report, BW did not show any correlation with serum concentrations of lipids except HDL-C or apolipoproteins. Thus, the difference in BW in the subjects studied cannot explain the difference in the present results. Because serum concentrations of lipids and apolipoproteins are affected by many factors such as nutrition, BW and so on, weak associations between BW and these parameters may be obscured by these environmental factors. The finding that the correlation of BW with HDL-C was significant only after adjusting for age, gender and BMI percentile may support our notion. Because BW was not a significant predictor of HDL-C in the present schoolchildren, the relationship between BW and HDL-C seems to have less physiological significance than those with adiponectin and uric acid.

### *Birthweight and insulin resistance*

With respect to the relation between BW and insulin resistance, several recent studies have reported different results.[10,13–20] In reports on infants and children, BW and postnatal catch-up growth have been found to have a significant inverse relationship with insulin secretion and insulin resistance.[13–16] In contrast, Whincup *et al.* reported that BW is not associated with insulin secretion or resistance in a study on schoolchildren.[17] In addition, racial difference has been reported in the relationship between BW and insulin resistance.[18] In the present studies, as in a previous study, BW is not associated with insulin secretion or resistance.[17] Recently, a U-shaped relation between BW and fasting insulin was found in Pima Indian children and adolescents.[19,20] Fasting insulin and HOMA2-IR in the present children showed a similar tendency, but this association was not significant (Table 1). Thus, we have demonstrated that BW is not associated with insulin resistance or sensitivity, at least in Japanese schoolchildren.

### *Study limitations*

Pubertal status can influence the biochemical parameters assessed in the present study. The pubertal stage of the present children was not defined, because most children were aged between 9 and 10 years (presumably prepubertal). However, we cannot exclude the possibility that some children were pubertal. Fortunately, the age distributions in the BW groups were similar and age and gender were adjusted for in the statistical analysis. Thus, the effect of pubertal status was likely to have been similar in the present subjects.

## Conclusions

BW was related to serum concentrations of adiponectin and uric acid and significantly predicted serum concentrations of adiponectin and uric acid. However, WV was a stronger determinant of serum adiponectin and uric acid levels than BW in Japanese schoolchildren. Thus, it may be important to control weight gain and reduce the prevalence of obesity to prevent the development of ACHD in children, especially in children with LBW.

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# Relationship Between Lipid Abnormalities and Insulin Resistance in Japanese School Children

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***Objective***—Dyslipidemia and insulin resistance (IR) are risk factors for coronary heart disease (CHD) in adults. To help prevent the development of CHD, it may be useful to understand the relationship between lipid abnormalities and IR during childhood.

***Methods and Results***—IR was assessed by the homeostasis model approximation index. We studied 1175 Japanese school children (642 boys and 533 girls), aged between 7 and 12 years. Obesity was defined by the body mass index standard deviation score (BMISD) (obese: BMISD ≥2.0). BMISD was most significantly associated with IR in nonobese children ($P$=0.000). Associations of IR with lipid-related parameters were affected by BMISD. After being corrected by BMISD, in nonobese children, log triglycerides (TG), apoB and low-density lipoprotein (LDL) size in boys and log TG, LDL size, and high-density lipoprotein (HDL) cholesterol in girls were still significantly associated with IR ($P$=0.000 to 0.017). In obese children, all parameters except for LDL cholesterol in boys and LDL size in girls were significantly associated with IR ($P$=0.000 to 0.030). Multiple regression analysis showed that log TG and LDL size in nonobese children, log TG in obese boys and LDL size in obese girls were independently associated with IR. Children with IIb and IV hyperlipidemia had significantly higher IR than those with normolipidemia and IIa, even after correcting for BMISD and age.

***Conclusion***—Our results suggest that in addition to controlling body weight, it may be important for school children to characterize lipid phenotypes to prevent progression to CHD and/or type 2 diabetes and to identify subjects who are at high risk for these disorders. (***Arterioscler Thromb Vasc Biol.* 2006;26:2781-2786.**)

**Key Words:** hyperlipidemia ■ insulin resistance ■ obesity ■ school children ■ type 2 diabetes

Dyslipidemia, insulin resistance (IR), and obesity are risk factors for atherosclerotic coronary heart disease (CHD) in adults.[1,2] Pathologically, atherosclerotic changes in coronary arteries originate during childhood, and the extent of atherosclerotic lesions in children and adolescent increases with the number of risk factors.[3,4] In our previous study, we showed the prevalence of dyslipidemia was 1.91% (familial hypercholesterolemia [FHD]: 0.19%, IIa: 0.87%, IIb: 0.26%, IV: 0.20%, low high-density lipoprotein [HDL]: 0.39%) in preschool Japanese children and children with dyslipidemia had more family or genetic background than adults.[5] Okada et al reported that >10% of Japanese school children had hypercholesterolemia (IIa and IIb).[6] Although these studies were performed in different regions, the prevalence of hypercholesterolemia in school children was much higher than that in preschool children. This suggests that the expression of hypercholesterolemia is severely affected by nongenetic factors (environmental factors). Among Japanese school children, the prevalence of type 2 diabetes increased from 0.2 to 7.3 per 100 000 children per year between 1976 and 1995.[7] The increase was attributed to changing dietary patterns and increase rates of obesity among these children.[7] Obese children have a higher prevalence of IR, type 2 diabetes, and dyslipidemia.[8,9] IR is also risk factor for impaired glucose tolerance and type 2 diabetes, even in children.[8] Furthermore, dyslipidemia, IR, and obesity are core phenotypes of the metabolic syndrome.[10–12] Thus, it seems important to clarify the relationship between dyslipidemia, IR, and obesity in children.

The metabolic syndrome is becoming a common disorder even in children, because of the increasing prevalence of obese children.[13–15] To help prevent the future development of CHD or type 2 diabetes, it is reasonable to identify children who are at high risk for these disorders. In the present study, as a first step in detecting a high-risk group, we investigated the relationship between dyslipidemia, IR, and obesity in Japanese school children.

## Methods

### Subjects

The present study was approved by the Review Board of the University of the Ryukyus. Informed consent was obtained from the

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**TABLE 1. Anthropometric and Chemical Characteristics in Non-Obese and Obese Children**

| | Boys | | | Girls | | |
|---|---|---|---|---|---|---|
| | Non-Obese (n=445) | *P* Value | Obese (n=197) | Non-Obese (n=389) | *P* Value | Obese (n=144) |
| Age, y | 9.7±1.6 | (*P*<0.0001) | 10.2±0.6 | 9.8±1.8 | (*P*<0.01) | 10.2±0.8 |
| BMISD | 0.42±1.03 | (*P*<0.0001) | 2.78±0.70 | 0.19±1.02 | (*P*<0.0001) | 2.78±0.77 |
| TC, mg/dL** | 177±28 | (*P*<0.01) | 184±30 | 174±26 | (*P*<0.0001) | 182±31 |
| TG, mg/dL*** | 73±41 | (*P*<0.0001) | 98±70 | 73±40 | (*P*<0.0001) | 101±55 |
| LDL-C, mg/dL** | 100±25 | (*P*<0.001) | 109±26 | 100±23 | (*P*<0.001) | 108±27 |
| HDL-C, mg/dL** | 63±12 | (*P*<0.0001) | 56±11 | 62±11 | (*P*<0.0001) | 54±10 |
| ApoB, mg/dL | 73±17 | (*P*<0.0001) | 81±18 | 72±16 | (*P*<0.0001) | 82±20 |
| LDL-size, nm | 27.14±0.96 | (*P*<0.0001) | 26.36±0.82 | 27.32±0.91 | (*P*<0.0001) | 26.42±0.82 |
| Glucose, mg/dL* | 88±7 | (*P*<0.0001) | 92±6 | 86±7 | (*P*<0.0001) | 91±8 |
| Insulin, μU/mL | 7.0±5.8 | (*P*<0.0001) | 15.9±9.3 | 6.7±5.5 | (*P*<0.0001) | 20.0±14.3 |
| HOMA-R | 1.55±1.33 | (*P*<0.0001) | 3.60±2.18 | 1.46±1.24 | (*P*<0.0001) | 4.59±3.67 |
| QUICKI | 0.38±0.05 | (*P*<0.0001) | 0.33±0.03 | 0.39±0.05 | (*P*<0.0001) | 0.32±0.03 |

Abbreviations as in text. Values are expressed as mean±SD. *To convert to mmol/L, divide by 18. **To convert to mmol/L, multiply by 0.0259. ***To convert to mmol/L, multiply by 0.0113.

parents of all of the children. We studied 1175 Japanese children (642 boys and 533 girls) aged 7 to 12 years, who underwent screening and were enrolled in a care program for lifestyle-related diseases since 2001 in Okinawa, Japan. Sex maturity stages in the children studied were equal to or less than Tanner Stage 3. The subjects were not patients who visited our hospital. Body mass index (BMI) was calculated as weight [kg]/height$^2$ [m$^2$]. BMI standard deviation scores (BMISD) adjusted for age and sex were obtained based on data on Japanese school children provided by the Ministry of Education, Culture, Sports, Science, and Technology (unpublished data). Obesity was defined as BMISD ≥2.0. None of the children studied were receiving therapy for weight reduction or drugs that might affect lipid metabolism. None had a smoking habit. Venous blood was drawn after an overnight fast.

## Laboratory Measurements

Serum insulin was measured by 2-step sandwich enzyme-linked immunosorbent assay (SRL, Inc, Hachioji, Japan). Routine chemical methods were used to determine the serum concentrations of total cholesterol (TC), HDL cholesterol (HDL-C), triglycerides (TG), and glucose. Low-density lipoprotein cholesterol (LDL-C) was calculated as [TC − HDL-C − TG/5]. Apolipoprotein B (apoB) was measured by the turbidity immunoassay method.[16] IR and insulin sensitivity were calculated using the homeostasis model approximation index (HOMA-R) and the quantitative insulin-sensitivity check index (QUICKI).[17,18] LDL size was evaluated by electrophoresis in nondenaturing polyacrylamide gradient gels on precast MULTIGEL-LP (2% to 15%) according to the procedure specified by the manufacturer (Daiichi Pure Chemicals Co, LTD, Tokyo, Japan). Standards used for size calibration included latex beads (37 nm) (Dow Chemical Company) and high-molecular-weight standards (Pharmacia). The stained gels were scanned with a laser scanning densitometer to provide a quantitative measurement of the size of the peak and its distance from the origin. Particle diameter was calculated from a plot of the log of the known diameters of the standards (latex beads 37 nm, thyroglobulin 17.0 nm, apoferritin 12.2 nm) on the y-axis against their positions from the origin of the gel (Rf) on the x-axis.

## Statistical Evaluation

The significance of differences in clinical and chemical data between nonobese and obese children were determined by the Mann-Whitney *U* test. The distributions of HOMA-R and levels of insulin and triglyceride were markedly skewed. Thus, these parameters were normalized by log-transformation. Pearson and partial correlation coefficients were then computed to assess the associations between log HOMA-R and various parameters. A stepwise multiple regression analysis was performed by entering the independent variable with the highest partial correlation coefficient at each step, until no variable remained with an F value of ≥4. Age-adjusted and BMISD-adjusted differences in parameters among subjects with normal, IIa, IIb, and IV were determined by an analysis of covariance. Parameters in these 4 groups were compared using Scheffe's multiple comparison test. Group differences or correlations with $P<0.05$ were considered to be statistically significant.

# Results

As shown in Table 1, adiposity-related differences were found in all of the parameters studied. Obese children showed more atherogenic lipid and apolipoprotein profiles and greater IR than nonobese children. Thus, we separated the data for nonobese and obese children in the following analysis. Tables 2 and 3 show Pearson and partial correlations between IR (log HOMA-R) and the other parameters studied. In nonobese boys, log HOMA-R was correlated with all of the parameters listed ($P=0.000$). In obese boys, log HOMA-R was correlated with BMISD, log TG, apoB and HDL-C ($P=0.000$ to 0.002). After being corrected by age and BMISD, log HOMA-R was correlated with log TG, apoB, and LDL size in nonobese boys ($P=0.000$ to 0.017). In obese boys, all parameters except for LDL-C were correlated with log HOMA-R ($P=0.000$ to 0.010), even after being corrected by BMISD and age. Age, BMISD, TC, log TG, LDL-C, LDL size, and HDL cholesterol (HDL-C) in nonobese girls and age, BMISD, and LDL size in obese girls were significantly correlated with log HOMA-R ($P=0.002$ to 0.003). After being corrected by age and BMISD, log TG, LDL size, and HDL-C in nonobese girls and LDL size in obese girls were significantly correlated with log HOMA-R ($P=0.000$ to 0.024). Because each of these parameters can potentially contribute directly to the regulation of log HOMA-R, we performed a stepwise multiple regression analysis with log HOMA-R as the dependent variable and the other parameters

**TABLE 2. Log HOMA-R and Variables in Boys**

| | Non-Obese Boys | | | | Obese Boys | | | |
|---|---|---|---|---|---|---|---|---|
| | Pearson Correlation | | Partial Correlation | | Pearson Correlation | | Partial Correlation | |
| | r | P | r | P | r | P | r | P |
| Age | 0.367 | 0.000 | — | — | −0.040 | 0.517 | — | — |
| BMISD | 0.666 | 0.000 | — | — | 0.124 | 0.001 | — | — |
| TC | 0.203 | 0.000 | 0.085 | 0.073 | 0.133 | 0.107 | 0.209 | 0.003 |
| Log TG | 0.260 | 0.000 | 0.245 | 0.000 | 0.459 | 0.000 | 0.459 | 0.000 |
| LDL-C | 0.222 | 0.000 | 0.055 | 0.247 | 0.050 | 0.485 | 0.082 | 0.252 |
| ApoB | 0.294 | 0.000 | 0.113 | 0.017 | 0.239 | 0.001 | 0.273 | 0.000 |
| LDL-size | −0.286 | 0.000 | −0.166 | 0.002 | −0.138 | 0.095 | −0.178 | 0.030 |
| HDL-C | −0.228 | 0.000 | −0.082 | 0.084 | −0.224 | 0.002 | −0.183 | 0.010 |

Abbreviations as in text. Partial correlation: variables corrected by age and BMISD.

listed in Tables 2 and 3 as independent variables. In nonobese boys, BMISD had the most significant association with log HOMA-R and accounted for 45.8% of the variability in log HOMA-R. Age, log TG, and LDL size had additional effects (6.2%, 2.7%, and 1.3%, respectively) (Table 4). In obese boys, log TG had the most significant association with log HOMA-R and accounted for 20.6% of the variability in log HOMA-R. BMISD had an additional effect (6.6%). In nonobese girls, BMISD had the most significant association with log HOMA-R and accounted for 32.7% of the variability in log HOMA-R. Age, log TG, and LDL size had additional effects (20.8%, 2.1%, and 1.1%, respectively) (Table 4). In obese girls, age had the most significant association with log HOMA-R and accounted for 17.1% of the variability in log HOMA-R. BMISD and LDL size had additional effects (11.3% and 4.5%, respectively).

To elucidate the relationship between lipid phenotypes and IR, we divided school children into normolipidemia (NL) and type IIa (IIa), IIb, and IV hyperlipidemia groups. We defined hyperlipidemia based on serum lipid levels in Japanese school children.[6] When serum concentrations of TC, TG, and LDL-C were >90th percentiles for the respective age-matched and gender-matched values, we considered the children to be hyper TC, hyper TG, and hyper LDL-C (IIa, hyper LDL-C alone; IIb, hyper LDL-C and hyper TG; IV, hyper TG alone). NL was defined as serum concentrations of LDL-C and TG of <90th percentiles. Table 5 shows BMISD-adjusted and age-adjusted chemical parameters in children with NL, IIa, IIb, and IV. In boys, serum concentrations of HDL-C in IIb and IV were significantly lower than those in NL and IIa ($P<0.0001$). LDL sizes in IIb and IV were significantly smaller than that in NL ($P<0.05$ to $0.0001$). LDL size in IIb was significantly smaller than that in IIa ($P<0.001$). Serum concentrations of glucose were significantly higher in IIa and IIb than in NL ($P<0.01$). Serum concentrations of insulin and the levels of HOMA-R in IIa, IIb, and IV were significantly higher than those in NL and those in IIb and IV were significantly higher than those in IIa ($P<0.01$ to $0.0001$). Differences between IIb and IV were not significant. The levels of QUICKI in IIa, IIb, and IV were significantly lower than that in NL ($P<0.0001$). Those in IIb and IV were significantly lower than that in IIa ($P<0.0001$). In girls, serum concentrations of HDL-C in IIb and IV were significantly lower than those in NL and IIa ($P<0.0001$). The difference between IIb and IV was not significant. LDL size in IIb and IV were significantly smaller than those in NL and IIa ($P<0.05$ to $0.0001$). Serum concentrations of glucose were similar in all groups. Serum concentrations of insulin and the levels of HOMA-R in IIb and IV were significantly higher than those in NL and IIa ($P<0.01$ to $0.0001$). Differ-

**TABLE 3. Log HOMA-R and Variables in Girls**

| | Non-Obese Girls | | | | Obese Girls | | | |
|---|---|---|---|---|---|---|---|---|
| | Pearson Correlation | | Partial Correlation | | Pearson Correlation | | Partial Correlation | |
| | r | P | r | P | r | P | r | P |
| Age | 0.468 | 0.000 | — | — | 0.414 | 0.002 | — | — |
| BMISD | 0.576 | 0.000 | — | — | 0.347 | 0.010 | — | — |
| TC | −0.146 | 0.008 | −0.078 | 0.158 | −0.020 | 0.861 | 0.024 | 0.832 |
| Log TG | 0.319 | 0.000 | 0.221 | 0.000 | 0.161 | 0.152 | 0.098 | 0.384 |
| LDL-C | −0.124 | 0.025 | −0.081 | 0.143 | −0.011 | 0.919 | 0.031 | 0.784 |
| ApoB | −0.084 | 0.327 | −0.019 | 0.866 | 0.056 | 0.623 | 0.079 | 0.483 |
| LDL-size | −0.283 | 0.000 | −0.173 | 0.002 | −0.326 | 0.003 | −0.250 | 0.024 |
| HDL-C | −0.260 | 0.000 | −0.145 | 0.008 | −0.126 | 0.264 | −0.071 | 0.529 |

Abbreviations as in text. Partial correlation: variables corrected by age and BMISD.

**TABLE 4. Stepwise Multiple Regression Models for Predicting Log HOMA-R**

| Independent Parameters | $r$ | $r^2$ |
|---|---|---|
| Boys | | |
| Non-Obese | | |
| Step 1 BMISD | 0.677 | 0.458 |
| Step 2 BMISD, Age | 0.721 | 0.520 |
| Step 3 BMISD, Age, Log TG | 0.740 | 0.547 |
| Step 4 BMISD, Age, Log TG, LDL size | 0.749 | 0.560 |
| Obese | | |
| Step 1 Log TG | 0.454 | 0.206 |
| Step 2 Log TG, BMISD | 0.521 | 0.272 |
| Girls | | |
| Non-Obese | | |
| Step 1 BMISD | 0.572 | 0.327 |
| Step 2 BMISD, Age | 0.731 | 0.535 |
| Step 3 BMISD, Age, Log TG | 0.746 | 0.556 |
| Step 4 BMISD, Age, Log TG, LDL size | 0.753 | 0.567 |
| Obese | | |
| Step 1 Age | 0.414 | 0.171 |
| Step 2 Age, BMISD | 0.533 | 0.284 |
| Step 3 Age, BMISD, LDL size | 0.573 | 0.329 |

$P$ values of all steps were $P<0.0001$.

ences between IIb and IV were not significant. The levels of QUICKI in IIb and IV were significantly lower than those in NL and IIa ($P<0.0001$). Those in IIb and IV were significantly lower than that in IIa ($P<0.0001$). The difference between IIb and IV was not significant.

## Discussion

Because the sample size in our study was large (total number of subject 1175), IR and insulin sensitivity were determined by HOMA-R and QUICKI, respectively. The accuracy and precision of HOMA-R and QUICKI as measures of IR and insulin sensitivity have been determined elsewhere by comparison with euglycemic and hyperglycemic clamps and the intravenous glucose tolerance test.[17,18] Recent data have shown that these indices are reliably sensitive and specific for evaluating IR even in children.[19,20] In the present study, we have shown that: (1) associations of IR (HOMA-R) with parameters of lipids and lipoproteins were affected by BMISD and age in our school children; (2) BMISD had the most significant association with IR in nonobese children. Among lipids and lipoprotein parameters, TG and LDL size were significantly associated with IR. Especially, TG had the most significant association with IR in obese boys. In obese girls, age had the most significant association with IR. Only LDL size among lipids and lipoprotein parameters was significantly associated with IR; and (3) both boys and girls with IIb and IV hyperlipidemia had significantly higher IR than those with IIa and NL.

Insulin regulates many aspects of lipoprotein metabolism. Resistance to the normal actions of insulin causes the hepatic overproduction of TG and apoB, which thereby enhances the secretion of very low-density lipoproteins from the liver.[21] In addition, IR decreases lipoprotein lipase activity, resulting in a delayed clearance of TG-rich lipoproteins.[22] It is generally believed that a delayed clearance of TG-rich lipoprotein is associated with the generation of small dense LDL and lower concentrations of HDL-C.[23,24] IR was significantly correlated with TG, apoB, HDL-C, and LDL size in our school children. Taken together, these findings suggest that IR may play an important role in lipid metabolism even in school children. However, BMISD and age were also significantly associated with IR in our school children. Adiposity, especially the accumulation of visceral fat, increases intraportal free fatty acid (FFA) levels and flux, thereby inhibiting insulin clearance and promoting IR.[25] In addition, an increased or de-

**TABLE 5. Chemical Data Adjusted for BMISD and Age Among Boys and Girls With Normolipidemia and Type IIa, IIb, and IV Hyperlipidemia**

| | NL | *P* Value | IIa | *P* Value | IIb | *P* Value | IV |
|---|---|---|---|---|---|---|---|
| Boys, n | 435 | | 119 | | 32 | | 56 |
| HDL-C, mg/dL | 63±12 | (ns) | 62±12 | ($P<0.0001$) | 50±8[d] | (ns) | 53±11[d,h] |
| LDL-size, nm | 27.00±0.95 | (ns) | 27.00±0.99 | ($P<0.001$) | 26.07±0.97[d] | (ns) | 26.58±0.99[a] |
| Glucose, mg/dL | 89±7 | ($P<0.01$) | 91±6 | (ns) | 93±6[b] | (ns) | 90±8 |
| Insulin, μU/mL | 7.9±6.5 | ($P<0.01$) | 10.2±7.3 | ($P<0.0001$) | 18.9±11.1[d] | (ns) | 16.7±11.8[d,h] |
| HOMA-R | 1.78±1.50 | ($P<0.01$) | 2.32±1.69 | ($P<0.0001$) | 4.38±2.66[d] | (ns) | 3.75±2.70[d,h] |
| QUICKI | 0.38±0.06 | ($P<0.0001$) | 0.35±0.04 | ($P<0.0001$) | 0.32±0.03[d] | (ns) | 0.33±0.04[d,h] |
| Girls, n | 387 | | 85 | | 27 | | 34 |
| HDL-C, mg/dL | 62±11 | (ns) | 61±10 | ($P<0.0001$) | 45±6 | (ns) | 52±9[d,h] |
| LDL-size, nm | 27.23±0.92 | (ns) | 27.20±0.99 | ($P<0.001$) | 26.25±0.89[d] | (ns) | 26.65±1.01[b,e] |
| Glucose, mg/dL | 87±8 | (ns) | 86±9 | (ns) | 89±8 | (ns) | 87±7 |
| Insulin, μU/mL | 9.1±9.4 | (ns) | 9.9±10.2 | ($P<0.001$) | 17.8±10.7[d] | (ns) | 17.0±16.2[d,g] |
| HOMA-R | 2.04±2.35 | (ns) | 2.24±2.49 | ($P<0.01$) | 3.92±2.40[d] | (ns) | 3.74±3.83[d,f] |
| QUICKI | 0.37±0.06 | (ns) | 0.37±0.06 | ($P<0.0001$) | 0.33±0.04[d] | (ns) | 0.33±0.03[d,h] |

n: No of subjects. NL: Normolipidemia. Abbreviations as in Text. a, $P<0.05$; b, $P<0.01$;c, $P<0.001$; d $P<0.0001$: significantly different from NL. e, $P<0.05$; f, $P<0.01$; g, $P<0.001$; h, $P<0.0001$: significantly different from IIa. ns: not significant.

creased in the secretion of adipocytokines form adipocytes, such as leptin, tumor necrosis factor (TNF)-$\alpha$, adiponectin, etc., may cause IR.[26-28] An age-related reduction in insulin receptor expression has also been reported.[25] Thus, to exclude effects of adiposity and age, BMISD and age were adjusted for by partial correlation. Because age did not affect the relationship between insulin resistance and lipid-related parameters (data not shown), the partial correlation in Tables 2 and 3 reflected the effect of BMISD. After being corrected by BMISD, correlations between IR and lipid-related parameters were weakened in girls and nonobese boys, and strengthened in obese boys (Tables 2 and 3). Although several parameters were significant after being corrected by BMISD, multiple regression analysis showed that only two (TG and/or LDL size) were independently associated with IR in our school children (Table 4). However, these parameters can only account for 3.3% to 4.5% of the variability in IR in girls and nonobese boys. In contrast, TG in obese boys was the strongest predictor for IR and accounted for 20.6% of the variability in IR. These findings suggest that weight gain (adiposity) can mostly explain the relationship between IR and lipid-related parameters in girls and nonobese boys. However, in obese boys, TG metabolism might be more important for IR than adiposity. Although further studies are needed, genetic factors may exacerbate TG metabolism (overproduction of very low-density lipoprotein or delayed clearance of TG-rich lipoprotein) in obese boys.

The question is whether increased TG or decreased LDL size precedes or follows IR. As mentioned, IR itself can induce hypertriglyceridemia and make LDL size smaller.[21-24] A substantial reduction of serum TG levels with fibrate treatment did not improve IR.[29,30] Improvement of IR reduced small dense LDL particles.[31] To date, no data are available on whether the improvement of LDL size can affect IR. Interestingly, the state of insulin resistance in familial combined hyperlipidemia (FCHL) is associated with the lipid phenotype.[32] Subjects with FCHL based on hyper TG (IV) or combined hyperlipidemia (IIb) are more insulin-resistant than FCHL subjects based on hyper TC (IIa) even after correcting for BMI.[32] As in FCHL, school children with IIb and IV showed more IR and smaller LDL size than those with NL and IIa (Table 5). Because a family study was not performed in the present study, we could not diagnose FCHL in our school children. However, the characteristics of school children with IIb and IV were very similar to those of FCHL patients. In addition, as shown in our previous study, most young children (preschool) with IIb were FCHL based on a familial study.[5] Taken together, these results might extend our previous finding (ie, that most young children with IIb are FCHL) to school children. If our notion is valid, a genetic background that regulates serum TG and/or LDL size such as in FCHL might contribute to the relationship between TG, LDL size, and IR. Weight gain may exacerbate IR and lipid abnormalities in these children.

With respect to the differences between boys and girls, it is well known that sex hormone affect the lipid metabolism. Because most of our children were pre-puberty, we did not measure sex hormone. However, age was strongly associated with IR especially in girls. This may suggest that subtle change of sex hormone may be responsible for the gender differences of our data. Further studies are needed to clarify a complex interplay between sex hormone, BMI, and insulin action.

In conclusion, TG and/or LDL size were significantly associated with IR, and lipid phenotypes (IIb and IV) showed higher IR, but neither of these associations could be fully explained by their BMISD. School children with types IIb and IV showed characteristics similar to those in subjects with FCHL. Thus, it is important for school children to control body weight to prevent progression to the metabolic syndrome and a familial study should be performed in children with IIb and IV to screen for those at high risk for CHD and/ or type 2 diabetes.

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## Disclosures

None.

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# Activation of diacylglycerol *O*-acyltransferase 1 gene results in increased tumor necrosis factor-α gene expression in 3T3-L1 adipocytes

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**Abstract The tumor necrosis factor-α (TNF-α) expression has been reported to be largely dependent on the size of adipocytes. We herein investigated the gene regulation of diacylglycerol *O*-acyltransferase (DGAT) in order to clarify the mechanism of TNF-α expression induced in large adipocytes. 3T3-L1 cells were cultured in the presence of 5 mM or 25 mM glucose to generate adipocytes from which the triglyceride content differs. The expression of TNF-α, DGAT1, and DGAT2 were upregulated in adipocytes cultured with 25 mM glucose. Furthermore, knockdown of DGAT1 gene significantly inhibited the TNF-α expression. Finally, the DGAT1 expression levels were closely related to the TNF-α level in 3T3-L1 adipocytes.**


*Keywords:* Adipocytes; Tumor necrosis factor-α; Diacylglycerol *O*-acyltransferase 1; Triglyceride synthesis; Insulin resistance

## 1. Introduction

Enlarged adipocytes isolated from either obese animals or human exhibit modified metabolic properties and which do not respond to exogenous stimuli as do adipocytes from lean controls. Enlarged adipocytes develop a decreased sensitivity to insulin [1–4] and produce increased quantities of secreted cytokine such as tumor necrosis factor-α (TNF-α) [5,6] or leptin [1,7]. An enlargement or accumulation of triglyceride (TG) seems to be associated with cellular functions in adipocytes. It is well known that the cytokine production from fat cells is mainly dependent on the cell size, and the greater the accumulation of TG, the larger the cell size that is induced. TG accumulation is largely induced by the increased TG synthesis. De novo lipogenesis is known to be one possible mechanism resulting in the accumulation of TG [8]. Among the many enzymes for the lipogenesis in adipocytes, diacylglycerol *O*-acyltransferase (DGAT) catalyzes the final step in TG synthesis [9,10]. Recent studies have suggested that an overexpression of DGAT may play a role in insulin resistance [11,12]. However, the relationship between TG accumulation and the gene expression in adipocytes has not yet been studied in relation to DGAT expression and activation. In the light of these observations, the aim of the current study was to characterize the relationship between the expression of DGAT and TNF-α genes in adipocytes.

## 2. Materials and methods

### 2.1. Cell culture

The 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA). The differentiation of 3T3-L1 preadipocytes to mature adipocytes was performed using insulin, dexamethasone, and 3-isobutyl-1-methyl-xanthine essentially as described [13] except that Dulbecco's modified Eagle's medium (DMEM; Sigma Chemicals, St. Louis, MO) contained either 5 mM or 25 mM glucose. Briefly, 2 days after reaching the confluence (day 0), differentiation was induced by switching the medium into DMEM containing 10 μg/ml insulin (Sigma Chemicals), 0.25 μM dexamethasone (Sigma Chemicals), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Chemicals) for 48 h. The cells were incubated with DMEM containing 5 μg/ml insulin for further 48 h, then the medium was changed back with original DMEM containing 10% FBS and changed every 2 days. The cells were used between days 14 and 21 after the induction of differentiation.

### 2.2. Oil red O staining

The 3T3-L1 adipocytes differentiated either with constant 5 mM or 25 mM glucose condition (adipocytes with 5 mM or 25 mM glucose, respectively) were fixed with 10% formaldehyde (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 15 min at room temperature. After fixation, cells were stained with 0.3% oil red O solution for 10 min at room temperature. The amount of TG was evaluated by measuring the area stained with oil red-O using the Scion Image software (Scion Corp., Fredrick, MD).

### 2.3. Quantitative RT-PCR analysis

Total RNA was extracted from 3T3-L1 cells using an RNeasy kit (Qiagen, Valencia, CA) and was reverse transcribed using the ExScript RT-PCR Kit (Takara Bio Inc., Shiga, Japan). Quantitataive RT-PCR amplifications were performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA) and Assays-on-Demand

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*Abbreviations:* TNF-α, tumor necrosis factor-α; TG, triglyceride; DGAT, diacylglycerol *O*-acyltransferase; DMEM, Dulbecco's modified Eagle's medium; Adipocytes with 5 mM glucose, adipocytes differentiated with constant 5 mM glucose condition; Adipocytes with 25 mM glucose, adipocytes differentiated with constant 25 mM glucose conditions; siRNA(s), small interfering RNA(s)

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Gene expression Assay Mix (PE Applied Biosystems). All PCRs were performed in an ABI PRISM 7000 sequence detection system (PE Applied Biosystems).

*2.4. Glucose treatment*

The 3T3-L1 adipocytes with 25 mM glucose were incubated with DMEM without glucose supplemented with 10% FBS for 24 h. The glucose-starved adipocytes were then treated with DMEM containing 10% FBS and various concentrations of glucose for 4–48 h. As an osmotic control, adipocytes with 25 mM glucose were exposed to mannitol instead of glucose.

*2.5. Suppression of DGAT1 and DGAT2 expression by small interfering RNAs (siRNAs)*

The 3T3-L1 adipocytes with 25 mM glucose were transfected with siRNA duplexes by electroporation. In brief, 3T3-L1 adipocytes with 25 mM glucose were detached from culture dishes with 0.05% trypsin and 0.02% EDTA (Sigma Chemicals), washed twice, and resuspended in phosphate-buffered saline. Approximately 5 million cells were then mixed with HP validated siRNA duplexes (Qiagen) either for DGAT1 or DGAT2, which were delivered to the cells by a pulse of electroporation with a Bio-Rad gene pulser XL system (Bio-Rad Laboratories, Hercules, CA) at the setting of 0.18 kV and 950 μF capacitance. After electroporation, cells were reseeded onto multiple-well plates, and incubated with DMEM containing 10% FBS and 25 mM glucose for 2 days. Glucose-starved adipocytes treated with siRNA were incubated either with 0 mM or 25 mM glucose for 24 h, and the levels of TNF-α and DGAT gene expression were analyzed by quantitative RT-PCR analyses. Non-silencing fluorescein-labeled siRNA duplexes (Qiagen) were used as the control for unrelated siRNA transfections.

*2.6. Statistical analysis*

The results are shown as means ± S.D. for each index, respectively. Statistical significance was determined by means of the Student's *t*-test or Dunnett's multiple range test followed by ANOVA among several groups; a value of $P < 0.05$ was considered as significant.

## 3. Results

*3.1. Increased TNF-α and DGAT gene expression in adipocytes with 25 mM glucose*

To generate and characterize of adipocytes of different sizes, we cultured 3T3-L1 cells with DMEM containing 5 mM or 25 mM glucose. A histological study using oil red O staining revealed that adipocytes with 25 mM glucose have enlarged adipocytes, and accumulated significantly much TG in comparison to the adipocytes with 5 mM glucose (Fig. 1A and B). We then examined mRNA expression levels for DGAT1 and DGAT2, which have been identified as the enzymes that catalyze the final step of TG synthesis. Both DGAT1 and DGAT2 mRNA expression were found to be significantly increased in adipocytes with 25 mM glucose compared to adipocytes with 5 mM glucose (DGAT1: 2.3-fold, DGAT2: 3.9-fold, Fig. 1C and D). We next examined TNF-α mRNA expression, which is a marker for functional changes in adipocytes [14–16]. The expression of TNF-α mRNA was 6.4-fold higher in adipocytes with 25 mM glucose than in adipocytes with 5 mM glucose (Fig. 2).

*3.2. Effects of glucose on TNF-α and DGAT gene expression in 3T3-L1 adipocytes*

To know the mechanism of the elevation of TNF-α and DGAT mRNA in adipocytes with 25 mM glucose, we examined the effects of glucose on TNF-α and DGAT mRNA expression in 3T3-L1 adipocytes. Treatment with 25 mM glucose (designated as high glucose) for 24 h caused a 2.3-fold increase of the TNF-α mRNA expression in 3T3-L1 adipocytes with 25 mM glucose (Fig. 3A). Treatment with high glucose for 24 h also induced both the DGAT1 and DGAT2 mRNA expression, to a maximum of 3.0-fold and 2.7-fold, respectively

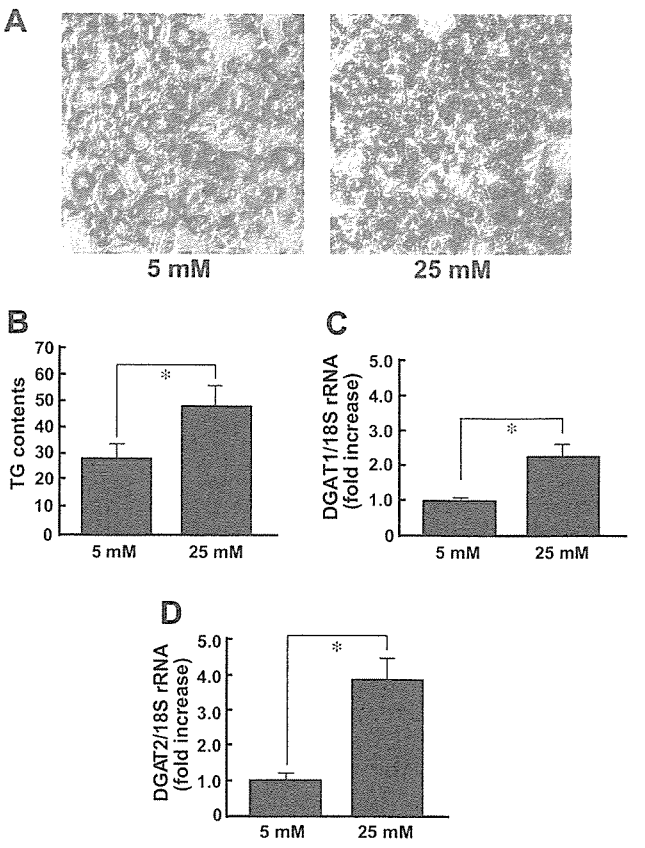


Fig. 1. Generation and characterization of adipocytes from which the TG content differs (A). 3T3-L1 cells were differentiated into adipocytes either with 5 mM or 25 mM concentration of glucose and were stained with oil-red O to identify the lipid droplet formation (magnification, ×20). (B) the quantification of the amount of TG. Data represent the means ± S.D. ($n = 5$). (C–D), the quantitation of DGAT1 (C) and DGAT2 (D) mRNA in adipocytes with 5 mM glucose and adipocytes with 25 mM glucose. The quantification of each mRNA is expressed in relation to the adipocytes with 5 mM glucose. 18S rRNA was used as an internal control. Data represent the means ± S.D. ($n = 4$). $^{*}P < 0.01$.

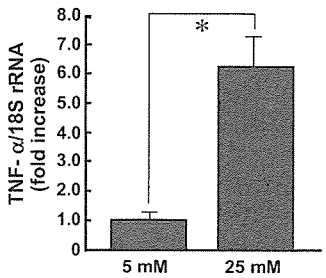


Fig. 2. The expression of the TNF-α gene is increased in adipocytes with 25 mM glucose in comparison to that in adipocytes with 5 mM glucose. The quantification of each mRNA is expressed in relation to the adipocytes with 5 mM glucose. Data represent the means ± S.D. ($n = 4$). $^{*}P < 0.01$.

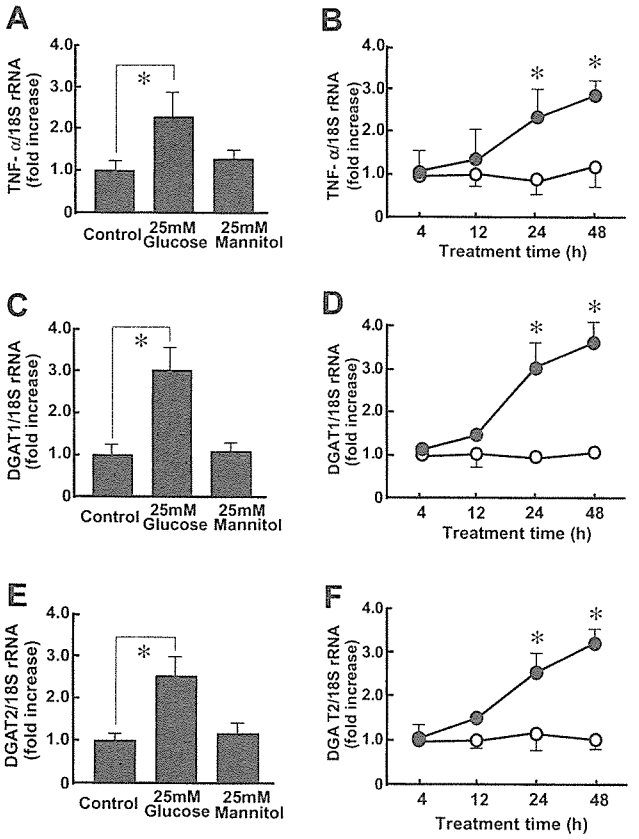


Fig. 3. High glucose induces both the TNF-α and DGAT mRNA expressions in 3T3-L1 adipocytes. After glucose-starvation for 24 h, 3T3-L1 adipocytes with 25 mM glucose were treated either with 25 mM glucose or mannitol for further 24 h ((A), (C), and (E)), or treated with 25 mM glucose for the indicated time up to 48 h ((B), (D), and (F)). ●, 25 mM glucose; ○, glucose-free (0 mM). Data represent the means ± S.D. ($n = 4$). $^{*}P < 0.01$ differences between the treatment groups and controls.

(Fig. 3C and E). As an osmotic control, 3T3-L1 adipocytes with 25 mM glucose were exposed to 25 mM mannitol instead of glucose, and no change in the expression of DGAT1, DGAT2 and TNF-α mRNA was observed (Fig. 3A, C and E). In the time course experiment, treatment with high glucose for 24 h significantly induced TNF-α mRNA, which then remained at those levels until 48 h after glucose addition (Fig. 3B). Similarly, treatment with high glucose increased the levels of DGAT1 and DGAT2 mRNA in a time-dependent manner to a maximum of 3.5-fold and 3.2-fold, respectively (Fig. 3D and F). These results indicate that the time-course induction is similar among DGAT1, DGAT2, and TNF-α mRNA expression in 3T3-L1 adipocytes.

### *3.3. siRNA-mediated knock down of DGAT1 gene decreases high glucose-induced TNF-α mRNA up-regulation*

To elucidate the direct association between TNF-α and DGAT gene expression, we tested whether the high glucose-induced TNF-α mRNA up-regulation would be responsible for that of DGAT. To knockdown the DGAT genes, we treated 3T3-L1 adipocytes with 25 mM glucose either with DGAT1 or DGAT2 specific siRNA duplexes. DGAT1 and DGAT2 siRNA reduced the expression of each isozyme to 15% and 5%, respectively (Fig. 4A and B). In addition, DGAT1 and

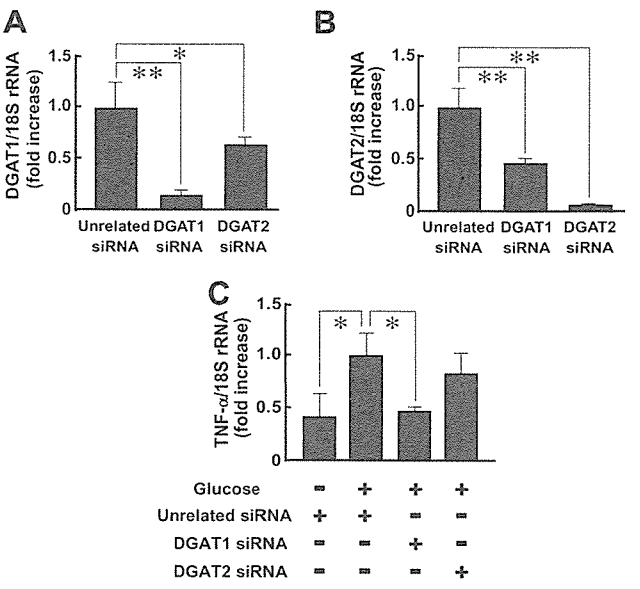


Fig. 4. High glucose induces TNF-α mRNA expression in 3T3-L1 adipocytes via the activation of the DGAT1 gene. DGAT1 (A) or DGAT2 (B) mRNA was knocked down by siRNA duplexes in 3T3-L1 adipocytes with 25 mM glucose as described in Section 2. Fluorescein-conjugated control siRNA was used as an unrelated control. The expression of DGAT1 (A) and DGAT2 (B) gene was measured by quantitative RT-PCR analyses. (C) The inactivation of the DGAT1 gene attenuates high glucose-induced TNF-α mRNA expression in 3T3-L1 adipocytes. The 3T3-L1 adipocytes were electroporated either with DGAT1 or DGAT2 siRNA, and both the basal (0 mM glucose) and high glucose (25 mM glucose)-treated levels of TNF-α mRNA expression were determined. $^{*}P < 0.01$, $^{**}P < 0.001$.

DGAT2 siRNA also reduced the expression levels of DGAT2 and DGAT1 by 51% and 46%, respectively (Fig. 4A and B). Unrelated siRNA did not affect the high glucose-induced TNF-α mRNA expression in 3T3-L1 adipocytes with 25 mM glucose, in which a 2.3-fold increase of TNF-α mRNA expression was observed as shown in Fig. 3A. The inhibition of the DGAT1 gene significantly suppressed high glucose-induced TNF-α gene expression to a closely similar level in the unrelated siRNA control. In contrast, DGAT2 inhibition did not significantly influence the high glucose-induced TNF-α mRNA expression (Fig. 4C). These data strongly suggest that DGAT1, but not DGAT2, contributes to the upregulation of TNF-α mRNA expression.

## 4. Discussion

In the current study, we generated and characterized the adipocytes of different sizes of 3T3-L1 cells. The cellular TG contents and the expression of markers such as DGAT1 and DGAT2 are increased in adipocytes with 25 mM glucose. Moreover, adipocytes with 25 mM glucose display the induction of TNF-α mRNA. In particular, we focused on the regulation of DGAT by glucose, especially in relation to the TNF-α gene expression. Glucose increased DGAT1, DGAT2, and TNF-α mRNA expression in 3T3-L1 adipocytes with 25 mM glucose. These results suggested that DGAT and TNF-α mRNA expression in 3T3-L1 adipocytes is closely related to each other. Finally, RNA interference experiments showed the DGAT1 expression or activation is important for

the TNF-α gene expression in adipocytes. We therefore propose that DGAT1 plays an important role in the regulation of functions for the TNF-α production in adipocytes.

DGAT1 and DGAT2 are both believed to catalyze the final reaction in the TG synthesis pathways in adipocytes. Although the ability to make TG is essential for normal physiology, excess accumulation of TG results in obesity and is associated with insulin resistance. Mice lacking DGAT1 have an increased energy expenditure and insulin sensitivity and thus are protected against diet-induced obesity and glucose intolerance [11]. The metabolic abnormality in the knockout mice could possibly be due to a disturbed function of the adipocytes, in addition to the degree of accumulated fat volume in the mice. As a result, the augmentation of TG synthesis in adipocytes by the transgenic overexpression of DGAT1 gene causes obesity and/or decreases insulin sensitivity. Two-fold greater DGAT activity levels in adipose tissue causes hyperglycemia, hyperinsulinemia, and glucose intolerance on a high-fat diet in mice[17]. Although DGAT2 knockout mice demonstrate decreased tissue triacylglycerol contents, similar to DGAT1 knockout mice, they have disparate phenotypes, thus suggesting that the two enzymes have functional differences. A recent study indicated that DGAT1 utilizes a variety of acyl acceptors as substrates in vitro [18]. These different activities between DGAT1 and DGAT2 may be relevant to the in vivo functions of DGAT1. Our finding of a tight link between DGAT1, and not DGAT2, and TNF-α expression may thus be caused by the different metabolic spectra between these enzymes. An over-accumulation of TG in adipocyte is well associated with the development of insulin resistance, possibly in part through the secretion of cytokines for the regulation of insulin sensitivity. An animal model showed that the increase in TG accumulation corresponded to an increase in the microsomal DGAT activity [19]. The expression of DGAT1 mRNA changes the most in the liver and adipose tissue, whereas the DGAT2 responses mainly induce changes in the muscle and intestine. Thus, DGAT1 may therefore be responsible for the elevated DGAT activity and the subsequent increase in de novo lipogenesis, and lead to the cytokine secretion from adipocytes.

The relationship between subcutaneous and visceral adipocyte metabolism and development has been extensively studied using primary cell cultures isolated from fat tissue [20]. We have shown that adipocytes which accumulated in mesenteric area, and not in the subcutaneous area, induce TNF-α gene expression, thus leading to an increased concentration of TNF-α in the serum using a cell transplantation method in mice [14]. In fact, the decrease in visceral fat accumulation is associated with a decrease in the serum concentration of TNF-α and insulin resistance [21]. Winkler et al. recently reported that TNF-α protein was expressed in the adipocytes of depots in humans, and the cell volume of both adipocyte depots was found to have a significant positive correlation with serum TNF-α level [22]. The cell volume of adipocyte is largely dependent on the accumulation of TG. DGAT is a key enzyme for the synthesis of TG in adipocytes. Therefore, in this study, we analyzed the modulation of DGAT regarding the TNF-α expression in adipocytes, independently of the cell size itself. Our results indicate that DGAT1 activation is closely related to the expression of TNF-α thus suggesting that DGAT1 plays an important role in the regulation of cytokine expression from adipocytes accumulated in the visceral area. The inhibition of DGAT1, but not DGAT2, drastically inhibited the TNF-α gene expression in adipocytes. Further attempts to elucidate the relationship between the activation of DGAT1 gene and the expression of TNF-α gene may help contribute to a better understanding of the disturbance of the functions in the adipocytes which accumulate in the visceral area, thus leading to insulin resistance in humans.

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# Catalytically Inactive Lipoprotein Lipase Overexpression Increases Insulin Sensitivity in Mice



## Abstract

Abnormalities in lipoprotein lipase (LPL) function contribute to the development of hypertriglyceridemia, one of the characteristic disorders observed in the metabolic syndrome. In addition to the hydrolyzing activity of triglycerides, LPL modulates various cellular functions via its binding ability to the cell surface. Here we show the effects of catalytically inactive LPL overexpression on high-fat diet (HFD)-induced decreased systemic insulin sensitivity in mice. The binding capacity of catalytically inactive G188E-LPL to $C_2C_{12}$ skeletal muscle cells was not significantly different from that of wild type LPL. Insulin-stimulated IRS-1 phosphorylation and glucose uptake were increased by addition of wild type or mutant LPL in $C_2C_{12}$ cells. After 10 weeks' of HFD feeding, mice had significantly higher blood glucose levels than chow-fed mice in insulin tolerance tests. The blood glucose levels after insulin injection was significantly decreased in mutated LPL-overexpressing mice (G188E mice), as well as in wild type LPL-overexpressing mice (WT mice). Overexpression of catalytically inactive LPL, as well as wild type LPL, improved impaired insulin sensitivity in mice. These results show that decreased expression of LPL possibly causes the insulin resistance, in addition to hypertriglyceridemia, in metabolic syndrome.

## Key words

High-fat diet · metabolic syndrome · insulin resistance · cell implantation · skeletal muscle



## Introduction

Metabolic syndrome is a high risk state for the development of cardiovascular diseases (CVD), which is characterized by an accumulation of systemic abnormalities, such as hypertriglyceridemia, hypoalphalipoproteinemia, glucose intolerance, elevated blood pressure and central obesity, even though each abnormality is not much disturbed as a single risk factor for the progression of atherosclerosis [1]. The accumulated metabolic abnormalities in metabolic syndrome are closely related to systemic insulin resistance [2]. Although the precise mechanism has not been elucidated, the hypertriglyceridemia is based on both increased secretion and decreased hydrolysis of triglycerides (TG)-rich lipoproteins [3, 4].

Lipoprotein lipase (LPL) is a key enzyme which catalyzes the hydrolysis of the TG component of circulating TG-rich lipoproteins such as, chylomicrons and VLDL [5]. The lipolytic function of enzyme is regulated by insulin at both mRNA and protein levels [6, 7]. Therefore, in the presence of systemic insulin resistance, transcriptionally and post-transcriptionally reduced LPL activity could contribute to the development of hypertriglyceridemia [8]. In addition to the hydrolytic activity, LPL mediates the binding of all classes of lipoproteins to a variety of cells, such

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as Hep G2 cells, fibroblast and macrophages [9–11]. The non-hydrolytic functions of enzyme accelerate incorporation of these lipoproteins into cells through specific receptors and/or by unclarified pathways. We have shown that LPL induces the chylomicrons uptake using LDL receptor-deficient cultured cells [11]. LPL can bridge between lipoproteins and cell-surface heparan sulfate proteoglycans [5], which does not depend on the hydrolyzing function of the enzyme [11,12].

We have previously reported that the TNF-α- or resistin-overexpressing animal model established by transplantation of the each cDNA-transfected cultured cells causes decreased insulin actions in muscle and systemic insulin resistance [13,14]. In the TNF-α-overexpressing mice, serum concentration of LPL mass was highly associated with the level of systemic insulin sensitivity [13]. In the current study, to find out the role of LPL in the systemic insulin resistance in metabolic syndrome, we have evaluated the effect of catalytically inactive LPL-overexpression using the mutated LPL cDNA-transfected cells in mice showing decreased insulin sensitivity caused by high-fat feeding.





## Materials and Methods

### Reagents, cell culture, and animals

Antibodies against IRS-1 and phosphotyrosine were obtained from Santa Cruz Biotechnology, Inc. (California). 2-[$^3$H]-Deoxyglucose (259 Gbq/mmol) was received from Amersham (Piscataway). Chinese hamster ovary (CHO) cells and $C_2C_{12}$ cells were obtained from the American Type Culture Collection (Manassas). CHO cells were grown in Ham's F-12 medium supplemented with 10% FBS. $C_2C_{12}$ cells were maintained using DMEM containing 10% FBS. Near-confluent $C_2C_{12}$ cells (~80% confluence) were differentiated by lowering the serum concentration to 2% horse serum for 3–4 days. Male ICR nude mice were purchased from Charles River, Inc. (Yokohama, Japan). The mice were fed standard rodent chow (CE-2; CLEA, Tokyo, Japan) or QuickFat (CLEA) as HFD for 8 or more weeks.

### Wild type or G188E mutant LPL-overexpressing CHO cells

Human G188E mutant LPL cDNA [15] was cloned via PCR from Human Liver Quick-Clone cDNA (BD Biosciences, Palo Alto), and verified by sequencing. In order to obtain a vector containing the human wild type LPL cDNA, *in vitro* site-directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla) and oligonucleotide primers (sense: 5'-CACATTCACCAGAGGGTCCCCTGGTCGAAG-3'; antisense: 5'-CTTCGACCAGGGGACCCTCTGGTGAATGTG-3'; the bases changed by mutagenesis are underlined). The cDNA fragment was subcloned, and the amplified sequence was confirmed on both sides. CHO cells were stably transfected with the human wild type LPL vector (CHO-WT-LPL), the G188E mutant LPL vector (CHO-G188E-LPL), or the vector alone (CHO cells), using a proprietary non-liposomal lipid (Effectane, Qiagen). Stable cells of wild type or mutant LPL were selected and subcloned.

### Preparation of wild type or mutant LPL from conditioned media

The immunological detection of wild type and G188E mutant LPL was performed using conditioned medium incubated with CHO-WT-LPL and CHO-G188E-LPL for 24 h. Fifty ml of conditioned medium from CHO-WT-LPL or CHO-G188E-LPL (for $5\times10^7$ cells) was collected, and applied on the column for conducting heparin-Sepharose affinity chromatography (HiTrap heparin-Sepharose column, Amersham). Eluted samples were kept at 4 °C until further use.

### Immunological detection of LPL

The immunoblot analysis for wild type or mutant LPL was performed using the above prepared samples. Monoclonal antibody against bovine milk LPL (1:200 dilution, Daiichi, Japan) was used for the immunodetection as described [16,17], and the blots were detected with ECL Western blotting detection reagents (Amersham).

### Binding of wild type and mutant LPL to skeletal muscle cells

Bovine milk LPL protein (Sigma) was biotinylated using EZ-Link NHS-biotin (Pierce, Rockford). Differentiated $C_2C_{12}$ cells, at $0.2\times10^5$ cells in a 96-well culture plate, were incubated with DMEM containing 3% BSA for 3 h at 37 °C. Biotinylated bovine milk LPL was added to wells in the presence of various concentration of G188E mutant LPL or wild type LPL, and the cells were incubated for 2 h at 4 °C. Cell-bound biotinylated bovine milk LPL was detected by using streptavidin-horseradish peroxidase (Prozyme, San Leandro) and TMB peroxidase substrate (Moss, Inc., Pasadena).

### Insulin-induced tyrosine phosphorylation of IRS-1 in skeletal muscle cells

Differentiated $C_2C_{12}$ cells were incubated in the absence or presence of G188E mutant and wild type LPL at 2 mg/ml, containing 2.5% horse serum for 4 days and further incubated for 4 h in DMEM without serum. Phosphorylated or total IRS-1 protein was detected by immunoblotting as described previously [18].

### Glucose transport into skeletal muscle cells

Differentiated $C_2C_{12}$ cells were incubated in the absence or presence of G188E mutant and wild type LPL at 2 μg/ml, containing 2.5% horse serum for 4 days and further incubated for 5 h in DMEM without serum. The cells were then incubated with 100 nmol/l human insulin for 30 min at 25 °C. Unlabeled 2-deoxyglucose and 2-[$^3$H]-deoxyglucose (3.7 kBq/ml) were added at a final concentration of 10 μmol/l, and the cells were incubated for 10 min. An aliquot of the extract was neutralized with 250 μl of 0.5 mol/l HCl. The $^3$H-labeled radioactivity was counted using a scintillation counter (LS-6500; Beckman Coulter, Inc., Fullerton).

### LPL-overexpressing mouse model

Eight-week-old ICR nude mice were injected subcutaneously with $1\times10^7$ CHO-WT-LPL (WT mice), $1\times10^7$ or $2\times10^7$ CHO-G188E-LPL (G188E mice-1 or mice-2), or CHO cells (control mice), as described previously [13,14,18]. Seven days after the cell transplantation, mice were fed on HFD diet instead of a standard chow diet for 10 weeks.

### Assay of LPL mass and activity, and tolerance tests

Mice were fasted for 16 h, and injected with a bolus injection of heparin at a dose of 50 U/kg and blood samples were collected 10 min after the injection. Human LPL mass in the culture media or mouse plasma was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using a kit with specific mono-

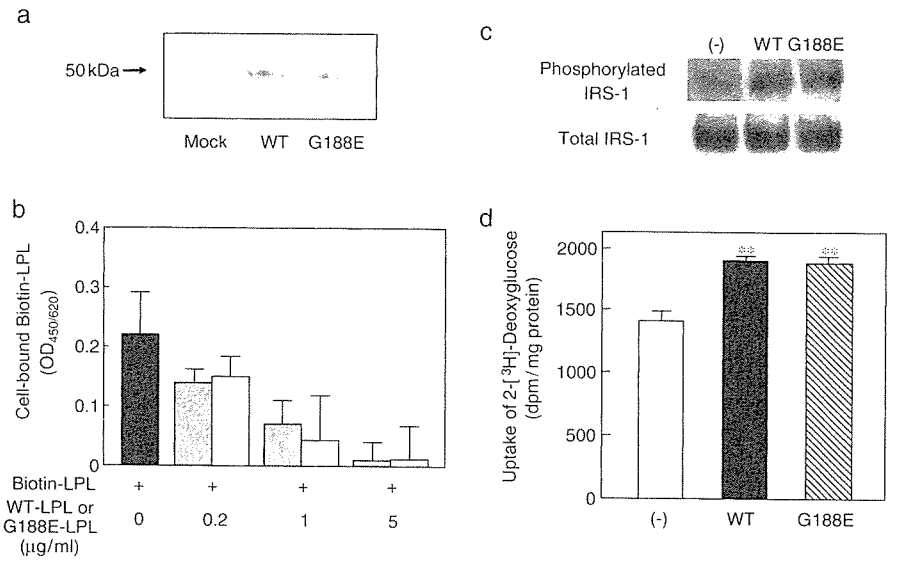


Fig. **1** Characterization of wild type LPL or G188E mutant LPL expressed by the respective cDNA-transfected CHO cells. Wild type or G188E mutant LPL protein sample was prepared from the media of cDNA-transfected cells using heparin-sepharose column affinity chromatography as described in Materials and Methods. The prepared samples (0.5 mg) were analyzed by gel electrophoresis by immunoblot staining using monoclonal antibody against bovine milk LPL (**a**). Effect of these prepared samples of transfected cells on binding (**b**, 0–5 mg/ml), insulin-stimulated tyrosine phosphorylation and protein levels of IRS-1 (**c**, 2 mg/ml), and uptake of 2-[$^3$H]-DOG (**d**, 2 mg/ml) were analyzed in a skeletal muscles cell line, differentiated $C_2C_{12}$ cells. In (**b**), cells were incubated with biotinylated bovine milk LPL in the presence of various concentration of wild type LPL or G188E mutant LPL (**b**). Data are expressed as means ± SEM. $^{**}p < 0.01$ *vs.* control.

clonal antibody against bovine milk LPL (Daiichi Pure Chemical, Tokyo) [13, 16, 17]. Glucose tolerance and insulin tolerance tests were performed in conscious mice 10 weeks after the transplantation of the LPL-producing cells, as described previously [13].

### Statistical analysis

Values are reported as means ± SEM. Statistical significance was determined using Student's *t*-test (2 tailed) with $p < 0.05$.

## Results

### Binding of catalytically inactive LPL to skeletal muscle cells

The CHO cells overexpressing the human wild type LPL and the catalytically inactive mutant LPL were established by stable transfection with the human wild type LPL vector (CHO-WT-LPL), and the catalytically inactive mutant LPL vector (CHO-G188E-LPL) [15, 19], respectively [13, 14, 18]. There was no difference in human LPL mass between the conditioned media from CHO-WT-LPL ($3.4 \pm 0.5$ ng · $10^6$ cell$^{-1}$) and CHO-G188E-LPL cells ($3.7 \pm 1.3$ ng · $10^6$ cell$^{-1}$). The LPL proteins purified by heparin-Sepharose column affinity chromatography showed the immunologically detectable proteins at the same mobility between G188E mutant and wild type LPL (Fig. **1a**). The specific activity of mutant G188E LPL showed only trace level of specific lipase activity ($0.02 \pm 0.01$ nmol/min/ng), obviously decreased compared with that in wild type LPL ($0.47 \pm 0.11$ nmol/min/ng).

We first investigated the binding ability of catalytically inactive LPL to skeletal muscle cells. Biotinylated bovine milk was bound to $C_2C_{12}$ cells. The specific binding of cell-bound Biotin-LPL was decreased in the presence of G188E-LPL, as well as wild type LPL, in a dose-dependent manner (Fig. **1b**).

### Effects of catalytically inactive LPL on insulin activities in skeletal muscle cells

The effects of catalytically active or inactive LPL on insulin-induced tyrosine phosphorylation of IRS-1 in skeletal muscle cells were studied. Differentiated $C_2C_{12}$ cells were incubated with the WT or G188E LPL protein, for 4 days. The levels of insulin-stimulated tyrosine phosphorylation of IRS-1 were increased in $C_2C_{12}$ cells treated with WT-LPL and G188E-LPL (Fig. **1c**).

We therefore evaluated the effects of catalytically active or inactive LPL on uptake of 2-[$^3$H]-deoxyglucose (2-[$^3$H]-DOG) into skeletal muscle cells. After four days of incubation in each of LPL proteins, differentiated $C_2C_{12}$ cells were stimulated with insulin, and glucose uptake assay was performed. Insulin-stimulated activation of glucose uptake was significantly increased in WT or G188E LPL treated $C_2C_{12}$ cells than in control (Fig. **1d**). These results suggest that both catalytically active and inactive LPL have an ability to modify insulin sensitivities in muscles.

### Metabolic markers and insulin sensitivity in HFD mice

In order to evaluate the effects of LPL on actions in tissues *in vivo*, we next transplanted the LPL-overexpressing cells into mice with decreased insulin sensitivity. For this purpose, we established a diet-induced animal model of insulin resistance and hypertriglyceridemia. After feeding HFD for 10 weeks, TG and insulin levels, but not glucose levels, in HFD-fed (HFD) mice were significantly higher than in standard chow-fed (chow) mice (Table **1** ). As a result, HFD mice exhibited significantly higher HOMA-IR than chow mice. These results suggested that the systemic insulin sensitivity is decreased, accompanied with hypertriglyceridemia in mice fed with HFD. Therefore, in order to know the systemic insulin sensitivity in the HFD-fed mice,

Table 1 Fasting glucose, TG and insulin levels in HFD and chow mice

| | *Glucose (mg/dl)* | *TG (mg/dl)* | *Insulin (pg/ml)* | *HOMA-IR* |
|---|---|---|---|---|
| Chow (n=5) | 125 ± 11 | 92 ± 8 | 349 ± 86 | 2.2 ± 0.3 |
| HFD (n=4) | 114 ± 3 | 140 ± 12[a] | 1387 ± 335[b] | 9.0 ± 2.0[b] |

Data are expressed as means ± SEM. [a]$p < 0.05$, [b]$p < 0.01$ *vs.* chow

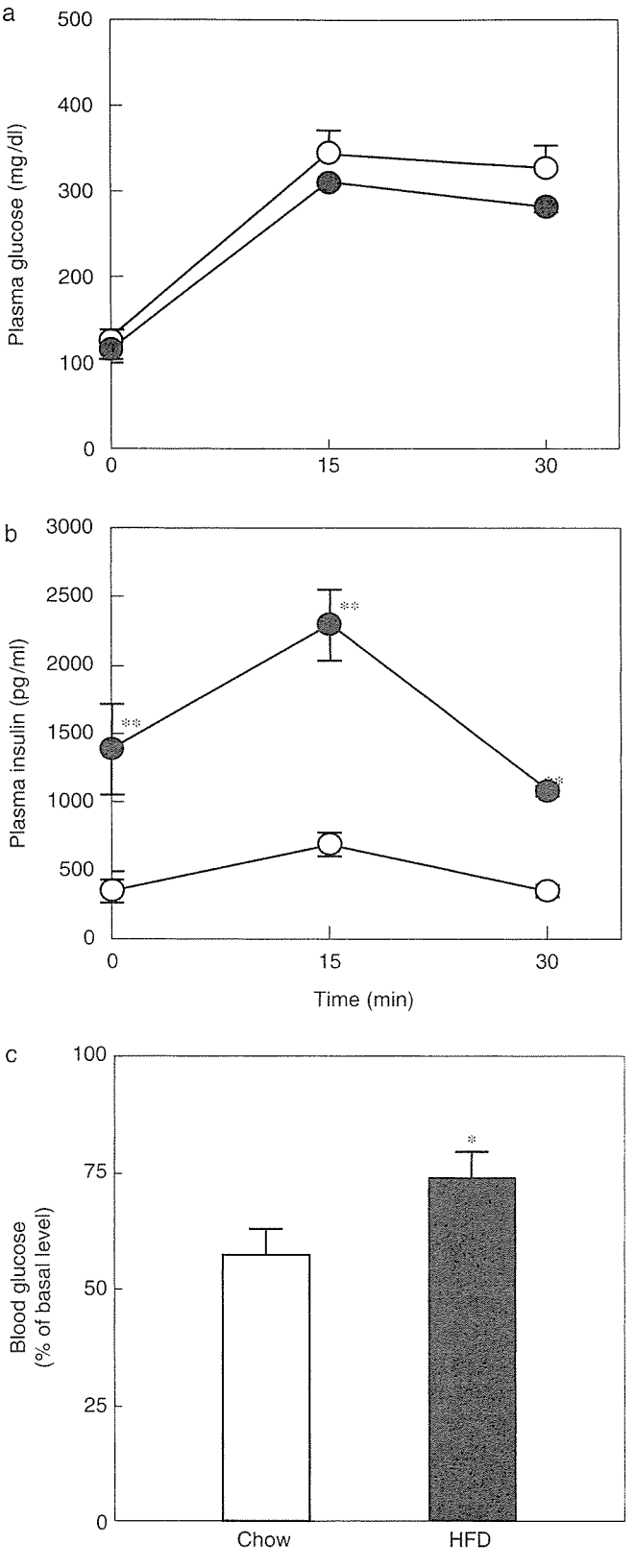


Fig. 2 Blood glucose (**a**) and plasma insulin (**b**) levels in standard chow-fed (chow, open circles) and HFD-fed (HFD, closed circles) mice after oral glucose administration. Data are expressed as means ± SEM; $n = 8$ and $n = 6$ for chow and HFD mice respectively. $^{**}p < 0.01$ *vs.* chow. **c**. Blood glucose levels in standard chow-fed (chow) and HFD-fed mice at 15 min after intraperitoneal insulin injection. Data (means ± SEM) are expressed as relative to basal (0 min) glucose levels, assigning a value of 100 % to the basal levels; $n = 4$ and $n = 6$ for chow and HFD mice respectively. $^{*}p < 0.05$ *vs.* chow.

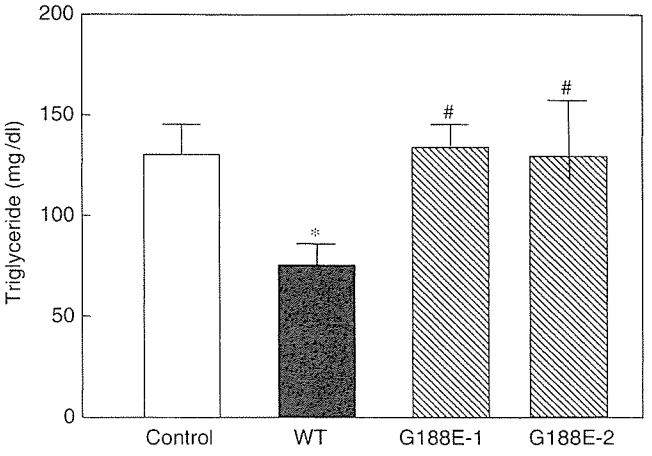


Fig. 3 Effect of overexpression of catalytically active or inactive LPL on plasma TG levels in HFD-fed mice. ICR nude mice were injected with CHO-WT-LPL (WT mice), CHO-G188E-LPL (G188E mice-1 and G188E mice-2), or CHO cells (control mice). Values are means ± SEM; $n = 9$ for each group. $^{*}p < 0.05$ *vs.* control. $\#p < 0.05$ *vs.* WT.

glucose and insulin tolerance tests were performed. Although there was no significant difference in blood glucose levels between chow and HFD mice (Fig. **2a**), HFD mice exhibited significantly higher plasma insulin levels than chow mice after oral glucose administration (Fig. **2b**). Insulin tolerance tests in 16 h fasted mice showed that the ratios of blood glucose levels at before and 15 min after insulin injection in HFD mice were significantly higher than those in chow mice (Fig. **2c**). Thus, HFD mice exhibited significantly higher insulin AUC values than chow mice (52.9 ± 5.9 *vs.* 15.6 ± 1.5 ng · min/ml).

### Effect of catalytically active and inactive LPL on insulin sensitivity in HFD mice

In order to investigate the effect of LPL overexpression on insulin sensitivity in HFD mice, ICR nude mice were transplanted subcutaneously with each of the LPL-producing cells (CHO-WT-LPL or CHO-G188E-LPL). Ten weeks after the cell inoculation, the mice had plasma human LPL concentrations of 12.6 ± 1.9 ng/ml (WT mice), 5.9 ± 1.1 ng/ml (G188E mice-1), and 11.9 ± 2.1 ng/ml (G188E mice-2). In control mice transplanted with CHO (mock) cells, no human LPL protein was detected by ELISA. As shown in Fig. **3**, plasma TG levels in WT mice were significantly lower than those in control mice. On the other hand, the TG levels both in G188E-1 and G188E-2 mice were not significantly different from that in control mice. Next, insulin tolerance tests were performed 10 weeks after transplantation. The ratios of blood glucose levels at before, and 15, 30 min after insulin injection for control, WT and G188E mice are shown in Fig. **4a**. WT mice exhibited significant lower ratios of glucose levels (54 %) than control mice (74 %) at 15 min after insulin injection (Fig. **4b**). Notably, G188E-1 and G188E-2 mice recovered the ratio to 55 % and 51 %, respectively, which are not significantly different from that of WT mice. These results show that both WT and G188E mutant LPL recover the decrease in insulin sensitivity caused by HFD feeding in mice.

## Discussion

In this study, we have shown the effects of catalytically active and inactive LPL on insulin sensitivity using mice fed with a HFD

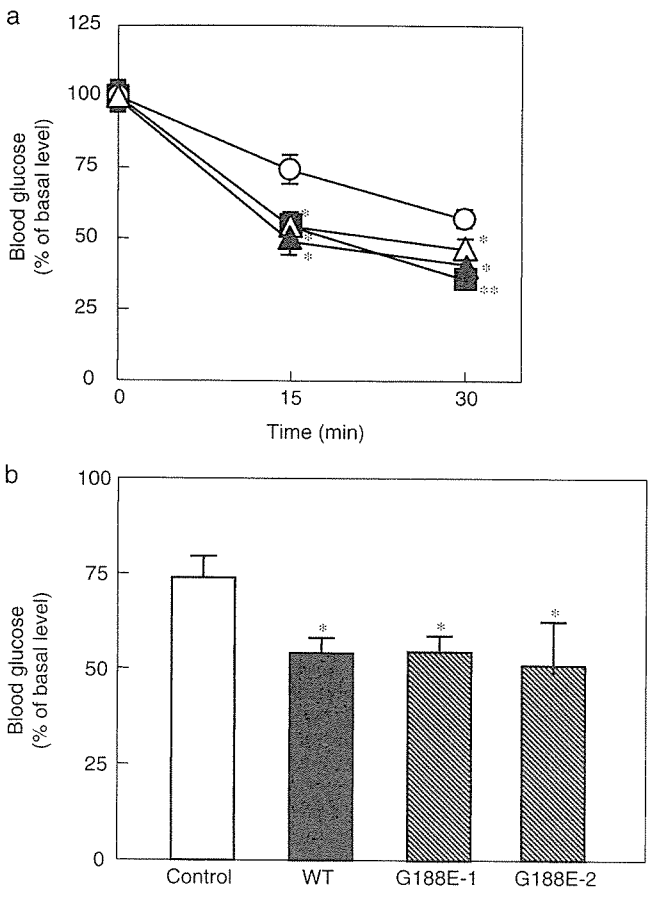


Fig. 4 Effect of overexpression of catalytically active or inactive LPL on insulin sensitivity in HFD mice. **a**. Blood glucose levels were determined for control (open circle), WT (closed square), G188E-1 (open triangle) and G188E-2 (closed triangle) mice at immediately before (0 min) and at 15, 30 min after intraperitoneal insulin injection. **b**. Data (means ± SEM) at 15 min after the injection are expressed as relative to basal (0 min) glucose levels, assigning a value of 100 % to the basal levels; $n = 6$, $n = 8$, $n = 9$ and $n = 9$ for control, WT, G188E-1, and G188E-2 mice respectively. $^{*}p < 0.05$ *vs.* control. $^{**}p < 0.01$ *vs.* control.

diet. In cultured skeletal muscle cells, insulin-induced IRS-1 phosphorylation and glucose uptake are upregulated by addition of catalytically inactive G188E-LPL protein [15,19], which binds to the cells as well as wild type LPL. Using our cell transplantation-based LPL-overexpressing method, we identified that LPL has the ability to improve insulin sensitivity impaired by HFD feeding through actions exclusive of TG hydrolysis. These findings suggest that the non-enzymatic function of LPL improves insulin sensitivity *in vivo*.

In order to establish a mouse model of impaired insulin sensitivity, ICR nude mice were fed on HFD diet for 10 weeks. In accordance with the observations that high-fat feeding induces obesity and diabetes in C57BL/6J mice [20,21], after 10 weeks' feeding, HFD mice exhibited significantly higher plasma insulin levels than standard chow mice before and after glucose administration (Table **1**, Fig. **2a**). Furthermore, insulin-induced reduction of blood glucose levels was impaired in HFD mice compared to chow mice (Fig. **2c**). Taken together, HFD feeding for 10 weeks seemed to be enough to reduce insulin sensitivity of ICR nude mice.

Several human-LPL-overexpressing animal models have been previously reported [22–26]. However, LPL functions on insulin resistance in mature mice have not been fully clarified because of the difficulty of growth when LPL function was disturbed. In this context, the cell transplantation method into mature mice makes it possible to evaluate the overexpression of wild type and catalytically inactive LPL on the progression or regression of insulin resistance in mice. Our LPL-overexpressing model mice exhibited protection against HFD-induced impaired insulin sensitivity. This is consistent with the recent findings using rabbits that systemic overexpression of LPL improves insulin sensitivity [25,26]. Systemic, but not tissue-specific [22–24], overexpression of LPL in mature mice may lead to increase in insulin-induced response.

In conclusion, the present study has revealed the beneficial and novel effects of LPL on the pathogenesis of insulin resistance. Further characterization needs to be performed to elucidate the molecular mechanisms in which LPL protein modulates insulin sensitivity by direct association with cells. The novel noncatalytic function of LPL observed in this study may contribute to clarify the pathogenesis of insulin resistance, in addition to the disturbed hydrolytic activity, in metabolic syndrome.


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# Modulation of Smooth Muscle Cell Migration by Members of the Low-Density Lipoprotein Receptor Family

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*Abstract*—Low-density lipoprotein receptor family members (LRs) play a key role in the catabolism of many membrane-associated proteins, such as complexes between proteinases and their receptors, in addition to being involved in lipoprotein metabolism as suspected by the hitherto well-established functions of low-density lipoprotein receptor, in a variety of tissues. Recent studies using receptor-deficient or -overexpressing animals and cells have suggested that certain LRs are important regulators of the migration (and proliferation) of vascular smooth muscle cells (SMCs). LR expression is markedly induced in intimal or medial SMCs during the formation of atherosclerotic lesions. Because LRs can modulate the activity of the urokinase-type plasminogen activator (uPA) receptor and possibly of the platelet-derived growth factor (PDGF) receptor, LRs may influence the migration of SMCs through functional modulation of these membrane receptors. Therefore, SMC migration may be regulated by time-restricted expression of LRs. In agreement with the concept of functional interaction between LRs and membrane signaling receptors, a negative regulator of uPA receptor protein catabolism, LR11, has been identified. Statins modulate the PDGF-induced migration of intimal SMCs via the LR11/uPA receptor cascade. Selective modification of the LRs/uPA receptor/PDGF receptor systems in SMCs may be important for suppression of atherosclerotic plaque formation as well as for preventing intimal thickening after angioplasty. (***Arterioscler Thromb Vasc Biol.* 2006;26:1246-1252.**)

**Key Words:** LDL receptor family ■ smooth muscle cells ■ migration ■ LR11 ■ urokinase-type plasminogen activator receptor ■ PDGF receptor

The members of the low-density lipoprotein receptor family (LRs) are characterized by distinct functional domains present in characteristic numbers and arrangements (Figure 1). The common structural domains in most LRs are the so-called low-density lipoprotein (LDL) receptor ligand binding repeats (type A), epidermal growth factor precursor homology repeats (type B1 and B2), epidermal growth factor precursor homology repeats with a consensus tetrapeptide, Tyr-Trp-Thr-Asp, and in the cytoplasmic region, signals for receptor internalization via coated pits. These LRs discovered to date are the LDL receptor, LDL receptor-related protein-1 (LRP-1), megalin, the very low-density lipoprotein (VLDL) receptor/LR8, apolipoprotein E receptor 2/LR8B, LR11, and, most recently, LRPs 3 through 7.[1–3] LRP-1 and megalin are giant LRs in which the amino acid sequence contains multiple repeats of each functional component of the LDL receptor.[4,5] The domain structures of VLDL receptor/LR8 and apolipoprotein E receptor 2/LR8B are most similar to that of the LDL receptor.[6–8] LRs indeed show considerable sequence identity (70% to 100%) between molecules harboring common structures and among a wide range of species. Such sequence conservation is thought to indicate evolution from an ancestral gene by duplication or exon shuffling. The avian VLDL receptor/LR8 is essential for reproduction as a receptor for the yolk accumulation.[8,9]

LRs play a key role in lipoprotein metabolism, as demonstrated by the well-established actions of the LDL receptor in a variety of tissues.[1] Extensive functional analyses have also revealed that LRs play an important role in the catabolism of many membrane-associated proteins such as complexes between proteinases and their receptors.[1–3] Recent studies using receptor-deficient or -overexpressing animals and cells have suggested that certain LRs are also important as regulators of the migration (and proliferation) of various cells such as fibroblasts, neurons, and vascular smooth muscle cells (SMCs).[10–17]

Histochemical studies have revealed that the expression of LRs, as well as scavenger receptors, is markedly induced during the development of atherosclerotic lesions.[1,18] For instance, the VLDL receptor/LR8 is highly expressed by SMCs, macrophages, and endothelial cells in rabbit atherosclerotic lesions, whereas the LDL receptor is not abundant in arterial walls.[18,19] LRP-1 expression is also induced in atheromatous plaques.[18–20] We identified strong LR11 expression inside plaques, particularly by intimal SMCs located at the interface between intima and media.[21,22] In addition, LRP-1B

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