Table 3. Fat distribution of women in low, median and high tertiles of lower-body fat mass in the middle tertile of trunk fat mass (A) and fat distribution in low, median and high tertiles of trunk fat mass in the middle tertile of lower-body fat mass (B)

A) Women with TFM in the median tertile	Tertiles of lower-body fat mass		
	Low (n=54)	Median (n=53)	High (n=54)
Body weight (kg)	52.4±5.4^{a}	53.7±5.1^{a}	56.7±6.5^{b}
Body fat mass (kg)	12.5±0.7^{a}	13.5±0.7^{b}	15.0±1.0^{c}
Trunk fat mass (kg)	6.3±0.5^{a}	6.4±0.4^{a}	6.6±0.5^{b}
Lower-body fat mass (kg)	4.6±0.4^{a}	5.5±0.2^{b}	6.5±0.5^{c}
B) Women with LFM in the median tertile	**Tertiles of trunk fat mass**		
	Low (n=54)	Median (n=54)	High (n=53)
Body weight (kg)	52.9±5.8^{a}	53.6±5.0^{a,b}	55.0±5.1^{b}
Body fat mass (kg)	12.2±0.6^{a}	13.6±0.7^{b}	15.5±1.3^{c}
Trunk fat mass (kg)	5.4±0.4^{a}	6.4±0.3^{b}	8.1±1.0^{c}
Lower-body fat mass (kg)	5.3±0.3^{a}	5.5±0.3^{b}	5.5±0.3^{b}

Mean±SD. Means not sharing common letters are significantly diferent at $p<0.05$ or less. TFM and LFM: trunk and lower-body fat mass, respectively.

tral obesity[30]. Lower concentrations of hepatic enzymes, including GGT levels[31], and higher adiponectin[32] appear to reflect lower liver fat content. Taken together, we speculate that liver fat may be a knot linking higher adiponectin, lower PAI-1 and GGT associated with larger LFM found in young women. A negative association between adiponectin and central fat mass accumulation and visceral fat mass[33, 34] was confirmed in the present study. Finally and unexpectedly, we found that larger TFM was associated with higher serum TNF-α even in young, healthy, slim women in the present study. We have no explanation for this finding because it is reported that there is no association between body fat distribution and serum TNF-α levels[35, 36], although they were increased in obesity[37, 38].

In addition to favorable effects on the adipokine profile, for a given level of TFM, larger LFM was associated with an advantageous serum lipid profile, i.e., higher HDL cholesterol and lower TG in young healthy, slim women. A favorable association of lower body fat with HDL cholesterol and TG has been reported in postmenopausal women[11, 12] and men[18]. Both serum adiponectin and insulin sensitivity have a mediating role between body fat distribution and blood lipids that may be manifested both in the liver and peripheral tissue[18]. LFM was positively associated with adiponectin in the current study although it was not associated with HOMA-IR, a crude marker of insulin resistance, in young women. Another explanation for favorable effects on the lipid profile is the opposite contributions of regional fat mass to plasma lipase activities[39]. Larger LFM and lower TFM in postmenopausal women were reported to be associated with higher lipoprotein lipase and lower hepatic lipase activities in post-heparin plasma[39], respectively, both of which contribute to higher HDL cholesterol and lower triglyceride[40]. In the present study, pre-heparin LPL was associated positively with LFM and negatively with TFM in young women, although most pre-heparin LPL is in an inactive form but might reflect somewhat the amount of LPL working in the body[41]. There was, however, no difference in pre-heparin LPL mass among the tertiles of both LFM and TFM in the current study.

Abnormal apolipoprotein B metabolism has been reported to occur in the early phase of normoglycemic and fasting normotriglyceridemic insulin-resistant women with abdominal obesity[42]. This may be consistent with our finding that larger TFM was associated with an increase in apolipoprotein B in young, slim women. In addition, elevated concentrations of RLP-cholesterol and triglycerides as well as apolipoprotein B, all of which were associated with larger TFM in young women in the present study, have been shown to be a characteristic feature of the atherogenic lipoprotein phenotype[42, 43]. Finally, larger TFM was associated with a greater ratio of apo B to A1, a strong,

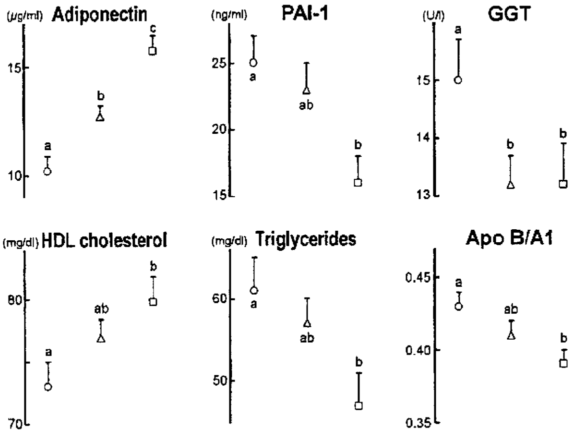

Fig. 1. Serum lipids and adipokines in low ($n=54$, circles), middle ($n=53$, triangles) and high ($n=54$, squares) tertiles of lower body fat mass in the middle tertile of trunk fat mass. Participants were divided into 9 groups according to tertiles of TFM and LFM. In the middle tertile of TFM, TMF ranged from 5.64 to 7.33 kg. Lower body fat mass averaged 4.6 kg in low, 5.5 kg in middle and 6.5 kg in high tertiles of lower-body fat. For details, see Table 3. Data are the mean ± SE. PAI-1: plasminogen activator inhibitor-1, GGT: gamma-glutamyltransferase, ApoB/A1: apolipoprotein B/A1. Means not sharing common letters are significantly different at $p<0.05$ or less.

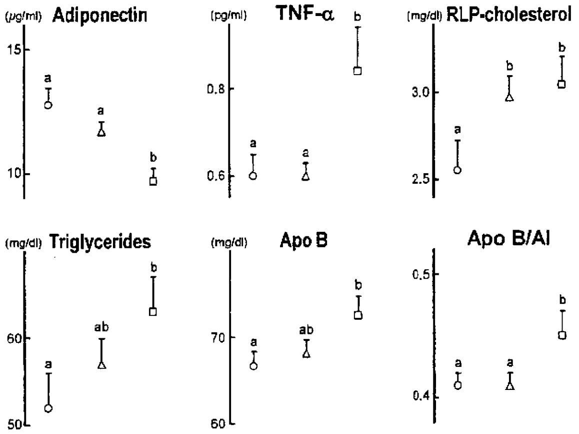

Fig. 2. Serum lipids and adipokines in low ($n=40$, circles), middle ($n=80$, triangles) and high ($n=41$, squares) tertiles of trunk fat mass in the middle tertile of lower-body fat mass. LFM ranged from 4.87 to 5.99 kg in the middle tertile of trunk fat mass. For details, see Table 3. Data are the mean ± SE. Means not sharing common letters are significantly different at $p<0.05$ or less. TNF-α: tumor necrosis factor-α, RLP-cholesterol: remnant-like particle cholesterol, ApoB: apolipoprotein B.

new risk factor for cardiovascular disease[44], in young women in the present study.

The main limitation of our study is that DXA does not allow separate quantification of intermuscular and subcutaneous fat in the legs, and visceral fat and subcutaneous fat in the trunk. The contribution of subcutaneous fat to the total amount of fat in the legs, however, is relatively large[45]; therefore, the associations found in our study with fat mass in the legs are probably mainly due to the subcutaneous fat depot. The cross-sectional design of the present study complicates the drawing of causal inferences, and a single measurement of biochemical variables may be susceptible to short-term variation, which would bias the results toward the null. We used several surrogates in the present study, which may be less accurate.

In summary, larger fat mass in the lower body has a considerable and favorable association with lipoprotein and adipokine metabolism even in healthy, slim, young women. These findings provide relevant new insight into the associations among obesity, body composition, and type 2 diabetes. Further investigation of the underlying pathophysiological mechanism is needed to explain the favorable association of leg fat with lipoprotein and adipokine metabolism.

Acknowledgments

This study was supported by the Open Research Center Project for Private University: Matching Fund Subsidy for Private Universities, The Ministries of Education, Culture, Sports, Science and Technology, Japan. We are indebted to all the participants for their dedicated and conscientious collaboration.

References

1) Rexrode KM, Carey VJ, Hennekens CH, Walters EE, Colditz GA, Stampfer MJ, Willett WC, Manson JE: Abdominal adiposity and coronary heart disease in women. JAMA, 1998; 280: 1843-1848
2) Kanaya AM, Vittinghoff E, Shlipak MG, Resnick HE, Visser M, Grady D, Barrett-Connor E: Association of total and central obesity with mortality in postmenopausal women with coronary heart disease. Am J Epidemiol, 2003; 158: 1161-1170
3) Carey VJ, Walters EE, Colditz GA, Solomon CG, Willett WC, Rosner BA, Speizer FE, Manson JE: Body fat distribution and risk of noninsulin-dependent diabetes mellitus

in Women. The Nurses' Health Study. Am J Epidemiol, 1997; 145: 614-619

4) Lissner L, Bjorkelund C, Heitmann BL, Seidell JC, Bengtsson C: Larger hip circumference independently predicts health and longevity in a Swedish female cohort. Obes Res, 2001; 9: 644-649

5) Heitmann BL, Frederiksen P, Lissner L: Hip circumference and cardiovascular morbidity and mortality in men and women. Obes Res, 2004; 12: 482-487

6) Snijder MB, Dekker JM, Visser M, Bouter LM, Stehouwer CD, Kostense PJ, Yudkin JS, Heine RJ, Nijpels G, Seidell JC: Associations of hip and thigh circumferences independent of waist circumference with the incidence of type 2 diabetes: the Hoorn study. Am J Clin Nutr, 2003; 77: 1192-1197

7) Seidell JC, Pérusse L, Deprés J-P, Bouchard C: Waist and hip circumferences have independent and opposite effects on cardiovascular disease risk: the Quebec family study. Am J Clin Nutr, 2001; 74: 315-321

8) Snijder MB, Zimmet PZ, Visser M, Dekker JM, Seidell JC, Shaw JE: Independent and opposite associations of waist and hip circumferences with diabetes, hypertension and dyslipidemia: the AusDiab study. Int J Obes Relat Metab Disord, 2004; 28: 402-409

9) Aasen G, Fagertun H, Halse J: Regional fat mass by DXA: High leg fat mass attenuates the relative risk of insulin resistance and dyslipidaemia in obese but not in overweight postmenopausal women. Scan L Clin Lab Invest, 2008; 68: 204-211

10) Williams MJ, Hunter GR, Kekes-Szabo T, Snyder S, Treuth MS: Regional fat distribution in women and risk of cardiovascular disease. Am J Clin Nutr, 1997; 5: 855-860

11) Van Pelt RE, Evans EM, Schechtman KB, Ehsani AA, Kohrt WM: Contribution of total and regional fat mass to risk for cardiovascular disease in older women. Am J Physiol Endocrinol Metab, 2002; 282: E1023-1028

12) Van Pelt RE, Jankowski CM, Gozansky WS, Schwartz RS, Kohrt WM: Lower-body adiposity and metabolic protection in postmenopausal women. J Clin Endocrinol Metab, 2005; 90: 4573-4578

13) Tatsukawa M, Kurokawa M, Tamari Y, Yoshimatsu H, Sakata T: Regional fat deposition in the legs is useful as a presumptive marker of antiatherogenicity in Japanese. Proc Soc Exp Bio Med, 2000; 223: 156-162

14) Tank LB, Bagger YZ, Alexandersen P, Larsen PJ, Christiansen C: Peripheral adiposity exhibits an independent dominant antiatherogenic effect in elderly women. Circulation, 2003; 107: 1626-1631

15) Snijder MB, Dekker JM, Visser M, Bouter LM, Stehouwer CD, Yudkin JS, Heine RJ, Nijpels G, Seidell JC; Hoorn study: Trunk fat and leg fat have independent and opposite associations with fasting and postload glucose levels. Diabetes Care, 2004; 27: 372-377

16) Snijder MB, Visser M, Dekker JM, Gooodpaster BH, Harris TB, Kritechevsky SB, De Rekeneire N, Kanaya AM, Newman AB, Tylavsky FA, Seidell JC; Health ABC Study: Low subcutaneous thigh fat is a risk factor for unfavourable glucose and lipid levels, independently of high abdominal fat. The health ABC study. Diabetologia, 2005; 48: 301-308

17) Snijder MB, Flyvbjerg A, Stehouwer CD, Frystyk J, Henry RM, Seidell JC, Heine RJ, Dekker JM: Relationship of adiposity with arterial stiffness as mediated by adiponectin in older men and women: the Hoorn Study. Eur J Endocrinol, 2009; 160: 387-395

18) Buemann B, Astrup A, Pedersen O, Black E, Holst C, Toubro S, Echwald S, Holst JJ, Rasmussen C, Sørensen TI; Possible role of adiponectin and insulin sensitivity in mediating the favorable effects of lower body fat mass on blood lipids. J Clin Endocrinol Metab, 2006; 91; 1698-1704

19) Bentley-Lewis R, Koruda K, Seely EW: The metabolic syndrome in women. Nat Clin Pract Endocrinol Metab, 2007; 3: 696-704

20) Boorsma W, Snijder MB, Nijpels G, Guidone C, Favuzzi AM, Mingrone G, Kostense PJ, Heine RJ, Dekker JM: Body composition, insulin sensitivity, and cardiovascular disease profile in healthy Europeans. Obesity (Silver Spring), 2008; 16: 2696-2701

21) Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, 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

22) Matsuda M, DeFronzo RA: Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care, 1999; 22: 1462-1470

23) Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem, 1972; 18: 499-502

24) Hirano T, Ito Y, Saegusa H, Yoshino G: A novel and simple method for quantification of small, dense LDL. J Lipid Res, 2003; 44: 2193-2201

25) Kobayashi J, Hashimoto H, Hashimoto H, Fukamachi I, Tashiro J, Shirai K, Saito Y, Yoshida S: Lipoprotein lipase mass and activity in severe hypertriglyceridemia. Clin Chim Acta, 1993; 216: 113-123

26) Van Harmelen V, Dicker A, Ryden M, Hauner H, Lonnqvist F, Naslund E, Arner P: Increased lipolysis and decreased leptin production by human omental as compared with subcutaneous preadipocytes. Diabetes, 2000; 51: 2029-2036

27) Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ, Rosato FE, Goldstein BJ: Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. J Endocrinol Metab, 2002; 87: 5662-5667

28) Manolopoulos KN, Karpe F, Frayn KN: Gluteofemoral body fat as a determinant of metabolic health. Int J Obes (Lond), 2010; 34: 949-959

29) Marie-Christine Alessi, Delphine Bastelica, Alenka Mavri, Pierre Morange, Bruno Berthet, Michel Grino, Irene Juhan-Vague: Plasma PAI-1 levels are more strongly related to liver steatosis than to adipose tissue accumulation. Arterioscler Thromb Vasc Biol, 2003; 23: 1262-1268

30) Alessi MC, Peiretti F, Morange P, Henry M, Nalbone G, Juhan-Vague I: Production of plasminogen activator inhibitor 1 by human adipose tissue: possible link

between visceral fat accumulation and vascular disease. Diabetes, 1997; 46: 860-867

31) Messier V, Karelis AD, Robillard ME, Bellefeuille P, Brochu M, Lavoie JM, Rabasa-Lhoret R: Metabolically healthy but obese individuals: relationship with hepatic enzymes. Metabolism, 2010; 1: 20-24

32) Koska J, Stefan N, Permana PA, Weyer C, Sonoda M, Bogardus C, Smith SR, Joanisse DR, Funahashi T, Krakoff J, Bunt JC: Increased fat accumulation in liver may link insulin resistance with subcutaneous abdominal adipocyte enlargement, visceral adiposity, and hypoadiponectinemia in obese individuals. Am J Clin Nutr, 2008; 87: 295-302

33) Alina Gavrila, Jean L. Chan, Nikos Yiannakouris, Meropi Kontogianni, Lisa C. Miller, Christine Orlova, Christos S. Mantzoros: Serum Adiponectin Levels Are Inversely Associated with Overall and Central Fat Distribution but Are Not Directly Regulated by Acute Fasting or Leptin Administration in Humans: Cross-Sectional and Interventional Studies. J Clin Endocrinol Metab, 2003; 88: 4823-4831

34) Hanley AJ, Bowden D, Wagenknecht LE, Balasubramanyam A, Langfeld C, Saad MF, Rotter JI, Guo X, Chen YD, Bryer-Ash M, Norris JM, Haffner SM: Associations of adiponectin with body fat distribution and insulin sensitivity in nondiabetic Hispanics and African-Americans. J Clin Endocrinol Metab, 2007; 92: 2665-2671

35) Zahorska-Markiewicz B, Janowska J, Olszanecka-Glinianowicz M, Zurakowski A: Serum concentrations of TNF-alpha and soluble TNF-alpha receptors in obesity. Int J Obes Relat Metab Disord, 2000; 24: 1392-1395

36) Katsuki A, Sumida Y, Murashima S, Murata K, Takarada Y, Ito K, Fujii M, Tsuchihashi K, Goto H, Nakatani K, Yano Y: Serum levels of tumor necrosis factor-alpha are increased in obese patients with noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab, 1998; 83: 859-862

37) Perry CD, Alekel DL, Ritland LM, Bhupathiraju SN, Stewart JW, Hanson LN, Matvienko OA, Kohut ML, Reddy MB, Van Loan MD, Genschel U: Centrally located body fat is related to inflammatory markers in healthy postmenopausal women. Menopause, 2008; 15: 619-627

38) Good M, Newell FM, Haupt LM, Whitehead JP, Hutley LJ, Prins JB: TNF and TNF receptor expression and insulin sensitivity in human omental and subcutaneous adipose tissue--influence of BMI and adipose distribution. Diab Vasc Dis Res, 2006; 3: 26-33

39) Bos G, Snijder MB, Nijpels G, Dekker JM, Stehouwer CD, Bouter LM, Heine RJ, Jansen H: Opposite contributions of trunk and leg fat mass with plasma lipase activities: the Hoorn study. Obes Res, 2005; 13: 1817-1823

40) Olivecrona G, Olivecrona T: Triglyceride lipases and atherosclerosis. Curr Opin Lipidol, 1995; 6: 291-305

41) Watanabe H, Miyashita Y, Murano T, Hiroh Y, Itoh Y, Shirai K: Preheparin serum lipoprotein lipase mass level: the effects of age, gender, and types of hyperlipidemias. Atherosclerosis, 1999; 145: 45-50

42) Pont F, Duvillard L, Florentin E, Gambert P, Vergès B: Early kinetic abnormalities of apoB-containing lipoproteins in insulin-resistant women with abdominal obesity. Arterioscler Thromb Vasc Biol, 2002; 22: 1726-1732

43) Twickler TB, Dallinga-Thie GM, Cohn JS, Chapman MJ: Elevated remnant-like particle cholesterol concentration: a characteristic feature of the atherogenic lipoprotein phenotype.Circulation, 2004; 109: 1918-1925

44) Walldius G, Jungner I: The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy--a review of the evidence. J Intern Med, 2006; 259: 493-519

45) Goodpaster BH, Thaete FL, Kelley DE: Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus. Am J Clin Nutr, 2000; 71: 885-892

Original Article

Associations of 18-Year-Old Daughters' and Mothers' Serum Leptin, Body Mass Index and DXA-Derived Fat Mass

Sanae Tanaka[1], Wu Bin[2], Mari Honda[2], Seiki Nanbu[1], Kazuhisa Suzuki[1, 2], Keisuke Fukuo[1, 2], and Tsutomu Kazumi[1, 2]

[1] Department of Food Sciences and Nutrition, School of Human Environmental Sciences, Hyogo, Japan
[2] Open Research Center for Lifestyle-related Diseases, Mukogawa Women's University, Hyogo, Japan

Aim and Methods: We assessed the relationship of the body mass index (BMI) of 187 college female students aged 18 years with the reported BMI of their middle-aged biological parents measured on 2 occasions: when the parents were 18–20 years old and at the time of the study. The relationships of fat mass measured using whole body dual energy X-ray absorptiometry (DXA) and serum leptin levels were also determined between 148 daughters and middle-aged parents (148 mothers and 59 fathers).

Results: The BMI of daughters was associated with their mothers' BMI ($r=0.30$, $p<0.0001$) but not with their fathers' BMI measured when they were 18 years old. Daughters' BMI showed a stronger association with the current BMI of their mothers BMI ($r=0.36$, $p<0.0001$) than that of their fathers' BMI ($r=0.19$, $p=0.01$). In addition, the serum leptin levels of daughters were correlated with their mothers' leptin values ($r=0.22$, $p=0.04$). Further, not only total body fat mass ($r=0.19$, $p<0.05$) but also fat mass in the trunk ($r=0.18$, $p<0.05$) and legs ($r=0.17$, $p<0.05$) was associated between daughters and their mothers.

Conclusion: The significant correlation between daughters' and mothers' BMI measured when their mothers were 18 years old did not result from shared environmental factors, including the intrauterine environment. The results in the present study therefore suggest that adiposity in 18-year-old daughters may be influenced by the maternal effect. The associations of serum leptin and DXA-derived fat mass between daughters and their mothers may support our hypothesis.

J Atheroscler Thromb, 2010; 17:1077-1081.

Key words; BMI, Leptin, Fat mass, DXA, Offspring-parent relation

Introduction

The contributions of genetic and environmental factors to obesity in humans have been investigated in a variety of family studies covering a wide range of age[1]. Genetic transmission as well as familial aggregation of obesity have been reported[2]. Pedigree studies provide one means for disentangling the genetic and environmental sources of covariation among traits. Inspection of significant familial patterns can lead to certain genetic and environmental inferences. For example, a pattern of significant correlations among siblings and between parents and offspring (who share about half their genes), but not between spouses (who share few genes, assuming random mating), suggests genetic heritability. Similarly, cross-trait familial correlations lead to the same type of genetic and environmental inferences. A pattern of significant cross-trait correlations between parents' body size and offspring's insulin level, and among siblings, but not spouses, would suggest that a common gene (or genes) influences both traits.

Because, as a general rule, mitochondrial DNA is exclusively maternally inherited[3] and because mitochondria are fundamental in mediating effects on

---

Address for correspondence: Tsutomu Kazumi, Department of Food Sciences and Nutrition, School of Human Environmental Sciences, Mukogawa Women's University, 6-46 Ikebiraki-cho, Nishinomiya, Hyogo 663-8558, Japan
E-mail: kazumi@mukogawa-u.ac.jp
Received: March 16, 2010
Accepted for publication: May 18, 2010

Table 1. Current BMI and BMI at age 18 in daughters and their parents

	Daughters (n=186)	Mothers (n=186)	Fathers (n=179)
Age (years)	19	48 ± 3	51 ± 4
BMI at age 18 (kg/m^2)	20.2 ± 2.4	20.0 ± 2.0	20.5 ± 2.3
Current BMI (kg/m^2)	20.1 ± 2.2	21.8 ± 2.7	23.7 ± 2.3

Mean ± SD

energy dissipation[4], we assessed the relationship between body mass index (BMI), a surrogate of body fat, in young women and their mothers, and compared with their fathers' BMI. These comparisons used BMI measured on 2 occasions; when parents were 18 years old and at the time of the study. We also measured serum leptin and fat mass using dual energy X-ray absorptiometry (DXA), a well-established technique of measuring body composition that has been validated against most other reference measures. Since age is one important factor known to affect mitochondrial function[5], we studied healthy, young and slim people as described below.

Methods

The study population consisted of 2 groups of young women and their biological parents. The young women were students of the Department of Food Sciences and Nutrition, Faculty of Environmental Sciences, Mukogawa Women's University (Nishinomiya, Japan). The study was approved by the MWU ethnic committee and written informed consent was obtained from all participants.

One group consisted of 208 female college students aged 18 years, who entered the Department of Food Sciences and Nutrition, School of Human Environmental Science, Mukogawa Women's University, in 2001. Height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively. Current BMI (weight in kg/ height in m) was calculated from these measurements. Anthropometric measurements were also performed 12 months later.

We asked their parents to recall their body weight at the age of 18 years and at the time of the study. Self-reported heights and weights, which were shown to respond closely to measured heights and weights[6-9], were available in 187 out of 208 students (**Table 1**).

Fat mass and serum leptin were measured in another set of 148 daughter-mother pairs and 59 daughter-father pairs (**Table 2**). Daughters were students of the University and the characteristics of the daughters[10] and their mothers[11] are described in detail elsewhere. Body composition was determined using whole-body DXA (QDR-2000, software version 7.20D; Hologic, Bedford, MA, USA) as previously reported[10]. The software provides estimates of lean tissue mass, fat mass, and bone mineral mass for the total body and for standard body regions. With the use of specific anatomic landmarks, regions of the head, trunk, arms, and legs were differentiated as shown in **Fig. 1**. Legs included both lower extremities and gluteal regions.

Table 2. Fat mass measured using DXA and serum leptin in daughters and their parents

	Daughters (n=148)	Mothers (n=148)	Fathers (n=59)
Age (years)	20.0 ± 0.8	50 ± 4	52 ± 5
BMI (kg/m^2)	20.4 ± 2.2	22.0 ± 2.8	24.1 ± 2.3
Body fat mass (kg)	14.4 ± 4.4	16.1 ± 5.9	15.5 ± 6.2
Trunk fat mass (kg)	7.0 ± 2.5	8.7 ± 3.6	9.7 ± 4.1
Leg fat mass (kg)	5.6 ± 1.5	5.3 ± 1.8	4.0 ± 1.6
Leptin (ng/mL)	8.6 ± 3.9	7.6 ± 4.9	3.6 ± 2.3

Mean ± SD

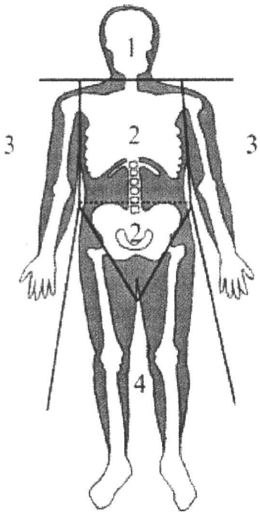

Fig. 1. Standard regions of DXA scanning: 1, head; 2, trunk; 3, arms; 4, legs.

Blood samples were obtained in the morning after a 12-hr overnight fast. Leptin concentrations were assessed by an RIA kit from LINCO research (St. Charles, MO, inter-assay CV=4.9%).

Statistical analysis was performed with Stat View. Data are presented as the means ± SD. Spearman's correlation coefficients were calculated to determine the

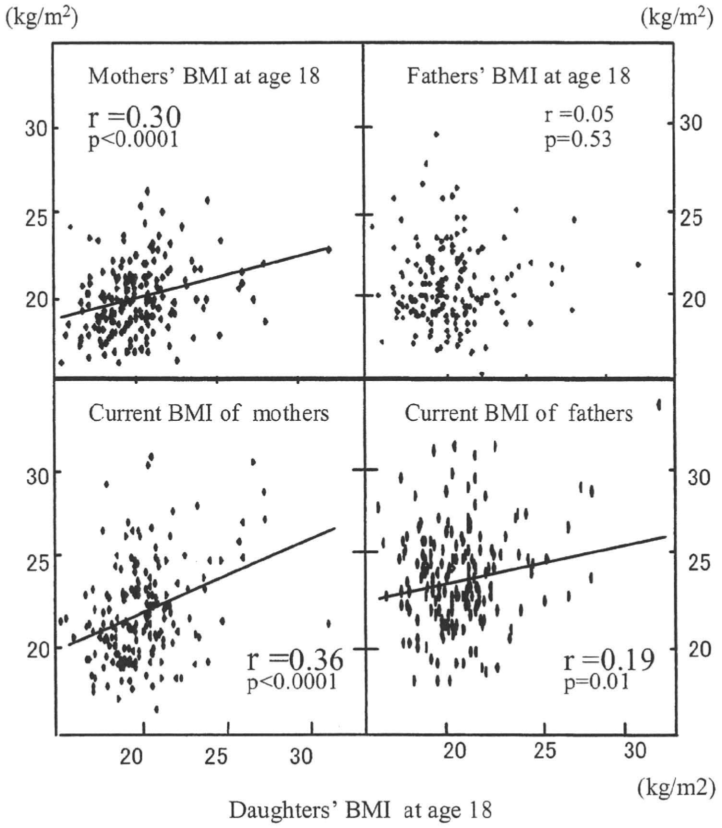

Fig. 2. Correlations between daughters and their biological parents of BMI at age 18 (upper panel) and current BMI (lower panel).

relation of parents' BMI to the blood pressure and BMI of their daughters. *P* values < 0.05 were considered significant.

Results

As shown in **Fig. 2**, 18-year-old daughters' BMI was associated with the current BMI of their mothers and fathers; however, it is noted that associations were stronger between daughters' and mothers' BMI than between daughters' and fathers' BMI. In addition, 18-year-old daughters' BMI was associated with their mothers' BMI measured when they were 18 years old. In contrast, there was no correlation between 18-year-old daughters' BMI and fathers' BMI measured when they were 18 years old. The results using students' BMI measured 12 months later were essentially same. Daughters' BMI at age 19 was strongly associated with their mothers' BMI at age 18 ($r = 0.35$, $p < 0.001$) and current BMI ($r = 0.34$, $p < 0.001$). Corresponding correlation coefficients with their fathers' BMI were 0.06 and 0.14 (not significant).

Table 3. Correlation coefficients of serum leptin and fat mass measured using DXA between daughters and their parents

		Daughter・Mother ($n = 148$)	Daughter・Father ($n = 59$)
BMI (kg/m²)		0.22^b	0.02
Serum leptin (ng/mL)		0.17^a	0.06
Fat mass (kg):	body	0.19^a	0.12
	trunk	0.18^a	0.11
	legs	0.17^a	0.12
	arm	0.06	-0.01

^{a}: $p < 0.05$, ^{b}: $p < 0.01$

Not only serum leptin levels but also total body fat mass measured using DXA were associated between daughters and their mothers, whereas there was no relation between daughters and their fathers (**Table 3**). In addition, trunk and leg fat mass of daughters were associated with those of their mothers but not with their fathers. Fat mass of the arms did not show a significant association between daughters and parents.

Discussion

In the present study, we found a significant association of 18-year-old daughters' BMI, a surrogate of total body fat, with their mother's BMI measured when they were 18 years old. In contrast, there was no association between daughters' BMI and fathers' BMI measured when they were 18 years old. In addition, not only serum leptin levels but also fat mass in the body, trunk and legs measured using DXA were associated in daughters and their mothers. There was no relation between daughters and fathers in serum leptin and fat mass in any regions measured in the present study. This discrepancy might be in part due to the smaller number of daughter-father pairs.

The underlying mechanisms of the associations of 18-year-old daughters' BMI with mothers' BMI measured when the mothers were 18 years old remain to be elucidated. We cannot rule out the possibility of genetic imprinting and sex-linked genetic transmission; however, significant correlations between 18-year-old daughters' BMI and mothers' BMI measured when mothers were 18 years old did not result from shared environmental factors, including intrauterine environment, although parent-offspring correlations in general do not allow the separation of genetic and environmental transmission. These results therefore suggest that their mothers' but not their fathers' mitochondrial function may influence adiposity in 18-year-old daughters because, as a general rule, mitochondrial DNA is exclusively maternally inherited[3] and because mitochondria are fundamental in mediating effects on energy dissipation[4]. Age[12] and exercise[13] are major factors known to affect the size and/or function of mitochondria. We therefore examined a homogeneous cohort of young healthy Japanese people. The mothers studied were only, on average, 30 years old when they passed their mitochondria on to their daughters, who were only 18 years old; therefore, the characteristics of these mitochondria could not be attributed to aging. In addition, 18-20-year-old college students are not sedentary, as compared to middle-aged individuals in the general population. Further, it was noted that the majority of the population (parents and daughters) did not suffer from metabolic syndrome, type 2 diabetes, or insulin resistance, but rather were slim, young and healthy people. We have recently reported that middle-aged mothers' BMI was associated with 18-year-old sons' BMI, asparate and alanine aminotransferase and gamma glutamyl transpeptidase, all of which are mitochondrial enzymes, whereas middle-aged fathers' BMI was not[14], suggesting that 18 year-old sons' adiposity may be influenced substantially by a maternal effect. The significant association between 18 year-old daughters' BMI and mothers' but not fathers' BMI measured when the parents were 18 years old found in the present study may imply that mothers' mitochondrial function may influence adiposity in 18-year-old daughters, as described above.

Significant associations between 18-year-old daughters' BMI and the current BMI of their parents, although correlation coefficients to mothers' BMI were consistently greater than those to fathers' BMI, might be related to the dominant influence of maternal genes. Mitochondrial-specific genes could be potential candidates[1], but imprinted genes, in which only the maternal allele is expressed, might be also implicated. In addition to a genetic effect, the potential contribution of environmental and behavioral components also needs to be considered[15, 16]. The effects of the intrauterine environment might contribute to the stronger association of BMI between daughters and mothers[15]. Further, a greater postnatal sharing of environmental factors between mothers and daughters than between fathers and daughters might also explain the stronger maternal effect[16].

In addition to BMI, serum leptin concentrations, another surrogate of total body fat[17], were correlated between daughters in early adulthood and middle-aged mothers. Furthermore, not only body fat mass but also fat mass in the trunk and legs measured using DXA, the gold standard to assess regional fat mass, were associated between daughters in early adulthood and middle-aged mothers. A strong genetic influence on BMI, leptin and fat mass has previously been reported[1, 2, 18, 19]. In contrast to the daughter-mother relationship, no significant association was found between daughters in early adulthood and middle-aged fathers, although these findings deserve further investigation, because daughter-father pairs were small in number.

We used recalled body weight and self-reported current weight. Self-reported weights at 50 years were reported to be accurate for both men and women7. In addition, recalling past weight was not significantly influenced by the passage of time, the numbers of years of education, or the accuracy of current weight reports[7]. In that report, correlations between recalling past weights and measured weights ranged from $r=0.87$ at 18 years to 0.95 at 40 years. In a validation study in the Nurses' Health Study II, the difference between measured and self-reported body weight at age 18 was, on average, only 1.4 kg[20]. The correlation coefficient between recalled weight at age 18 and measured weight in physical examination records at age 18 has been reported to be 0.87.

Acknowledgements

This study was supported in part by the Open Research Center Project for Private Universities: Matching Fund Subsidy for Private Universities, The Ministries of Education, Culture, Sports, Science and Technology, Japan. We are indebted to all the participants for their dedicated and conscientious collaboration. We also thank Ms. Mami Toyazaki-Adachi for her help in preparing this manuscript.

References

1) Maes HHM, Neale MC, Eaves LJ: Genetic and environmental factors in relative body weight and human adiposity. Behav Genet, 1997; 27: 325-351
2) Grilo CM, Pogue-Geile MF. The nature of environmental influences on weight and obesity: a behavior genetic analysis. Psychol Bull, 1991; 110: 520-537
3) DiMauro S, Schon EA: Mitochondria respiratory-chain diseases. N Engl J Med, 2003; 348: 2656-2668
4) Bradford B, Lowell, Bruce M, Spiegelman BM, Towards a molecular understanding of adaptive thermogenesis. Nature, 2000; 404: 652-660
5) Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI: Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science, 2003; 300: 1140-1142
6) Stunkard AJ, Albaum JM: The accuracy of self-reported weights. Am J Clin Nutr, 1981; 34: 1593-1599
7) Casey VA, Dwyer JT, Berkey CS, Coleman KA, Gardner J, Valadian. Long-term memory of body weight and past weight satisfaction: a longitudinal follow-up study. Am J Clin Nutr, 1991; 53: 1493-1498
8) Schlichting P, Hoilund-Carlsen PF, Quaade F: Comparison of self-reported height and weight with controlled height and weight in women and men. Int J Obes, 1981; 5: 67-76
9) Coates TJ, Jeffrey RW, Wing RR: The relationship between persons' relative body weights and the quality and quantity of food stored in their homes. Addict Behav, 1978; 3: 179-184
10) Wu B, Fukuo K, Suzuki K, Yoshino G, Kazumi T: Relationships of systemic oxidative stress to body fat distribution, adipokines and inflammatory markers in healthy middle-aged women. Endocr J, 2009; 56: 773-782
11) Miyanaga K, Fukuo K, Akasaka H, Katsuya T, Fukada R, Rakugi H, Kazumi T: C allele of angiotensin II type 1 receptor gene A1166C polymorphism affects plasma adiponectin concentrations in healthy young Japanese women. Hypertens Res, 2009; 32: 901-995
12) Lithell H, Lindgärde F, Hellsing K, Lundqvist G, Nygaard E, Vessby B, Saltin B: Body weight, skeletal muscle morphology, and enzyme activities in relation to fasting serum insulin concentration and glucose tolerance in 48-year-old men. Diabetes, 1981; 30: 19-25
13) Essig DA: Contractile activity-induced mitochondrial biogenesis in skeletal muscle. Exerc Sport Sci Rev, 1996; 24: 289-319
14) Kazumi T, Kawaguchi A, Yoshino G: Associations of middle-aged mother's but not father's body mass index with 18-year-old son's waist circumferences, birth weight, and serum hepatic enzyme levels. Metabolism, 2005; 54: 466-470
15) Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med, 1996; 334: 292-295
16) Gluckman PD, Hanson MA, Cooper C, Thornburg KL: Effect of in utero and early-life conditions on adult health and disease. N Engl J Med, 2008; 3; 359: 61-73
17) Oliveria SA, Ellison RC, Moore LL, Gillman MW, Garrahie EJ, Singer MR: Parent-child relationships in nutrient intake: the Framingham Children's Study. Am J Clin Nutr, 1992; 56: 593-598
18) Narkiewicz K, Szczech R, Winnicki M, Chrostowska M, Pawlowski R, Lysiak-Szydlowska W, Choe I, Kato M, Sivitz WI, Krupa-Wojciechowska B, Somers VK: Heritability of plasma leptin levels: a twin study. J Hypertens, 1999; 17: 27-31
19) Zhang S, Liu X, Yu Y, Hong X, Christoffel KK, Wang B, Tsai HJ, Li Z, Liu X, Tang G, Xing H, Brickman WJ, Zimmerman D, Xu X, Wang X: Association of plasma leptin concentrations with adiposity measurements in rural Chinese adolescents. J Clin Endocrinol Metab. 2009; 94: 3497-3504
20) Troy LM, Hunter DJ, Manson JE, Colditz GA, Stampfer MJ, Willett WC: The validity of recalled weight among younger women. Int J Obes Relat Metab Disord, 1995; 19: 570-572

Nifedipine Inhibits Vascular Smooth Muscle Cell Dedifferentiation via Downregulation of Akt Signaling

Taeko Kaimoto, Osamu Yasuda, Mitsuru Ohishi, Masaki Mogi, Yukihiro Takemura,
Toshimitsu Suhara, Toshio Ogihara, Keisuke Fukuo, Hiromi Rakugi

Abstract—Calcium is an essential signaling molecule that controls vascular smooth muscle cell (VSMC) contraction, proliferation, and differentiation. Here, we show that the calcium antagonist nifedipine inhibits VSMC dedifferentiation in vitro and in vivo. Differentiated VSMCs cultured on laminin-coated dishes were transferred to laminin-free dishes to induce dedifferentiation. Induction of dedifferentiation resulted in the upregulation of nonmuscle myosin heavy chain expression, a marker of dedifferentiation, and the downregulation of smooth muscle myosin heavy chain expression, a marker of differentiation. Nifedipine significantly inhibited both the induction of these phenotypic changes and upregulation of Akt signaling in these cells. Administration of nifedipine at a low concentration that did not affect blood pressure could inhibit the increase in nonmuscle myosin heavy chain expression and decrease in smooth muscle myosin heavy chain expression in a rat balloon-injury model. Furthermore, nifedipine suppressed neointimal hyperplasia and upregulation of Akt signaling. However, phospho-Akt expression was not suppressed in the regenerating arterial endothelium of the nifedipine-treated rats. The inhibitory effect of the downregulation of Akt signaling by dominant-negative Akt on the induction of VSMC dedifferentiation in the intima was identical to that of nifedipine. In contrast, upregulation of Akt signaling by transfection of the cells with a constitutively active Akt reversed the nifedipine-induced inhibition of VSMC dedifferentiation. In conclusion, nifedipine inhibits VSMC dedifferentiation by suppressing Akt signaling, thereby preventing neointimal thickening. (***Hypertension*. 2010;56:247-252.**)

Key Words: calcium antagonist ■ hypertension ■ vascular smooth muscle cell ■ Akt ■ dedifferentiation

Excessive proliferation of vascular smooth muscle cells (VSMCs) plays a major role in the pathogenesis of vascular diseases. Unlike skeletal or cardiac muscle cells that have undergone terminal differentiation, VSMCs of adult animals retain plasticity and can shuttle between a quiescent, contractile phenotype and a proliferative, synthetic phenotype in response to various physiological and pathological stimuli.[1] Phenotypic changes of VSMCs are known to be critical in the genesis of atherosclerosis, as well as in neointimal thickening after angioplasty. Growth factors, inflammatory cytokines, and extracellular matrix proteins have been reported as factors that mediate such phenotypic changes of VSMCs.[2–4] These changes are accompanied by alterations in the expression of phenotypic markers, such as smooth muscle α-actin, smooth muscle myosin heavy chain (SM2), and nonmuscle myosin heavy chain (SMemb).[1,5] The phosphatidylinositol 3-kinase/Akt signaling pathway is involved in regulating the phenotypic changes of VSMCs. The Akt signaling is activated by certain growth factors, such as platelet-derived growth factor (PDGF), which influences the phenotype of VSMCs.[6]

Calcium antagonists are widely used to treat angina pectoris and hypertension. There is evidence that calcium antagonist therapy reduces cardiovascular morbidity and mortality and the progression of atherosclerosis in hypertensive patients,[7–9] partly because of an antioxidant effect and amelioration of free radical damage.[10,11] One of the most widely used calcium antagonists, nifedipine, suppresses the development of atherosclerosis in cholesterol-fed rabbits without reducing hypercholesterolemia.[12] Nifedipine dose-dependently reduces the expression of proliferative cell nuclear antigen in the thoracic aorta after balloon injury and inhibits neointimal thickening.[13] However, the influence of calcium antagonists on the phenotypic changes of VSMCs remains unclear. We hypothesized that calcium antagonists may inhibit dedifferentiation of VSMCs by modulating the phosphatidylinositol 3-kinase/Akt signaling pathway.

Methods

More information on Materials and Methods can be found in the online Data Supplement (please see http://hyper.ahajournals.org).

Received January 3, 2010; first decision January 25, 2010; revision accepted May 14, 2010.

From the Department of Geriatric Medicine (T.K., M.O., Y.T., T.S., T.O., H.R.), Osaka University Graduate School of Medicine, Osaka, Japan; Department of Cardiovascular Clinical and Translational Research (O.Y.), Kumamoto University Hospital, Kumamoto, Japan; Department of Molecular Cardiovascular Biology and Pharmacology (M.M.), Ehime University Graduate School of Medicine, Ehime, Japan; Department of Food Sciences and Nutrition (K.F.), School of Human Environmental Sciences, Mukogawa Women's University, Hyogo, Japan.

Correspondence to Osamu Yasuda, Department of Cardiovascular Clinical and Translational Research, Kumamoto University Hospital, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail o-yasuda@kumamoto-u.ac.jp

***Hypertension* is available at http://hyper.ahajournals.org**

DOI: 10.1161/HYPERTENSIONAHA.110.149781

Animals

Male Sprague-Dawley rats were obtained from SLC Japan (Shizuoka, Japan). Animals were maintained at room temperature on a 12-hour light/dark cycle and given access to food and water ad libitum. The experimental protocols were approved by the Osaka University Medical School Animal Care and Use Committee and were performed according to the Osaka University Medical School Guidelines for the Care and Use of Laboratory Animals.

Cell Culture

Human aortic smooth muscle cells were purchased from Kurabo and cultured in HuMedia-SG2 medium (Kurabo) supplemented with 5% FBS, 50 μg/mL of gentamicin, and 50 ng/mL of amphotericin B at 37°C in a humidified atmosphere containing 5% CO_2.

Immunohistochemistry

Differentiation marker was visualized by the streptavidin-biotin method[14] using a Labeled Streptavidin Biotin kit (Dako Cytomation). Mounted sections were preincubated with 0.1 mol/L of PBS containing 1% albumin for 30 minutes at room temperature and then incubated overnight at 4°C with mouse anti-α smooth muscle actin antibody (Clone1A4) or mouse anti-SMemb, mouse anti-SM2, or rabbit antiphosho-Akt (Ser473) antisera diluted with PBS containing 1% albumin. Then the samples were washed in PBS and incubated for an additional 1 hour at room temperature with biotinylated goat antirabbit IgG or antimouse IgG, followed by further incubation with horseradish peroxidase–labeled streptavidin for 1 hour at room temperature. After washing in PBS, the reaction products were visualized by incubation with 0.020% (weight/volume) 3,3′-diaminobenzidine and 0.005% (volume/volume) H_2O_2 in 50 mmol/L of Tris-HCl buffer for 5 to 15 minutes. Finally, the samples were dehydrated and cover slips were applied with Permount (Eentellan Neu, Merck).

Western Blot Analysis for α-Actin, SMemb, SM2, and Phospho-Akt

VSMCs were washed twice in PBS, harvested by scraping, and were adjusted to 10^6 cells per 10 μL of lysis buffer (1% SDS, 100 mmol/L of NaCl, 50 mmol/L of Tris-HCl [pH 8.0], and 20 mmol of EDTA). The protein concentration of the lysate was determined with a Bio-Rad protein assay kit (Bio-Rad). Samples containing 20 μg of protein were run on a 7% to 10% SDS-PAGE gel and electroblotted onto a polyvinyldine diflouride membrane (Immobilon-P, Millipore). Blots were blocked for 1 hour with 3% skim milk in PBS containing 0.1% Tween 20; incubated overnight at 4°C with antibodies for α-smooth muscle actin (Clone1A4), SMemb, SM2, or phospho-Akt (Ser473); and washed 3 times with PBS containing 0.1% Tween 20. Then, blocking with 3% skim milk in PBS containing 0.1% Tween 20 was done for 1 hour, followed by incubation with the peroxidase-conjugated secondary antibodies (antimouse or antirabbit IgG, Promega) for 2 hours at room temperature and washing 3 times with PBS containing 0.1% Tween 20. Immunoblots were developed with an ECL Western blotting detection system (Amersham International plc), and the blots were then reprobed with α-tubulin to confirm equal loading of protein into each well.

Immunocytochemistry

VSMCs grown in 2-chamber culture dishes (Laboratory-Tek, Nunc, Inc) were fixed for 5 minutes with 4% paraformaldehyde in PBS at room temperature, washed twice for 5 minutes each with PBS, and then preincubated with PBS containing 1% albumin for 30 minutes. The labeled streptavidin biotin kit (Dako) was used for immunostaining.

Adenoviral Constructs

To modulate Akt activity, we used 2 adenoviral constructs tagged with the hemagglutinin epitope, as described previously.[15,16] In the dominant-negative Akt construct (DN-Akt), 2 phosphorylation sites (serine 473 and threonine 308) were both mutated to alanine, resulting in a form of Akt that could not be phosphorylated. A replication-defective adenovirus vector expressed the constitutively active form of murine Akt (CA-Akt) under the control of the cytomegalovirus promoter, whereas Adenovirus–β-galactosidase expressed the LacZ gene under the cytomegalovirus promoter as a control vector.[17] All of the viral constructs were grown in 293 cells and purified by CsCl gradient ultracentrifugation. Viral titers were determined by the plaque assay.

Statistical Analysis

Statistical analysis was performed by 1-way ANOVA. Results are expressed as the mean±SEM, and $P<0.05$ was considered significant.

Results

Nifedipine Inhibits VSMC Dedifferentiation In Vitro

To investigate the influence of nifedipine on the VSMC phenotype, we examined whether nifedipine modulates dedifferentiation of VSMCs in culture. Differentiated VSMCs were obtained by culture on laminin-coated dishes, as reported previously.[18,19] Then, dedifferentiation of these cells was induced by transfer to uncoated culture dishes, with the cells being incubated in the presence or absence of nifedipine. In the absence of nifedipine, the percentage of SMemb-positive cells increased from 6 to 72 hours (Figure 1A and 1B), whereas that of SM2-positive cells decreased (Figure 1C and 1D). These phenotypic changes were significantly inhibited by nifedipine. Western blot analysis also showed that exposure to nifedipine significantly suppressed both upregulation of SMemb expression (Figure S1) and downregulation of SM2 expression (Figure S2) induced by dedifferentiation of VSMCs. These findings indicate that nifedipine inhibits dedifferentiation of VSMCs in culture.

Nifedipine Inhibits the Akt Signaling in VSMCs In Vitro

We next examined whether nifedipine influences Akt signaling during dedifferentiation of VSMCs in vitro. Western blot analysis showed that transfer of differentiated VSMCs to uncoated dishes resulted in elevated phospho-Akt expression, which peaked at 6 hours after the transfer. Nifedipine treatment significantly suppressed this elevation induced by dedifferentiation for as long as 3 to 24 hours after induction of dedifferentiation (Figure 2). Nifedipine treatment also significantly suppressed the increase in phospho-Akt expression induced by exposure of cultured VSMCs to PDGF-BB (Figure S3). Furthermore, a different calcium antagonist, amlodipine, significantly suppressed the insulin-like growth factor 1–induced increase in phospho-Akt expression (Figure S4), indicating that both nifedipine and amlodipine inhibit Akt signaling.

Downregulation of Akt Inhibits VSMC Dedifferentiation In Vitro

To examine the direct role of the Akt signaling in dedifferentiation of VSMCs in culture, the adenoviral construct expressing DN-Akt was transfected into differentiated VSMCs cultured on laminin-coated dishes. Then, these cells were transferred to uncoated dished to induce dedifferentiation. Suppression of the Akt signaling by DN-Akt signifi-

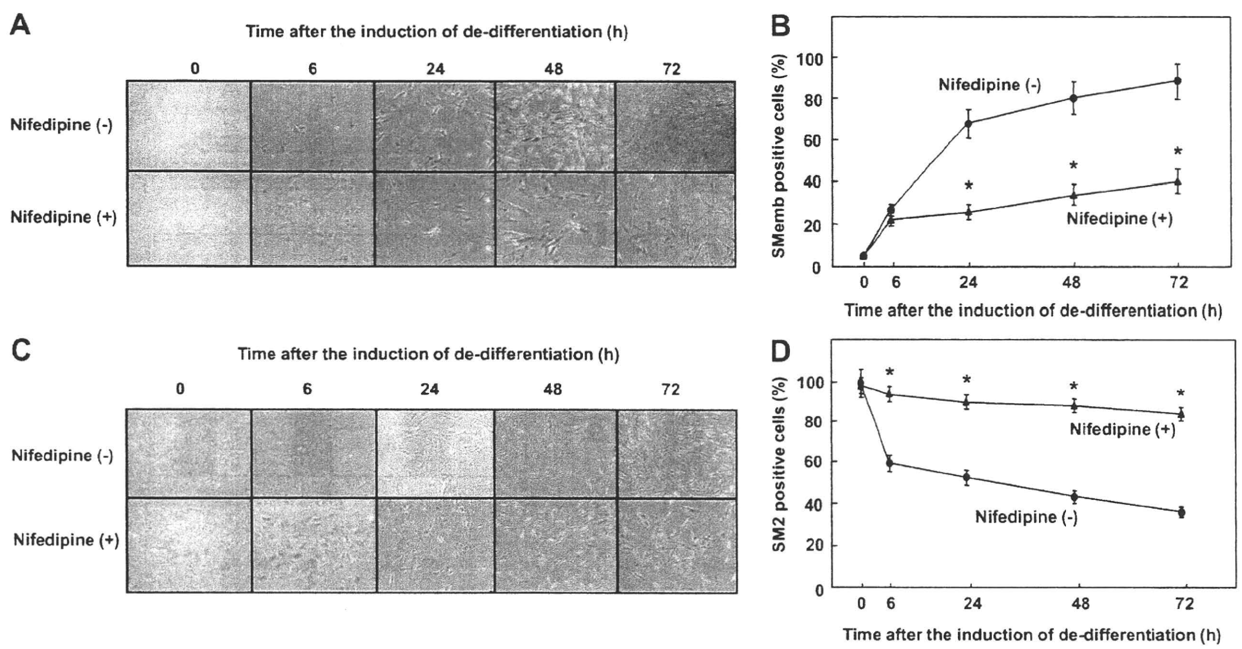

Figure 1. Nifedipine inhibits VSMC dedifferentiation in culture. Differentiated VSMCs cultured on laminin-coated dishes were transferred to laminin-uncoated dishes to induce dedifferentiation and then cultured in medium containing 5% FBS in the presence or absence of nifedipine (50 μmol/L). Cells were then immunostained with SMemb (A) and SM2 (C) at 6, 24, 48, and 72 hours after induction of dedifferentiation. Percentages of positive cells stained for SMemb (B) or SM2 (D) were counted after the immunostaining procedure. *$P<0.05$ vs VSMCs without nifedipine.

cantly inhibited both upregulation of SMemb (Figure 3A and 3B) expression and downregulation of SM2 (Figure 3C and 3D) expression induced by dedifferentiation. These findings indicate that the Akt signaling is involved in dedifferentiation of VSMCs in culture.

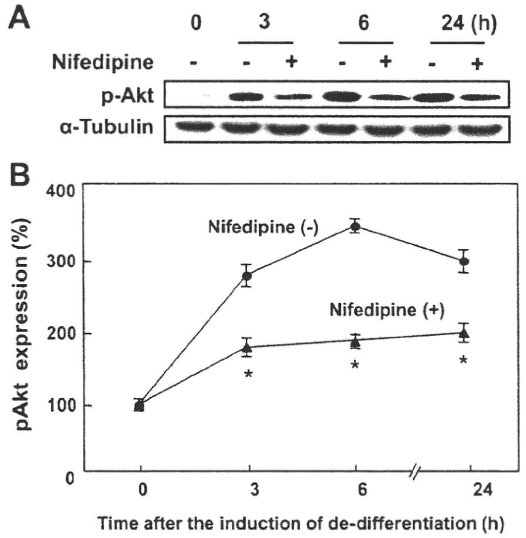

Figure 2. Nifedipine inhibits the Akt signaling upregulated by VSMC dedifferentiation. A, Phosho-Akt expression after induction of VSMC dedifferentiation with or without nifedipine (50 μmol/L) was determined by Western blot analysis. B, Phospho-Akt expression was quantified by densitometric analysis from 4 independent experiments. Data are normalized for α-tubulin and expressed as the mean±SEM (n=5). *$P<0.05$ vs VSMCs without nifedipine.

Nifedipine Inhibits the Akt Signaling in the Intimal VSMCs In Vivo

Next, we examined whether nifedipine inhibits the Akt signaling during VSMC dedifferentiation in vivo. As shown in Figure 4, phospho-Akt was highly expressed by actin-positive VSMCs in the intima. Nifedipine treatment significantly inhibited the Akt signaling in intimal VSMCs. In contrast, phospho-Akt–positive endothelial cells were present in the regenerating endothelium of nifedipine-treated arteries, suggesting that the Akt signaling in the endothelium is not affected by nifedipine.

Nifedipine and Downregulation of Akt Inhibit VSMC Dedifferentiation In Vivo

To test whether nifedipine inhibits VSMC dedifferentiation through downregulation of the Akt signaling in vivo, we evaluated the effect of nifedipine treatment and Akt gene transfer on VSMC dedifferentiation in the balloon injury model. Nifedipine treatment significantly suppressed the intimal hyperplasia (Figure 5A and 5B) and inhibited upregulation of SMemb expression and downregulation of SM2 expression in the intima (Figure 5C). Downregulation of Akt by transfer with DN-Akt was as effective as nifedipine at inhibiting hyperplasia and VSMC dedifferentiation in the intima. Conversely, upregulation of Akt by transfer with CA-Akt reversed the inhibition of VSMC dedifferentiation. These findings may provide causal evidence that the modulation of the Akt signaling is responsible for inhibiting VSMC dedifferentiation by nifedipine treatment.

Discussion

Calcium antagonists are widely used in the treatment of hypertension and angina pectoris. Recent evidence suggests

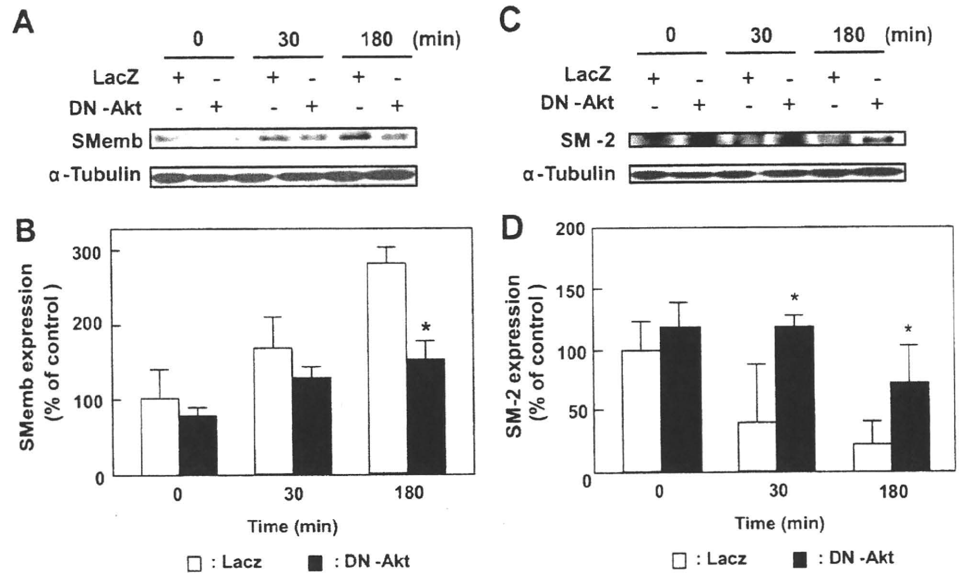

Figure 3. Downregulation of Akt inhibits VSMC dedifferentiation. Differentiated VSMCs were infected with LacZ or DN-Akt for 48 hours. VSMC dedifferentiation was induced in these cells, and VSMCs were then harvested at 0, 30, and 180 minutes after induction of dedifferentiation. SMemb expression (A) and SM2 expression (C) by were determined by Western blot analysis. Then, the blot was reprobed for α-tubulin to confirm equal loading of protein in each well. SMemb expression (B) and SM2 expression (D) were quantified by densitometric analysis from 5 independent experiments. Data are normalized for α-tubulin and expressed as the mean±SEM. *$P<0.05$ vs VSMCs treated with LacZ.

that these drugs improve the clinical outcome in patients with certain cardiovascular diseases.[20] Generally, nifedipine and other dihydropyridine derivatives are considered to retard VSMC proliferation by reducing the cellular availability of calcium and interfering with the calcium-calmodulin complex to inhibit VSMC proliferation and migration. In addition, nifedipine has been shown to modulate low-density lipoprotein metabolism by macrophages. However, it has not been clear whether the Akt signaling is involved in the beneficial effect of calcium antagonists on the development of cardiovascular diseases.

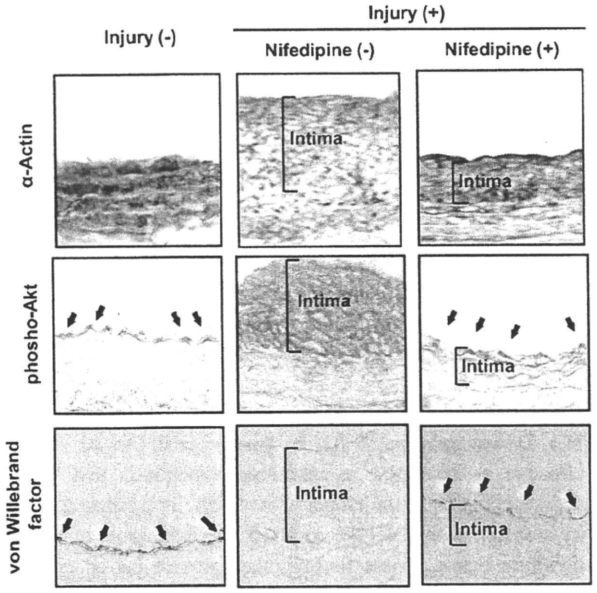

Figure 4. Nifedipine inhibits upregulation of the Akt signaling in intimal VSMCs but not in the endothelium after the rat balloon injury model. Nifedipine was administered for 3 weeks at 0.3 mg/kg per day. The balloon injury was performed 1 week after the administration of nifedipine. Expression for α-actin, phosphor-Akt, and von Willebrand factor was immunostained in injured arteries at 2 weeks after the balloon injury.

In the present study, we showed that nifedipine inhibits VSMC dedifferentiation and suppresses neointimal thickening after balloon injury. The nifedipine concentration used in this study does not reduce the blood pressure; this suggests that it has an antiatherogenic effect that is independent of its influence on blood pressure. This finding is consistent with that of studies that have reported the inhibition of atherosclerosis through a direct antioxidant effect of calcium antagonists on endothelial cells[10,11]; these studies have also reported that calcium antagonists exhibit an antiatherogenic action without causing any reduction in the blood pressure or changes in the plasma lipid profile.[21]

PDGF-BB was overexpressed at sites of VSMC proliferation after balloon injury of the vessels and at sites of atherosclerosis, suggesting its role in the development of intimal thickening. In fact, blocking of PDGF-BB or its receptor has been reported to inhibit neointimal thickening.[1] Therefore, PDGF-BB seems to be responsible for the phenotypic changes and VSMC dedifferentiation in vivo. Recently, costimulation with PDGF-BB and interleukin 1β has been proved to induce sustained activation of Akt and p70S6K.[22] In the present study, we showed that nifedipine suppresses PDGF-induced increases in phospho-Akt expression. Our preliminary experiments also showed that nifedipine inhibits increases in both phosho-Akt1 and phospho-Akt2 (data not shown). These findings suggest that nifedipine inhibits PDGF-induced neointimal thickening by suppressing Akt phosphorylation and VSMC dedifferentiation.

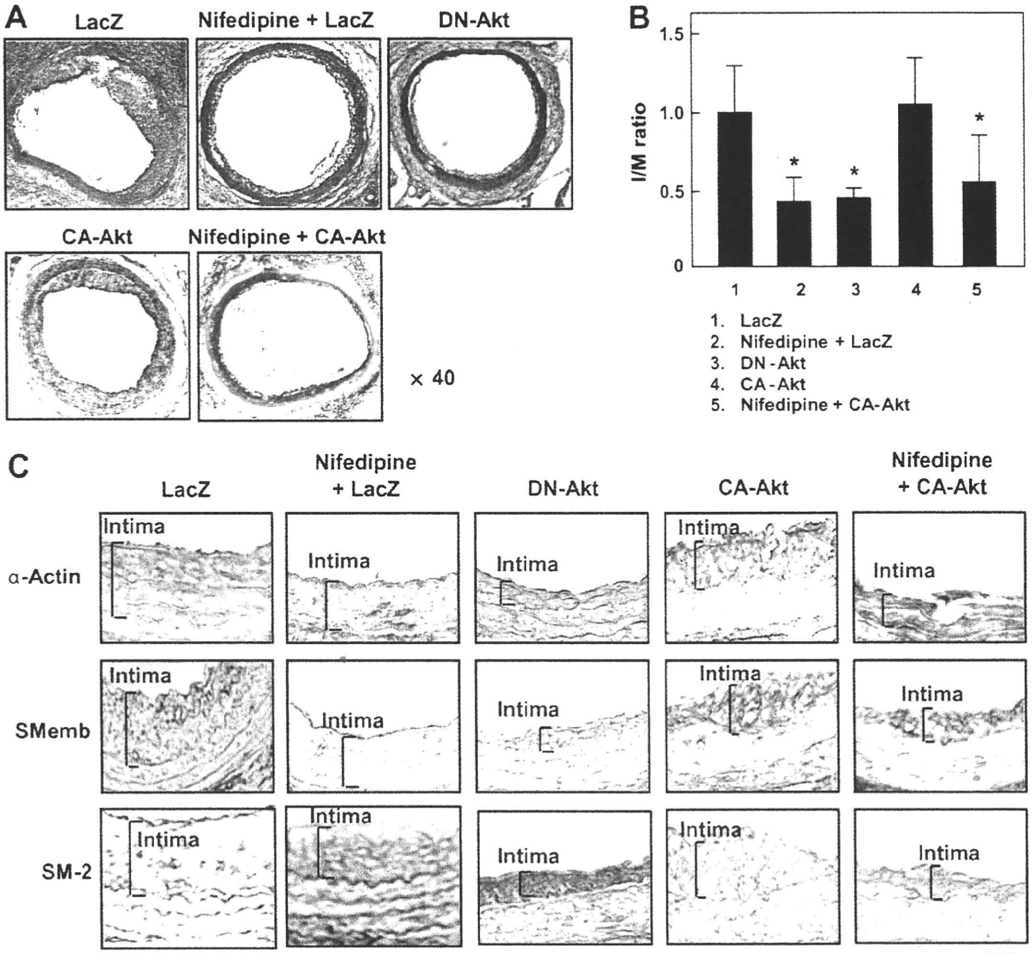

Figure 5. Nifedipine or DN-Akt transfer inhibits both VSMC proliferation and dedifferentiation in the intima after the balloon injury. Nifedipine (0.3 mg/kg per day) was administrated for 1 week before the balloon injury. DN-Akt gene transfer significantly suppressed neointimal thickening similar to nifedipine treatment. After the staining for hematoxylin-eosin (A), intima:media ratio (I/M ratio; B) was calculated (n=5 in each group). *$P<0.05$ vs the I/M ratio of the LacZ or Ad-CA-Akt groups. C, Expression levels for α-actin, SM-2, and SMemb in the intima were compared among 5 groups (LacZ, nifedipine+LacZ, DN-Akt, CA-Akt, and nifedipine+CA-Akt) after the immunostaining.

Nifedipine suppressed the increased phospho-Akt expression in vitro. Moreover, treatment of injured arteries with nifedipine reduced Akt phosphorylation in the neointimal VSMCs in vivo. These results indicate that nifedipine suppresses the activation of Akt signaling and thereby inhibits VSMC dedifferentiation. In contrast, nifedipine did not affect the Akt signaling in the endothelial cells of injured arteries. This finding is reasonable because endothelial cells are not known to have any receptors for calcium antagonists. This result is also supported by our previous finding that nifedipine indirectly enhances NO production by endothelial cells by stimulating vascular endothelial growth factor release from the VSMCs.[23] Our present findings have the important implication that regeneration of endothelial cells and upregulation of endothelial NO synthase expression via Akt signaling activated by vascular endothelial growth factor and other growth factors may not be suppressed by calcium antagonists.[24,25]

The number of functional L-type calcium channels significantly decreased in dedifferentiated VSMCs and increased on differentiation.[26] This is consistent with our finding that nifedipine inhibits the dedifferentiation of differentiated VSMCs, thereby suppressing the development and progression of atherosclerosis.

Perspectives

We demonstrated that nifedipine, an L-type calcium channel antagonist, inhibits upregulation of the Akt signaling in VSMCs but not in the regenerating endothelium. Our results also suggest that modulation of the Akt signaling by nifedipine leads to an inhibition of VSMC dedifferentiation in injured arteries. These findings may provide new insights into the mechanisms underlying the beneficial effects of calcium antagonists in the treatment of cardiovascular diseases.

Sources of Funding

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan.

Disclosures

None.

References

1. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767–801.
2. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809.
3. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995;75:487–517.
4. Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2008;28:812–819.
5. Zanellato AM, Borrione AC, Tonello M, Scannapieco G, Pauletto P, Sartore S. Myosin isoform expression and smooth muscle cell heterogeneity in normal and atherosclerotic rabbit aorta. *Arteriosclerosis*. 1990;10:996–1009.
6. Brazil DP, Yang ZZ, Hemmings BA. Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci*. 2004;29:233–242.
7. Hansson L, Lindholm LH, Ekbom T, Dahlof B, Lanke J, Schersten B, Wester PO, Hedner T, de Faire U. Randomised trial of old and new antihypertensive drugs in elderly patients: cardiovascular mortality and morbidity the Swedish Trial in Old Patients With Hypertension-2 Study. *Lancet*. 1999;354:1751–1756.
8. Group TAOaCftACR. Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs diuretic: the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *JAMA*. 2002;288:2981–2997.
9. Brown MJ, Palmer CR, Castaigne A, de Leeuw PW, Mancia G, Rosenthal T, Ruilope LM. Morbidity and mortality in patients randomised to double-blind treatment with a long-acting calcium-channel blocker or diuretic in the International Nifedipine GITS Study: Intervention as a Goal in Hypertension Treatment (INSIGHT). *Lancet*. 2000;356:366–372.
10. Mak IT, Boehme P, Weglicki WB. Antioxidant effects of calcium channel blockers against free radical injury in endothelial cells: correlation of protection with preservation of glutathione levels. *Circ Res*. 1992;70:1099–1103.
11. Mak IT, Boehme P, Weglicki WB. Protective effects of calcium channel blockers against free radical-impaired endothelial cell proliferation. *Biochem Pharmacol*. 1995;50:1531–1534.
12. Henry PD, Bentley KI. Suppression of atherogenesis in cholesterol-fed rabbit treated with nifedipine. *J Clin Invest*. 1981;68:1366–1369.
13. Hirata A, Igarashi M, Yamaguchi H, Suwabe A, Daimon M, Kato T, Tominaga M. Nifedipine suppresses neointimal thickening by its inhibitory effect on vascular smooth muscle cell growth via a MEK-ERK pathway coupling with Pyk2. *Br J Pharmacol*. 2000;131:1521–1530.
14. Guesdon JL, Ternynck T, Avrameas S. The use of avidin-biotin interaction in immunoenzymatic techniques. *J Histochem Cytochem*. 1979;27:1131–1139.
15. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem*. 1999;274:16349–16354.
16. Fujio Y, Guo K, Mano T, Mitsuuchi Y, Testa JR, Walsh K. Cell cycle withdrawal promotes myogenic induction of Akt, a positive modulator of myocyte survival. *Mol Cell Biol*. 1999;19:5073–5082.
17. Smith RC, Branellec D, Gorski DH, Guo K, Perlman H, Dedieu JF, Pastore C, Mahfoudi A, Denefle P, Isner JM, Walsh K. p21CIP1-mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene. *Genes Dev*. 1997;11:1674–1689.
18. Glukhova M, Koteliansky V, Fondacci C, Marotte F, Rappaport L. Laminin variants and integrin laminin receptors in developing and adult human smooth muscle. *Dev Biol*. 1993;157:437–447.
19. Pauly RR, Passaniti A, Crow M, Kinsella JL, Papadopoulos N, Monticone R, Lakatta EG, Martin GR. Experimental models that mimic the differentiation and dedifferentiation of vascular cells. *Circulation*. 1992;86:III68–III73.
20. Packer M, O'Connor CM, Ghali JK, Pressler ML, Carson PE, Belkin RN, Miller AB, Neuberg GW, Frid D, Wertheimer JH, Cropp AB, DeMets DL. Effect of amlodipine on morbidity and mortality in severe chronic heart failure: Prospective Randomized Amlodipine Survival Evaluation Study Group. *N Engl J Med*. 1996;335:1107–1114.
21. Waters D, Lesperance J, Francetich M, Causey D, Theroux P, Chiang YK, Hudon G, Lemarbre L, Reitman M, Joyal M, Gosselin G, Dyrda I, Macer J, Havel RJ. A controlled clinical trial to assess the effect of a calcium channel blocker on the progression of coronary atherosclerosis. *Circulation*. 1990;82:1940–1953.
22. Chen CN, Li YS, Yeh YT, Lee PL, Usami S, Chien S, Chiu JJ. Synergistic roles of platelet-derived growth factor-BB and interleukin-1β in phenotypic modulation of human aortic smooth muscle cells. *Proc Natl Acad Sci U S A*. 2006;103:2665–2670.
23. Fukuo K, Yang J, Yasuda O, Mogi M, Suhara T, Sato N, Suzuki T, Morimoto S, Ogihara T. Nifedipine indirectly upregulates superoxide dismutase expression in endothelial cells via vascular smooth muscle cell-dependent pathways. *Circulation*. 2002;106:356–361.
24. Lu J, Jiang W, Yang JH, Chang PY, Walterscheid JP, Chen HH, Marcelli M, Tang D, Lee YT, Liao WS, Yang CY, Chen CH. Electronegative LDL impairs vascular endothelial cell integrity in diabetes by disrupting fibroblast growth factor 2 (FGF2) autoregulation. *Diabetes*. 2008;57:158–166.
25. Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, Nakao K. Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo. *FEBS Lett*. 2003;544:86–92.
26. Gollasch M, Haase H, Ried C, Lindschau C, Morano I, Luft FC, Haller H. L-type calcium channel expression depends on the differentiated state of vascular smooth muscle cells. *FASEB J*. 1998;12:593–601.

日本臨床栄養学会雑誌
32(3)：159-166, 2011

《原　著》

内皮細胞におけるミトコンドリア局在たん白質 Apop-1 による グルコースのミトコンドリア活性酸素種産生増大作用

河端　真実[1]　竹村　幸宏[3]　深田　瑠美[2]
山本　遥菜[2]　安田　修[4]　福尾　惠介[1,2]

要旨　最近，糖尿病による動脈硬化の病態にミトコンドリア活性酸素種の産生増大が関与すると報告されている．今回我々は，内皮細胞において，ミトコンドリア局在たん白質 Apop-1 がグルコースによるミトコンドリア活性酸素種の産生増大を増強する可能性を検討した．内皮細胞を高濃度グルコース処理すると，ミトコンドリアのスーパーオキシド産生増大やミトコンドリア障害が誘導された．しかし，活性酸素種消去剤 NAC の添加により，高濃度グルコース処理によるミトコンドリア障害の誘導が阻害された．また，高濃度グルコース処理をした内皮細胞で，Apop-1 たん白質発現量が増加したが，Apop-1 siRNA を導入し，内因性の Apop-1 発現を抑制すると，ミトコンドリアのスーパーオキシド産生が有意に抑制された．以上より，内皮細胞において，グルコースによるミトコンドリア活性酸素種の産生増大に，Apop-1 が関与することが示唆された．この結果から，Apop-1 は糖尿病による動脈硬化の進行に関与する新しいミトコンドリア局在たん白質であると推察された．

キーワード：Apop-1，ミトコンドリア，活性酸素種，内皮細胞

緒　言

動脈硬化の予防には，内皮細胞の機能保持が重要である[1]．内皮細胞より産生される一酸化窒素（NO）は，動脈硬化進行の抑制に重要な役割を果たしている[2]．しかし，動脈硬化のリスク因子である高血圧，脂質代謝異常症，または糖尿病などを有する患者の動脈では，活性酸素種が多量に産生されるため，NO 作用が不十分となり，動脈硬化進行促進をみる[3]．

ミトコンドリアは，エネルギー代謝と関係の深い細胞内小器官であるが，活性酸素種の産生器官でもある[4,5]．重要なことに，高血糖による内皮細胞のミトコンドリア活性酸素種の産生増大は，糖尿病による血管障害や動脈硬化の発症に関与する[6]．しかし，高血糖がどのようなメカニズムでミトコンドリア活性酸素種の産生を増大させるかは明らかではない．

Apop-1 は，動脈硬化進行に伴って特異的に発現亢進するミトコンドリア局在たん白質である．Apop-1 は血管平滑筋細胞に過剰発現すると，ミトコンドリアのチトクローム C 遊離を促進し，アポトーシスを誘導する[7]．今回我々は，内皮細胞においてミトコンドリア局在たん白質である Apop-1 がグルコースによるミトコンドリア活性酸素種の産生増大を増強する可能性を検討した．

方　法

1）細胞培養

ヒト臍帯静脈由来内皮細胞（HUVEC）（クラボウバイオメディカル，大阪）を用い，10％ ウシ胎仔血清（FBS）を含む専用培地 HuMedia-EG 2（クラボウバイオメディカル，大阪）で培養した．

2）ミトコンドリアのスーパーオキシド産生量の測定

ミトコンドリアのスーパーオキシド産生量を，蛍光指示薬 MitoSOX（Invitrogen，東京）で測定した[8]．すなわち，2 Well スライドチャンバーで内皮細胞を培養し，高濃度グルコース溶液（最終濃度 15，33 mM）またはマンニトール溶液（最終濃度 33 mM）を添加して 48 時

---

[1] 武庫川女子大学大学院　生活環境学研究科　食物栄養学専攻

[2] 武庫川女子大学　生活環境学部　食物栄養学科

[3] 大阪大学大学院　医学系研究科　老年・腎臓内科学講座

[4] 熊本大学医学部附属病院　循環器臨床研究先端医療寄附講座

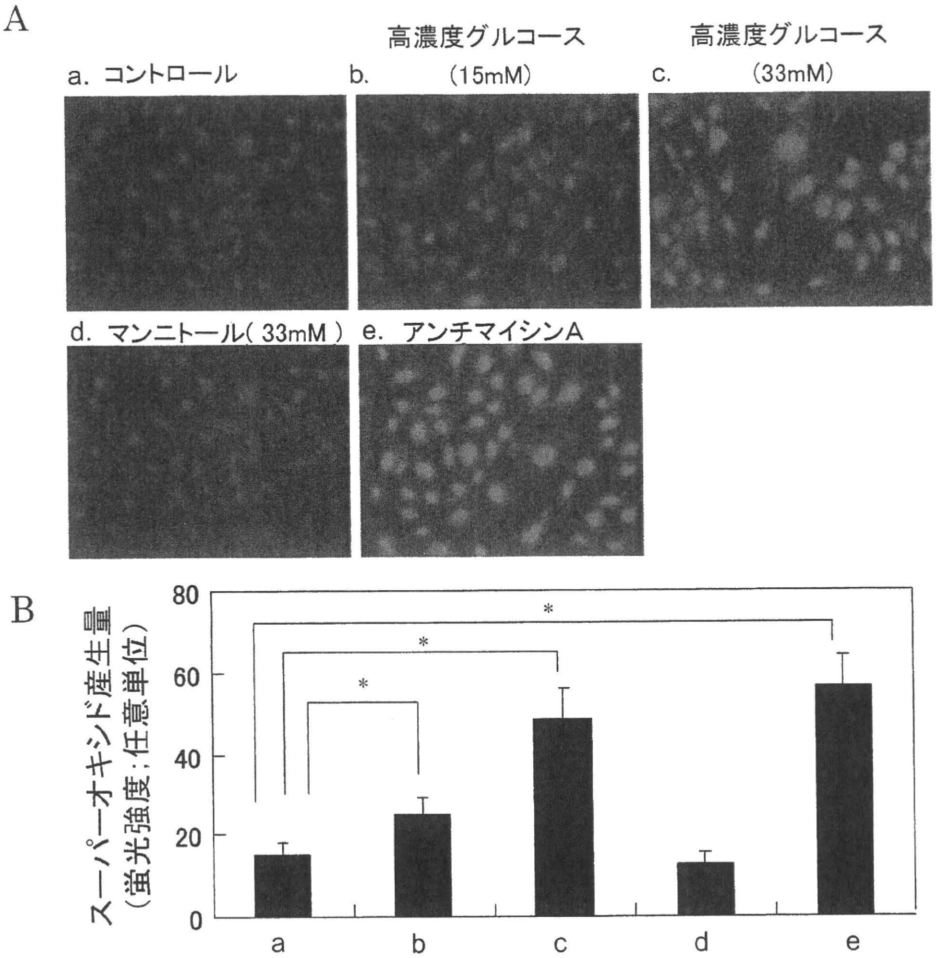

Fig. 1　高濃度グルコース処理によるミトコンドリアのスーパーオキシド産生増大
A：内皮細胞を，高濃度グルコース溶液（15 mM，33 mM），マンニトール溶液（33 mM）でそれぞれ48時間処理した．MitoSOX処理後，アンチマイシンA（100 μM）を30分間処理し，ミトコンドリアのスーパーオキシド産生量を測定した．コントロール群の培養液中には5.5 mM グルコースが含まれる．B：蛍光強度を定量化し，スーパーオキシド産生量を数値化し，平均値 ± 標準偏差（n = 5）で表した．（$^*p < 0.01$：コントロール群との比較）

間培養後，MitoSOX（最終濃度5 μM）を添加し，30分間処理した．アンチマイシンA（SIGMA，東京）（最終濃度100 μM）を，MitoSOX処理した内皮細胞に添加し，30分間処理した．その後，細胞をPBSで2回洗浄し，2% パラフォルムアルデヒド（PFA）で5分間室温にて固定後，VECTASHIELD Mounting Medium with DAPI（フナコシ，東京）で封入し，蛍光顕微鏡 ECLIPSE TE 2000-S（Nikon，東京）で蛍光強度を測定した．siRNA導入細胞での実験は，導入後24時間あるいは48時間後の内皮細胞を用いて，高濃度グルコース処理した．

3）ミトコンドリア障害（膜電位変動）の測定

ミトコンドリア障害を，膜電位変動で測定した．蛍光指示薬JC-1（Cell Technology Inc，米国）は，ミトコンドリアに取り込まれる色素で，ミトコンドリアが正常の高い膜電位を持つ状態では赤色蛍光を発し，障害を受けて低い膜電位状態では緑色蛍光を発する[9]．4 Well スライドチャンバーで内皮細胞を培養し，高濃度グルコース溶液（最終濃度33 mM）またはマンニトール溶液（最終濃度33 mM）を添加して60時間培養した．内皮細胞を，2,3-Dimethoxy-1-naphthoquinone（DMNQ）（SIGMA，東京）（最終濃度5μM）で30分間処理した．また，内皮細胞を，活性酸素種消去剤 N-Acetyl-L-cysteine（NAC）（SIGMA，東京）（最終濃度100 mM）で30分間処理後，高濃度グルコース処理した．その後，JC-1（2 μg/*ml*）を添加し，15分間処理した．細胞をPBSで2回洗浄し，2% PFAで5分間室温にて固定後，VECTASHIELD

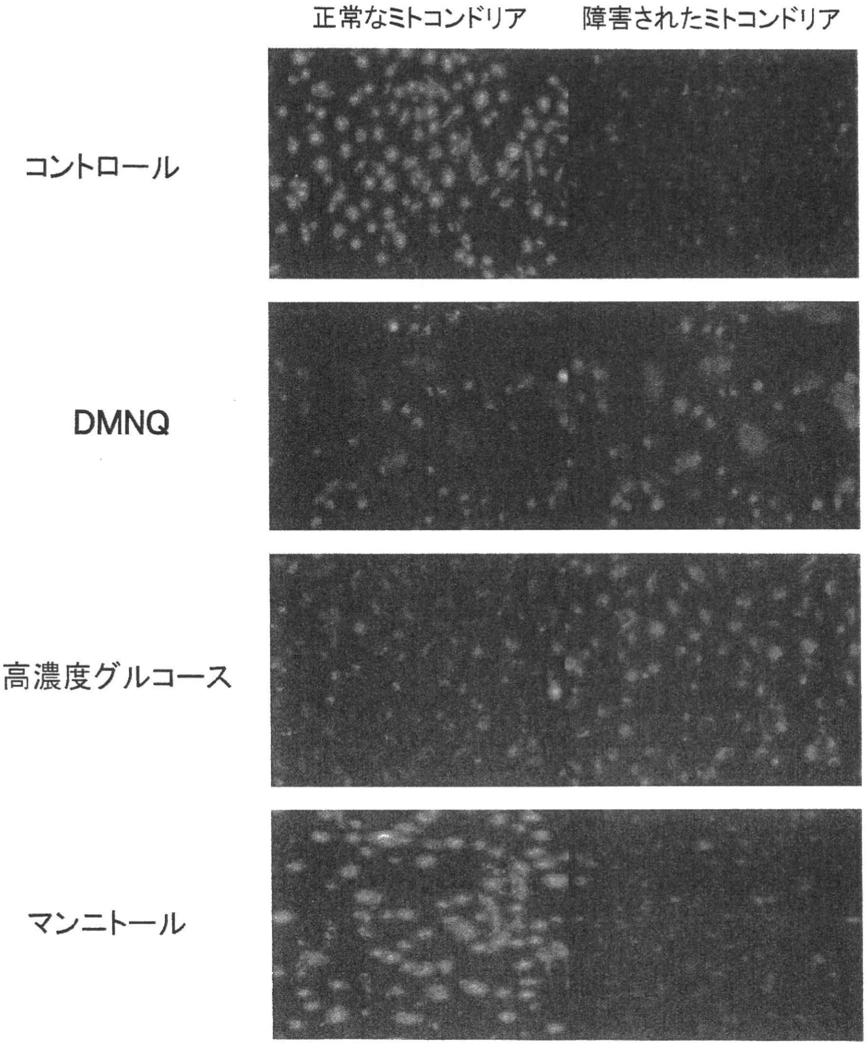

Fig. 2 高濃度グルコース処理によるミトコンドリア障害の誘導
内皮細胞を，高濃度グルコース溶液（33 mM），マンニトール溶液（33 mM），DMNQ（5 μM）でそれぞれ 60 時間処理し，蛍光指示薬 JC-1 を用いてミトコンドリア障害を測定した．赤色蛍光は正常なミトコンドリア膜電位を示し，緑色蛍光は障害されて変動したミトコンドリア膜電位を示す．

Mounting Medium with DAPI で封入し，蛍光顕微鏡 ECLIPSE TE2000-S で，蛍光強度を測定した．

4）Apop-1 発現の抑制

Apop-1 発現抑制を目的に合成された Apop-1 siRNA（QIAGEN，東京）を，Nucleofector II Device（Lonza，東京）を用い，エレクトロポレーション法により内皮細胞に導入した．スクランブル siRNA を導入した内皮細胞をコントロールとした．また，ウエスタンブロット法を用いて，Apop-1 たん白質発現の siRNA による抑制を確認した．

5）ウエスタンブロット法による Apop-1 たん白質の定量

Apop-1 と α-tublin のたん白質発現量を，それぞれ抗 Apop-1 抗体（Yasuda O 作成，熊本）と抗 α-tublin 抗体（CALBIOCHEM，独国）を用いて，ウエスタンブロット法で定量した．すなわち，Apop-1 siRNA 導入 24 時間後あるいは 48 時間後，高濃度グルコース溶液（最終濃度 33 mM）を添加して 48 時間培養した内皮細胞を PBS で 2 回洗浄した．その後，lysis buffer（50 mM Tris-HCl pH 8.0，20 mM EDTA，1% SDS，100 mM NaCl）を加えて細胞膜を破壊し，超音波処理で均一化後，3000 rpm で 5 分間遠心分離した上清を細胞溶解液とした．

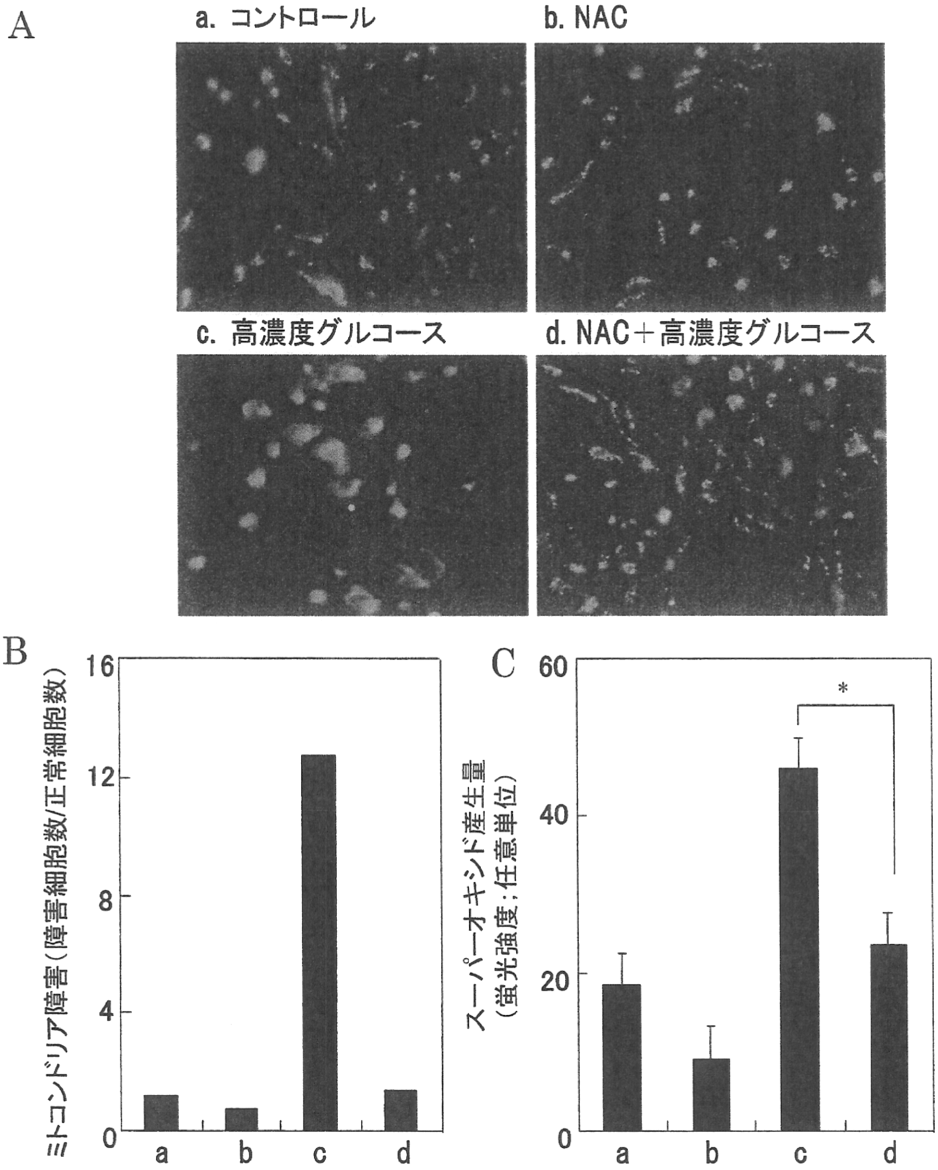

Fig. 3　活性酸素種消去剤によるミトコンドリア障害の誘導阻害
A：内皮細胞を，高濃度グルコース溶液（33 mM），あるいは高濃度グルコース溶液＋NAC（100 mM）で60時間処理し，蛍光指示薬JC-1を用いてミトコンドリア膜電位変動を測定した．JC-1の赤と緑の蛍光を重ねて示した．B：JC-1が示す赤の正常なミトコンドリアに対し，緑のミトコンドリア障害が起こった細胞の比率を定量化した．C：蛍光指示薬MitoSOXを用いてミトコンドリアのスーパーオキシド産生量を数値化し，平均値 ± 標準偏差（n = 3）で表した．（$^*p < 0.01$；高濃度グルコース群との比較）

Bio-Rad Protein Assay Kit（Bio-Rad，東京）~~細胞溶解~~液でたん白質を定量後，12.5%　e-PAGEL（ATTO，東京）を用いてSDS-polyacrylamide gel electrophoresis（SDS-PAGE）でたん白質を分画し，Polyvinylidene fluoride（PVDF）膜Immobilon-P（Millipore，米国）に転写した．転写膜を，3% スキムミルクを含むPBSでブロックし，一次抗体希釈液を加えて4℃で一晩振とうした．転写膜を洗浄後，HRP標識二次抗体（Wako，大阪）希釈液を加えて室温で1時間反応させた．最後に，転写膜を洗浄後，ECL Western Blotting Detection Reagents（GE Healthcare Bio-Sciences，東京）を用いた化学発光法でApop-1たん白質を検出した．画像解析には，Lumino Imaging Analyzer（Toyobo，東京）及びGel-Pro Analyzer（Media Cybernetics，米国）を用いた．

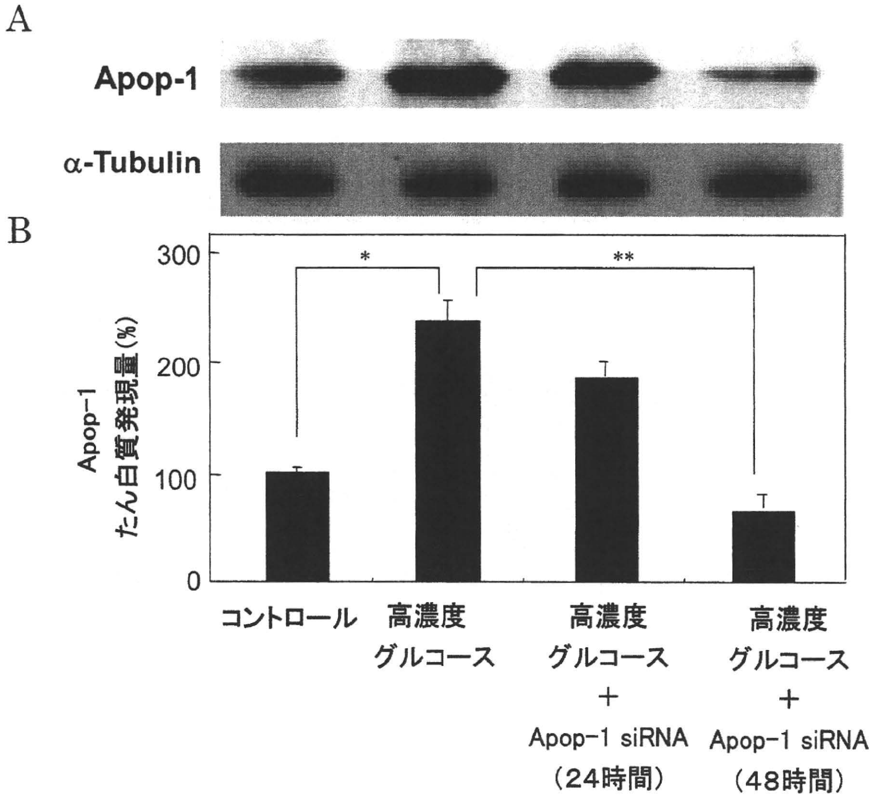

Fig. 4 高濃度グルコース処理による Apop-1 たん白質発現増大と siRNA による Apop-1 発現の抑制
A：内皮細胞を，高濃度グルコース溶液（33 mM）で 48 時間処理し，ウエスタンブロット法を用いて Apop-1 たん白質発現量を測定した．siRNA 導入 24 時間後あるいは 48 時間後に，高濃度グルコース処理した．コントロール群には，スクランブル siRNA を導入した．B：検出された Apop-1 たん白質のバンドの濃さを Image Analyzer を用いて数値化し，平均値 ± 標準偏差（n = 3）で表した．（* $p < 0.01$：コントロール群との比較，** $p < 0.01$：高濃度グルコース群との比較）α-tubulin の発現量を 1 ウエルあたり加えた試料のたん白質量が一定であることを示す内部標準とした．

6）統計解析

解析ソフト SPSS 15.0 J for Windows を用いた t 検定法により，データを統計学的に処理し，有意性を検定した．

結　果

1）高濃度グルコース処理による内皮細胞のミトコンドリアのスーパーオキシド産生増大

ミトコンドリアの活性酸素種産生を誘導するミトコンドリア complex III inhibitor アンチマイシン A[10)] は，内皮細胞において，ミトコンドリアのスーパーオキシド産生を増大した（Fig. 1A，1B）．また，内皮細胞に高濃度グルコース溶液（15，33 mM）を 48 時間処理すると，5.5 mM グルコース溶液で培養したコントロール群に比し，ミトコンドリアのスーパーオキシド産生が有意に増大した．これに対し，浸透圧のみを上昇させたマンニトール溶液（33 mM）で培養した細胞では，スーパーオキシドの産生増大は認められなかった．

2）高濃度グルコース処理によるミトコンドリアのスーパーオキシド産生を介したミトコンドリア障害の誘導

次に，ミトコンドリアで産生増大したスーパーオキシドがミトコンドリア障害を誘導する可能性を検討した．内皮細胞を，高濃度グルコース溶液（最終濃度 33 mM）で 60 時間処理すると，正常なミトコンドリア膜電位を示す JC-1 の赤色蛍光強度が低下し，障害されたミトコンドリア膜電位を示す緑色蛍光強度が増大した（Fig. 2）．これに対し，マンニトール溶液（最終濃度 33 mM）