10) Menzaghi C, Ercolino T, Di Paola R, Berg AH, Warram JH, Scherer PE, Trischitta V, and Doria A: A haplotype at the adiponectin locus is associated with obesity and other features of the insulin resistance syndrome. *Diabetes*, 2002; 51:2306-2312
- 11) Hara K, Bautin P, Mori Y, Tobe K, Dina C, Yasuda K, Yamauchi T, Otabe S, Okada T, Eto K, Kadowaki H, Hagura R, Akanuma Y, Yazaki Y, Nagai R, Taniyama M, Matsubara K, Yoda M, Nakano Y, Tomita M, Kimura S, Ito C, Froguel P, and Kadowaki T: Genetic variation in the gene

- encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. *Diabetes*, 2002; 51:536-540
- 12) Lacquemant C, Froguel P, Lobbens S, Izzo P, Dina C, and Ruiz J: The adiponectin gene SNP +45 is associated with coronary artery disease in Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetic Medicine*, 2004; 21:776-781
 - 13) The Examination Committee of Criteria for 'Obesity Disease' in Japan, Japan Society for the Study of Obesity: New criteria for 'obesity disease' in Japan. *Circ J*. 2002; 66:987-992
 - 14) Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*, 1997; 20:1183-1197
 - 15) Matthews DR, Hosker JP, Rudenski AS, and Naylor BA: Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985; 28:412-419
 - 16) Yoshizumi T, Nakamura T, Yamane M, Islam AHMW, Menju M, and Yamasaki K: Abdominal fat: Standardized technique for measurement at CT. *Radiology*, 1999; 211:283-286
 - 17) Nagai Y, Kitagawa K, Yamagami H, Kondo K, Hougaku H, Hori M, and Matsumoto M: Carotid artery intima-media thickness and plaque score for the risk assessment of stroke subtypes. *Ultrasound Med Biol*, 2002; 28:1239-1243
 - 18) Stumvoll M, Tschritter O, Fritsche A, Staiger H, Renn W, Weisser M, Machicao F, and Haring H: Association of the T-G polymorphism in adiponectin (exon 2) with obesity and insulin sensitivity: interaction with family history of type 2 diabetes. *Diabetes*, 2002; 51:37-41
 - 19) Kondo H, Shimomura I, Matsukawa Y, Kumada M, Takahashi M, Matsuda M, Ouchi N, Kihara S, Kawamoto T, Sumitsuji S, Funahashi T, and Matsuzawa Y: Association of adiponectin mutation with type 2 diabetes: a candidate gene for the insulin resistance syndrome. *Diabetes*, 2002; 51:2325-2328
 - 20) Ukkola O, Ravussin E, Jacobson P, and Bouchard C: Mutations in the adiponectin gene in lean and obese subjects from the Swedish obese subjects cohort. *Metabolism*, 2003; 52:881-884
 - 21) Bouatia-Naji N, Meyre D, Lobbens S, Seron K, Fumeron F, Balkan B, Heude B, Jouret B, Scherer P, Dina C, Weill J, and Froguel P: ACDC/Adiponectin polymorphism are associated with severe childhood and adult obesity. *Diabetes*, 2006; 55:545-549
 - 22) Murase Y, Asano A, Kobayashi J, Yamaaki N, and Mabuchi H: Impact of adiposity on carotid atherosclerosis in Japanese males with metabolic syndrome. *J Intern Med*, 2005; 257:311-312
 - 23) Butte NF, Hopkinson JM, Wong WW, Smith EO, and Ellis KJ: Body composition during the first 2 years of life: an updated reference. *Pediatr Res*, 2000; 47:578-585
 - 24) Taylor RW, Jones IE, Williams SM, and Goulding A: Body fat percentages measured by dual-energy X-ray absorptiometry corresponding to recently recommended body mass index cutoffs for overweight and obesity in child and adolescents aged 3-18 y. *Am J Clin Nutr*, 2002; 76:1416-1421
 - 25) Marin P and Bjorntorp: Endocrine-metabolic patterns and adipose tissue distribution. *Horm Res*, 1993; 39:81-85
 - 26) Rosenbaum M and Leibel RL: Role of gonadal steroids in the sexual dimorphisms in body composition and circulating concentrations of leptin. *J Clin Endocrinol Metab*, 1999; 84:1784-1789
 - 27) Cooke PS and Naaz A: Role of estrogens in adipocyte development and function. *Exp Biol Med*, 2004; 229:1127-1135
 - 28) Clegg D, Brown L, Woods S, and Benoit S: Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes*, 2006; 55:978-987
 - 29) Simoncini T, Maffei S, and Basta G: Estrogens and glucocorticoids inhibit endothelial vascular cell adhesion molecule-1 expression by different transcriptional mechanisms. *Circ Res*, 2000; 87:19-25
 - 30) Hsu SM, Chen YC, and Jiang MC: 17β -estradiol inhibits tumor necrosis factor- α -induced nuclear factor- κ B activation by increasing nuclear factor- κ B p105 level in MCF-7 breast cancer cells. *Biochem Biophys Res Commun*, 2000; 279:47-52
 - 31) Takahashi M, Arita Y, Yamagata K, Matsukawa Y, Okutomi K, Horie M, Shimomura I, Hotta K, Kuriyama H, Kihara S, Nakamura T, Yamashita S, Funahashi T, and Matsuzawa Y: Genomic structure and mutations in adipose-specific gene, adiponectin. *Int J Obes Relat Metab Disord*, 2000; 24:861-868
 - 32) Fu Y, Luo N, Klein R, and Garvey W: Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *Journal of Lipid Research*, 2005; 46:1369-1379
 - 33) Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schultness T, Engel J, Brownlee M, and Scherer PE: Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin: implications for metabolic regulation and bioactivity. *J Biol Chem*, 2003; 278:9073-9085
 - 34) Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doeber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, and Scherer PE: Complex distribution, not absolute amount of adiponectin, correlate with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem*, 2004; 279:12152-12162
 - 35) Lara-Castro C, Luo N, Wallace P, Klein R, and Garvey W: Adiponectin multimeric complexes and the metabolic syndrome trait cluster. *Diabetes*, 2006; 55:249-259

Reduction of Diabetes-Induced Oxidative Stress, Fibrotic Cytokine Expression, and Renal Dysfunction in Protein Kinase C β -Null Mice

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Diabetes induces the activation of several protein kinase C (PKC) isoforms in the renal glomeruli. We used PKC- $\beta^{-/-}$ mice to examine the action of PKC- β isoforms in diabetes-induced oxidative stress and renal injury at 8 and 24 weeks of disease. Diabetes increased PKC activity in renal cortex of wild-type mice and was significantly reduced (<50% of wild-type) in diabetic PKC- $\beta^{-/-}$ mice. In wild-type mice, diabetes increased the translocation of PKC- α and - β 1 to the membrane, whereas only PKC- α was elevated in PKC- $\beta^{-/-}$ mice. Increases in urinary isoprostane and 8-hydroxydeoxyguanosine, parameters of oxidative stress, in diabetic PKC- $\beta^{-/-}$ mice were significantly reduced compared with diabetic wild-type mice. Diabetes increased NADPH oxidase activity and the expressions of p47^{phox}, Nox2, and Nox4 mRNA levels in the renal cortex and were unchanged in diabetic PKC- $\beta^{-/-}$ mice. Increased expression of endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)- β , connective tissue growth factor (CTGF), and collagens IV and VI found in diabetic wild-type mice was attenuated in diabetic PKC- $\beta^{-/-}$ mice. Diabetic PKC- $\beta^{-/-}$ mice were protected from renal hypertrophy, glomerular enlargement, and hyperfiltration observed in diabetic wild-type mice and had less proteinuria. Lack of PKC- β can protect against diabetes-induced renal dysfunction, fibrosis, and increased expressions of Nox2 and -4, ET-1, VEGF, TGF- β , CTGF, and oxidant production. *Diabetes* 55:3112–3120, 2006

Diabetic nephropathy is characterized by glomerular hyperfiltration, extracellular matrix accumulation, glomerular enlargement, mesangial expansion, and intertubular fibrosis, resulting ultimately in diabetic glomerulosclerosis and progressive

renal insufficiency (1,2). Hyperglycemia-induced metabolic and hemodynamic factors are thought to be mediators of this injury, which is associated with the diabetic state (3). The hemodynamic factors implicated in the pathogenesis of diabetic nephropathy include increased systemic and intraglomerular pressure and activation of various vasoactive hormone pathways including the renin-angiotensin system and endothelins (3). This may interact with metabolic pathways activating signaling pathways that lead to renal injury. Multiple biochemical pathways have been proposed to explain the adverse effects of hyperglycemia. Activation of diacylglycerol (DAG)-protein kinase C (PKC) pathway (4), enhanced polyol pathway (5), increased oxidative stress (6), and overproduction of advanced glycation end products (AGEs) (7) have all been proposed as potential cellular mechanisms by which hyperglycemia induces chronic diabetes complications.

We and others (8–10) have previously reported that multiple PKC isoforms are activated in each vascular tissue of diabetic animal models, and activation of the DAG-PKC pathway is a key mediator of diabetes vascular complications. Immunoblotting studies have reported that PKC- α and - β 1 isoforms were increased in vivo in membranous fractions (activated pool) of diabetic rat glomeruli and in vitro in mesangial cells exposed to elevated glucose levels (10), whereas PKC- β 2 was reported to be preferentially activated in the aorta and heart of diabetic rats (8). Whiteside and Dlugosz (11) reported that PKC- β and - δ isoforms were also increased in the membrane pool in the glomeruli of diabetic rats. Treatment of diabetic animals with a selective PKC- β isoform inhibitor (LY333531 or ruboxistaurin [RBX]) was associated with normalization of hemodynamic changes, extracellular matrix, and histological features of glomerular damage in animal models of diabetes (10,12,13). Phase two clinical trial results suggested that RBX can decrease the loss of glomerular filtration rate (GFR) and proteinuria in diabetic patients already treated with inhibitors of angiotensin actions (14). Recently, activation of NADPH oxidase and increased reactive oxygen species (ROS) production have been proposed as important mediators of renal dysfunction in diabetes (15,16). We have reported that inhibition of PKC- β by RBX can also normalize diabetes or hyperglycemia-induced oxidative stress (17). PKC- β has been noted to contribute to NADPH oxidase activations in multiple cells, including endothelial and mesangial cells (18). However, it is still unclear which of the renal abnormalities are induced by PKC- β isoforms as compared with other PKC isoforms and which of the potential downstream biochem-

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8-OHdG, 8-hydroxydeoxyguanosine; AGE, advanced glycation end product; CTGF, connective tissue growth factor; DAG, diacylglycerol; ET-1, endothelin-1; FF, filtration fraction; GFR, glomerular filtration rate; PAH, para-aminohippurate; PKC, protein kinase C; RBX, ruboxistaurin; ROS, reactive oxygen species; RPF, renal plasma flow; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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TABLE 1
General characteristics of the four experimental mouse groups

	Wild-type	Wild-type diabetic	PKC- $\beta^{-/-}$	PKC- $\beta^{-/-}$ diabetic
At 8 weeks of diabetes				
<i>n</i>	6	6	6	6
Body weight (g)	29.8 \pm 2.1	25.2 \pm 2.4*	28.3 \pm 2.5	24.6 \pm 2.7*
Blood glucose (mg/dl)	100 \pm 10	434 \pm 50†	98 \pm 13	445 \pm 49†‡
SBP (mmHg)	106 \pm 18	108 \pm 13	111 \pm 18	114 \pm 16
DBP (mmHg)	79 \pm 17	84 \pm 4	83 \pm 15	84 \pm 17
At 24 weeks of diabetes				
<i>n</i>	6	6	6	6
Body weight (g)	50.9 \pm 4.8	39.4 \pm 2.9†	47.7 \pm 3.2	38.5 \pm 4.7†‡
Blood glucose (mg/dl)	101 \pm 10	412 \pm 81†	99 \pm 11	436 \pm 42†‡
SBP (mmHg)	108 \pm 8	110 \pm 8	105 \pm 14	105 \pm 11
DBP (mmHg)	81 \pm 16	76 \pm 13	76 \pm 13	76 \pm 16

* $P < 0.05$ versus wild-type control; † $P < 0.01$ versus wild-type control; ‡ $P < 0.01$ versus nondiabetic PKC- $\beta^{-/-}$. DBP, diastolic blood pressure; SBP, systolic blood pressure.

ical pathways are activated to cause the functional and pathological changes. In the present study, we used the PKC- $\beta^{-/-}$ mouse to clarify the role of PKC- β isoform activation on the various biochemical, physiological, and pathological abnormalities in the glomeruli induced by diabetes and hyperglycemia. This information could be important to evaluate the use of PKC- β inhibitors in the treatment of diabetic nephropathy by identifying the specific renal end points for determining efficacy.

RESEARCH DESIGN AND METHODS

PKC- β inhibitors were provided by Dr. Michael Leitges. The derivation of PKC- $\beta^{-/-}$ mice has been described previously (19). Wild-type mice with the same genetic background (129 \times C57BL6) as the knockout mice were used as control animals. Diabetes was induced in 6-week-old male PKC- $\beta^{-/-}$ and wild-type mice fasted for 12 h with intraperitoneal injections of streptozotocin in citrate buffer (70 mg/kg body wt) (Sigma Chemical, St Louis, MO) on 2 consecutive days. The diabetic state was confirmed with blood glucose levels exceeding 250 mg/dl. Control animals received an injection of sterile citrate buffer. All experiments were conducted in accord with the Joslin Diabetes Center's Animal Care and Use Committee guidelines. Blood pressure was monitored by tail-cuff plethysmography (Ueda Electric Works, Tokyo, Japan). Glucose levels from tail blood samples were measured with a Glucometer Elite XL (Bayer, Elkhart, IL). At 8 and 24 weeks after diabetes, mice were placed in metabolic cages for collection of urine. The samples were stored frozen at -80°C . Albuminuria, urinary isoprostane, and 8-hydroxydeoxyguanosine (8-OHdG) were all measured by enzyme-linked immunosorbent assay kits from Exocell (Philadelphia, PA), Oxis International (Foster City, CA), and Genox (Baltimore, MD), respectively.

Measurement of GFR. Renal clearance studies were performed at 8 weeks of diabetes as previously described, with modification for mice (20). Briefly, mice were anesthetized with an intraperitoneal injection of thiopental sodium. A catheter was inserted into the left jugular vein for continuous infusion of inulin and para-aminohippurate (PAH) solution. The urinary bladder was catheterized for urine collection. After equilibration, blood samples from mice tail and urine samples were obtained for measurement of inulin and PAH at timed intervals. The concentrations of inulin in plasma and urine were measured by cysteine-tryptophan reaction, and PAH was determined by a calorimetric technique as described previously (20). GFR was calculated from inulin clearance and renal plasma flow (RPF) from PAH clearance and filtration fraction from a standard formula, the GFR-to-RPF ratio.

Histological assessment. For morphometric analysis of glomeruli, kidneys from animals were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm . Sections were stained with hematoxylin and periodic acid Schiff. Sections were coded and read by an observer unaware of the experimental protocol applied. In each animal, glomerular area was measured in 50 consecutive glomeruli encountered in a back and forth scan between the outer and inner cortex. The images were imported into Image-Pro Plus (Media Cybernetics, Silver Spring, MD), and the glomerular tuft area was quantified for each glomerular cross-section.

Measurement of PKC activity. To evaluate PKC activity in the renal cortex, a modified in situ PKC assay was used (20). To assess activation of different

isoforms of PKC, expression of PKC isoforms in the cytosolic and membranous fractions of the renal cortex were assessed by immunoblot analysis. The fractionation of the renal cortex was performed with a method involving ultracentrifugation steps as previously described (10).

The cytosolic and membranous fractions obtained were then used for immunoblotting according to methods described previously (10). Antibodies to the various PKC isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). p47^{phox} antibody was supplied from Abcam (Cambridge, MA).

Gene expression. Real-time PCR was performed to evaluate the mRNA expression of transforming growth factor (TGF)- β , connective tissue growth factor (CTGF), type IV and VI collagens, endothelin-1 (ET-1), and vascular endothelial growth factor (VEGF) in the renal cortex of various groups of mice. Total RNA extraction and reverse transcription were performed with the RNeasy Mini kit (Qiagen, Valencia, CA) and the First-Strand cDNA Synthesis Kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Quantitative real-time PCR of TGF- β 1, CTGF, and collagen IV and VI was performed with a TaqMan Universal PCR MasterMix and TaqMan Gene Expression assays (TGF- β 1, Mm 00441724_m1; CTGF, Mm 00515790_g1; collagen IV, Mm 00802372_m1; and collagen VI, Mm 00487160_m1) (Applied Biosystems, Forest City, CA). Primer and probe sequences of NADPH oxidase subunits are listed as follows: p47^{phox} (MN 010876.2, forward 5'-ACCTTCAT TCGCCATATCGCCCT-3', reverse 5'-TTCTGTAGACCACCTTCTCCGACA-3', and probe 5'-6FAM-CCCAGCCAGCACTATGTACATGTT-TAMRA3'), Nox2 (MN 007807.2, forward 5'-TGCAGCCTGCCTGAATTTCAACTG-3', reverse 5'-AGATGTGCAATTGTGTGGATGGCG-3', and probe 5'-6FAM-TGCGTGTTC TCGACAAGGATTCGAA-TAMRA3'), and Nox4 (NM_015760, forward 5'-TGT TGGCCCTAGGATTGTGTT-3', reverse 5'-AAAAGGATGAGGCTGCAGTTG-3', and probe 5'-6FAM-AAGCAGAGCATCTGCATCTGTCCTCAACC-TAMRA 3'). Primers and probes for ET-1 and VEGF were previously described (21). 18S ribosomal RNA expression was used for normalization.

Statistics. All data are presented as means \pm SD. Comparisons between groups were performed using one-way ANOVA, with post hoc correction by Tukey's method. Comparisons of urinary albumin excretion between groups were made with the Mann-Whitney nonparametric test, since the values were not normally distributed. Comparisons between two groups were performed using an unpaired *t* test. $P < 0.05$ was considered significantly different.

RESULTS

General characteristics. Both diabetic wild-type and PKC- $\beta^{-/-}$ mice exhibited marked hyperglycemia during the experimental periods (8 and 24 weeks) (Table 1). Diabetic wild-type and PKC- $\beta^{-/-}$ mice had significantly lower body weight at the end of the experimental period compared with nondiabetic counterparts, regardless of genotype, but higher than at beginning of the study. There was no significant difference between wild-type and PKC- $\beta^{-/-}$ mice in body weight, blood glucose, or blood pressure. Similarly, there was no significant difference in these physiological parameters between diabetic wild-type and PKC- $\beta^{-/-}$ mice at 8 and 24 weeks.

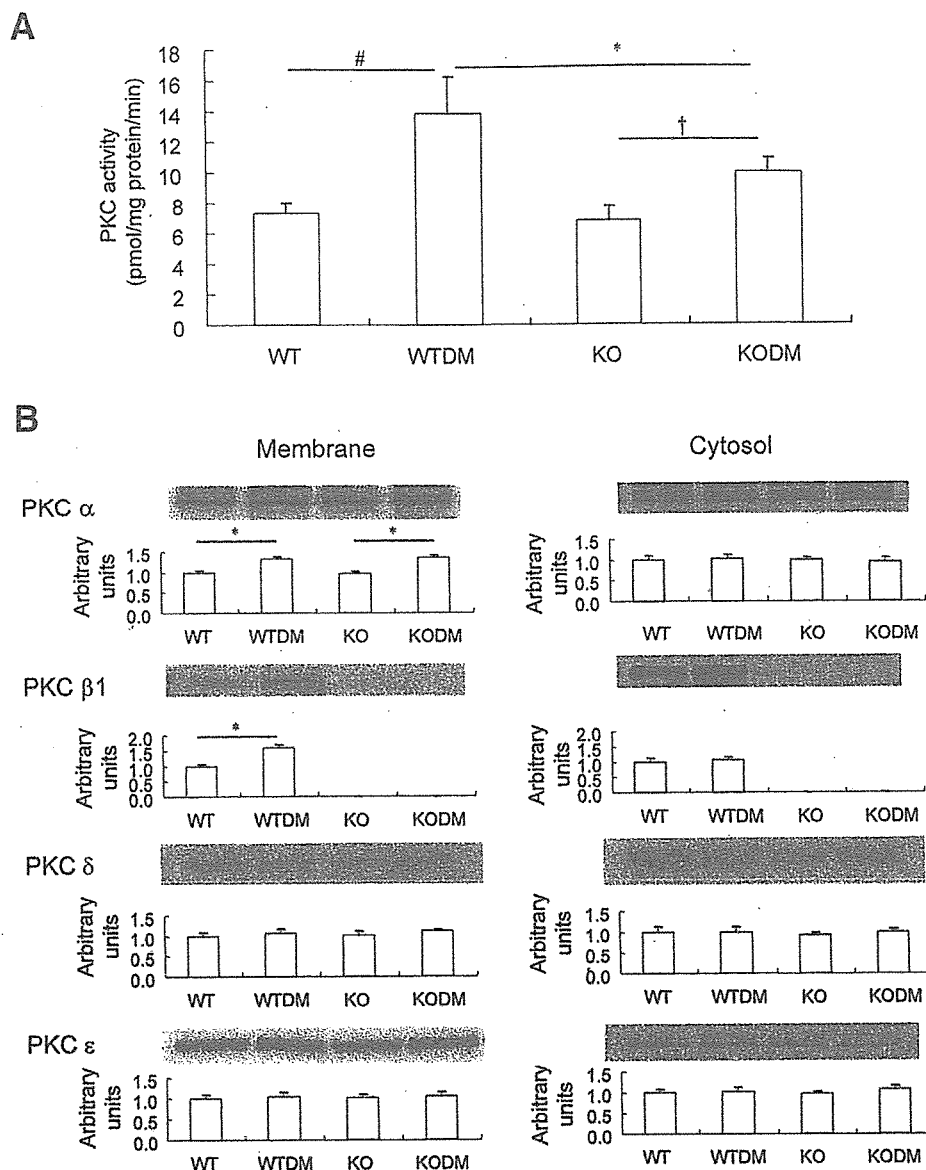


FIG. 1. A: PKC activity in renal cortex of wild-type and PKC- $\beta^{-/-}$ mice as measured by in situ PKC assay. *n* = 5 for each group. B: Expression of various PKC isoforms in the membranous and cytosolic fractions of renal cortex assessed by immunoblotting. Values for protein expression are expressed as fold change versus wild-type. *n* = 4 for each group (also indicates the number of mice studied). KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. †*P* < 0.05, **P* < 0.01, #*P* < 0.001.

Reduced PKC activation associated with diabetes in PKC- $\beta^{-/-}$ mice. Induction of diabetes for 8 weeks was associated with elevated PKC activity in the renal cortex in wild-type mice, from 7.35 ± 0.66 to 13.9 ± 2.39 pmol \cdot mg protein $^{-1} \cdot$ min $^{-1}$, an increase of 89% (*P* < 0.001). When diabetes was induced in PKC- $\beta^{-/-}$ mice, activated PKC pool was only elevated by 46% to 9.96 ± 0.9 pmol \cdot mg protein $^{-1} \cdot$ min $^{-1}$, which was significantly less than the increase in wild-type mice (*P* < 0.01, Fig. 1A). To compare the relative contribution of different isoforms of PKC to the elevated PKC activity observed in the diabetic state, expression of various isoforms of PKC in membranous and cytosolic fractions of renal cortex were examined (Fig. 1B). In wild-type mice, diabetes increased PKC- β 1 expression by 58% (*P* < 0.01) in the membranous fraction, consistent with activation of this isoform. There was no detectable PKC- β 1 in the PKC- $\beta^{-/-}$ mice. Diabetes increased the expression of PKC- α by 31% (*P* < 0.01) in the membranous fractions, which was observed in both wild-

type and PKC- $\beta^{-/-}$ mice. There was no difference in expression of PKC- α , - δ , and - ϵ isoforms in the cytosolic fractions between nondiabetic wild-type and PKC- $\beta^{-/-}$ mice.

Amelioration of renal dysfunctions in diabetic PKC- $\beta^{-/-}$ mice. Renal function was evaluated by GFR and filtration fraction (FF) following 8 weeks of diabetes (Fig. 2). GFR and FF increased in diabetic wild-type mice by 55 and 57%, respectively, compared with nondiabetic wild-type mice (*P* < 0.05). In contrast, increases in GFR and FF were not observed in diabetic PKC- $\beta^{-/-}$ mice for the same duration. Furthermore, PKC- $\beta^{-/-}$ mice with diabetes had significantly lower GFR (*P* < 0.05) and FF (*P* < 0.05) than wild-type mice with diabetes.

After 8 weeks of diabetes, urinary albumin excretion increased 10-fold in wild-type mice (Fig. 3, *P* < 0.01). Induction of diabetes in PKC- $\beta^{-/-}$ mice was also associated with an increase in urinary albumin excretion by 4.4-fold, which was significantly less than that observed in

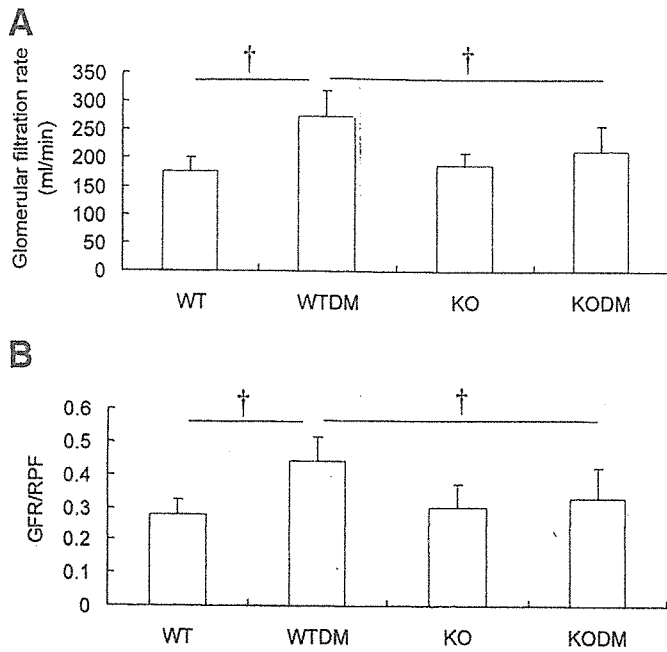


FIG. 2. GFR (A) and filtration fraction (GFR/RPF) (B) in wild-type and PKC- $\beta^{-/-}$ mice at 8 weeks of diabetes. KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. $n = 6$ for each group (also indicates the number of mice studied individually). † $P < 0.05$.

diabetic wild-type animals ($P < 0.01$). Similarly, following 24 weeks of diabetes, albuminuria remained significantly increased in both diabetic wild-type and PKC- $\beta^{-/-}$ mice. However, the amount of albuminuria in diabetic PKC- $\beta^{-/-}$ mice was significantly less than in wild-type mice exposed to diabetes of the same duration (Fig. 3, $P < 0.01$).

Protection against diabetes-induced oxidative stress in PKC- $\beta^{-/-}$ mice. Elevation in oxidative stress has been associated with diabetic nephropathy as measured by increases in the levels of several urinary oxidants (22). Thus, we measured the urinary excretions of isoprostane and 8-OHdG. After 8 weeks of diabetes, urinary isoprostane levels were increased 3.6-fold in diabetic wild-type mice as compared with nondiabetic wild-type mice ($P < 0.001$). This increase in urinary isoprostane was significantly attenuated in the diabetic PKC- $\beta^{-/-}$ mice (Fig. 4A, $P < 0.001$). Similarly, after 24 weeks of diabetes, diabetic PKC- $\beta^{-/-}$ mice continued to excrete significantly less isoprostane when compared with diabetic wild-type mice

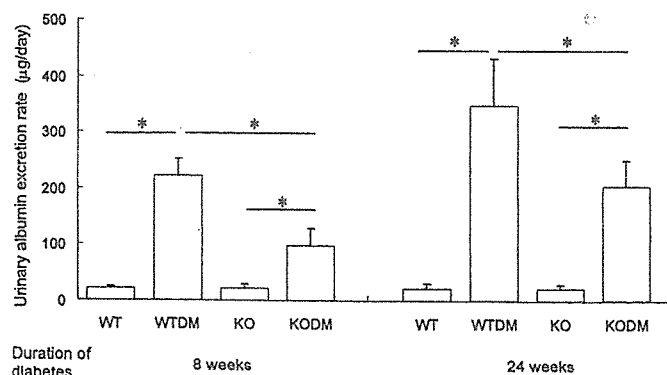


FIG. 3. Urinary albumin excretion rate in wild-type and PKC- $\beta^{-/-}$ mice after 8 and 24 weeks of diabetes. KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. $n = 5$ for each group. * $P < 0.01$.

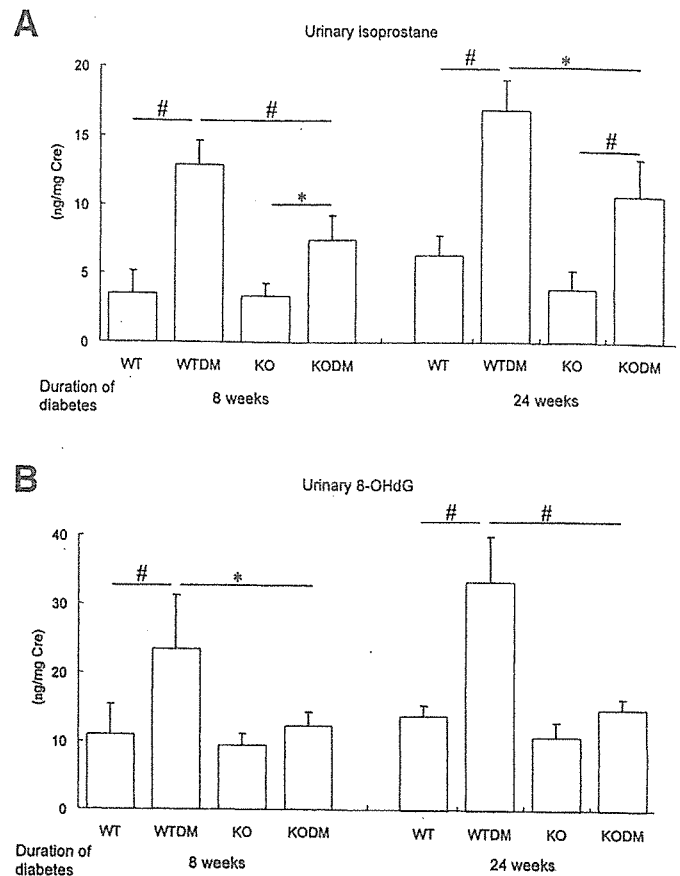


FIG. 4. Markers of oxidative stress, urinary isoprostane (A) and urinary 8-OHdG (B), in wild-type and PKC- $\beta^{-/-}$ mice after 8 and 24 weeks of diabetes. The levels of these two markers were normalized by urinary creatinine content. Cre, creatinine; KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. $n = 5$ for each group (also indicates the number of mice studied individually). * $P < 0.01$, # $P < 0.001$.

($P < 0.01$). Urinary 8-OHdG levels were not significantly increased in diabetic wild-type mice compared with nondiabetic wild-type control mice. Urinary 8-OHdG levels were significantly reduced in diabetic PKC- $\beta^{-/-}$ mice compared with diabetic wild-type mice ($P < 0.01$, Fig. 4B). **Reduced activation of NADPH oxidase in PKC- $\beta^{-/-}$ mice with diabetes.** Since activation of NADPH oxidase has been reported to be induced by diabetes and is associated with increased oxidant production induced by hyperglycemia, we assessed changes in several components of this enzyme complex (15). As shown in Fig. 5A, there was increased membranous translocation of p47 subunit of NADPH oxidase by 1.73 ± 0.4 -fold ($P < 0.01$) in diabetic wild-type mice compared with wild-type control. This activation was not observed in diabetic PKC- $\beta^{-/-}$ mice. Furthermore, diabetes also significantly increased the mRNA expressions of p47 subunit of NADPH oxidase, Nox2, and Nox4 as compared with wild-type control mice. These increases were not seen in diabetic PKC- $\beta^{-/-}$ mice (Fig. 5B-D). Expression of Nox1 mRNA level was too low to be assessed credibly (data not shown).

Altered expression of ET-1 and VEGF in PKC- $\beta^{-/-}$ mice with diabetes. Elevation of ET-1 in glomeruli has been postulated to be involved in diabetes-induced alteration of hemodynamics and changes in extracellular matrix (23). As reported previously, expression of ET-1 mRNA in renal cortex of diabetic wild-type mice was significantly

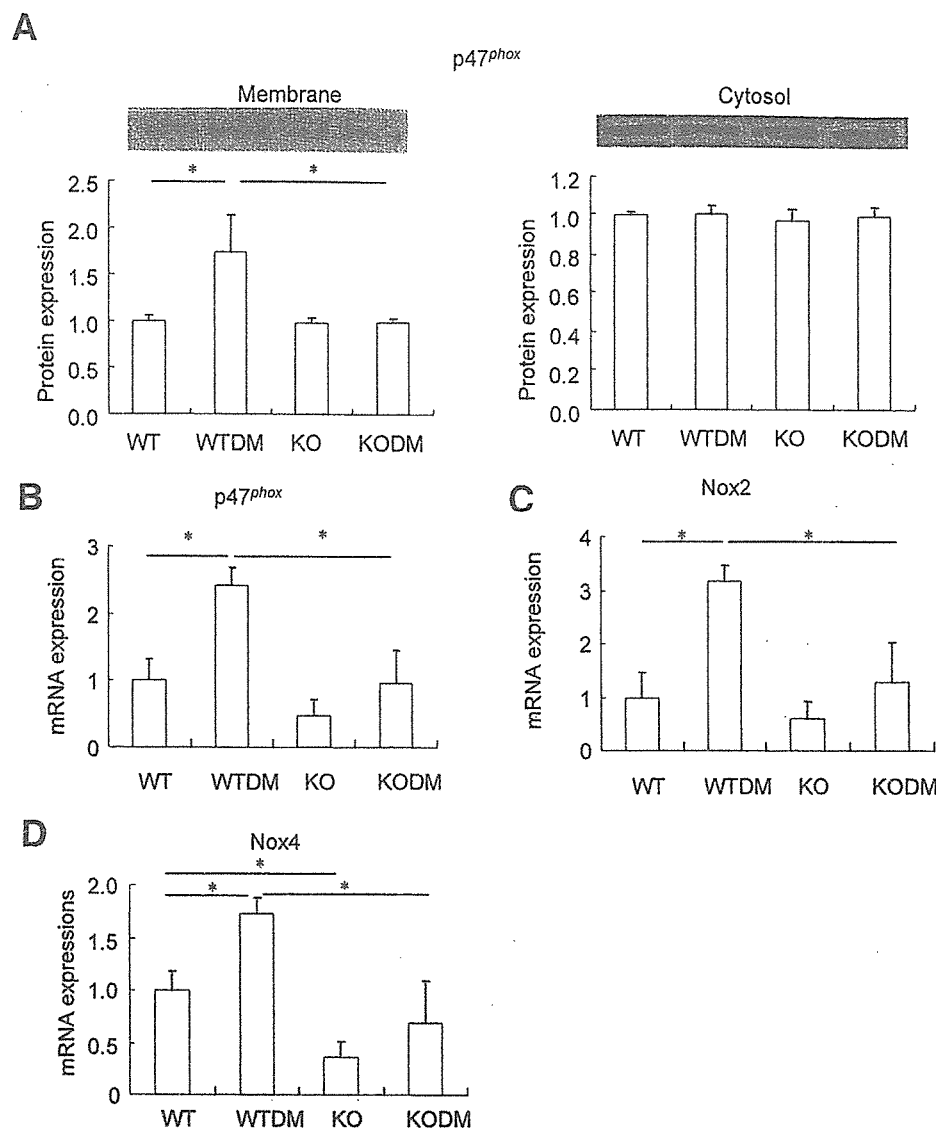


FIG. 5. Activation of NADPH oxidase by hyperglycemia in wild-type and PKC- $\beta^{-/-}$ mice after 8 weeks of diabetes. **A:** Immunoblot analysis of p47 subunit of NADPH oxidase in membranous and cytosol fractions of renal cortex. Values for protein expression are expressed as fold change versus wild-type. $n = 4$ for each group. Renal cortical mRNA expression of p47^{phox} (**B**), Nox2 (**C**), and Nox4 (**D**). Values for mRNA levels are expressed as fold change versus wild-type. $n = 5$ for each group (also indicates the number of mice studied). KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. * $P < 0.01$.

increased by twofold (1.90 ± 0.21 -fold change versus nondiabetic wild-type mice, $P < 0.01$) as compared with wild-type control mice. In contrast, the mRNA level of ET-1 was not increased in diabetic PKC- $\beta^{-/-}$ mice (fold change compared with nondiabetic wild-type mice: nondiabetic PKC- $\beta^{-/-}$ 0.31 ± 0.21 -fold versus diabetic PKC- $\beta^{-/-}$ 0.37 ± 0.16 -fold, $P > 0.05$), in which expression level was significantly less compared with that observed in diabetic wild-type mice (diabetic PKC- $\beta^{-/-}$ 0.37 ± 0.16 -fold versus diabetic wild-type 1.90 ± 0.21 -fold, $P < 0.01$). The baseline expression of ET-1 in renal cortex of nondiabetic PKC- $\beta^{-/-}$ mice was also significantly less than that of nondiabetic wild-type mice (0.31 ± 0.21 -fold of nondiabetic wild-type mice, $P < 0.01$).

Similarly, diabetes induced a significant increase in the renal cortical expression of VEGF mRNA in diabetic wild-type mice (1.31 ± 0.16 -fold of nondiabetic wild-type mice, $P < 0.05$) but not in diabetic PKC- $\beta^{-/-}$ mice (fold change compared with nondiabetic wild-type: nondiabetic PKC- $\beta^{-/-}$ 0.43 ± 0.14 -fold versus diabetic PKC- $\beta^{-/-}$

0.67 ± 0.28 -fold, $P > 0.05$). Ablation of the PKC- β gene reduced the basal expression of VEGF mRNA in the renal cortex of nondiabetic PKC- $\beta^{-/-}$ mice as compared with nondiabetic wild-type mice (0.43 ± 0.14 -fold of nondiabetic wild-type mice, $P < 0.01$).

Reduced glomerular hypertrophy and accumulation of extracellular matrix components in renal cortex of diabetic PKC- $\beta^{-/-}$ mice. Increases in the expressions of fibrotic factors, such as TGF- β and CTGF, and extracellular matrix proteins, such as collagens, are believed to be partly responsible for the glomerular enlargements and fibrosis (24). Wild-type mice with diabetes had increased mRNA expression of TGF- β (191%, $P < 0.01$), CTGF (63%, $P < 0.01$), collagen IV (134%, $P < 0.01$), and collagen VI (102%, $P < 0.01$), which were observed following 8 weeks of diabetes (Fig. 6). In contrast, there was no significant increase in mRNA expression of CTGF and collagen IV and VI among diabetic PKC- $\beta^{-/-}$ mice after 8 weeks (Fig. 6B-D). There was significant increase in expression of TGF- β after 8 weeks of diabetes in the PKC- $\beta^{-/-}$ mice, although

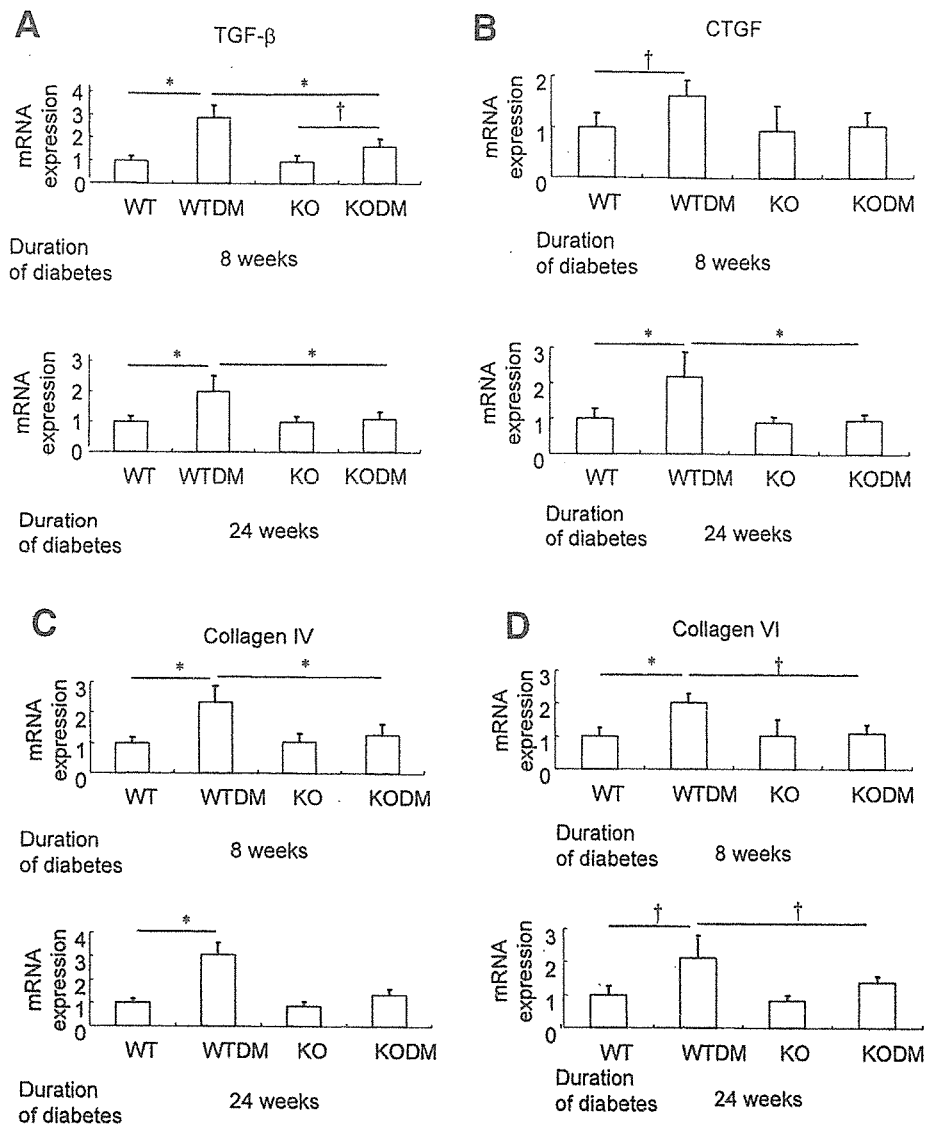


FIG. 6. Expression of genes associated with fibrosis in the renal cortex of wild-type and PKC- $\beta^{-/-}$ mice after 8 and 24 weeks of diabetes. Renal cortical mRNA expression of TGF- β (A), CTGF (B), collagen IV (C), and collagen VI (D). Values are expressed as fold change versus wild-type. KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. $n = 5$ for each group (also indicates the number of mice studied). * $P < 0.01$, † $P < 0.05$.

this was significantly less than the increase observed in wild-type mice (Fig. 6A). Following 24 weeks of diabetes, there was a sustained increase in the expression of TGF- β , CTGF, and collagen IV and VI in the diabetic wild-type mice (Fig. 6). Long duration of diabetes was associated with increased expression of only collagen VI in the PKC- $\beta^{-/-}$ mice, although the increase was less than in the wild-type mice.

Kidney weight was significantly increased (24%, $P < 0.01$) in wild-type mice following 8 weeks of diabetes (Fig. 7A). This increase in kidney weight was not found in diabetic PKC- $\beta^{-/-}$ mice for the same duration. Likewise, while there was significant increase in kidney weight in wild-type mice after 24 weeks of diabetes, there was significantly less renal enlargement in PKC- $\beta^{-/-}$ mice with diabetes of the same duration (Fig. 7A).

Morphometric analysis showed that glomerular size was increased by 24.2% ($P < 0.05$) in wild-type mice following 24 weeks of diabetes. In contrast, there was no glomerular enlargement observed in PKC- $\beta^{-/-}$ mice exposed to the same duration of diabetes (Fig. 7B).

DISCUSSION

Previous studies have used RBX to determine which of the various renal dysfunctions and pathologies are due to the activation of PKC- β isoforms (10,12,13). However, the results are not definitive, since RBX is an isoform-selective inhibitor, which at the dose used may inhibit PKC- α activation by 10–20% (10). We showed that PKC- $\beta^{-/-}$ mice could be used to test the specific effects of PKC- β isoforms on renal function, since immunoblot analysis of the renal cortex demonstrated that diabetes can still activate PKC- α isoform in these mice, unlike the wild-type mice where both PKC- α and - β 1 were increased. These results show for the first time that PKC- α and - β are activated independently by diabetes or hyperglycemia; several reports have shown that PKC- β activation may increase PKC- α expression (25). This suggestion of PKC- α and - β isoforms being independently activated is more than a biochemical interest because it indicates that a selective PKC- β isoform inhibitor may not completely prevent all renal pathologies of diabetes unless it can also partially inhibit the activation of PKC- α .

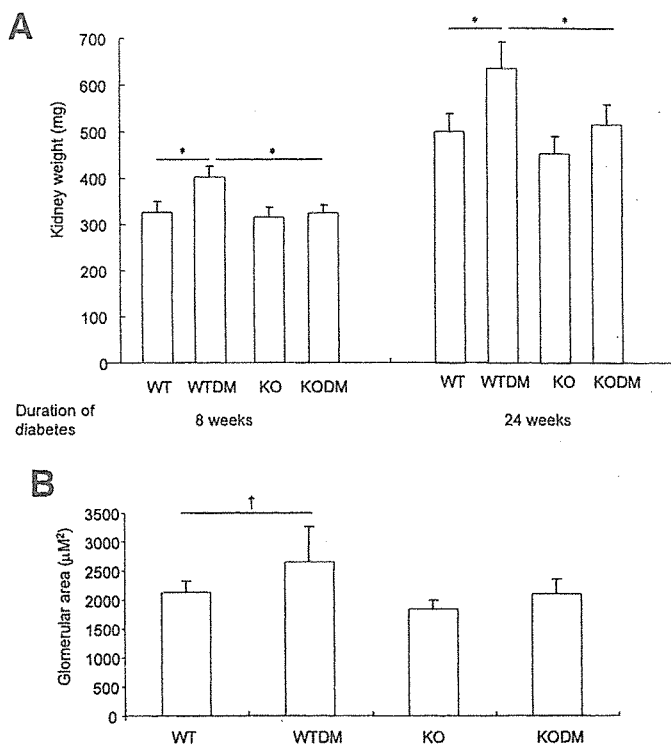


FIG. 7. A: Kidney weight of wild-type and PKC- $\beta^{-/-}$ mice after 8 and 24 weeks of diabetes. $n = 6$ for each group (also indicates the number of mice studied). **B:** Glomerular area measured by morphometric analysis of glomeruli in kidneys of wild-type and PKC- $\beta^{-/-}$ mice after 24 weeks of diabetes. KO, PKC- $\beta^{-/-}$ nondiabetic mice; KODM, PKC- $\beta^{-/-}$ diabetic mice; WT, wild-type mice; WTDM, wild-type diabetic mice. * $P < 0.01$, † $P < 0.05$.

One of the most interesting findings in this study is the protection from hyperglycemia-induced oxidative stress in mice lacking PKC- β isoforms, as shown by the significant decreases in urinary isoprostane and 8-OHdG, which were maintained even after 24 weeks of diabetes. This lowering of oxidative stress appears to be partially due to renal origin, since several subunits and isoforms of NADPH oxidase, a major producer of oxidants (26), were found to be decreased in their expression in the renal cortex of PKC- $\beta^{-/-}$ mice. The decreases in Nox activities appear to be due to lowering of both activation and expression, since the translocation of p47^{phox} and the expressions of Nox2 and -4 were significantly reduced in the diabetic PKC- $\beta^{-/-}$ mice. These data suggest that PKC- β is a major inducer of oxidative stress in diabetes, possibly via the activation of Nox complex, although confirmation by NADPH oxidase activities would be needed to support the changes in mRNA levels. This is consistent with recent work suggesting that diabetes leads to increased expression of NADPH oxidase subunits Nox4 and p22^{phox}, which may be responsible for overproduction of ROS and the renal damage seen in diabetes (15,16). Nox4-derived ROS were found to induce renal hypertrophy and increase fibronectin expression (27). In addition, chronic inhibition of NADPH oxidase was found to prevent podocyte apoptosis, albuminuria, and mesangial expansion in a mouse model of diabetes (28). Amelioration of several markers of renal damage in mice lacking PKC- β , along with absent activation of NADPH oxidase, suggests NADPH oxidase is a downstream target of PKC- β . Previous studies have demonstrated that glucose and fatty acids increase ROS production via PKC-dependent activation of NADPH oxi-

dase (29). Recent in vitro studies using inhibitors specific for PKC- β isoforms suggest that PKC- β is the main isoform responsible for activating Nox (30). Furthermore, the increased activation of NADPH oxidase and increased urinary 8-hydroxyguanosine excretion in diabetic animals can be improved by treatment with the PKC- β inhibitor ruboxistaurin mesylate (22). Our in vivo studies confirmed the key role of PKC- β in diabetes-induced NADPH oxidase activation and the protective effect of PKC- β against ROS-induced renal damage. This is consistent with the renoprotective effect observed in both animal and human studies using RBX (10,12,14). However, the results from the present study also showed that the increases in isoprostane were not completely suppressed in diabetic PKC- $\beta^{-/-}$ mice, which is different from findings when diabetic animals were treated with RBX. This suggests that PKC- α can also induce oxidant production either via Nox or other pathways. Further studies are needed to determine cellular sites of oxidant production by measuring intracellular oxidative markers such as nitrosylated proteins and 4-hydroxynonenal adducts in renal tissues and cells.

The source of ROS production remains controversial, with evidence suggesting that several pathways, including enhanced formation of AGEs (6), altered polyol pathway activity (31), increased PKC activity (29), and increased superoxide release from the mitochondria (6), may be involved. Recently, there is accumulating evidence that NADPH oxidase is the most important source of ROS production in vascular tissues and may be involved in vascular damage caused by hypercholesterolemia, hypertension, and atherosclerosis (32–34). The kidney is vulnerable to oxidative damage and is known to express NADPH oxidase and generate ROS (26). Increased production of ROS within the glomeruli decreases NO bioactivity on mesangial contraction and arteriolar tone and may contribute directly to the renal hemodynamic and vascular abnormalities observed during the initiation and established phase of diabetic nephropathy (28). The beneficial effects of ACE inhibitors (35) and angiotensin receptor blockers (36) could also be related to reduction of ROS production, since angiotensin has been reported to induce or activate NADPH oxidase (32–34).

PKC- β activation can also affect hemodynamic changes by increasing the expression of ET-1 possibly via excessive oxidant production. ET-1 has been implicated in diabetic kidney injury and may contribute to glomerular injury and interstitial tubular changes in diabetes (23,37). Changes in the expression of ET-1 in PKC- $\beta^{-/-}$ mice are consistent with previous observations suggesting that PKC- β activation can regulate both basal and diabetes-induced ET-1 expression (38). A similar pattern of changes is noted for VEGF expression, which was increased in diabetic wild-type but not changed in the diabetic PKC- $\beta^{-/-}$ mice. The physiological role of VEGF overexpression in the renal cortex is not clear but will likely have effects on renal plasma flow (39). It is interesting to note that diabetes-induced ET-1 and VEGF expressions were completely suppressed in the diabetic PKC- $\beta^{-/-}$ mice, unlike the oxidative parameters and Nox2 and Nox4 expressions, suggesting that PKC- β activation is more important for the induction of ET-1 and VEGF than PKC- α isoform.

Renal enlargement in diabetes results especially from tubular hypertrophy and also from glomerular enlargement and accumulation of extracellular matrix (24). This hypertrophy usually precedes the development of irrevers-

ible renal changes including glomerulosclerosis and tubulointerstitial fibrosis. The present studies demonstrate that PKC- β plays a critical role in diabetes-induced tubular hypertrophy and glomerular enlargement, since diabetic PKC- $\beta^{-/-}$ mice did not show the expected increase in kidney weight or glomerular area. TGF- β and CTGF, in particular, have been identified as key cytokines mediating the changes that lead to extracellular matrix accumulation and glomerular enlargement (40,41). Whereas induction of diabetes led to marked increases in the expression of TGF- β and CTGF in wild-type mice, this increase in TGF- β expression was attenuated at 8 weeks of diabetes and was absent at 6 months of disease, whereas the increased expression of CTGF was completely absent in PKC- $\beta^{-/-}$ mice. This may account for the lack of glomerular enlargement and nephromegaly and marked attenuation in diabetes-induced increase in expression of collagen IV and VI seen in the PKC- $\beta^{-/-}$ mice. These findings are consistent with previous observations suggesting that PKC- β isoforms can mediate extracellular matrix accumulation in the diabetic kidney (10,13,42) and in other tissues (43).

In contrast to the complete lack of renal and glomerular hypertrophy in diabetic PKC- $\beta^{-/-}$ mice, diabetes in the PKC- $\beta^{-/-}$ mice was associated with only partial protection from increased urinary albumin excretion. In addition to extracellular matrix accumulation and mesangial expansion, several other factors are known to be important in determining albuminuria. This includes reduced glomerular nephrin expression, effacement of podocyte foot processes, as well as apoptosis and progressive loss of glomerular podocytes (24). Interestingly, effects of PKC activation on podocyte biology and functions are not known. Other pathways in addition to PKC- β may also mediate some of these processes. Recently, it was shown that hyperglycemia-induced downregulation of the negatively charged basement membrane heparin sulfate proteoglycan perlecan was prevented in mice lacking PKC- α , which also had a reduction in diabetes-associated albuminuria (44). Our data on PKC activity in renal cortex and activation of the α isoform in the PKC- $\beta^{-/-}$ mice would be consistent with the view that activation of PKC- α may also contribute to albuminuria observed in diabetes despite it not being involved in causing extracellular matrix accumulation and renal hypertrophy.

In summary, the results derived from using the PKC- $\beta^{-/-}$ mice have identified the specific role of PKC- β in mediating diabetes-induced increases in the expression of CTGF and TGF- β with the resultant glomerular and renal enlargement. These effects of PKC- β may be partially due to its actions on enhancing the actions or expressions of the Nox complex of oxidases. In addition, these results have also strongly suggested that PKC- α or other pathways may be partly responsible for causing diabetes-induced increases in oxidative stress and elevated albuminuria. Strategies to target the DAG-PKC pathway using isoform-specific inhibitors of PKC- β may need to take in to consideration that some inhibition of PKC- α may also be necessary to prevent most hyperglycemia-induced injury in the renal glomeruli. Inhibition of PKC- β isoforms does not appear to have significant clinical side effects. However, inhibiting both PKC- β and - α isoforms may have more side effects than inhibiting a single isoform, since PKC activation has been implicated in essential physiological functions, even for insulin secretion (45).

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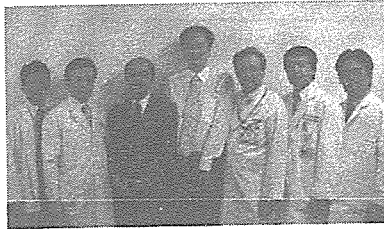
REFERENCES

- Raptis AE, Viberti G: Pathogenesis of diabetic nephropathy. *Exp Clin Endocrinol Diabetes* 109 (Suppl. 2):S424-S437, 2001
- Mauer SM, Steffes MW, Goetz FC, Sutherland DE, Brown DM: Diabetic nephropathy: a perspective. *Diabetes* 32 (Suppl. 2):52-55, 1983
- Cooper ME: Interaction of metabolic and haemodynamic factors in mediating experimental diabetic nephropathy. *Diabetologia* 44:1957-1972, 2001
- Koya D, King GL: Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859-866, 1998
- Obrosova IG, Minchenko AG, Vasupuram R, White L, Abatan OI, Kumagai AK, Frank RN, Stevens MJ: Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. *Diabetes* 52:864-871, 2003
- Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
- Wendt T, Harja E, Bucciarelli L, Qu W, Lu Y, Rong LL, Jenkins DG, Stein G, Schmidt AM, Yan SF: RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. *Atherosclerosis* 185:70-77, 2006
- Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL: Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* 89:11059-11063, 1992
- Craven PA, Studer RK, Negrete H, DeRubertis FR: Protein kinase C in diabetic nephropathy. *J Diabetes Complications* 9:241-245, 1995
- Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL: Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 100:115-126, 1997
- Whiteside CI, Dlugosz JA: Mesangial cell protein kinase C isozyme activation in the diabetic milieu. *Am J Physiol Renal Physiol* 282:F975-F980, 2002
- Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunctions in diabetic rats by an oral PKCbeta inhibitor. *Science* 272:728-731, 1996
- Koya D, Haneda M, Nakagawa H, Ishiki K, Sato H, Maeda S, Sugimoto T, Yasuda H, Kashiwagi A, Wada DK, King GL, Kikkawa R: Amelioration of accelerated diabetic mesangial expansion by treatment with a PKCbeta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* 14:439-447, 2000
- Tuttle KR, Bakris GL, Toto RD, McGill JB, Hu K, Anderson PW: The effect of ruboxistaurin on nephropathy in type 2 diabetes. *Diabetes Care* 28:2686-2690, 2005
- Etoh T, Inoguchi T, Kakimoto M, Sonoda N, Kobayashi K, Kuroda J, Sumimoto H, Nawata H: Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibility by interventional insulin treatment. *Diabetologia* 46:1428-1437, 2003
- Satoh M, Fujimoto S, Haruna Y, Arakawa S, Horike H, Komai N, Sasaki T, Tsujioka K, Makino H, Kashiwara N: NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 288:F1144-F1152, 2005
- Abiko T, Abiko A, Clermont AC, Shoelson B, Horio N, Takahashi J, Adamis AP, King GL, Bursell SE: Characterization of retinal leukostasis and hemodynamics in insulin resistance and diabetes: role of oxidants and protein kinase-C activation. *Diabetes* 52:829-837, 2003
- Dekker LV, Leitges M, Altschuler G, Mistry N, McDermott A, Roes J, Segal AW: Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. *Biochem J* 347:285-289, 2000
- Leitges M, Schmedt C, Guinamard R, Davoust J, Schaal S, Stabel S,

- Tarakhovskiy A: Immunodeficiency in protein kinase c β -deficient mice. *Science* 273:788–791, 1996
20. Koya D, Lee IK, Ishii H, Kanoh H, King GL: Prevention of glomerular dysfunction in diabetic rats by treatment with d-alpha-tocopherol. *J Am Soc Nephrol* 8:426–435, 1997
 21. Vicent D, Ilany J, Kondo T, Naruse K, Fisher SJ, Kisanuki YY, Bursell S, Yanagisawa M, King GL, Kahn CR: The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *J Clin Invest* 111:1373–1380, 2003
 22. Kitada M, Koya D, Sugimoto T, Isono M, Araki S, Kashiwagi A, Haneda M: Translocation of glomerular p47phox and p67phox by protein kinase C- β activation is required for oxidative stress in diabetic nephropathy. *Diabetes* 52:2603–2614, 2003
 23. Sorokin A, Kohan DE: Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285:F579–F589, 2003
 24. Wolf G, Chen S, Ziyadeh FN: From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *Diabetes* 54:1626–1634, 2005
 25. Collazos A, Diouf B, Guerineau NC, Quittau-Prevostel C, Peter M, Coudane F, Hollande F, Joubert D: A spatiotemporally coordinated cascade of protein kinase C activation controls isoform-selective translocation. *Mol Cell Biol* 26:2247–2261, 2006
 26. Geiszt M, Kopp JB, Varnai P, Leto TL: Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 97:8010–8014, 2000
 27. Gorin Y, Block K, Hernandez J, Bhandari B, Wagner B, Barnes JL, Abboud HE: Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. *J Biol Chem* 280:39616–39626, 2005
 28. Susztak K, Raff AC, Schiffer M, Bottinger EP: Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes* 55:225–233, 2006
 29. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49:1939–1945, 2000
 30. Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A: Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes* 52:2795–2804, 2003
 31. Chung SS, Ho EC, Lam KS, Chung SK: Contribution of polyol pathway to diabetes-induced oxidative stress. *J Am Soc Nephrol* 14:S233–S236, 2003
 32. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86:494–501, 2000
 33. Li JM, Shah AM: Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. *Cardiovasc Res* 52:477–486, 2001
 34. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK: Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 105:1429–1435, 2002
 35. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy: the Collaborative Study Group. *N Engl J Med* 329:1456–1462, 1993
 36. Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, Shahinfar S: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869, 2001
 37. Mishra R, Emancipator SN, Kern TS, Simonson MS: Association between endothelin-1 and collagen deposition in db/db diabetic mouse kidneys. *Biochem Biophys Res Commun* 339:65–70, 2006
 38. Yokota T, Ma RC, Park JY, Isshiki K, Sotiropoulos KB, Rauniyar RK, Bornfeldt KE, King GL: Role of protein kinase C on the expression of platelet-derived growth factor and endothelin-1 in the retina of diabetic rats and cultured retinal capillary pericytes. *Diabetes* 52:838–845, 2003
 39. Ichinose K, Maeshima Y, Yamamoto Y, Kitayama H, Takazawa Y, Hirokoshi K, Sugiyama H, Yamasaki Y, Eguchi K, Makino H: Antiangiogenic endostatin peptide ameliorates renal alterations in the early stage of a type 1 diabetic nephropathy model. *Diabetes* 54:2891–2903, 2005
 40. Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K: Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020, 2000
 41. Sakharova OV, Taal MW, Brenner BM: Pathogenesis of diabetic nephropathy: focus on transforming growth factor-beta and connective tissue growth factor. *Curr Opin Nephrol Hypertens* 10:727–738, 2001
 42. Cohen MP, Ziyadeh FN, Lautenslager GT, Cohen JA, Shearman CW: Glycated albumin stimulation of PKC-beta activity is linked to increased collagen IV in mesangial cells. *Am J Physiol* 276:F684–F690, 1999
 43. Way KJ, Isshiki K, Suzuma K, Yokota T, Zvagelsky D, Schoen FJ, Sandusky GE, Pechous PA, Vlahos CJ, Wakasaki H, King GL: Expression of connective tissue growth factor is increased in injured myocardium associated with protein kinase C β 2 activation and diabetes. *Diabetes* 51:2709–2718, 2002
 44. Menne J, Park JK, Boehne M, Elger M, Lindschau C, Kirsch T, Meier M, Gueler F, Fiebeler A, Bahlmann FH, Leitges M, Haller H: Diminished loss of proteoglycans and lack of albuminuria in protein kinase C α -deficient diabetic mice. *Diabetes* 53:2101–2109, 2004
 45. Sjöholm A: Glucose stimulates islet α -cell mitogenesis through GTP-binding proteins and by protein kinase C-dependent mechanisms. *Diabetes* 46:1141–1147, 1997

Angiographic flow grading and graft arrangement of arterial conduits

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Objective: We sought to delineate the effects of competitive and reverse flow on the intermediate-term patency of arterial conduits and examined graft arrangements for maximizing antegrade bypass flow.

Methods: The angiograms of 2083 bypass grafts in 570 patients who underwent off-pump total arterial revascularization without aortic manipulation since December 2000 were reviewed. The blood flow in the bypass grafts were graded A (antegrade), B (competitive), C (reverse), or O (occlusion). The mean number of distal anastomoses was 3.65 ± 0.94 per patient.

Results: In the early angiography 91.3% (1901/2083) of the bypasses were grade A. Thirty (1.4%) bypasses were grade O, whereas 2.9% (61/2083) were grade B, and 4.4% (91/2083) were grade C. In the multivariate analysis the end-to-side anastomosis ($P < .0001$), 4 or more distal anastomoses of the conduit ($P = .01$), native coronary stenosis of less than 75% ($P < .0001$), and target branch location of the right coronary artery territory ($P < .0001$) and left circumflex artery territory ($P = .02$) significantly correlated with grade non-A. The patency rate in the late angiography of the bypasses graded B or C in the early angiography was 7 (28.0%) of 25, whereas that of the bypasses graded A was 164 (89.1%) of 184 ($P < .0001$). The actuarial graft patency rate of the bypasses graded A was 72.3% at 3 years and was significantly higher than that of the bypasses graded B or C (28.6% at 3 years after surgical intervention, $P < .0001$).

Conclusions: The sufficient antegrade bypass flow had a favorable effect on the graft patency of arterial conduits. The graft arrangement should be adjusted for each patient so as to maximize the antegrade bypass flow and to confirm the advantage of arterial grafts.

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The use of the in situ internal thoracic artery (ITA) graft to the left anterior descending artery (LAD) is widely accepted as a standard strategy that provides long-term patency and improves late survival. The radial artery is also useful as a free graft for coronary artery bypass grafting (CABG). For the radial artery, the graft patency mostly depended on the severity of the native coronary stenosis rather than on the proximal anastomotic site^{1,2} and was equivalent to that of ITA, even when the target coronary branch was small in diameter or had severe atherosclerosis.^{3,4}

In the bypass conduits having 2 or more distal anastomoses, dominant reversal flow is not quite rare. Because the bypass grafts with reverse flow do not contribute to the coronary perfusion in the grafted territory, the efficacy of CABG might be unpromising, even when the bypass graft is anatomically patent. In addition, as a consequence of inappropriate graft flow, graft failure, such as diffuse narrowing or occlusion, can occur because it has been reported that the arterial materials have shown adaptability of their own diameter to the circumstances of the blood flow in the graft lumen.⁵⁻⁹