Changes in Waist Circumference and Body Mass Index in Relation to Changes in Serum Uric Acid in Japanese Individuals

NOBUKAZU ISHIZAKA, YUKO ISHIZAKA, AKIKO TODA, MIZUKI TANI, KAZUHIKO KOIKE, MINORU YAMAKADO, and RYOZO NAGAI

ABSTRACT. ***Objective.*** Studies have shown that obesity is associated with an increase in serum uric acid; and few data are available on the relationship between changes in measures of obesity and changes in uric acid concentrations. We investigated the relationship among percentage changes in waist circumference (%dWC), body mass index (%dBMI), and serum uric acid (%dUA).

Methods. The data of 3153 individuals [1968 men, 1185 women (536 premenopausal, 649 postmenopausal)] who underwent general health screening over a 2-year period and were not taking antihyperuricemic medication were analyzed.

Results. Stepwise multiple regression analysis showed that %dBMI was associated positively with %dUA in postmenopausal women and men, and the association retained statistical significance after adjustment for changes in blood pressure and in renal function. Association between %dBMI and %dUA was not significant in premenopausal women. In men, %dWC was a predicting factor for %dUA, although it did not remain significant when %dBMI was used as a covariate in the statistical model. Multivariate logistic regression analysis showed that the odds ratio of the association between the lowest %dBMI quartile (%dBMI < −1.86) and the lowest %dUA quartile (%dUA < −7.41) was 2.04 (95% CI 1.35–3.07) in postmenopausal women and 1.46 (95% CI 1.14–1.86) in men.

Conclusion. Weight loss may represent an effective nonmedical strategy for reducing serum UA levels, especially in postmenopausal women and men. (First Release Dec 23 2009; J Rheumatol 2010;37:410–6; doi:10.3899/jrheum.090736)

Key Indexing Terms:
WAIST CIRCUMFERENCE BODY MASS INDEX URIC ACID
GLOMERULAR FILTRATION RATE BLOOD PRESSURE

Obesity and serum uric acid (UA) are both associated with enhanced insulin resistance and incidence of metabolic syndrome[1-3]. In addition, measures of obesity have been reported to be positively associated with serum levels of UA[4,5], an association that may be caused by impaired renal clearance of UA in the condition of obesity[6]. The finding that a reduction in weight, and thus in body mass index (BMI), may have a significant effect on serum UA[7] and renal urate excretion[6] suggests that changes in weight may play a role in the regulation of serum UA levels, although the reverse scenario might also be possible[8]. In our study, by analyzing individuals who underwent general health screening, we examined the influence of changes in waist circumference (WC) and BMI on changes in UA, and the dependency/independency on changes in either blood pressure or renal function, which is the possible critical factor affecting serum UA levels in healthy subjects[9].

MATERIALS AND METHODS

Study population. The study was approved by The Ethical Committee of Mitsui Memorial Hospital. Between October 2005 and October 2006, 11,558 individuals underwent general health screening at our institute. Of these, 3326 individuals (2113 men, 1213 women) underwent general health screening during this period (first visit) and again the following year (second visit). Among these 3326 individuals, 3179 (1968 men, 1211 women) reported taking no antihyperuricemic drugs at either visit. Among the 1211 women, 1185 (98%) answered the questionnaire concerning whether they still had menstruation, and were enrolled for study. Therefore, we analyzed data of 3153 individuals (1968 men, 1185 women). The mean ± standard deviation (SD) interval between the 2 visits of the individuals enrolled was

From the Department of Cardiovascular Medicine and Department of Gastroenterology, University of Tokyo Graduate School of Medicine, Tokyo; and Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital, Tokyo, Japan.

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N. Ishizaka, MD, Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine; Y. Ishizaka, MD; A. Toda, MD; M. Tani, MD, Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital; K. Koike, MD, Department of Gastroenterology, University of Tokyo Graduate School of Medicine; M. Yamakado, MD, Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital; R. Nagai, MD, Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine.

Address correspondence to Dr. N. Ishizaka, Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Hongo 7-3-1 Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: nobuishizka-tky@umin.ac.jp

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356.2 ± 51.7 days. WC, BMI, UA, systolic blood pressure, and estimated glomerular filtration rate (eGFR) at the first visit were designated WC1, BMI1, UA1, BPs1, and eGFR1, respectively, and at the second visit WC2, BMI2, UA2, BPs2, and eGFR2.

The percentage differences between values of WC1 and WC2, BMI1 and BMI2, UA1 and UA2, BPs1 and BPs2, and eGFR1 and eGFR2 were designated %dWC, %dBMI, %dUA, %dBPs, and %deGFR. All participants were seen after an overnight fast. Height and weight were determined, and BMI was expressed as weight (kilograms) divided by the square of height (meters). With the subject standing, waist circumference was measured at the umbilical level to the nearest 1 cm by trained physicians and technicians[10].

Laboratory analysis. Blood samples were taken after an overnight fast. Serum levels of low density lipoprotein (LDL), high density lipoprotein (HDL) cholesterol, and triglycerides were determined enzymatically. Serum UA was measured by the uricase-peroxidase method, and hemoglobin A_{1C} by latex agglutination immunoassay. Creatinine was measured by TBA-200FR (Toshiba Medical Systems, Tochigi, Japan) using a commercial kit. Accuras Auto CRE (Shino-test, Tokyo, Japan) eGFR was calculated by the equation: $eGFR = 194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287}$ (× 0.739 if female)[11]. This equation was recently determined by a multicenter study, and differs from the equation[12] that we used in previous studies[13-15]. Blood pressure was measured after about 10 min of rest with an automated sphygmomanometer.

Statistical analysis. Data are expressed as the mean ± SD unless stated otherwise. Analyses of variance with trend analysis and stepwise multiple regression analysis were conducted as appropriate to assess the statistical significance of differences between groups using SPSS II (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was taken to be statistically significant.

RESULTS

Baseline characteristics. We enrolled 536 premenopausal women, 649 postmenopausal women, and 1968 men for study. At the first visit the mean age of premenopausal women was 43.1 ± 5.51 years, postmenopausal women 59.1 ± 6.8 years, and men 53.3 ± 10.2 years. The sex-nonspecific range of the first to the fourth %dUA quartiles (maximum/minimum) was –47.2/–7.5, –7.4/–1.2, 0.0/7.1, and 7.2/77.8 (Table 1). A plot of WC1 and BMI1 compared to UA1 is shown in Figure 1. In both men and women, there was a statistically significant correlation between WC1 or BMI1 and UA1. The correlation coefficient between WC1 and BMI1 was 0.626 ($p < 0.001$) in premenopausal women, 0.563 ($p < 0.001$) in postmenopausal women, and 0.838 ($p < 0.001$) in men.

Relationship between %dWC, %dBMI, and %dUA. The relationship between %dBMI and %dUA, although very weak, was significant in postmenopausal women and men, and the relationship between %dWC and %dUA was significant only in men (Figure 2). In premenopausal women, the relationships between %dWC and %dUA and between %dBMI and %dUA were not statistically significant. The correlation coefficient between %dWC and %dBMI was 0.267 ($p < 0.001$) in premenopausal women, 0.221 ($p < 0.001$) in postmenopausal women, and 0.484 ($p < 0.001$) in men.

We next performed stepwise multiple regression analysis (Table 2). In a model in which age, UA1, WC1, and %dWC were used as independent variables (model 1), %dWC was found to have independent predictive value for %dUA in men, but not in women. However, after adding BMI1 and %dBMI as independent variables, %dWC in men was no longer a predictor for %dUA (model 2). %dBMI was found to be a predictor for %dUA in postmenopausal women and men even after using either or both %dBPs and %deGFR as independent variables (models 3-5). On the other hand, in premenopausal women, %dBMI was not a significant predictor value for %dUA in any of these models. In addition, in a model in which age, UA1, BMI1, and %dBMI were used as independent variables, %dBMI again was not found to have significant predictive value for %dUA (data not shown). In model 5, variance inflation factor (VIF) scores of all the independent variables were less than 10 (data not shown).

Logistic regression analysis. When the highest %dUA quartile (%dUA ≥ 7.25) was used as a dependent variable, logistic regression analysis showed that the highest %dBMI quartile (%dBMI ≥ 1.47) had a significant positive association in postmenopausal women and in men after adjusting for UA1 and BMI1 (Table 3, model 1). In these groups, statistical significance was retained even after further adjustment for %dBPs and %deGFR (model 2). On the other hand, in premenopausal women, the highest %dBMI quartile was, unexpectedly, negatively associated with the highest %dUA quartile, although statistical significance was lost after further adjustment for %dBPs and %deGFR. In all 3 subgroups tested, %deGFR was negatively associated with the highest quartile of %dUA.

Logistic regression analysis showed that the lowest %dBMI quartile (%dBMI < –1.86) had a significant positive association with the lowest %dUA quartile (%dUA < –7.41) in postmenopausal women and men, and this remained statistically significant even after further adjustment for %dBPs and %deGFR (model 2). But in premenopausal women, association between the lowest %dBMI quartile and lowest %UA was not statistically significant regardless of this further adjustment.

DISCUSSION

Analyzing data of individuals who underwent general heath screening and who were taking no antihyperuricemic medication, we found that correlation between percentage changes in BMI (%dBMI) and in UA (%dUA) was statistically significant in postmenopausal women and in men, but not in premenopausal women.

Stepwise multiple regression analysis showed that %dWC is a significant independent variable for %dUA in men, where UA1, WC1, and %dWC was used as possible independent variables (model 1); however, the relationship lost statistical significance after further adjustment for BMI1 and %dBMI (Table 3). %dWC was not found to be a

Table 1. Baseline characteristics at the first visit according to %dUA quartiles.

Variables	Total, n	%dUA Quartile First (range –47.2/–7.5)	Second (range –7.4/–1.2)	Third (range 0.0/7.1)	Fourth (range 7.2/77.8)	p for Trend
Women/men	1185/1968	290/496	252/483	327/514	316/475	
Baseline data at visit 1						
Age, yrs	52.8 ± 10.2	52.6 ± 10.6	53.3 ± 10.1	52.9 ± 10.0	52.3 ± 10.2	0.225
Waist circumference, cm	82.3 ± 9.2	82.8 ± 9.6	82.7 ± 9.1	82.2 ± 8.7	81.7 ± 9.3	0.074
Body mass index, kg/m^2	22.8 ± 3.1	23.0 ± 3.3	22.8 ± 3.0	22.8 ± 3.0	22.6 ± 3.1	0.056
Systolic blood pressure, mmHg	123 ± 19	124 ± 20	123 ± 19	122 ± 19	123 ± 19	0.636
LDL-cholesterol, mg/dl	129.1 ± 31.2	130.2 ± 31.3	129.4 ± 29.5	129.8 ± 31.3	126.9 ± 32.2	0.147
HDL-cholesterol, mg/dl	60.7 ± 15.3	60.7 ± 15.8	60.5 ± 15.5	60.8 ± 14.8	60.9 ± 15.1	0.980
Triglyceride, mg/dl	111 ± 72	112 ± 75	115 ± 81	109 ± 67	109 ± 65	0.249
Uric acid, mg/dl	5.5 ± 1.3	5.8 ± 1.5	5.7 ± 1.2	5.4 ± 1.3	5.1 ± 1.2	< 0.001
Fasting glucose, mg/dl	96 ± 21	97 ± 20	96 ± 19	95 ± 18	96 ± 25	0.486
Hemoglobin A1c, %	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.9	0.941
Blood urea nitrogen, mg/dl	14.1 ± 3.5	14.6 ± 4.0	14.3 ± 3.2	14.1 ± 3.3	13.6 ± 3.5	< 0.001
Serum creatinine, mg/dl	0.77 ± 0.29	0.79 ± 0.42	0.78 ± 0.15	0.76 ± 0.15	0.75 ± 0.34	0.068
Estimated glomerular filtration rate, ml/min/1.73m^2	68.3 ± 11.8	67.3 ± 11.8	67.8 ± 11.5	68.1 ± 11.4	70.2 ± 12.4	< 0.001
Antihypertensive medication, n (%)	306 (9.7)	78 (9.9)	78 (10.6)	76 (9.0)	74 (9.4)	0.736
Antidiabetic medication, n (%)	74 (2.3)	19 (2.4)	14 (1.9)	24 (2.9)	17 (2.1)	0.632
Postmenopause (female), n (%)	649 (54.8)	152 (52.4)	141 (56.0)	194 (59.3)	162 (51.3)	0.165
Current smoker, n (%)	734 (23.3)	180 (22.9)	173 (23.5)	197 (23.4)	184 (23.3)	0.992
Percent change between 2 visits						
%dBMI	–0.28 ± 3.1	–0.70 ± 3.41	–0.46 ± 3.06	0.01 ± 2.88	–0.03 ± 2.98	< 0.001
%dWC	0.17 ± 6.12	–0.02 ± 6.33	–0.35 ± 6.14	0.64 ± 6.14	0.35 ± 5.83	0.008
%deGFR	1.8 ± 10.0	6.3 ± 10.0	2.6 ± 9.0	0.9 ± 9.1	–2.5 ± 10.0	< 0.001

predictive value for %dUA in premenopausal or postmenopausal women. By contrast, %dBMI was found to be a predictor for %dUA, even after adjustment for WC1, %dWC, %dBPs, and %deGFR in postmenopausal women and men, although it was not significant in premenopausal women. In premenopausal women, %dBMI was not a significant predictive value for %dUA in the model in which age, UA1, BMI1, and %dBMI were used as independent variables; therefore, failure of %dBMI as a predictor for %dUA in premenopausal women may not fully be explained by the multicollinearity between %dWC and %dBMI. These findings collectively indicate that mode of association between change in BMI and change in UA differs between premenopausal and postmenopausal women.

There are several previous studies in which changes in obesity measures have been analyzed in relation to the changes in UA over a certain period of time. For example, Heyden, *et al* showed that there was a stepwise progression from decreased UA levels associated with maximum weight loss to increased levels with maximum weight gain[16]. In addition, Rathmann, *et al*[7] analyzed the data of 1249 male and 1362 female subjects aged 17–35 years from the Coronary Artery Risk Development in Young Adults (CARDIA) Study who attended a 10-year followup. They reported that changes in BMI and WC were associated with changes in UA in a statistical model adjusted for age and baseline UA levels[7]. In contrast, we found that %dBMI was, but %dWC was not, significantly associated with %dUA in multiple linear regression (Table 2, models 2-5). This might be because statistical significance had been weakened after %dWC and %dBMI were simultaneously included into the statistical model; however, %dWC was not significantly associated with %dUA in women even before the adjustment for %dBMI (model 1). From our epidemiological study, we cannot determine what would have caused the different observations between the findings of Rathmann, *et al*[7] and our own. However, considering that circulating insulin levels may have potential to regulate serum UA levels[17], the difference might derive from the difference in insulin sensitivity[18,19] and/or difference in the effect of obesity on insulin resistance[20] among various ethnicities. This possibility should be investigated in future studies. Choe, *et al* found that mean changes in BMI, but not in WC, were statistically different between subjects who had decreased or had no change in UA and those with increased UA during a 1-year followup in men who underwent health promotion screening[9]. They also found that changes in serum creatinine levels, but not in systolic or diastolic blood pressure, were significantly different between subjects who had decreased or unchanged UA levels and those with increased UA[9] — a finding that is, in one sense, in agreement with our observations.

What would be the possible underlying mechanisms that explain the difference in the mode of association between

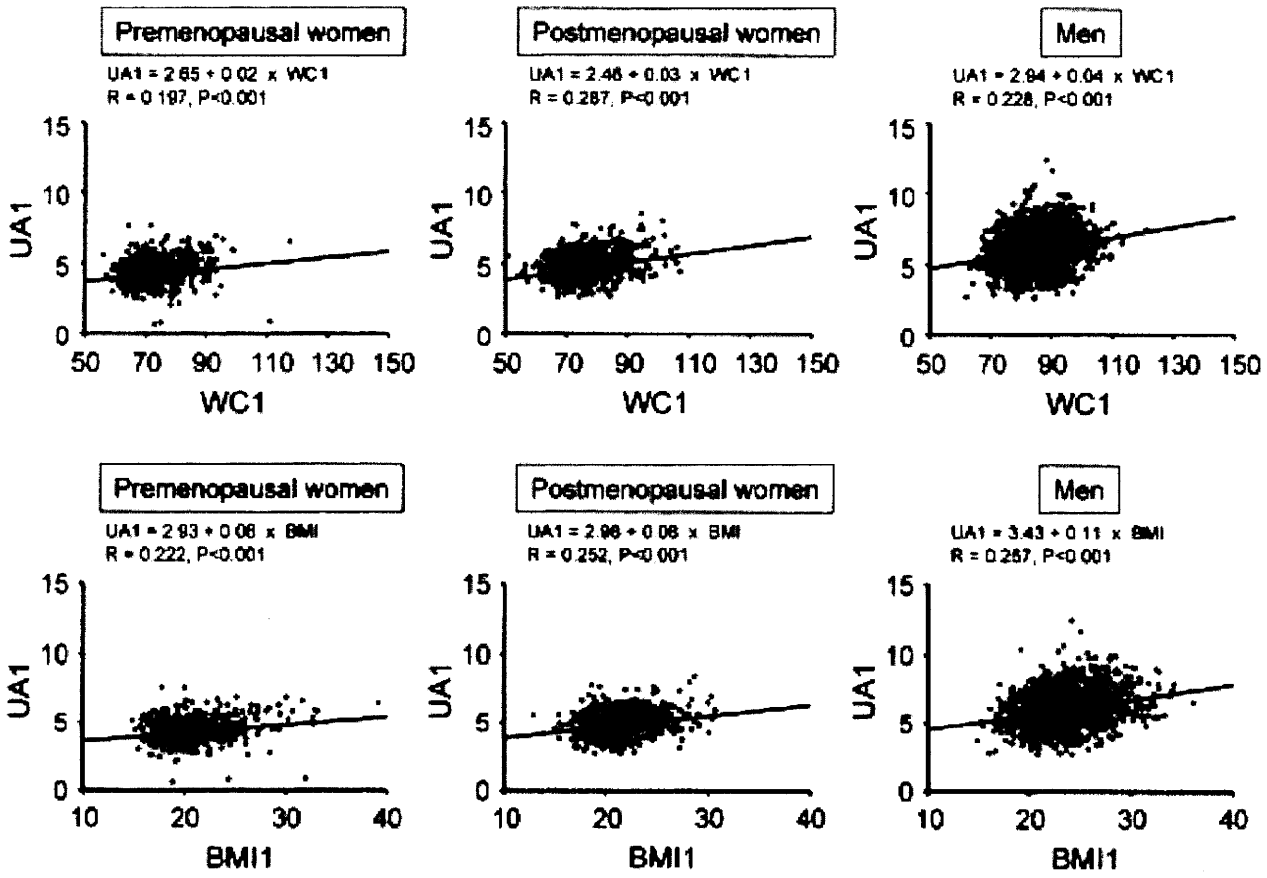

Figure 1. Scatterplot and linear regression between waist circumference at the first visit (WC1) and uric acid at the first visit (UA1) and those between body mass index at the first visit (BMI1) and UA1 in premenopausal and postmenopausal women and in men. Serum uric acid values were not adjusted for age or other possible confounders.

%dBMI and %dUA between premenopausal and postmenopausal women? It has been reported that certain alterations in UA metabolism may occur after the menopause; menopause leads to an increase in serum UA levels[21,22], and this may be in part attributed to decreased estrogen production and subsequent reduction of the fractional excretion of UA[23]. In addition, a recent study suggested that association between insulin resistance and serum UA levels may be greater in postmenopausal women than premenopausal women[24]. Whether these phenomena are related to the difference in the mode of association between %dBMI and %dUA of premenopausal and postmenopausal women remains to be investigated.

We previously showed that obesity or overweight was significantly associated with chronic kidney disease[14], and that changes in obesity measures may be associated with changes in eGFR and urinary excretion of albumin[25]. The strength of the current study was that we demonstrate that change in BMI was positively associated with change in UA in postmenopausal women and men independent of change in eGFR. In addition, we show that mode of association between %dBMI and %dUA was different between premenopausal and postmenopausal women, which may have relation with the fact that menopause causes the elevation of serum UA[21,22,26]. However, controlling BMI is neither unnecessary nor ineffective in keeping the metabolic measures in optimal ranges in "premenopausal" women, because weight gain may result in the reduced insulin sensitivity and aggravation of cardiovascular risk also in premenopausal women[27].

Data for visceral fat volume measured by computed tomography were not available in our study. Recent reports showed that subcutaneous fat accumulation is related to impaired urinary UA excretion[6], whereas visceral fat accumulation is linked closely to the overproduction of uric acid[28], and that serum UA levels are increased both in individuals with subcutaneous fat obesity and in those with visceral fat obesity[28]. It remains to be determined whether changes of WC will lead to an increase in urinary UA excretion in our population, and whether there is a relationship between %dUA and change in visceral fat volume.

Our study has several potential limitations. First, we had no information on the extent to which modifications of lifestyle and dietary habits affected observed changes in

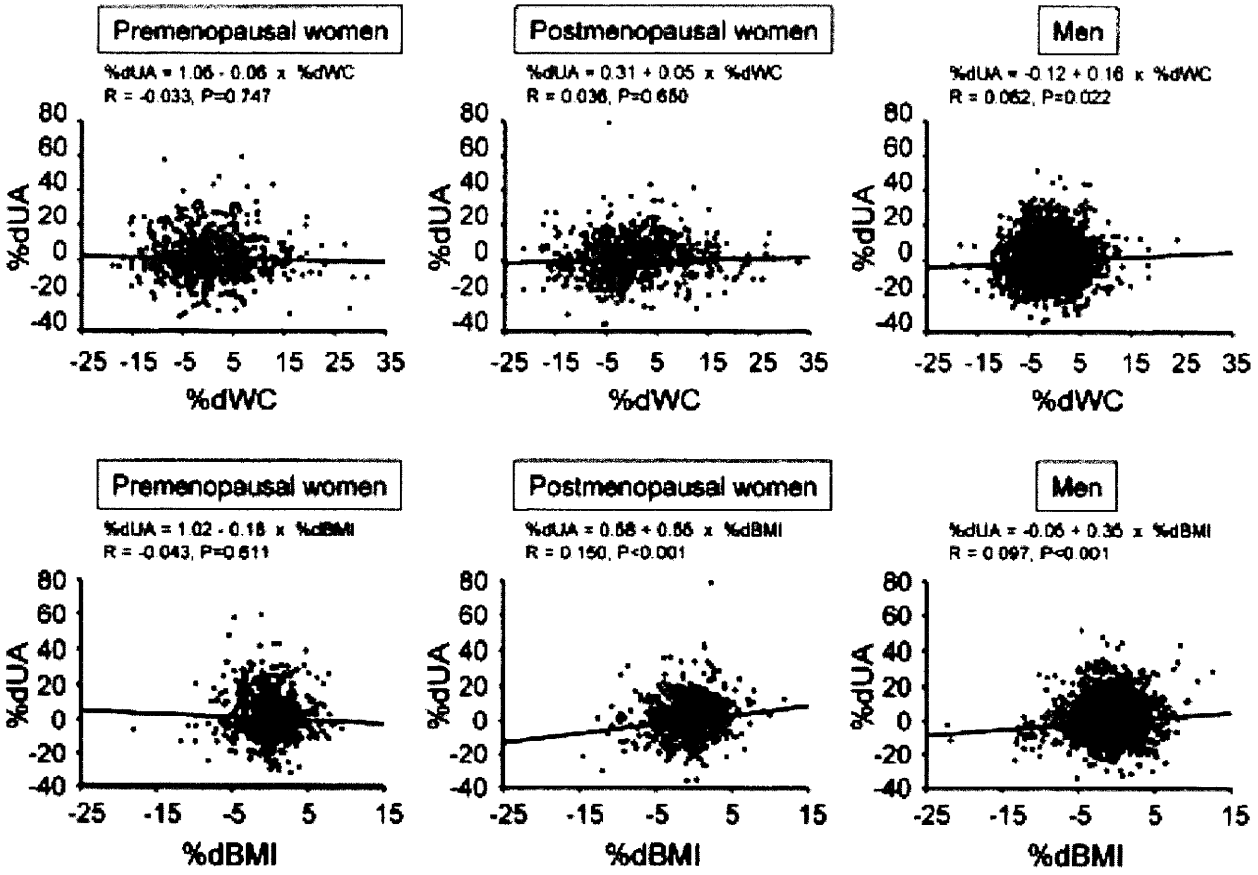

Figure 2. Scatterplot and linear regression between percentage change in waist circumference (%dWC) and percentage change in uric acid (%dUA), and those between percentage change in BMI (%dBMI) and %dUA in premenopausal and postmenopausal women and in men. Serum uric acid values were not adjusted for age or other possible confounders.

general/abdominal obesity, as no program to reduce weight was conducted by our institute. Second, we did not take into account participants' level of alcohol consumption or number of cigarettes smoked; both may affect serum UA levels[29,30]. Third, blood samples were taken from individuals in fasting condition, which may have affected their serum creatinine levels, and thus eGFR.

In summary, during a 1-year period, percentage changes in BMI (%dBMI) were associated positively with percentage changes in serum UA levels (%dUA) in postmenopausal women and men, but not in premenopausal women. This relationship was, at least in part, independent of changes in blood pressure and renal function. Weight loss may represent an effective strategy to decrease serum UA levels without use of antihyperuricemic medications, especially in postmenopausal women and men.

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Table 2. Stepwise multiple regression analysis using %dUA as the dependent variable.

	ß	(95% CI)	Standardized ß	p
Premenopausal women				
Model 1				
UA1	–4.21	(–5.42, –3.00)	–0.28	< 0.001
Model 2				
UA1	–4.21	(–5.42, –3.00)	–0.28	< 0.001
Model 3				
UA1	–4.21	(–5.42, –3.00)	–0.28	< 0.001
Model 4				
%deGFR	–0.43	(–0.53, –0.33)	–0.34	< 0.001
UA1	–3.70	(–4.84, –2.56)	–0.25	< 0.001
Model 5				
%deGFR	–0.43	(–0.53, –0.33)	–0.34	< 0.001
UA1	–3.70	(–4.84, –2.56)	–0.25	< 0.001
Postmenopausal women				
Model 1				
UA1	–3.09	(–4.05, –2.13)	–0.24	< 0.001
Model 2				
UA1	–3.04	(–4.00, –2.09)	–0.24	< 0.001
%dBMI	0.53	(0.26, 0.81)	0.14	< 0.001
Model 3				
UA1	–3.04	(–4.00, –2.09)	–0.24	< 0.001
%dBMI	0.53	(0.26, 0.81)	0.14	< 0.001
Model 4				
%deGFR	–0.33	(–0.41, –0.25)	–0.30	< 0.001
UA1	–2.83	(–3.73, –1.92)	–0.22	< 0.001
%dBMI	0.55	(0.29, 0.81)	0.15	< 0.001
Model 5				
%deGFR	–0.33	(–0.41, –0.25)	–0.30	< 0.001
UA1	–2.83	(–3.73, –1.92)	–0.22	< 0.001
%dBMI	0.55	(0.29, 0.81)	0.15	< 0.001
Men				
Model 1				
UA1	–2.51	(–2.90, –2.12)	–0.27	< 0.001
%dWC	0.14	(0.04, 0.25)	0.06	0.008
Model 2				
UA1	–2.49	(–2.88, –2.10)	–0.27	< 0.001
%dBMI	0.32	(0.17, 0.48)	0.09	< 0.001
Model 3				
UA1	–2.51	(–2.89, –2.12)	–0.27	< 0.001
%dBMI	0.38	(0.22, 0.54)	0.10	< 0.001
%dBPs	–0.06	(–0.10, –0.02)	–0.06	0.006
Model 4				
%deGFR	–0.36	(–0.40, –0.31)	–0.32	< 0.001
UA1	–2.29	(–2.66, –1.92)	–0.25	< 0.001
%dBMI	0.35	(0.21, 0.50)	0.10	< 0.001
Model 5				
%deGFR	–0.36	(–0.40, –0.31)	–0.32	< 0.001
UA1	–2.29	(–2.66, –1.92)	–0.25	< 0.001
%dBMI	0.35	(0.21, 0.50)	0.10	< 0.001

Model 1. Independent variables include age, UA1, WC1, and %dWC. Model 2. Independent variables include Model 1 + BMI1 and %dBMI. Model 3. Independent variables include Model 2 + %dBPs. Model 4. Independent variables include Model 2 + %deGFR. Model 5. Independent variables include Model 2 + %dBPs and %deGFR. UA: uric acid; BMI: body mass index; BPs: systolic blood pressure; eGFR: estimated glomerular filtration rate.

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Table 3. Logistic regression analysis using the highest or lowest %dUA quartile as the dependent variable.

	Independent Variable			
	%dUA ≥ 7.2%		%dUA < −7.5%	
	OR (95% CI)	p	OR (95% CI)	p
Premenopausal women				
Model 1				
%dBMI quartile				
First			1.19 (0.73, 1.94)	0.474
2 and 3	1 Reference			
4	0.65 (0.42, 0.99)	0.046	1.00 Reference	
Model 2				
%dBMI quartile				
First			1.41 (0.85, 2.34)	0.181
2 and 3	1.00 Reference			
4	0.71 (0.45, 1.10)	0.126	1.00 Reference	
%deGFR	0.93 (0.91, 0.95)	< 0.001	1.06 (1.04, 1.08)	< 0.001
%dBPs	0.99 (0.97, 1.01)	0.225	1.00 (0.98, 1.02)	0.804
Postmenopausal women				
Model 1				
%dBMI quartile				
First			2.04 (1.37, 3.03)	< 0.001
2 and 3	1.00 Reference			
4	1.60 (1.06, 2.41)	0.025	1.00 Reference	
Model 2				
%dBMI				
First			2.04 (1.35, 3.07)	0.001
2 and 3	1.00 Reference			
4	1.72 (1.12, 2.63)	0.013	1.00 Reference	
%deGFR	0.95 (0.93, 0.97)	< 0.001	1.05 (1.03, 1.07)	< 0.001
%dBPs	0.98 (0.97, 1.00)	0.039	1.00 (0.98, 1.01)	0.684
Men				
Model 1				
%dBMI quartile				
First			1.35 (1.07, 1.69)	0.011
2 and 3	1.00 Reference			
4	1.38 (1.08, 1.76)	0.010	1.00 Reference	
Model 2				
%dBMI quartile				
First			1.46 (1.14, 1.86)	0.002
2 and 3	1.00 Reference			
4	1.49 (1.15, 1.92)	0.002	1.00 Reference	
%deGFR	0.94 (0.92, 0.95)	< 0.001	1.07 (1.06, 1.08)	< 0.001
%dBPs	1.00 (0.99, 1.01)	0.300	1.00 (0.99, 1.01)	0.715

Model 1. Independent variables include age, UA1, BMI1, and %dBMI quartiles. Model 2. Independent variables include Model 1 + %dBPs and %deGFR. UA: uric acid; BMI: body mass index; BPs: systolic blood pressure; eGFR: estimated glomerular filtration rate.

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ORIGINAL ARTICLE

Effects of the AT_1 receptor blocker losartan and the calcium channel blocker benidipine on the accumulation of lipids in the kidney of a rat model of metabolic syndrome

Nobukazu Ishizaka[1], Makiko Hongo[1], Gen Matsuzaki[1], Kyoko Furuta[1], Kan Saito[1], Ryota Sakurai[1], Aiko Sakamoto[1], Kazuhiko Koike[2] and Ryozo Nagai[1]

Unfavorable lipid accumulation may occur in the kidneys in the presence of metabolic syndrome and diabetes. The aim of this study was to investigate whether excess lipids would accumulate in the kidneys of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of metabolic syndrome. From 34 weeks of age, OLETF rats were treated orally with a calcium channel blocker, benidipine (3 mg kg^{-1} per day), or an AT1 receptor blocker, losartan (25 mg kg^{-1} per day), for 8 weeks. Blood pressure was slightly but significantly higher in the untreated OLETF rats (149 ± 4 mm Hg) than in Long-Evans Tokushima Otsuka (LETO) rats (136 ± 2 mm Hg), and both losartan (135 ± 3 mm Hg) and benidipine (138 ± 3 mm Hg) reduced blood pressure in OLETF rats to a level comparable to that in LETO rats. Tissue content of triglycerides (TG) was greater in OLETF rats than in LETO rats (6.24 ± 3.77 and 2.85 ± 1.32 μg mg^{-1} · tissue, respectively), and both losartan and benidipine reduced these values. Histological analysis showed lipid droplets in tubular cells in which increased dihydroethidium fluorescence was present. Expression of peroxisome proliferator-activated receptor-α, PGC-1α and uncoupling protein-2 was found to be higher in OLETF rats than in LETO rats; however, the expression of these genes was not altered by treatment with either antihypertensive drug. In contrast, both losartan and benidipine increased the amount of total and phosphorylated forms of AMP kinase and the expression of carnitine palmitoyltransferase-1 (CPT-1). In conclusion, treatment of OLETF rats with losartan and benidipine reduced the tissue content of TG, decreased the production of superoxide and regulated the expression of genes related to fatty acid oxidation such as AMP-activated protein kinase and CPT-1 in the kidneys.
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Keywords: calcium channel blocker; diabetes; kidney; lipotoxicity; renin angiotensin system

INTRODUCTION

Unfavorable lipid accumulation in the kidneys may occur in animal models of diabetes,[1–3] aging,[4] diet-induced obesity[5,6] and nephrectomy.[7] Although the precise mechanism by which lipid content is increased in the kidneys is still not fully elucidated, it may include upregulation of lipogenic gene expression in the kidneys[1,7,8] and uptake of filtered albumin-bound fatty acids by renal tubular cells when increased urinary excretion of albumin is present.[9] Transfer of lipogenic genes induced deposition of lipids and upregulation of fibrosis-related gene expression in the kidneys of diabetic animals, whereas lipogenic gene knockdown had the opposite effect,[1,8] suggesting that the accumulation of excessive lipid in the kidneys is one factor in the pathophysiological process of diabetic nephropathy.[6]

We reported earlier that administration of angiotensin II upregulates the expression of lipogenic genes and increases lipid content in the kidneys,[10] suggesting that activation of the renin angiotensin system may have a role in lipid accumulation in the kidney. It has been reported that lipid content is increased in the liver[11] and pancreas[12] of OLETF rats. In this study, therefore, we investigated whether excessive lipid accumulation occurs in the kidneys of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which exhibit features of metabolic syndrome,[13] and if present, whether angiotensin II receptor blockers and calcium channel blockers could exert similar effects on renal lipid content in OLETF rats. We used benidipine as the calcium channel blocker because this drug has been reported to reduce the extent of proteinuria in OLETF rats.[14]

[1]Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Tokyo, Japan and [2]Department of Gastroenterology, University of Tokyo Graduate School of Medicine, Tokyo, Japan
Correspondence: Dr N Ishizaka, Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Bunkyo-ku, Hongo 7-3-1, Tokyo 113-8655, Japan.
E-mail: nobuishizka-tky@umin.ac.jp
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METHODS

Animals

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Male OLETF and age-matched Long-Evans Tokushima Otsuka (LETO) rats, a genetic control, were obtained from the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and maintained under constant temperature and lighting conditions with free access to food and water. At 34 weeks of age, the OLETF rats were given 3 mg kg^{-1} benidipine or 25 mg kg^{-1} losartan per day orally, which was continued for 8 weeks. One day before sacrifice, the rats were kept in a metabolic cage, and urine was collected for 24 h under fasting conditions. Systolic blood pressure and heart rate were measured in conscious rats by tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan).

Measurement of lipid content in the serum and kidney

Serum levels of total cholesterol (TC), triglycerides (TG) and non-esterified fatty acid were measured by enzymatic methods (SRL, Tokyo, Japan). Contents of TG and TC in the kidney were measured from homogenate extracts by enzymatic colorimetric determination using the Triglyceride-E Test, the Cholesterol-E Test and the Free cholesterol-E Test, respectively (Wako Pure Chemicals, Osaka, Japan).

Histological analysis

Oil red O staining was performed on sections of unfixed, freshly frozen kidney samples (3 μm in thickness). For semi-quantification of lipid deposition, images of each specimen stained with oil red O were taken with an Olympus BX51 microscope and a DP12 digital camera system (Olympus, Tokyo, Japan). Five images taken in the cortical region of each sample were analyzed. The ratio of the areas of lipid deposition to the total tissue region area was calculated using Adobe Photoshop image analysis software (Adobe Systems, San Jose, CA, USA). *In situ* superoxide production was estimated using the oxidative fluorescent dye dihydroethidium (DHE) in unfixed frozen kidney specimens as described earlier.[10] Images were obtained from at least five fields in each section, and signal intensity was presented as a percentage of that in OLETO rats.

Western blot analysis

Western blot analysis was performed as described earlier.[15] Antibodies against total and phosphorylated forms of AMP-activated protein kinase (AMPK) (Cell Signaling Technology, Danvers, MA, USA), sterol regulatory element-binding protein (SREBP)-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and total and phosphorylated forms of acetyl-CoA carboxylase (ACC) (Cell Signaling Technology) were used at a dilution of 1/1000.

Real time RT-PCR

The mRNA expression of lipid metabolism-related genes was analyzed by real-time quantitative PCR performed using a LightCycler together with Hybri-Probe technology (Roche Diagnostics, Basel, Switzerland). The expression of target genes was normalized to the mRNA expression of the endogenous control, glyceraldehyde-3-phosphate dehydrogenase. The target genes were SREBP-1c, fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A reductase, peroxisome proliferator-activated receptor (PPAR)-γ, PPAR-α, PPAR-γ coactivator (PGC)-1α, CD36, carnitine palmitoyltransferase (CPT)-1 and uncoupling protein (UCP)-2. The forward and reverse primers used were described earlier.[16]

Statistical analysis

Data are expressed as mean ± s.e.m. ANOVA and Kruskal–Wallis analyses followed by a *post hoc* multiple comparison test were performed using the statistical analysis software Dr. SPSS II (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was taken to be statistically significant.

RESULTS

Characteristics of the experimental animals

Body weight, blood pressure, heart rate and blood levels of lipids and glucose in each group have been described elsewhere.[17] Blood pressure was slightly but significantly higher in the untreated OLETF rats (149 ± 4 mm Hg, n=11, P=0.012) than in LETO rats (136 ± 2 mm Hg, n=11), and both losartan (135 ± 3 mm Hg, n=6) and benidipine (138 ± 3 mm Hg, n=11) reduced blood pressure in OLETF rats to a level comparable to that in LETO rats. Treatment of OLETF rats with either antihypertensive drug had no significant effect on circulating levels of triglyceride and glucose, which were higher in OLETF than in LETO rats. Compared with LETO rats, kidney weight was greater in the untreated OLETF rats and the OLETF rats treated with either losartan or benidipine, but no significant difference was observed in creatinine clearance among the groups examined (Figure 1). Urinary protein excretion was greater in OLETF rats than in LETO rats, and both losartan and benidipine reduced proteinuria to a similar extent.

Accumulation of lipids in the kidney

The content of TG in the kidney was significantly greater in untreated OLETF rats than in LETO rats, and both losartan and benidipine treatment reduced renal TG content in the OLETF rats (Figure 2a). The content of TC in the kidney was not significantly different between LETO and untreated OLETF rats; however, both antihyper-

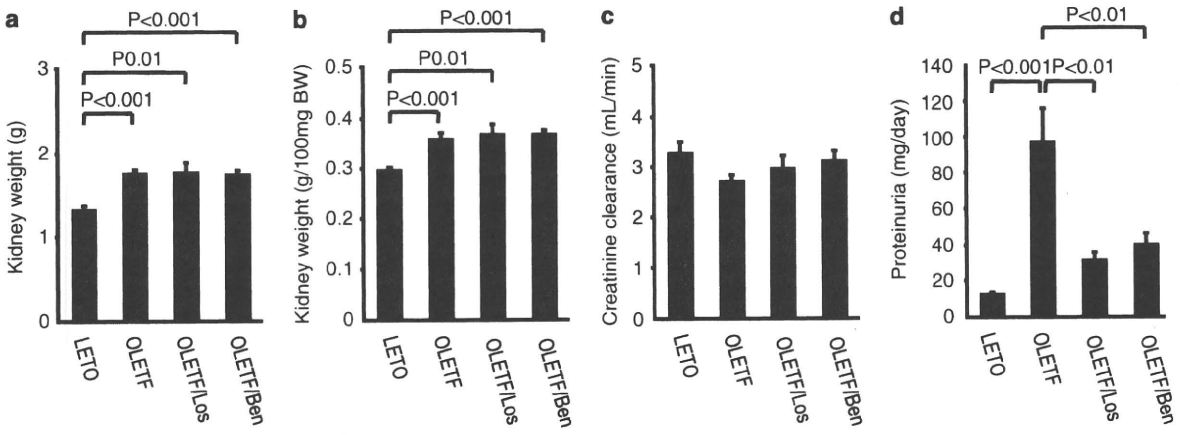

Figure 1 Kidney weight, creatinine clearance and proteinuria in LETO rats and untreated and antihypertensive drug-treated OLETF rats. Absolute values of kidney weight (**a**) and kidney weight expressed per 100 g body weight (**b**). Creatinine clearance (**c**) and daily excretion of urinary protein (**d**). Summary of data from four to six rats in each group.

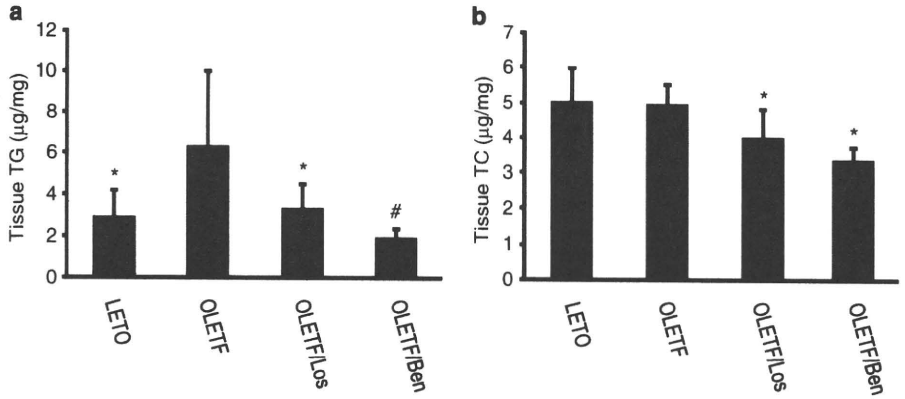

Figure 2 Tissue lipid content in the kidneys of LETO rats and untreated and antihypertensive drug-treated OLETF rats. Content of triglycerides (TG) (**a**) and total cholesterol (TC) (**b**) in the kidneys of LETO (n=4), OLETF (n=8), OLETF/Los (n=8) and OLETF/Ben (n=8) groups is shown.

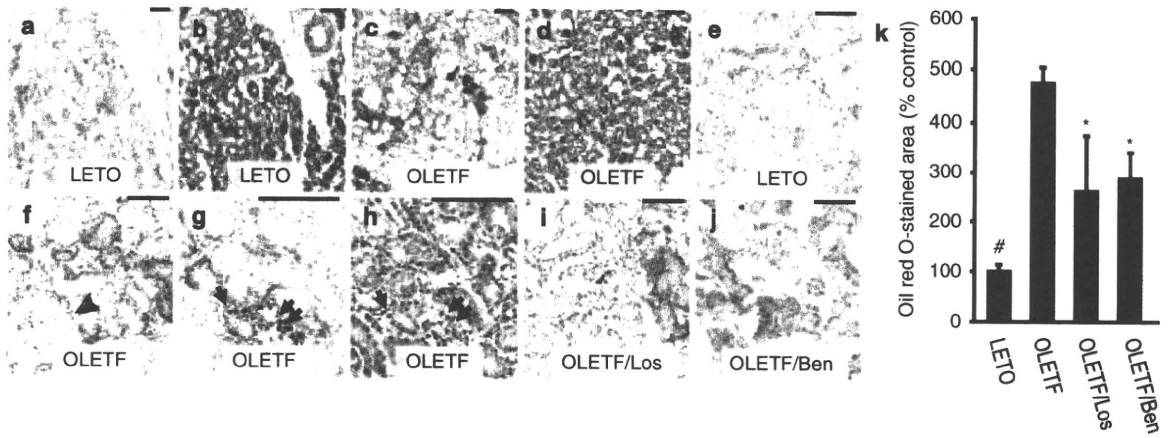

Figure 3 Accumulation of lipids in the kidney. (**a**, **b**, **e**) Kidney sections from LETO rats. (**c**, **d**, **f–h**) Kidney sections from untreated OLETF rats. (**i**, **j**) Kidney sections from OLETF rats treated with losartan (**i**) and benidipine (**j**). (**a**, **c**, **e–g**, **i**, **j**) Oil red O staining. (**b**, **d**, **h**) Hematoxylin and eosin staining; (**a**, **b**), (**c**, **d**) and (**g**, **h**) are serial sections. Lipid droplets were observed in the tubular (**c**, **g**), but not glomerular (**f**, arrowhead), regions of kidney from untreated OLETF rats. In some hematoxylin and eosin-stained specimens, lipid droplets could be identified by unstained small vesicles (**g**, **h**, arrows). The extent of lipid accumulation in the tubular cells was diminished by treatment of OLETF rats with either losartan (**i**) or benidipine (**j**). Scale bars indicate 100 μm. (**k**) Semi-quantification of the oil red O-stained area. Summary of data from five to seven experiments in each group. Kruskal–Wallis analyses followed by a *post hoc* multiple comparison test were performed. $^{*}P<0.05$ and $^{\#}P<0.01$ versus untreated OLETF rats.

tensive drugs reduced renal TC content in OLETF rats (Figure 2b). Histological analysis showed that only a trace amount of oil red O-positive lipid droplets was present in the kidneys of LETO rats (Figure 3). By contrast, increased lipid droplets were observed in the tubular and interstitial regions of untreated OLETF rats. Some lipid droplets appeared as small cavities on hematoxylin and eosin-stained frozen specimens (Figures 3g and h, arrows). Areas of oil red O-positive deposits were significantly reduced by the treatment of OLEFT rats with losartan or benidipine (Figures 3i–k). The correlation coefficients between tissue TG content and the extent of proteinuria and between the oil red O stained area and the extent of proteinuria were 0.40 ($P<0.05$, n=33) and 0.65 ($P<0.001$, n=36), respectively.

Localization of superoxide

Fluorescent signals on DHE staining were greater in untreated OLETF rats than in LETO rats and were reduced by treatment with either losartan or benidipine (Figure 4). In untreated OLETF rat kidney, DHE signals were found to be increased in tubular epithelial (Figures 4e–j, arrowheads) and vascular wall cells (Figures 4e–g, arrows), the former of which contained lipid droplets.

Regulation of genes related to lipid metabolism

The expression of mature SREBP-1 protein did not significantly differ among the four groups examined (Figure 5). Compared with LETO rats, expression of both total and phosphorylated forms of AMPK α was increased in OLETF rats treated with either losartan or benidipine, although it was not increased in untreated OLETF rats. The expression of total ACC protein was unaffected by losartan or benidipine in OLETF rats; however, treatment with either antihypertensive drug significantly increased the amount of the phosphorylated form of ACC.

Among the genes tested, mRNA expression of SREBP-1c, FAS, 3-hydroxy-3-methylglutaryl coenzyme A reductase, PPAR-γ, LDL-r and CD36 did not significantly differ between untreated OLETF and

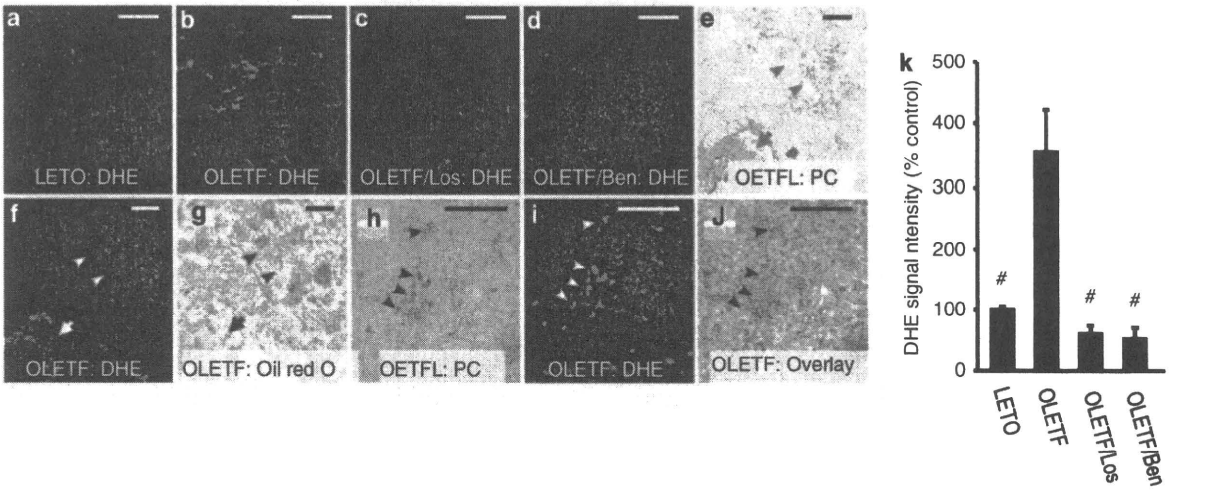

Figure 4 Localization of lipid droplets and superoxide in kidney sections. (**a**) Kidney sections from LETO rats. (**b**, **e**–**j**) Kidney sections from untreated OLETF rats. (**c**, **d**) Kidney sections from OLETF rats treated with losartan (**c**) and benidipine (**d**). (**a**–**d**, **f**, **i**) Dihydroethidium (DHE) staining. (**e**, **h**) Phase contrast (PC) microscopic images. (**j**) PC microscopic image overlaid with DHE stained images. (**g**) Oil red O staining; (**e**, **f**) and **h**–**j**) are the same section; (**e**–**g**) are serial sections. DHE signals were more intense in OLETF kidneys (**b**) than in LETO kidneys (**a**), and both losartan (**c**) and benidipine (**d**) reduced DHE signal intensity. Granular droplets could be observed in the tubular regions, presumably lipid droplets, by PC imaging (**e**, arrowheads) of the unstained specimens, and DHE-stained superoxide was increased in these regions and in vascular wall cells (arrow, **e**, **f**). Oil red O staining (**h**) of the serial specimen confirmed that granular materials were lipid droplets. Higher magnification imaging demonstrated that DHE signals were increased in cells with granular droplets. Scale bars indicate 100 μm. (**k**) Semi-quantification of the DHE signals. Summary of data from five to seven experiments in each group. Kruskal–Wallis analyses followed by a *post hoc* multiple comparison test were performed. $^{\#}P<0.01$ versus untreated OLETF rats.

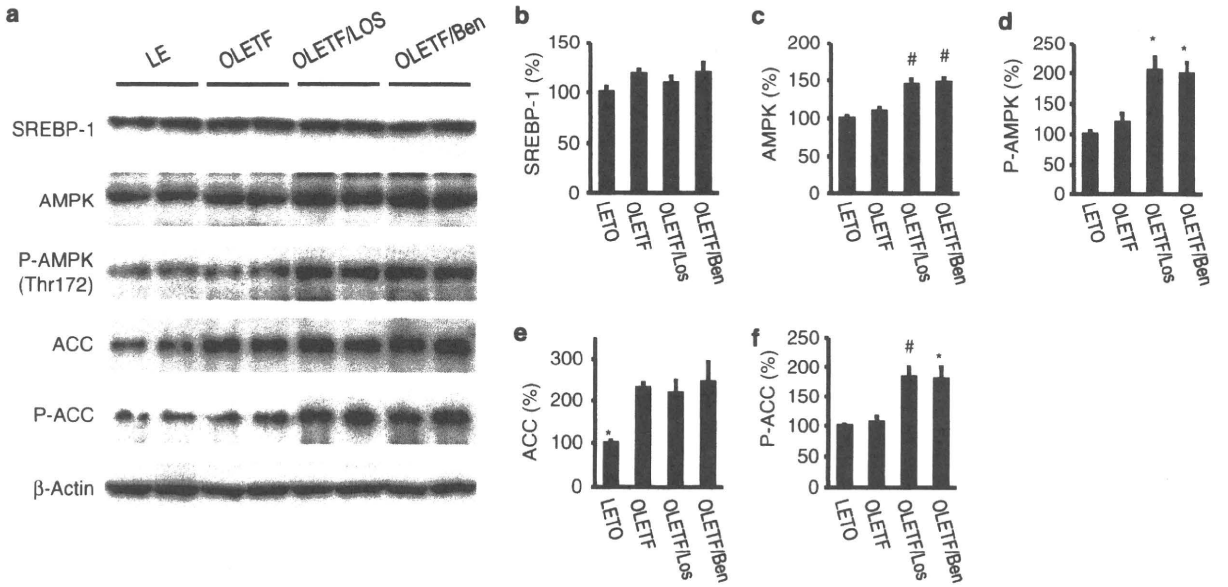

Figure 5 Western blot analysis. Western blot of sterol regulatory element-binding protein-1 (SREPB-1), AMP-activated protein kinase α (AMPK), the phosphorylated (activated) form of AMPK α (P-AMPK), acetyl-CoA carboxylase (ACC) and phosphorylated ACC. (**a**–**f**) Summary of data from four to six experiments in each group. $^{*}P<0.01$ and $^{\#}P<0.05$ versus untreated OLETF rats by Dunnett's *post hoc* analysis.

LETO rats (Figure 6). By contrast, mRNA expression of PPAR-α and UCP-2 was greater, whereas that of PGC-1α was lower, in untreated, losartan-treated and benidipine-treated OLETF rats than in LETO rats. Although the mRNA expression of CPT-1 did not differ between untreated OLETF and LETO rats, treatment of OLETF rats with either losartan or benidipine significantly increased CPT-1 expression.

DISCUSSION

In this study, we found that TG and TC contents were increased in the kidneys of untreated OLETF rats compared with LETO rats. In the kidneys of OLETF rats, oil red O-positive lipid droplets were observed mainly in tubular epithelial cells, in which increased superoxide was present. Treatment of OLETF rats with either losartan or benidipine,

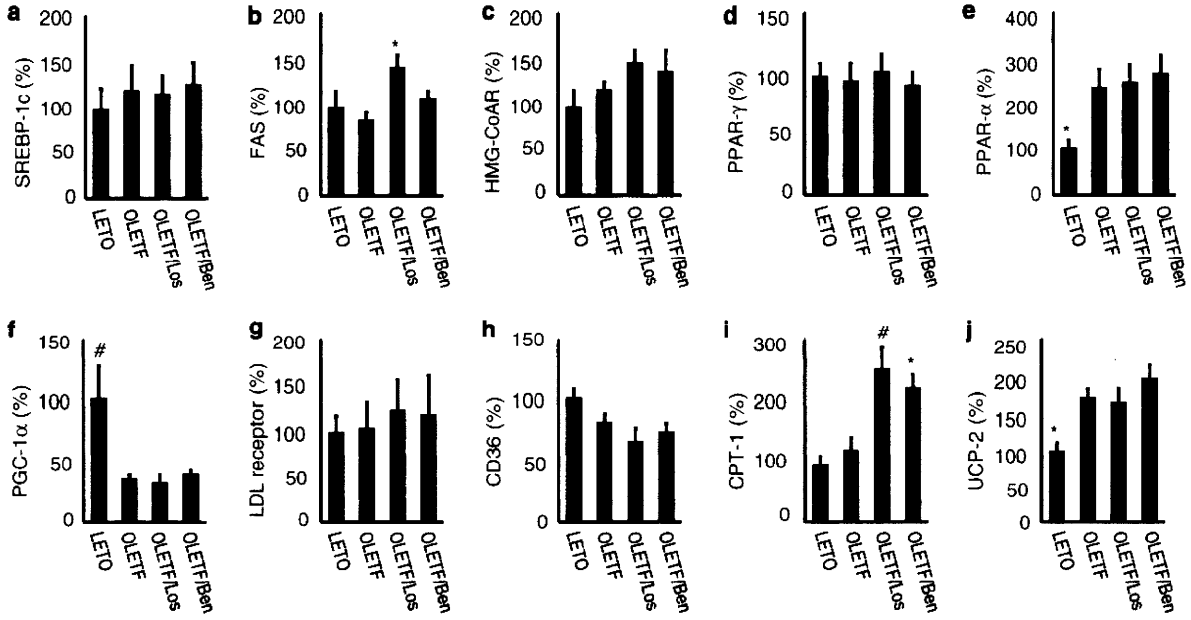

Figure 6 mRNA expression and regulation of lipid metabolism-related genes. (**a**) Sterol regulatory element-binding protein-1c (SREBP-1c). (**b**) Fatty acid synthase (FAS). (**c**) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR). (**d**) Peroxisome proliferator-activated receptor (PPAR)-γ. (**e**) PPAR-α. (**f**) PPAR-γ coactivator (PGC)-1α. (**g**) LDL receptor. (**h**) CD36. (**i**) Carnitine palmitoyltransferase (CPT)-1. (**j**) Uncoupling protein (UCP)-2. Summary of data from six to ten experiments in each group $^{*}P<0.01$ and $^{\#}P<0.05$ versus untreated OLETF rats by Dunnett's *post hoc* analysis.

both of which lowered blood pressure to a similar extent, reduced tissue TG content, oil red O-positive deposits, superoxide signals and urinary protein excretion in the kidneys to a similar extent.

The mechanisms underlying lipid accumulation in the kidney in various animal models and in humans have not been fully elucidated; however, the presumed mechanisms include the regulation of genes related to the uptake, biosynthesis, catabolism and efflux of lipids[1,3,4,10,18] and the uptake of fatty acids carried on filtered albumin by the renal tubules.[7,9] What is the possible mechanism underlying the reduction in the renal lipid content of OLETF rats caused by both losartan and benidipine? We reported earlier that long-term administration of angiotensin II upregulates the expression of SREBP-1 and FAS and increases the renal content of lipids, which was suppressed by losartan. This effect of losartan may, at least in part, be independent of its depressor effect.[19] Therefore, it is possible that benidipine may also reduce renal lipid content through a mechanism independent of its depressor effect. To our knowledge, however, no earlier studies have shown that a calcium channel blocker can regulate the expression of certain lipogenic gene and/or reduce tissue lipid content. Toblli *et al.*[20] reported that renin angiotensin system inhibition, but not calcium channel blockade, suppressed lipid deposition in the heart[20] and liver[21] in obese Zucker rats.

In this study, the expression of SREBP-1 and FAS was not upregulated in the kidneys of OLETF rats compared with LETO rats, and neither losartan nor benidipine reduced the expression of these genes in the kidneys of OLETF rats. Therefore, it can be stated that the mechanism by which lipid content increased in the kidneys of OLETF rats may be, at least in part, different from that in angiotensin II-infused rats. This could be the reason why losartan was not more effective than benidipine in terms of reducing lipid content in the kidney.

As enhanced urinary albumin excretion may lead to the subsequent tubular absorption of lipid-bound albumin,[7,9] another possibility is that both losartan and benidipine reduced renal lipid content by their anti-proteinuric effect. Several studies have reported that benidipine may have a greater anti-proteinuric effect than other CCBs such as amlodipine,[22] and the renoprotective effect of benidipine may be, in part, mediated by the preservation of an essential cofactor of nitric oxide synthase, (6R)-5,6,7,8–tetrahydrobiopterin.[14] As both losartan and benidipine reduced the extent of proteinuria, both drugs might reduce the transport of the albumin-bound fatty acid to tubular cells. The close relationship between the extent of lipid deposition and proteinuria observed in this study may support this notion. Furthermore, the reduction of blood pressure *per se* might have a role in the modulation of lipid content in the kidney.

In this study, tubular cells that contained lipid droplets were positive for DHE signals (Figure 4). A spatial relationship between lipid droplets and superoxide was also observed in the kidneys and heart of angiotensin II-induced hypertensive animals,[10,16] indicating that these two phenomena have a relationship[23] under conditions of hypertension and metabolic syndrome, although a causal and resultant relationship has not yet been determined. Taking these observations into account, future studies should examine whether other antihypertensive drugs such as calcium channel blockers of other subclasses, anti-oxidative agents[24] and other drugs that may have an anti-proteinuric effect are effective in reducing lipid content in the kidneys of OLETF rats.

In this study, we also found several differences between OLETF and LETO rats in terms of the expression of lipid regulatory genes. The expression of PPAR-α and UCP-2 (mRNA) and ACC (protein) was higher, whereas that of PGC-1α (mRNA) was lower, in untreated OLETF rats than in LETO rats. Several earlier studies have shown

altered expression of these genes under conditions of diabetes or metabolic syndrome in the kidney or other organs. Proctor *et al.* reported that PPAR-α expression is decreased in the kidneys of diabetic animals compared to their non-diabetic counterparts, which results in decreased fatty acid oxidation.[2] In addition, the expression of UCP-2 was found to be increased in diabetic kidneys.[25] The mRNA expression of UCP-2 was also found to be upregulated in the liver, skeletal muscles, heart and aorta in OLETF rats compared to LETO rats.[26,27] Downregulation of PGC-1α mRNA expression in skeletal muscles was also reported in diabetes,[28] which might have a role in reducing mitochondrial function, leading to muscular lipotoxicity.[29]

Although regulation of the expression of these genes may have played a role in the increased lipid accumulation in OLETF rat kidneys, treatment with either losartan or benidipine did not significantly alter the mRNA expression of PPAR-α, PGC-1α or UCP-2 in OLETF rats. Compared with untreated OLETF rats, OLETF rats treated with either losartan or benidipine showed increased phosphorylation of AMPK (activated form) and ACC (inactivated form) and increased expression of CPT-1 mRNA, which may result in increased β-oxidation.[11] Therefore, it is possible that changes in the expression of these genes may have a role in the anti-steatotic effects of losartan and benidipine.

In summary, lipid content in the kidney was increased in untreated OLETF rats compared with LETO rats. Oil red O-stainable lipid droplets were primarily found in tubular epithelial cells, which also showed increased superoxide production. Treatment of OLETF rats with either losartan or benidipine, both of which suppressed proteinuria, reduced tissue TG content and modulated the expression of several lipid regulatory genes such as the total and phosphorylated forms of AMPK and CPT-1. These data collectively suggest that losartan and benidipine are both effective in suppressing proteinuria and normalizing lipid homeostasis in the kidneys of a rat model of metabolic syndrome. The underlying mechanisms by which these antihypertensive agents reduced lipid content in the kidney should be investigated in future studies.

ACKNOWLEDGEMENTS

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Cell Metabolism

Impaired Insulin Signaling in Endothelial Cells Reduces Insulin-Induced Glucose Uptake by Skeletal Muscle

Tetsuya Kubota,[1,3,5,18] Naoto Kubota,[1,3,6,18,*] Hiroki Kumagai,[1,18] Shinichi Yamaguchi,[1] Hideki Kozono,[1] Takehiro Takahashi,[1] Mariko Inoue,[1,3] Shinsuke Itoh,[1] Iseki Takamoto,[1,6] Takayoshi Sasako,[1] Katsuyoshi Kumagai,[1,6] Tomoko Kawai,[1,3] Shinji Hashimoto,[1] Tsuneo Kobayashi,[7] Maki Sato,[8] Kumpei Tokuyama,[8] Satoshi Nishimura,[2] Masaki Tsunoda,[9] Tomohiro Ide,[9] Koji Murakami,[9] Tomomi Yamazaki,[4] Osamu Ezaki,[4] Koichi Kawamura,[10] Hirotake Masuda,[10] Masao Moroi,[5] Kaoru Sugi,[5] Yuichi Oike,[11] Hiroaki Shimokawa,[12] Nobuyuki Yanagihara,[13] Masato Tsutsui,[14] Yasuo Terauchi,[15] Kazuyuki Tobe,[16] Ryozo Nagai,[2,6] Katsuo Kamata,[7] Kenji Inoue,[17] Tatsuhiko Kodama,[6,17] Kohjiro Ueki,[1,6] and Takashi Kadowaki[1,3,6,*]

[1]Department of Diabetes and Metabolic Diseases

[2]Department of Cardiovascular Diseases

Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan

[3]Clinical Nutrition Program

[4]Nutritional Science Program

National Institute of Health and Nutrition, Tokyo 162-8636, Japan

[5]Division of Cardiovascular Medicine, Toho University Ohashi Medical Center, Tokyo 113-8655, Japan

[6]Translational Systems Biology and Medicine Initiative, University of Tokyo, Tokyo 153-8515, Japan

[7]Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Tokyo 142-8501, Japan

[8]Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba 305-8577, Japan

[9]Bioscience Division 1, Metabolic Discovery Research Laboratories, Kyorin Pharmaceutical Co. Ltd., Shimotsuga 329-0014, Japan

[10]Second Department of Pathology, Akita University School of Medicine, Akita 010-8543, Japan

[11]Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan

[12]Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

[13]Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807-8555, Japan

[14]Department of Pharmacology, Faculty of Medicine, University of the Ryukyu, Okinawa 903-0215, Japan

[15]Department of Diabetes and Endocrinology, Yokohama City University, School of Medicine, Kanagawa 236-0004, Japan

[16]First Department of Internal Medicine, Faculty of Medicine, University of Toyama, Toyama, 930-0194, Japan

[17]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan

[18]These authors contributed equally to this work

*Correspondence: kadowaki-3im@h.u-tokyo.ac.jp (T.K.), nkubota-tky@umin.ac.jp (N.K.)

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SUMMARY

In obese patients with type 2 diabetes, insulin delivery to and insulin-dependent glucose uptake by skeletal muscle are delayed and impaired. The mechanisms underlying the delay and impairment are unclear. We demonstrate that impaired insulin signaling in endothelial cells, due to reduced Irs2 expression and insulin-induced eNOS phosphorylation, causes attenuation of insulin-induced capillary recruitment and insulin delivery, which in turn reduces glucose uptake by skeletal muscle. Moreover, restoration of insulin-induced eNOS phosphorylation in endothelial cells completely reverses the reduction in capillary recruitment and insulin delivery in tissue-specific knockout mice lacking Irs2 in endothelial cells and fed a high-fat diet. As a result, glucose uptake by skeletal muscle is restored in these mice. Taken together, our results show that insulin signaling in endothelial cells plays a pivotal role in the regulation of glucose uptake by skeletal muscle. Furthermore, improving endothelial insulin signaling may serve as a therapeutic strategy for ameliorating skeletal muscle insulin resistance.

INTRODUCTION

Skeletal muscle is one of the major target organs of insulin actions and plays an essential role in insulin-induced glucose uptake (DeFronzo et al., 1979; Bergman, 1989). Insulin, secreted by the pancreatic β cells, is delivered into the capillaries and crosses the endothelial barrier to enter the interstitial spaces (Vincent et al., 2005). It then binds to the insulin receptors on the skeletal muscle cell surface, activating intracellular pathways in the skeletal muscle (White and Kahn, 1994; Petersen et al., 2004; Long and Zierath, 2008). Recent evidence indicates that insulin delivery to the skeletal muscle interstitium is the rate-limiting step in insulin-stimulated glucose uptake by the skeletal muscle, and is much slower in obese insulin-resistant subjects than in normal subjects (Sherwin et al., 1974; DeFronzo et al., 1979; Yang et al., 1989; Jansson et al., 1993; Miles et al., 1995; Sjostrand et al., 2002; Barrett et al., 2009). Andres and colleagues have shown that insulin is distributed more slowly

to the compartment corresponding to the skeletal muscle than to other compartments, and the time course of insulin equilibration with this pool closely paralleled the glucose infusion rate (GIR) in their compartmental model (Sherwin et al., 1974; DeFronzo et al., 1979). Moreover, the dynamics of insulin concentrations in the skeletal muscle lymphatics, which is derived from the interstitial fluid in the skeletal muscle, is actually slow, and is significantly correlated with the peripheral glucose uptake after insulin infusion (Yang et al., 1989). In obese subjects, the appearance of insulin in the interstitial fluid of the skeletal muscle and onset of insulin action after insulin infusion were found to be significantly delayed in comparison with the results in control subjects (Sjostrand et al., 2002). Although the slow onset of insulin action on glucose disposal in vivo could be secondary to a slow response by the cellular machinery within the myocytes, which are the cells responsible for glucose uptake in the skeletal muscle, insulin-induced glucose uptake by myocytes in vitro appears to be fully activated within 2–5 min, much earlier than that in vivo (Karnieli et al., 1981). Moreover, Bergman et al. demonstrated that injection of insulin directly into the interstitium of the skeletal muscle is followed by a prompt increase in the glucose uptake (Chiu et al., 2008). Based on these findings, insulin delivery may determine the in vivo time course of insulin-induced glucose uptake, which may be much slower than the insulin-induced glucose uptake in vitro.

Two discrete steps have been reported to increase insulin delivery to the skeletal muscle: an increase in the available capillary surface area (capillary recruitment), and an increase in the transendothelial transport of insulin. Clark and colleagues measured capillary recruitment following insulin infusion and found that it was associated with increased glucose uptake by the skeletal muscle (Rattigan et al., 1997; Vincent et al., 2004). The insulin-induced capillary recruitment and glucose uptake were reported to be significantly impaired in obese subjects and diabetic models (Wallis et al., 2002; Keske et al., 2009). In addition to the vasoactive actions of insulin on the capillaries, insulin may promote its own movement across the endothelial barrier. Recent in vivo evidence suggests that transendothelial transport of insulin in the skeletal muscle increased along with elevation of the plasma insulin levels, and that these levels were diminished in the presence of high-fat (HF) diet-induced insulin resistance (Hamilton-Wessler et al., 2002; Ellmerer et al., 2006; Wang et al., 2008). It is still unclear whether capillary recruitment and transendothelial transport of insulin may be related or may function independently (Clark, 2008).

We hypothesized that an insulin signaling defect in the endothelial cells impairs insulin-induced capillary recruitment and insulin delivery in the skeletal muscle in obesity. In HF diet-fed obese mice, the expression levels of insulin receptor substrate (Irs)2, which is the major Irs isoform expressed in the endothelial cells (Kubota et al., 2003), were markedly reduced, and insulin-induced eNOS phosphorylation, capillary recruitment, and insulin delivery were markedly impaired. Consistent with these results, mice with Irs2 deletion in the endothelial cells (ETIrs2KO mice) also exhibited a reduction of insulin-induced eNOS phosphorylation, capillary recruitment, and insulin delivery. Moreover, restoration of the insulin-induced eNOS phosphorylation in the endothelial cells completely reversed the reduction of the capillary recruitment and insulin delivery in both the HF diet-fed and ETIrs2KO mice. We conclude that a genetically and/or environmentally induced insulin signaling defect in the endothelial cells causes skeletal muscle insulin resistance as a consequence of the impaired insulin-induced capillary recruitment and insulin delivery.

RESULTS

Insulin Signaling Was Significantly Impaired in the Endothelial Cells of the ob/ob and HF Diet-Fed Mice

To investigate insulin signaling in the endothelial cells in mouse models of obesity, we first measured the expression levels of the insulin receptor (Ir) and Ir substrate (Irs) in mice with genetically (ob/ob mice) or environmentally induced (8-week HF diet-fed mice) obesity. Although there were no significant differences in the mRNA or protein expression levels of Ir and endothelin (ET)-1 (see Figures S1A and S1B available online), the mRNA and protein expression levels of Irs1 were reduced by approximately 50% and those of Irs2, the major Irs isoform expressed on the endothelial cells (Kubota et al., 2003), by approximately 80% in the endothelial cells of both the mouse models of obesity (Figures 1A and 1B). To elucidate the mechanisms by which the expressions of Irs1 and Irs2 in the endothelial cells were downregulated, we investigated the effects of FFA on the expressions of Irs1 and Irs2 following continuous administration of intralipid for 24 hr in C57Bl/6 mice. Although a 7-fold increase of the plasma FFA levels was observed after the intralipid treatment, the expression levels of Irs1 and Irs2 measured before and after the intralipid treatment were not significantly different (Figure S1C). We next investigated whether the Irs1 and Irs2 gene expressions in the endothelial cells might be altered by hyperinsulinemia induced by continuous administration of insulin for 24 hr using a miniosmotic pump in C57Bl/6 mice. After continuous administration of insulin for 24 hr, a 7-fold increase of the plasma insulin levels was noted (Figure 1C). In regard to the expression levels of the Irs1 and Irs2, on the other hand, while no change in the expression of endothelial Irs1 was observed, the expression of endothelial Irs2 was significantly decreased (Figure 1C). These data suggest that hyperinsulinemia may be one of the mechanisms underlying the decrease in the expression of endothelial Irs2 observed in the ob/ob and HF diet-fed mice, although the reason for the downregulated Irs1 expression in these mice still remains unexplained. The insulin signaling cascade activates Akt, which in turn phosphorylates and activates eNOS in the endothelial cells (Muniyappa and Quon, 2007). Consistent with the results for the expression levels of Irs1 and Irs2, insulin-stimulated phosphorylations of Akt and eNOS were decreased by 70%–80% in the endothelial cells of the ob/ob and HF diet-fed mice (Figure 1D), indicating that insulin signaling was impaired in the endothelial cells of these obesity models. We then investigated whether impaired insulin signaling in the endothelial cells might be involved in the HF diet-induced, obesity-linked impairment of glucose uptake by the skeletal muscle. Although an increase of the capillary blood volume at 10 min after the insulin infusion in the hyperinsulinemic-euglycemic clamp study was observed in the normal chow-fed mice, no such increase was observed in the HF diet-fed mice (Figure 1E). On the other hand, no increase of

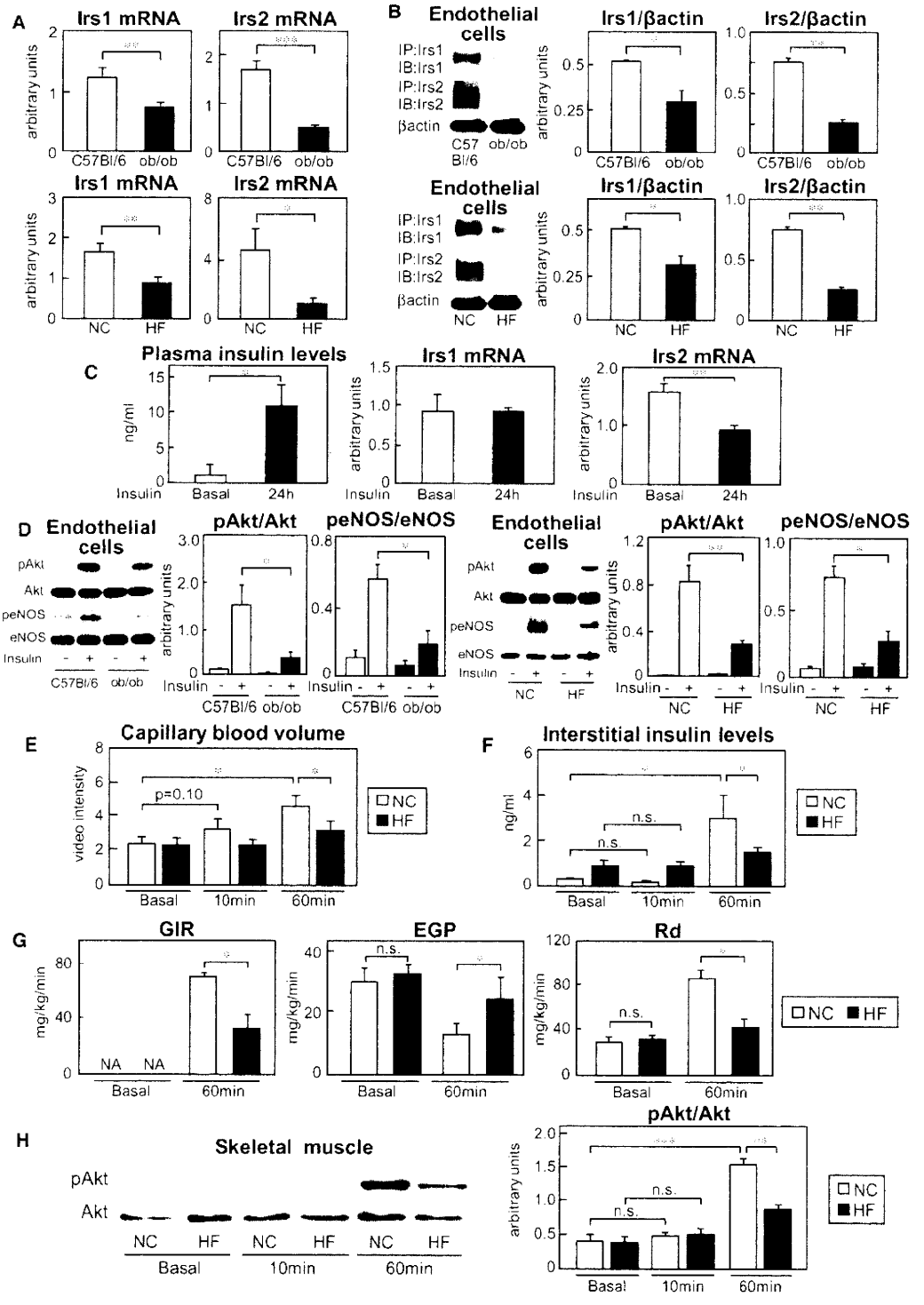


Figure 1. In the HF Diet-Fed Obese Mice, which Show Impaired Glucose Uptake by the Skeletal Muscle, Decreased Insulin-Induced Phosphorylation of eNOS in the Endothelial Cells, along with Downregulation, Mainly of Irs2, Was Associated with Attenuation of the Insulin-Induced Increase of the Capillary Blood Volume and of the Increase of the Interstitial Concentrations of Insulin

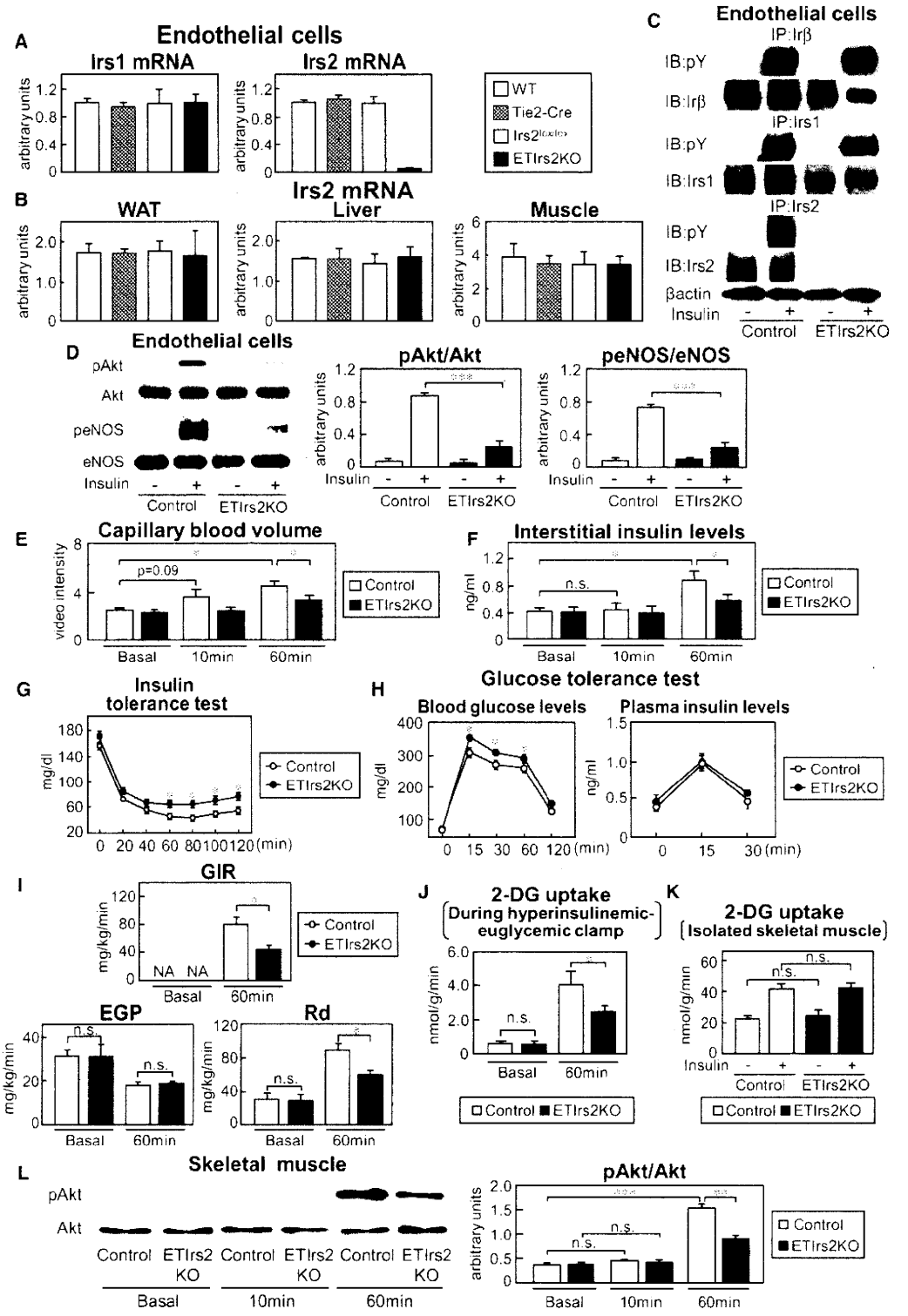
(A and B) Expression levels of Irs1 or Irs2 mRNA and protein in the endothelial cells of the ob/ob and HF diet-fed mice (n = 3–11).

the interstitial concentrations of insulin was observed at 10 min after the insulin infusion in either the normal chow-fed or the HF diet-fed mice (Figure 1F). These data suggest that capillary recruitment starts to increase well before the increase of the interstitial insulin concentrations, consistent with the results of previous studies (Miles et al., 1995; Vincent et al., 2005). Insulin-induced increases of the capillary blood volume and interstitial concentrations of insulin were significantly impaired at 60 and 120 min after the insulin infusion in the hyperinsulinemic-euglycemic clamp study (Figures 1E and 1F; Movies S1A and S1B, and data not shown), even when the plasma insulin levels were adjusted to be the same in both the normal chow-fed and HF diet-fed mice (Figure S1D and data not shown). A significant decrease of the GIR and glucose disappearance rate (Rd), as also a significant increase of the endogenous glucose production (EGP), were found in the animals at 60 and 120 min after the insulin infusion in the hyperinsulinemic-euglycemic clamp study (Figure 1G and data not shown). Consistent with these results, the phosphorylation levels of Akt in the skeletal muscle in the normal chow-fed mice began to increase by 30 min and reached its peak about 60 min after the insulin infusion, whereas this increase of the Akt phosphorylation level from 30 min onward after the insulin infusion in the hyperinsulinemic-euglycemic clamp study was significantly impaired in the HF diet-fed mice (Figure 1H; Figure S1E and data not shown). These data suggest that the decreased phosphorylation level of eNOS induced by insulin in the endothelial cells associated with downregulation, mainly of Irs2, appears to be involved, along with the impaired insulin-induced capillary recruitment and increase of the interstitial concentrations of insulin, in the impaired glucose uptake by the skeletal muscle in the HF diet-fed obese mice.

ETIrs2KO Mice Exhibited Impaired Insulin-Induced Glucose Uptake by the Skeletal Muscle

In order to elucidate the causal relationship between insulin signaling in the endothelial cells and the glucose uptake by the skeletal muscle, we generated ETIrs2KO mice. The endothelial Irs2 mRNA levels were reduced by approximately 95% in the ETIrs2KO mice, whereas the Irs2 expression levels in the white adipose tissue (WAT), liver, and skeletal muscle remained unchanged (Figures 2A and 2B). The Irs2 protein expression and insulin-stimulated tyrosine phosphorylation of Irs2 were almost completely abrogated in the endothelial cells of the ETIrs2KO mice (Figure 2C). There were no significant differences in the mRNA and protein levels of eNOS or ET-1 between the control and ETIrs2KO mice (Figures S2A and S2B). The basal blood glucose and plasma insulin levels were indistinguishable between the control and ETIrs2KO mice (Figure S2C). Insulin-stimulated phosphorylations of Akt and eNOS were found to be significantly reduced in the endothelial cells of the ETIrs2KO mice (Figure 2D). Although an increase of the capillary blood volume was observed in the control mice by 10 min after the insulin infusion in the hyperinsulinemic-euglycemic clamp study, no such increase was observed in the ETIrs2KO mice (Figure 2E). In contrast, no increase in the interstitial concentrations of insulin was observed at 10 min after the insulin infusion in either the control or the ETIrs2KO mice (Figure 2F). The insulin-induced increases of the capillary blood volume and interstitial concentrations of insulin were significantly impaired at 60 and 120 min after insulin infusion in the hyperinsulinemic-euglycemic clamp study (Figures 2E and 2F and data not shown), even when the plasma insulin levels were adjusted to be the same in both the control and ETIrs2KO mice (Figure S2D and data not shown). Furthermore, the whole-body insulin sensitivity and glucose tolerance were also significantly impaired in the ETIrs2KO mice (Figures 2G and 2H). The GIR and Rd at 60 and 120 min after insulin infusion in the hyperinsulinemic-euglycemic clamp study were significantly reduced in the ETIrs2KO mice (Figure 2I and data not shown). The decreased Rd observed in the ETIrs2KO mice during the hyperinsulinemic-euglycemic clamp, however, indicates impairment of glucose uptake by mainly the skeletal muscle, but also by other tissues containing nonfenestrated endothelial cells forming tight junctions. Thus, we also measured the insulin-induced glucose uptake by the skeletal muscle during the hyperinsulinemic-euglycemic clamp study using 2-deoxy-[^{3}H]glucose (2-[^{3}H]DG). Consistent with the results obtained for the Rd, the skeletal muscle glucose uptake was significantly reduced in the ETIrs2KO mice (Figure 2J), suggesting that the glucose uptake through the capillaries, at least, was impaired in the skeletal muscle of the ETIrs2KO mice. In contrast, glucose uptake by the isolated skeletal muscle from the ETIrs2KO mice was not impaired (Figure 2K), indicating that glucose uptake by the skeletal muscle per se was not impaired in the ETIrs2KO mice. Consistent with these results, the phosphorylation level of Akt in the skeletal muscle in the control mice began to increase by 30 min and reached its peak about 60 min after insulin infusion in the hyperinsulinemic-euglycemic clamp study, whereas this increase in the Akt phosphorylation level was significantly impaired in the ETIrs2KO mice (Figure 2L; Figure S2E and data not shown). Moreover, the phosphorylation levels of Irβ, as well as those of Irs1 and Akt, in the skeletal muscle were also significantly decreased in the ETIrs2KO mice at 60 min after insulin infusion into the inferior vena cava (Figure S2F). In contrast, no significant differences in the phosphorylation levels of Irβ, Irs1, Irs2, or Akt in the liver were observed between the control and ETIrs2KO mice (Figure S2G), even though Irs2 was also deleted from the hepatic endothelial cells of the ETIrs2KO mice (Figure S2H). Quantitative analysis revealed no significant difference in the β cell mass between the control and ETIrs2KO mice (Figure S2I). These data suggest that Irs2 deletion in the endothelial cells causes an insulin

(C) Expression levels of Irs1 and Irs2 mRNA after insulin infusion (n = 3–4).
(D) Phosphorylation levels of Akt and eNOS in the ob/ob and HF diet-fed mice (n = 4-7).
(E and F) Capillary blood volume (E) and interstitial insulin levels (F) in the HF diet-fed mice (n = 3–10).
(G) GIR, EGP, and Rd in the HF diet-fed mice after insulin infusion in the hyperinsulinemic-euglycemic clamp study (n = 7–9).
(H) Phosphorylation levels of Akt (ser473) in the skeletal muscle of the HF diet-fed mice after insulin infusion in the hyperinsulinemic-euglycemic clamp study (n = 3–4). "NC" indicates normal chow-fed mice. "NA" indicates not applicable. Where error bars are shown, the results represent the means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.


A
Endothelial cells
Irs1 mRNA
Irs2 mRNA
arbitrary units
WT
Tie2-Cre
Irs2loxP/loxP
ETIrs2KO
B
WAT
Irs2 mRNA
Liver
Muscle
C
Endothelial cells
IP:Irβ
IB:pY
IB:Irβ
IP:Irs1
IB:Irs1
IP:Irs2
IB:Irs2
βactin
Insulin
Control
ETIrs2KO
D
Endothelial cells
pAkt
Akt
peNOS
eNOS
pAkt/Akt
peNOS/eNOS
E
Capillary blood volume
video intensity
p=0.09
Basal
10min
60min
F
Interstitial insulin levels
ng/ml
n.s.
G
Insulin tolerance test
mg/dl
H
Glucose tolerance test
Blood glucose levels
Plasma insulin levels
I
GIR
mg/kg/min
NA
EGP
Rd
J
2-DG uptake
(During hyperinsulinemic-euglycemic clamp)
nmol/g/min
K
2-DG uptake
(Isolated skeletal muscle)
L
Skeletal muscle
pAkt
Akt
Control
ETIrs2 KO

signaling defect in the cells that results in impairment of insulin-induced eNOS phosphorylation, capillary recruitment, and increase of the interstitial concentrations of insulin, consequently impairing the skeletal muscle glucose uptake.

Insulin-Induced Glucose Uptake by the Skeletal Muscle Was Not Impaired in the ETIrs1KO Mice, but Was Significantly Reduced in the ETIrs1/2DKO Mice, as Compared with that in the ETIrs2KO Mice

To investigate the role of Irs1 expressed in the endothelial cells in the regulation of insulin-induced glucose uptake by the skeletal muscle, we generated mice with endothelial-cell-specific Irs1 knockout (ETIrs1KO mice). Although the Irs1 mRNA and protein levels were almost completely abrogated in the endothelial cells of the ETIrs1KO mice (Figures 3A and 3B), no significant differences in the expression levels of Irs2, eNOS, or ET-1 mRNA and protein were observed between the control and ETIrs1KO mice (Figures 3A and 3B). No significant difference in the insulin-stimulated phosphorylation levels of Akt or eNOS was observed between the control and ETIrs1KO mice (Figure 3C). Furthermore, no significant differences in the results of the insulin tolerance test or glucose tolerance test were observed between the control and ETIrs1KO mice (Figures 3D and 3E). Consistent with these results, there were also no significant differences in the results of the hyperinsulinemic-euglycemic clamp study between the control and ETIrs1KO mice (Figure 3F).

We next generated mice with endothelial-cell-specific Irs1/Irs2 double-knockout (ETIrs1/2DKO), in an attempt to elucidate whether insulin signaling in the endothelial cells might be exclusively mediated by Irs1 and Irs2. Although the Irs1 and Irs2 mRNA and protein levels were almost completely abrogated in the endothelial cells of the ETIrs1/2DKO mice (Figures 3G and 3H), no significant differences in the mRNA or protein levels of eNOS and ET-1 were observed between the control and ETIrs1/2DKO mice (Figures 3G and 3H). The insulin-stimulated phosphorylation levels of Akt and eNOS were significantly reduced in the ETIrs1/2DKO mice (Figure 3I). The insulin tolerance test revealed that the glucose-lowering effect of insulin was significantly attenuated in the ETIrs1/2DKO mice (Figure 3J), and the glucose tolerance test revealed significantly elevated plasma glucose levels in the ETIrs1/2DKO mice (Figure 3K). Moreover, the GIR and Rd in the ETIrs1/2DKO mice were significantly reduced as compared with the values not only in the control mice but also in the ETIrs2KO mice (Figure 3L). These data suggest that Irs1 may play a significant role in endothelial cell insulin signaling, which becomes evident especially when Irs2 expression is downregulated, such as in the ETIrs2KO and HF diet-fed mice.

Restoration of Insulin-Induced eNOS Phosphorylation in the Endothelial Cells Restored Glucose Uptake by the Skeletal Muscle in the ETIrs2KO Mice

To determine whether restoration of the insulin-induced eNOS phosphorylation in the endothelial cells might restore the glucose uptake by the skeletal muscle, we administered beraprost sodium (BPS), a stable prostaglandin $(PGI)_2$ analog (Kainoh et al., 1991), to the ETIrs2KO mice; this agent has been reported to increase the expression levels of eNOS mRNA and protein through the cyclic adenosine monophosphate (cAMP)-, protein kinase A-, and cAMP-responsive element-mediated pathways (Niwano et al., 2003). Indeed, this treatment increased the eNOS mRNA and protein expression levels in the endothelial cells (Figures 4A and 4B). BPS treatment restored insulin-induced phosphorylation of eNOS in the BPS-treated ETIrs2KO mice to a level similar to that observed in the saline-treated control mice (Figure 4B), despite the absence of any change in the ratio of phosphorylated eNOS to total eNOS (Figure 4B); also, no change in the insulin-induced phosphorylation of Akt was observed in these mice (Figure S3A). The insulin-induced increase of the capillary blood volume and interstitial concentrations of insulin were restored at 60 and 120 min after insulin infusion in the hyperinsulinemic-euglycemic clamp study (Figures 4C and 4D and data not shown) under comparable plasma insulin concentrations (Figure S3B and data not shown). The restoration of the insulin-induced increase of the capillary blood volume by BPS treatment in the ETIrs2KO mice was completely blocked by administration of the NOS inhibitor, N^G-nitro-$_L$-arginine methyl ester ($_L$-NAME) (Figure 4E), suggesting that the restoration of the insulin-induced capillary recruitment by BPS treatment was eNOS dependent. To rule out the possibility that BPS and $_L$-NAME regulated the skeletal muscle glucose uptake through an eNOS-independent mechanism, we next administered BPS and $_L$-NAME to eNOS-knockout (eNOSKO) mice. There were no significant differences in the insulin-induced increases of the capillary blood volume or interstitial insulin concentrations among the saline-treated, BPS-treated, and $_L$-NAME-treated eNOSKO mice, either at the basal

Figure 2. Irs2 Deletion in the Endothelial Cells Caused an Insulin Signaling Defect Resulting in a Reduction of the Insulin-Induced Increase of the Capillary Blood Volume and Interstitial Concentrations of Insulin and, as a Consequence, Impaired Glucose Uptake by the Skeletal Muscle

(A) Expression levels of Irs1 and Irs2 mRNA in the endothelial cells of the WT, Tie2-Cre, Irs2lox/lox, and ETIrs2KO mice (n = 4–8).
(B) The expression levels of Irs2 in the WAT, liver, and skeletal muscle of the WT, Tie2-Cre, Irs2lox/lox, and ETIrs2KO mice (n = 5–6).
(C) Insulin-stimulated tyrosine phosphorylation levels of Irβ, Irs1, and Irs2 in the endothelial cells of the ETIrs2KO mice.
(D) Phosphorylation levels of Akt and eNOS in the endothelial cells of the ETIrs2KO mice (n = 8–12).
(E and F) Capillary blood volume (E) and interstitial insulin levels (F) in the ETIrs2KO mice (n = 3–10).
(G and H) Insulin tolerance test (G) and glucose tolerance test (H) in the ETIrs2KO mice (n = 8–10).
(I) GIR, EGP, and Rd in the ETIrs2KO mice after insulin infusion in the hyperinsulinemic-euglycemic clamp study (n = 3–9).
(J) Glucose uptake by the skeletal muscle in the ETIrs2KO mice after insulin infusion in the hyperinsulinemic-euglycemic clamp study (n = 3–9).
(K) Glucose uptake by the isolated skeletal muscle in the ETIrs2KO mice (n = 3).
(L) Phosphorylation levels of Akt (ser473) in the skeletal muscle of the ETIrs2KO mice after insulin infusion in the hyperinsulinemic-euglycemic clamp study (n = 3–4). "Control" indicates $Irs2^{lox/lox}$ mice. "NA" indicates not applicable. Where error bars are shown, the results represent the means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

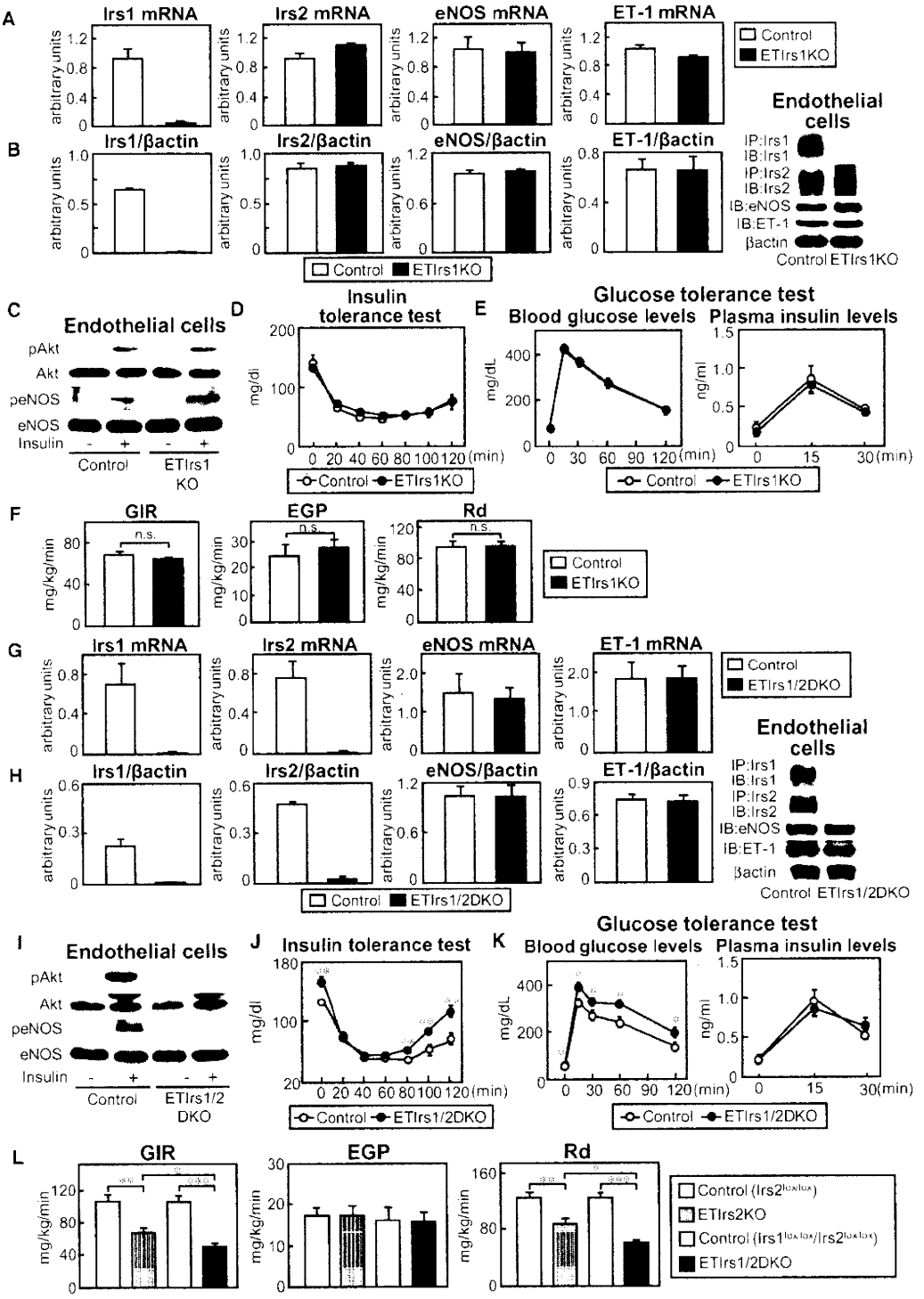


Figure 3. In the ETIrs1/2DKO, but Not ETIrs1KO, Mice, the GIR and Rd Were Significantly Reduced as Compared with the Values Seen Not Only in the Control Mice, But Also in the ETIrs2KO Mice, in Addition to the Impairment of Insulin Signaling in the Endothelial Cells, Whole-Body Insulin Sensitivity, and Glucose Tolerance

(A and B) Expression levels of Irs1, Irs2, eNOS, or ET-1 mRNA and protein in the endothelial cells of the ETIrs1KO mice (n = 3–6).