III. 研究成果の刊行に関する一覧表

雑誌

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Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: November 18, 2010
Published online: January 11, 2011

Journal of Atherosclerosis and Thrombosis Vol.18, No.●

Original Article

Associations of Lower-Body Fat Mass with Favorable Profile of Lipoproteins and Adipokines in Healthy, Slim Women in Early Adulthood

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Aim: We determined the association of lower-body fat mass (LFM) and trunk fat mass (TFM) with cardiometabolic risk factors and adipokines in young, healthy, slim women.
Methods: A total of 481 college female students underwent the following: regional body fat distribution as assessed by dual energy X-ray absorptiometry (DXA), a 75g oral glucose tolerance test (OGTT) and fasting blood sampling for measurement of lipids, lipoproteins, apolipoproteins (apo), liver enzymes and adipokines.
Results: After adjusting for TFM, LFM was positively associated with HDL cholesterol, adiponectin, pre-heparin lipoprotein lipase and insulin sensitivity, as estimated by the Matsuda index, whereas it was negatively related to triglycerides, apo B, apo B/A1 ratio, small dense LDL, FFA, glucose and insulin at 2h during OGTT, area under the curve of insulin response during OGTT and the white blood cell count. Participants were divided into 9 groups according to tertiles of TFM and LFM. In the middle tertile of TFM, HDL cholesterol and adiponectin increased and triglycerides, apoB/A1 ratio and plasminogen-activator inhibitor-1 decreased from the low to high LFM tertiles. Gamma-glutamyltransferase levels in middle and high LFM tertiles were lower than in the lower LFM tertile.
Conclusion: For a given level of trunk fat mass, a higher lower-body fat mass is associated with an advantageous profile of not only blood lipoproteins but also serum adipokines, even in healthy, slim women in early adulthood.

J Atheroscler Thromb, 2011; 18:000-000.

Key words; Lower-body fat, Trunk fat, Adiponectin, HDL, Triglyceride

Introduction

Increases in abdominal circumference have been demonstrated to be associated with an increased risk of both cardiovascular diseases and type 2 diabetes[1-3]. On the other hand, increases in hip circumference seem to have protective effects against these conditions[4-8]. Studies in postmenopausal women have revealed that trunk fat mass (TFM) measured using dual energy X-ray absorptiometry (DXA) is a strong independent predictor of insulin resistance and dyslipidemia[9-12]. Several studies have also suggested that in contrast with trunk fat, peripheral adiposity may attenuate both insulin resistance and dyslipidemia[10-15] or just lipid levels[16] in normal and overweight postmenopausal women. Although these differences may be due to differences in adipokine secretion from regional adipose tissue, a few studies have determined serum adipokine levels[17,18].

The majority of the above studies using DXA have been limited to females with a mean BMI of

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Received: September 26, 2010
Accepted for publication: November 18, 2010

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: November 18, 2010
Published online: January 11, 2011

around 26 kg/m^2, but have also included senior subjects. Because obesity and ageing are known to affect glucose and lipid metabolism, specifically in women[19], and because very little information is available on the relationship between regional body composition and cardiovascular risk factors in very young and lean adults, we studied healthy, very young and slim women as described below to avoid confounding factors. The aim of this study was first whether fat mass is associated with numerous cardiometabolic risk factors and adipokines in healthy, slim women in early adult life with a narrow age span. A second aim was to investigate the association between regional fat mass by DXA and risk factors and adipokines.

Subjects and Methods

Participants

481 female students at Mukogawa Women's University were enrolled; all were Japanese and aged 18-24 years. Subjects with clinically diagnosed acute or chronic inflammatory diseases, endocrine, cardiovascular, hepatic, renal diseases, hormonal contraception, or unusual dietary habits were excluded. No subject was taking any medication. The study was approved by the ethics committees of the university and written informed consent was obtained from all participants.

Anthropometry and Body Composition

Body weight, height, and waist circumference (WC) were measured following standard procedures and BMI was calculated. Whole-body DXA was performed using fan beam technology (Hologic QDR-2000, software version 7.20D; Waltham, MA). This software provides estimates of lean tissue mass, fat mass, and bone mineral mass for the total body and for standard body regions. Using specific anatomic landmarks, regions of the head, trunk, arms, and legs were distinguished as previously reported[20].

Insulin, Glucose, and Insulin Resistance

Blood samples were obtained in the morning after a 12-hr overnight fast. The oral glucose tolerance test (OGTT) was performed with 75-g glucose administration in 165 participants. Blood samples were taken at 0, 30, 60, and 120 min for glucose and insulin analysis. Plasma glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase method [interassay coefficient of variation (CV) <2%]. Serum insulin was measured by ELISA with narrow specificity, excluding des-31, des-32, and intact proinsulin (interassay CV <6%). Insulin resistance was determined by homeostasis model assessment (HOMA-IR) using fasting plasma glucose and insulin levels[21] and Matsuda's index (insulin sensitive index, ISI) using glucose and insulin levels during OGTT[22]. Area under the curve during OGTT (AUC) was calculated using the trapezoidal method.

Lipids, Lipoproteins, Apolipoproteins and Liver Enzymes

Serum triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol and liver enzymes were measured in fasted blood samples using an autoanalyzer (AU5232; Olympus, Tokyo, Japan). Apolipoprotein A-1 (apoA1) and apolipoprotein B-100 (apoB) were measured by their respective commercially available kits using an Olympus autoanalyzer (AU600; Mitsubishi Chemicals, Tokyo, Japan). Low-density lipoprotein (LDL) cholesterol was calculated using Friedwald's formula[23]. Small dense LDL was measured by a precipitation method[24]. Free fatty acid (FFA) was measured using enzymatic colorimetric methods (Wako, Tokyo, Japan). Remnant-like particle (RLP) cholesterol was measured by an immunoaffinity separation method (RLP-C assay; Otsuka, Japan). Preheparin serum lipoprotein lipase (LPL) mass was measured by sandwich ELISA using a specific monoclonal antibody against bovine milk LPL, as described by Kobayashi *et al.*[25]. Using a commercial kit from Daiichi Pure Chemicals (Tokyo, Japan, interassay CV= 2.8%) was used.

Adipokines, Inflammation and Oxidative Stress Markers

Adiponectin was assayed by a sandwich enzyme-linked immunosorbent assay (Otsuka Pharmaceutical Co., Ltd., Tokushima City, Japan). Intra- and inter-assay CV were 3.3% and 7.5%, respectively. Leptin was assessed by an RIA kit from LINCO research (St. Charles, MO, interassay CV=4.9%). Highly sensitive C-reactive protein (hs-CRP) was measured by an immunoturbidometric assay using reagents and calibrators from Dade Behring Marburg GmbH (Marburg, Germany; interassay CV <5%). TNF-α was measured by immunoassays (R&D Systems, Inc., Minneapolis, MN, interassay CV <6%). Plasminogen activator inhibitor-1 (PAI-1) was measured by ELISA (Mitsubishi Chemicals, interassay CV <8%). For statistical analysis, serum concentrations of hs-CRP and TNF-α below the limit of detection were assigned a value of 0.05 mg/L and 0.50 pg/mL (lowest limit of detection), respectively. Adipokines and inflammatory markers were measured in fasted blood samples.

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: November 18, 2010
Published online: January 11, 2011

Statistics

Data are presented as the mean ± SD unless otherwise stated. Due to deviations from normal distribution, CRP was logarithmically transformed for analysis. Mean differences across tertiles were compared by the nonparametric Mann-Whitney *U* test. Bivariate correlations of regional fat mass with cardiometabolic parameters were evaluated by both Pearson and Spearman correlation analysis. The two methods gave practically identical results, thus only Pearson correlation coefficients are presented. A two-tailed value of $p < 0.05$ was considered significant. Statistical analysis was performed with SPSS system 17.0 (SPSS Inc., Chicago, IL).

Results

Participants were lean, normoglycemic, normolipidemic and normotensive and had normal liver function tests (**Tables 1** and **2**). After adjusting for TFM (**Table 2**), LFM was positively associated with HDL cholesterol, adiponectin, pre-heparin LPL and insulin sensitivity, as estimated by the Matsuda index, whereas it was negatively related to triglycerides, apolipoprotein B, small dense LDL, FFA, glucose and insulin concentrations at 2h during OGTT, in the AUC of insulin and the white blood cell count. In contrast to LFM, TFM showed opposite associations with all variables described above. TFM was also positively associated with fasting insulin, HOMA-IR, RLP-cholesterol, PAI-1 and log(CRP). LFM was negatively associated with glucose AUC and TNF-α.

Participants were divided into 9 groups according to tertiles of TFM and LFM. In the middle tertile of TFM (TMF range: 5.64-7.33 kg), women with LFM in the top tertile had very small but significantly greater TFM than women in low and median tertiles (**Table 3A**). Similarly, in the middle tertile of LFM (LMF range: 4.87-5.99 kg), women with TFM in the middle and top tertiles had small but significantly greater TFM than women in the low tertile (**Table 3B**).

After adjusting for TFM (**Fig. 1**), increases in LFM were associated with stepwise increases in HDL cholesterol (73 ± 2.2, 77 ± 1.5, 80 ± 2.1 mg/dL) and adiponectin (9.2 ± 0.7, 11.6 ± 0.5, 14.7 ± 0.7 μg/mL) whereas they were accompanied by stepwise decreases in triglycerides (61 ± 4, 57 ± 3, 47 ± 4 mg/dL), the apoB/A1 ratio (043 ± 001 vs. 041 ± 001 and 039 ± 001) and PAI-1 (25.1 ± 2.5, 22.9 ± 1.7, 15.7 ± 2.3 ng/mL). Gamma-glutamyltranspeptidase levels in middle and high LFM tertiles (13.2 ± 0.5 and 13.2 ± 0.7 units/L, respectively) were lower than in the low LFM tertile (15.0 ± 0.7 units/L). There was no difference in lean tissue mass in any regions measured in the middle tertile of TFM (data not shown).

Table 1. Anthropometric characteristics of very young, healthy, slim women

Age (year)	20.3 ± 1.3
Weight (kg)	54.3 ± 7.5
BMI (kg/m^2)	20.9 ± 2.2
Waist circumeference(cm)	72.1 ± 5.8
Total fat mass (kg)	14.1 ± 4.3
Total fat mass (%)	26.0 ± 5.9
Trunk fat mass (kg)	6.8 ± 2.4
Trunk fat mass (%)	26.7 ± 6.8
Lower-body fat mass (kg)	5.6 ± 1.5
Lower-body fat mass (%)	28.6 ± 5.7
Total lean mass (kg)	37.3 ± 5.5
Trunk lean mass (kg)	17.6 ± 2.6
Lower-body lean mass (kg)	12.9 ± 2.3

Mean ± SD. $n = 481$

In the middle tertile of LFM (LFM range: 4.87-5.99 kg), women with TFM in median and top tertiles had small but significantly greater LFM than women in the low tertile (**Table 3B**). After adjusting for LFM (**Fig. 2**), young women in the top TFM tertile had greater triglycerides (63 ± 4 vs. 52 ± 4 and 57 ± 3 mg/dL), apo B (72 ± 2 vs. 67 ± 2 and 68 ± 1 mg/dL), apoB/A1 ratio (045 ± 002 vs. 041 ± 001 and 041 ± 001) and TNF-α (0.84 ± 0.10 vs. 0.60 ± 0.05 and 0.60 ± 0.03 ng/mL) than the other 2 groups. RLP-cholesterol levels were higher in the middle and high than low TFM tertiles (3.0 ± 0.2 and 3.0 ± 0.1 vs. 2.6 ± 0.2 mg/dL). In contrast, increases in TFM were associated with a stepwise decrease in adiponectin (12.8 ± 0.70, 11.7 ± 0.43, 9.7 ± 0.50 μg/mL, **Fig. 2**).

Only serum leptin showed positive associations with both TFM and LFM but the association was stronger with TFM than with LFM in young slim women (**Table 2**). Serum leptin increased in a stepwise fashion as LFM (7.0 ± 0.4, 7.2 ± 0.3, 8.6 ± 0.4 ng/mL) and TFM (6.4 ± 0.3, 7.2 ± 0.3, 9.3 ± 0.4 ng/mL) increased in the middle tertile of TFM and LFM, respectively.

Discussion

The main finding of this study is that even in healthy, slim women in early adult life, larger LFM was associated with a favorable lipid profile, whereas larger TFM was associated with an unfavorable lipid profile. Furthermore, larger LFM was associated with a favorable serum adipokine profile, whereas larger

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: November 18, 2010
Published online: January 11, 2011

Table 2. Cardiometabolic characteristics of young women and partial Pearson's correlation coefficient (r) of trunk fat mass (TFM) and lower-body fat mass (LFM) after adjusting for LFM and TFM, respectively

	Mean ± SD	partial r	
		TFM	LFM
Fasting glucose (mg/dL)	84±7	−0.072	0.094*
2-h glucose (mg/dL)	89±20	0.168*	−0.186**
Fasting insulin (μU/mL)	6.0±3.9	0.157***	−0.056
2-h insulin (μU/mL)	32.2±23.6	0.234***	−0.211**
Matsuda index	10.7±5.3	−0.144	0.150*
HbA1c (%)	4.8±0.2	−0.041	0.030
HOMA-IR	1.27±1.11	0.199***	−0.082
Triglyceride (mg/dL)	58±30	0.195***	−0.173***
Total cholesterol (mg/dL)	182±27	0.078	−0.014
HDL cholesterol (mg/dL)	76±13	−0.202***	0.137**
LDL cholesterol (mg/dL)	95±23	0.160***	−0.052
Small dense LDL (mg/dL)	11.9±5.2	0.207***	−0.140*
Apolipoprotein A1 (mg/dL)	167±21	−0.167***	0.089
Apolipoprotein B (mg/dL)	70±14	0.234***	−0.124**
RLP-cholesterol (mg/dL)	2.9±2.0	0.118**	−0.078
FFA (mEq/L)	0.52±0.22	0.051	−0.189**
Preheparin LPL (ng/mL)	76±19	−0.287***	0.320***
Leptin (ng/mL)	7.8±3.7	0.389***	0.094*
Adiponectin (μg/mL)	11.5±4.3	−0.399***	0.368***
PAI-1 (ng/mL)	21.0±12.1	0.128**	−0.063
CRP (μg/dL)	30±72	0.129**	−0.070
TNF-α (pg/mL)	0.65±0.45	0.080	−0.124**
Systolic blood pressure (mmHg)	106±10	0.088*	−0.003
Diastolic blood pressure (mmHg)	60±7	0.075	−0.008
AST (U/dL)	19±8	−0.061	0.033
ALT (U/dL)	13±7	0.070	−0.049
GGT (U/dL)	14±5	0.088	−0.051

n=481, except for 2-h glucose and insulin and Matsuda index in oral glucose tolerance tests, which were performed in 168 women. HOMA-IR: homeostasis model assessment of insulin resistance, RLP: remnant-like lipoprotein, FFA: free fatty acid, LPL: lipoprotein lipase, PAI-1: plasminogen activator inhibitor-1, CRP: C-reactive protein, TNF-α: tumor-necrosis factor-α, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: γ-glutamyltransferase, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

TFM was associated with an unfavorable serum adipokine profile. It is worth noting that in the present study these observations were found in a homogeneous sample of very young female college students, most of whom were nonobese, normolipidemic, nondiabetic, and normotensive. In addition, no subject reported a smoking habit or alcohol intake and no subject was receiving any medications.

There are some known regional differences in adipokine secretion between abdominal subcutaneous and visceral fat[26, 27], but less is known about differences between gluteal and abdominal subcutaneous fat[28]. Although several studies have reported the protective effects of lower-body fat on lipid metabolism [See Ref. 28], and a few studies have examined the associations of lower body fat with serum adiponectin as far as we know, no study has examined the associations with serum PAI-1 or TNF-α.

In the present study employing young, slim women, larger LFM resulted in increased serum adiponectin. Several other studies with direct measurement of different fat depots by DXA found a positive association of adiponectin with LFM[17, 18]. Furthermore, larger LFM was associated with decreased PAI-1 and lower GGT in addition to higher adiponectin in young, healthy, slim women. Plasma PAI-1 levels have been shown to be more strongly related to liver steatosis than to adipose tissue accumulation[29] although abdominal adipose tissue is an important source of plasma PAI-1 in insulin resistance syndrome with cen-

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: November 18, 2010
Published online: January 11, 2011

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,

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: November 18, 2010
Published online: January 11, 2011

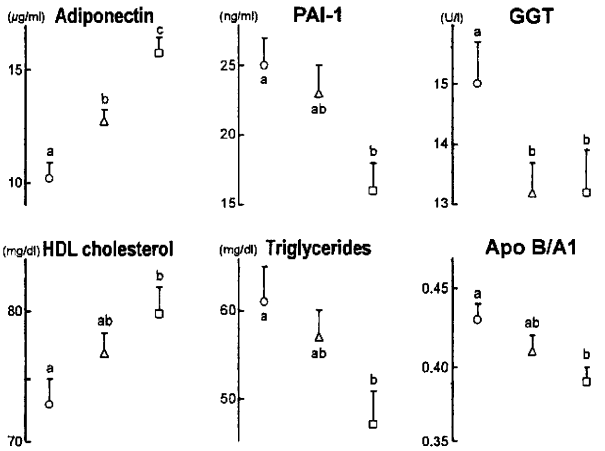

Fig. 1. Serum lipids and adipokines in low ($n=54$, circles), middle ($n=53$, triangles) and high ($n=54$, squares) tertiles of lower body fat mass in the middle tertile of trunk fat mass. Participants were divided into 9 groups according to tertiles of TFM and LFM. In the middle tertile of TFM, TMF 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.

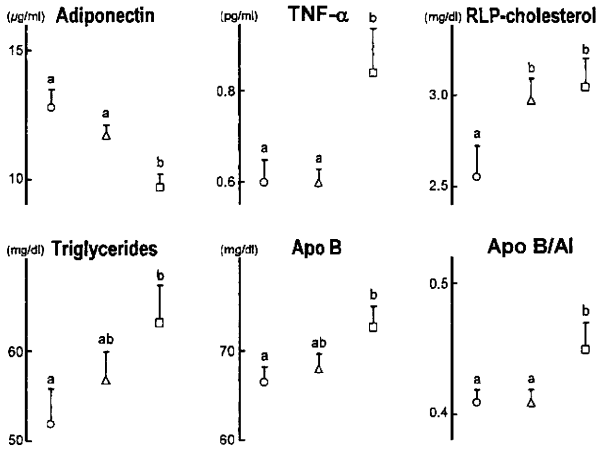

Fig. 2. Serum lipids and adipokines in low ($n=40$, circles), middle ($n=80$, triangles) and high ($n=41$, squares) tertiles of trunk fat mass in the middle tertile of lower-body fat mass. LFM ranged from 4.87 to 5.99 kg in the middle tertile of trunk fat mass. For details, see Table 3. Data are the 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.

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Accepted for publication: November 18, 2010
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Original Article

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

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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

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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

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

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

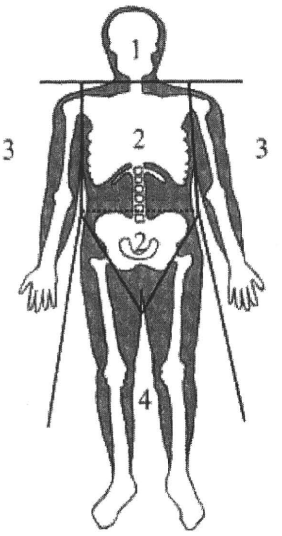

Fig. 1. Standard regions of DXA scanning: 1, head; 2, trunk; 3, arms; 4, legs.

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.

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

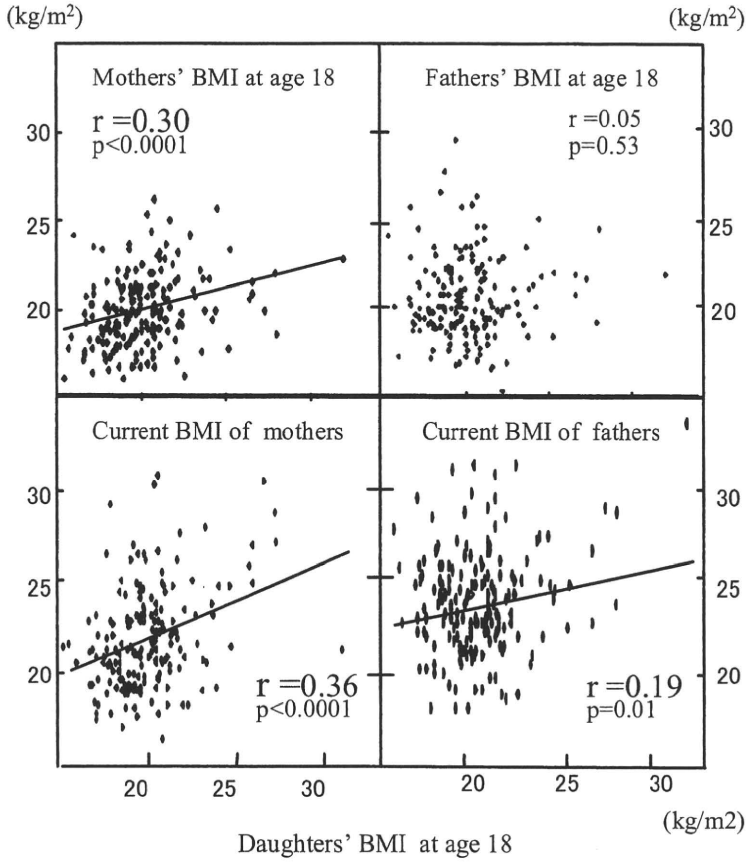

Fig. 2. Correlations between daughters and their biological parents of BMI at age 18 (upper panel) and current BMI (lower panel).

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 sig-

nificant 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.

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

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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.

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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-