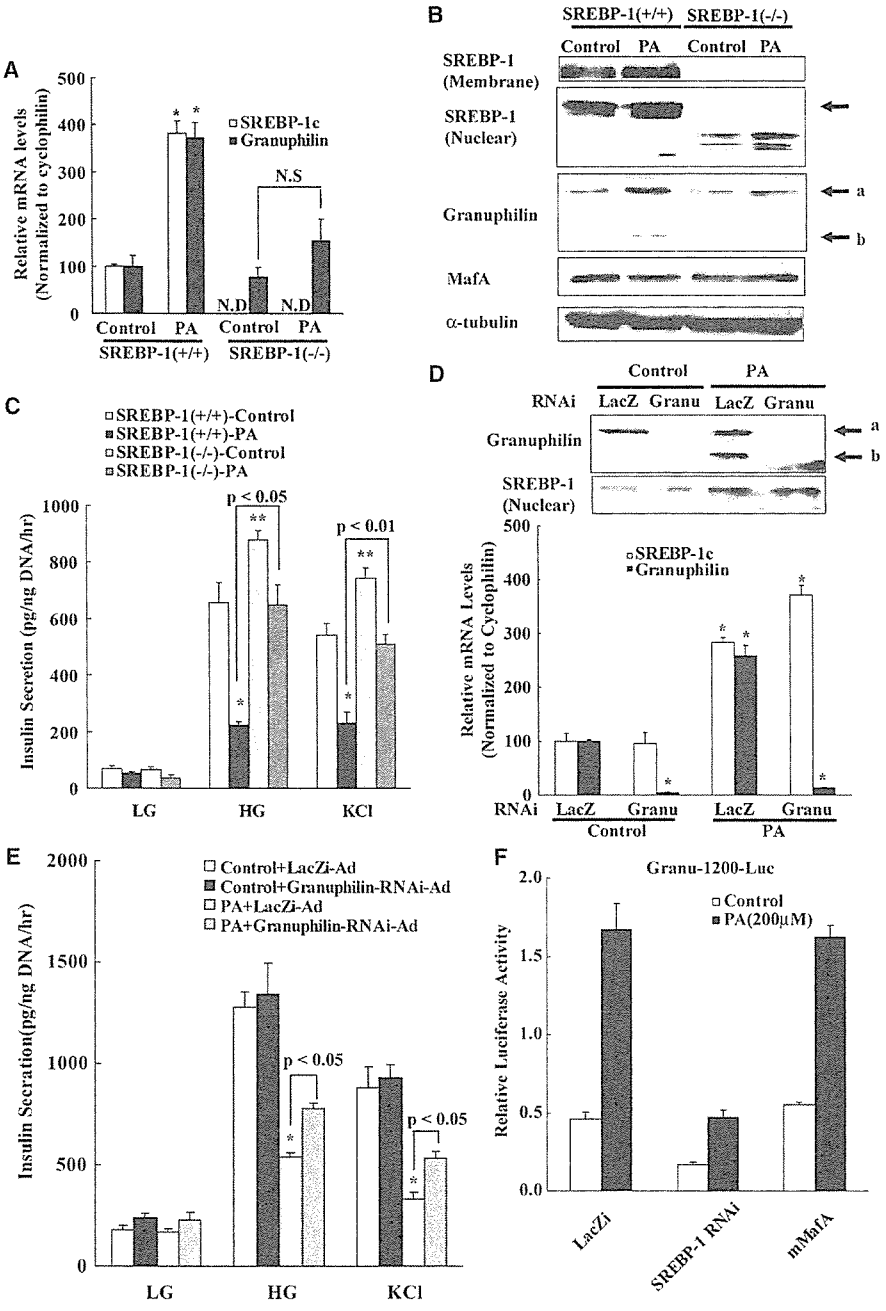

Figure 6. Involvement of granuphilin in insulin secretion from isolated murine islets treated with palmitate

Islets were isolated from male age-matched (13- to 17-week-old) SREBP-1-null, SREBP-1(−/−), and wild-type littermate, SREBP-1 (+/+), mice. The islets were incubated with 400 μM palmitate (PA) (**A–E**) and were infected with adenoviral-LacZ-RNAi or -granuphilin-RNAi (500 MOI) for 48 hr (**D** and **E**).

A) mRNA levels of SREBP-1c (white bars) and granuphilin (black bars) from the indicated islets as estimated by real-time RT-PCR. * $p < 0.01$ (versus SREBP-1(+/+)-Control). ND, not detectable.

B) Immunoblot analysis of SREBP-1, MafA, and mouse granuphilin from indicated islets with α-tubulin as a loading control.

C) LG-, HG-, and KCl-stimulated insulin secretion in SREBP-1(+/+)-control group (white bars), -PA group (black bars), SREBP-1(−/−)-control group (light gray bars), and -PA group (dark gray bars). *$p < 0.01$ and **$p < 0.05$ (versus SREBP-1(+/+)-Control).

D) Upper panel: immunoblot analysis of mouse granuphilin and SREBP-1. Lower panel: expression of SREBP-1c (white bars) and granuphilin (black bars) from the indicated islets as estimated by real-time RT-PCR. *$p < 0.01$ (versus Control-LacZ-RNAi).

E) LG-, HG-, and KCl-stimulated insulin secretion in islets untreated (Control) or treated with palmitate (PA), and infected with Adeno-LacZ-RNAi (LacZi-Ad) or Adeno-granuphilin RNAi (granuphilin RNAi-Ad). *$p < 0.01$ (versus Control-LacZi).

F) Effects of SREBP-1-RNAi and mutant MafA on Granu-1200-Luc in PA treated Min6 cells. The luciferase activity was normalized to *Renilla* luciferase activity and performed in triplicate.

Results were normalized to cellular DNA content (**C** and **E**) and are expressed by mean ± SEM. Studies were performed in triplicate with sets of islets pooled from three to four mice per replicate.

develop in these situations. We also show that the granuphilin promoter is activated by MafA. MafA has recently been established to be crucial for normal structure and function of β cells and insulin gene expression (Hagman et al., 2005; Kajihara et al., 2003) and thus is likely to be responsible for β cell-specific basal expression of granuphilin. Supportively, basal expression of granuphilin was severely diminished in islets from MafA knockout mice (S.T., unpublished data), while it was only 50% reduced in homozygous SREBP-1-null islets.

Granuphilin activation inhibits insulin secretion

Granuphilin has been thought to be a component of the fusion machinery for exocytosis of insulin granules. However, activation of granuphilin leads to inhibition of insulin secretion. Although the exact molecular mechanisms involved are yet unknown, various gain- and loss-of-function experiments from our current data as well as published reports strongly suggest that this Rab27a effector is a potent inhibitor of insulin secretion (Coppola et al., 2002; Gomi et al., 2005; Torii et al., 2002). SREBP-1c and thus granuphilin was activated in islets from SREBP-1c transgenic, KK-Ay, ob/ob, and DIO mice, as well as in islets treated with PA. Decreased KSIS in these islets were all restored by granuphilin knockdown, demonstrating that granuphilin plays a crucial role in the disturbance of insulin secretion post-ATP production. In combination with granuphilin activation, syntaxin-1a and Munc18-1, components of this docking machinery, were decreased by SREBP-1c activation and thus, may also be involved in the secretion impairment. Dysregulation

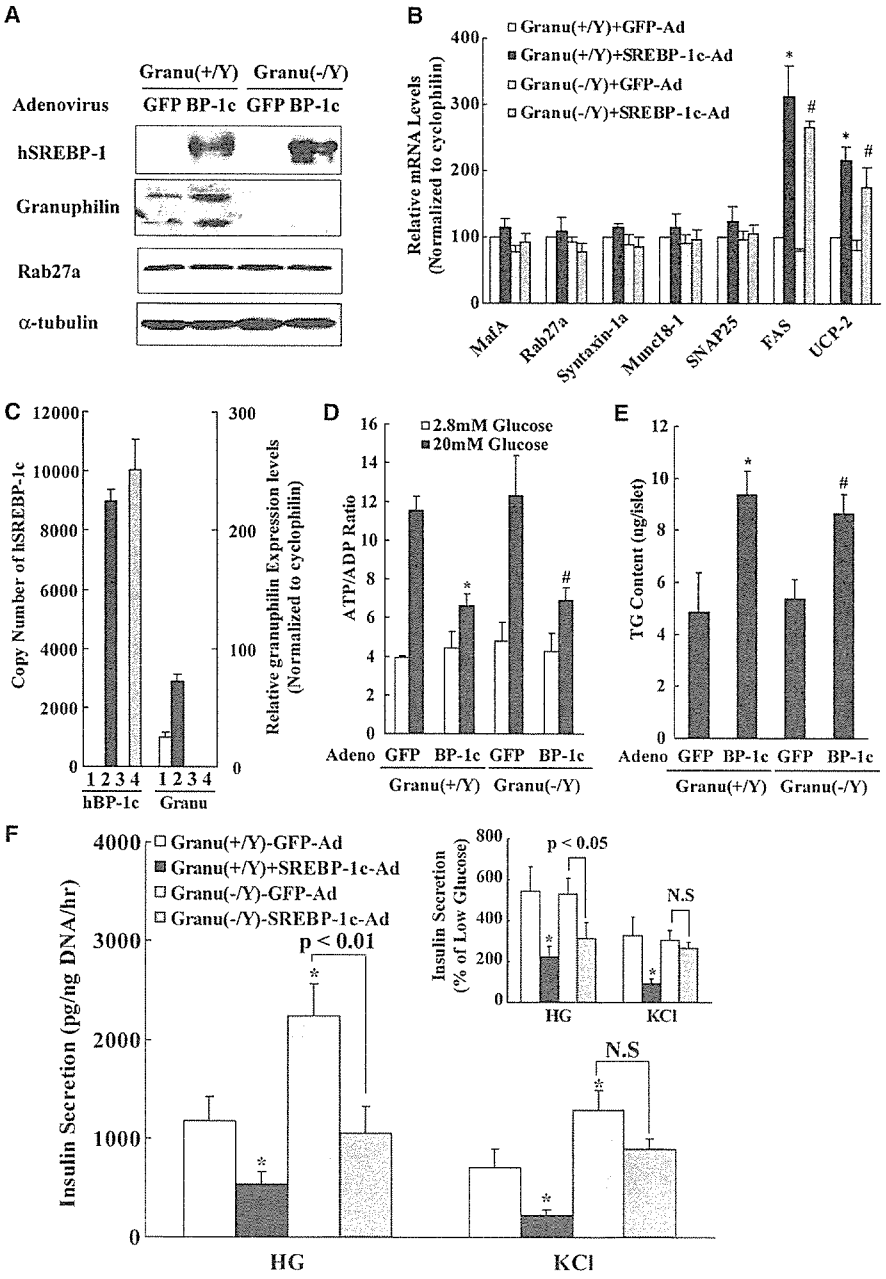

Figure 7. Effect of overexpression of nuclear SREBP-1c on insulin secretion in pancreatic islets from granuphilin-null mice

Islets were isolated from male age-matched (30- to 32-week-old) granuphilin-null (Granu(−/Y)) and wild-type littermate (Granu(+/Y)) mice. The islets were infected with adenoviral-GFP or -SREBP-1c (500 MOI) for 48 hr.

A) Immunoblot analysis of SREBP-1, granuphilin, and Rab27a from the indicated islets with α-tubulin as a loading control.

B) mRNA levels of indicated genes from the indicated islets as estimated by real-time RT-PCR. Granu(+/Y)-GFP (white bars), Granu(+/Y)-SREBP-1c (black bars), Granu(−/Y)-GFP (light gray bars), and Granu(−/Y)-SREBP-1c (dark gray bars).

C) mRNA levels of human SREBP-1c (hBP-1c) and granuphilin (Granu) from the indicated islets as estimated by real-time RT-PCR. Lane 1, Granu(+/Y)-GFP; lane 2, Granu(+/Y)-SREBP-1c; lane 3, Granu(−/Y)-GFP; lane 4, Granu(−/Y)-SREBP-1c.

D) Cellular ATP/ADP ratio from the indicated islets.

E) Cellular TG content from the indicated islets.

F) Upper panel: HG and KCl-stimulated insulin secretion ratio, normalized by LG-stimulated insulin secretion. Lower panel: HG- and KCl-stimulated insulin secretion in Granu(+/Y)-GFP (white bars), Granu(+/Y)-SREBP-1c (black bars), Granu(−/Y)-GFP (light gray bars), and Granu(−/Y)-SREBP-1c (dark gray bars).

Results were normalized to cellular DNA content (**F**) and are expressed by mean ± SEM. *p <0.01 (versus Granu(+/Y)-GFP) and #p < 0.01 (versus Granu(−/Y)-GFP). Studies were performed in triplicate with sets of islets from 3–4 mice per replicate.

of these three proteins may impair insulin secretion by disturbing the balance of vesicle transport component concentrations.

Potential involvement of SREBP-1c/granuphilin pathway in β cell lipotoxicity

We showed that upregulation of granuphilin is observed in β cells not only from transgenic mice overexpressing SREBP-1c but also from obesity-associated insulin resistant diabetic mice such as KK-Ay, ob/ob, and DIO mice, leading to a reduction of KSIS. It has been believed that impaired GSIS, due to deterioration of glucose metabolism characterized by decreased ATP/ADP ratio and/or insulin production in β-cells, is responsible for the insulin secretion defect in type 2 diabetes (Weir and Bonner-Weir, 2004). Our current data demonstrate that additional disturbances can also occur at the exocytosis stage of insulin secretion in diabetic models. Impaired KSIS was primarily mediated through activation of granuphilin, providing another mechanism for development of diabetes. Overexpression of granuphilin also inhibited GSIS in normal islets, but this was because the path to GSIS also includes the process of KSIS, and disturbance causing decreased KSIS should result in impaired GSIS as well. Supportively, granuphilin knockdown efficiently restored impaired KSIS in SREBP-1c transgenic and diabetic islets but only partially restored GSIS, indicating that the SREBP-1c/granuphilin effect was specific to the process post-ATP production.

Our data also provide further evidence that SREBP-1c is intimately involved in β cell dysfunction. In islets from the mice with lipotoxicity and diabetes, SREBP-1c is upregulated and contributes to disturbances in ATP production leading to impaired GSIS

and also to upregulation of granuphilin leading to attenuated KSIS. Various approaches using gene engineering and adenovirus consistently supported this hypothesis. Thus, the SREBP-1c/granuphilin pathway should be added to the list of potential mechanisms for β cell lipotoxicity in diabetes and could be a potential target for therapy of obesity-related diabetes. In contrast, it was reported that granuphilin as well as Rab27a and 3 were suppressed in a glucotoxic state of INS-1 cells through induction of ICER (Abderrahmani et al., 2006). Further studies are needed to elucidate the precise molecular role of granuphilin in insulin secretion and to determine the extent to which granuphilin contributes to diabetes in humans.

Experimental procedures

Materials

All chemical compounds were obtained from Sigma chemicals. Enhanced chemiluminescence Western blot detection kit and redivue [α-^{32}P] dCTP were purchased from Amersham Pharmacia and restriction enzymes were from Takara Bio Inc.

Animals

All animal studies were approved by the Animal Care Committee of University of Tsukuba. Male C57BL/6, ob/ob, db/m, and db/db mice were purchased from Charles River Laboratories. KK and KK-Ay were purchased from Clea. RIP-human nuclear SREBP-1c transgenic mice (Takahashi et al., 2005), SREBP-1-null mice (Shimano et al., 1997b), and Granuphilin-null mice (Gomi et al., 2005) were generated as previously described. Animals were adapted to environments for 1 week before isolation of pancreatic islets. The mice were housed in colony cages and maintained on a 12 hr light/12 hr dark cycle and given free access to water and a standard chow diet (MF, Oriental yeast).

Expression plasmids

All expression vectors were produced as previously described (Amemiya-Kudo et al., 2000, 2005; Ide et al., 2004; Kajihara et al., 2003; Yamamoto et al., 2004).

Reporter plasmid

The reporter plasmid Granu-1200-Luc contains a fragment of the granuphilin promoter from −1200 to +87 bp cloned into the MluI/XhoI sites of the pGL3 basic vector (Promega) containing the coding sequences of firefly luciferase cDNA. Other constructs were produced by PCR using this construct as a DNA template, and the PCR products were inserted into pGL3 basic vector. The primers used for PCR were as follows: 5′ primers Granu-1200-Luc 5′-taagcgttgagaaatgaatg-3′, Granu-500-Luc 5′-agggttgcttacagggcgat-3′, Granu-300-Luc 5′-tcgctgaacaaaaaggcaag-3′, Granu-265-Luc 5′-ttcctacccc caccccgac-3′, Granu-174-Luc 5′-tgctaactcaggaggaaattc-3′, Granu-150-Luc 5′-aattcctccagtgccttaag-3′, and 3′ primer 5′-ggtccgggtgccgaatg-3′. Restriction sites MluI and XhoI were added to each 5′ primer and 3′ primer, respectively. The site-directed mutagenesis constructs mSRE- and mMARE-Luc were produced by PCR with the following primers: mSRE-5′ 5′-attcccat tcc*gta*ccccaccccgacccc-3′, -3′ 5′-ggggtcgggggtgggg*tac*ggaatgggaat-3′, mMARE-5′ 5′-tcaaactct*cag*aactcaggaggaaattcc-3′, and -3′ 5′-ggaatttcctcc tgagttc*tga*gagtttga-3′. SRE-Luc was produced as previously described (Amemiya-Kudo et al., 2000).

Cell cultures, transfection, and luciferase assays

Min6 cells were cultured at 37°C in atmosphere of 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) with 25 mM glucose supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and β-mercaptoethanol. Each expression plasmid (0–0.5μg), luciferase reporter plasmid (0.2 μg), and pSV40-*Renilla* plasmid (0.05 μg) were cotransfected using the LipofectAMINE reagent (Invitrogen) according to manufacture's instructions. Min6 cells were seeded in 24-well plates at a density of 3 × 10^5 cells/well. The total amount of DNA was adjusted to 0.5–1 μg/well with empty vector DNA. After 48 hr of transfection, cells were washed with phosphate-buffer saline (PBS) and harvested. Luciferase assays were carried out according to the manufacture's instructions (Promega), and luciferase activity was quantified by using ALOVA 1420 multilabel counter (Perkin Elmer Life Sciences). As the internal standard, SV40 *Renilla* luciferase control vector was also cotransfected to normalize for transfection efficiency.

Gel mobility shift assay

Gel mobility shift assays (EMSA) were performed as previously described (Amemiya-Kudo et al., 2005; Nakagawa et al., 2006; Yamamoto et al., 2004). In brief, recombinant SREBPs (SREBP-1a, -1c,and -2) and MafA proteins were produced using the T_NT T7 quick-coupled transcription/translation system (Promega). The reaction mixture were incubated with in vitro synthetic protein lysate and then analyzed on 4% polyacrylamide gels. Super-shift reactions were performed by adding antibody (anti-SREBP-1, sc-8984 Santa Cruz) to the reaction mixture. The DNA probes for EMSA are followed: granuphilin-SRE, aactttattcccattcctacccccacccccgaccccaatc atctaagaga, granuphilin-mSRE, aactttattcccattcc*gta*ccccaccccgaccccaat catctaagaga, granuphilin-MARE, cctcaaactctgctaactcaggaggaaattcctccagtg cc, and granuphilin-mMARE, cctcaaactcc*ag*taactcaggaggaaattcctccagtgcc.

Isolation of mouse pancreatic islets and insulin secretion experiments

Isolation of islets from mice was carried out using the Ficol-Conray protocol as previously described(Scharp et al., 1973; Takahashi et al., 2005). Briefly, 4 mg/ml collagenase was injected into the pancreatic duct and incubated at 37°C for 20 min. The islets were subsequently purified by Ficol gradient and hand picking. The remaining islets were cultured for 2 hr at 37°C in a humidified atmosphere containing 5% CO_2 and in regular RPMI-1640 medium supplemented with 10% FCS (pH 7.4) prior to the experiments. Insulin release from islets was measured as previously described (Takahashi et al., 2005). In brief, groups of 10 islets of similar size for each condition were preincubated in Krebs-Ringer bicarbonate buffer (KRBH [pH 7.4]) containing 0.5% BSA at 2.8mM glucose for 30 min. Islets were then incubated with 1 ml of KRBH with 0.5% BSA at 2.8mM glucose (low glucose; LG), 20mM glucose (high glucose; HG), 30mM KCl with 2.8 mM glucose (KCl), 3mM Arginine with 2.8 mM glucose, forscolin (10μM) with 2.8 mM glucose, or TPA (0.5 μM) with 2.8 mM glucose, respectively. Subsequently, medium were replaced with fresh medium for insulin determination followed by total islet insulin extraction by 0.5ml of cold acid ethanol mixture (75% ethanol with 0.2M HCl). Hoechst-33258 staining of sonicated islets was performed to determine the islet DNA content.

Determination of ATP/ADP ratio and triglyceride contents of islets

ATP and ADP content in isolated islets were determined as previously described (Schultz et al., 1993; Takahashi et al., 2005). In brief, after preincubation at 37°C for 30 min in KRBH buffer containing 2.8 mM glucose, groups of 25 islets were incubated at 37°C for 30 min in KRBH buffer containing 20 mM glucose. The conversion from ADP to ATP was performed in the reaction buffer (20mM HEPES and 3mM $MgCl_2$ [pH 7.75]) containing 2.3 U/ml pyruvate kinase and 1.5mM phosphoenolpyruvate at room temperature for 15 min. ATP and ADP were extracted from islets with 100 μl of 5% trichloroacetic acid (TCA). After centrifugation, the supernatants were neutralized with NaOH. ATP content was measured using CellTiter-Glo luminescent cell viability assay kit (Promega).

Real-time PCR and immnoblot analysis

Comparative analysis of mRNA levels was performed with fluorescence-based real-time PCR. Total RNA extraction and first-strand cDNA synthesis were previously as described (Takahashi et al., 2005). Quantitative real-time PCR was performed using Sybr-Green Dye (Nihon Gene) in an ABI 7000 PCR instrument (Applied Biosystems). Relative abundance for each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of a cDNA sample and normalized to cyclophilin. Primer sequences are available upon request. For immnoblot blots, total or nuclear cell extracts of isolated islets (Hagman et al., 2005; Takahashi et al., 2005) and nuclear extracts from Min6 cells (Amemiya-Kudo et al., 2005) were prepared as described previously, separated by SDS-PAGE, transferred to immobilon-P membranes (Millipore), and were probed with polyclonal anti-SREBP-1 (sc-8984), anti-Rab27a (sc-22756), anti-α-tubulin (sc-5282, Santa Cruz), anti-MafA (A-300-611, Bethyl Lab. Inc.), anti-syntaxin-1a (S1172, Sigma), anti-Vamp2 (627724, Calbiochem), anti-Rab3 (R35520), anti-Munc18-1 (M32320), and

anti-SNAP25 (S35020, BD) antibodies. Detection was performed using an ECL advance Western blotting ECL detection kit and Hyperfilm (Amersham Biosciences). Anti-granuphilin-a/b antibody was as previously described (Gomi et al., 2005).

Northern blot analysis

Northern blot analysis was performed as previously described (Amemiya-Kudo et al., 2005; Matsuzaka et al., 2004). cDNA probe for granuphilin was prepared from reverse transcriptase for PCR of Min6 cells total RNA with the following primers: 5′-cgagatggaaagggatttga-3′ for sense and 5′-gtctgc tgaaggagggactg-3′ for antisense of granuphilin.

Preparation of recombinant adenovirus

The construct of granuphilin-a coding cDNA was generated by PCR amplification and into the pShuttle-CMV vector (Ide et al., 2004; Nakagawa et al., 2006). The following primers were used: 5′ primer, 5′-atgtcggagatactagacc tctctt-3′, and 3′ primer 5′-catacacccagcttctgcttgaccat-3′. Generation of recombinant adenoviral plasmid was produced by homologous recombination with the pAdEasy-1 plasmid. The siRNA construct for mouse granuphilin and SREBP-1 was generated in the coding sequence; Granuphilin-RNAi-784 5′-gagcgagagtctggatagctaca-3′, Granuphilin-RNAi-1955 5′-aggctggaagatct acaacaaat-3′, and SREBP-1-RNAi 5′-ggcaaaggaggcactacag-3′. Oligonucleotide containing this sequence was subcloned into U6/RNAi empty vector (Invitrogen). Granuphilin RNAi adenoviruses were generated using BLOCK-iT Adenoviral RNAi Expression System (Invitrogen). Production of recombinant adenoviruses was performed by CsCl gradient centrifugation as previously described (Ide et al., 2004).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Amemiya-Kudo et al., 2005; Nakagawa et al., 2006). To amplify the granuphilin promoter region containing SRE and MARE, the following primer sets were used: for SRE 5′-gtactttactattggacaac-3′ for sense, 5′-ccttaaggcactggaggaatttc-3′ for antisense, and for MARE 5′-gtgagcaaagata gactaga-3′ for sense, 5′-tggagcggggaggagctggactc-3′ for antisense. The PCR conditions were 5 min at 95°C and 30 cycles of 30 s at 94°C, 30 s at 57°C and 1 min 72°C for SRE region, and 10 min at 94°C and 30 cycles of 10 s at 98°C, 30 s at 54°C, and 1 min 72°C for MARE region.

Palmitate treatment

Palmitate was dissolved in ethanol as 100 mM stock solution with 0.5% fatty acid free BSA (Sigma). For using experiments, palmitate was diluted in DMEM supplemented with 0.5% fatty acid free BSA to a final concentration of 400 μM for islets study or 200 μM for Luc-assay.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using analysis of variance (ANOVA).

Supplemental data

Supplemental data include five figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/2/143/DC1/.

Acknowledgments

This work was supported by grants in aid from the Ministry of Science, Education, Culture, and Technology of Japan.

Received: February 2, 2006
Revised: May 5, 2006
Accepted: June 28, 2006
Published: August 8, 2006

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methods

A novel method for measuring human hepatic lipase activity in postheparin plasma

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Abstract The objective of this study was to establish a hepatic lipase (HL) assay method that can be applied to automatic clinical analyzers. Seventy-four hyperlipidemic subjects (men/women 45/29) were recruited. Lipase activity was assayed measuring the increase in absorbance at 546 nm due to quinonediimine dye production. Reaction mixture R-1 contained 50 mM Tris-HCl (pH 9.5), 0.5 mM glycerol-1,2-dioleate, 0.4% (unless otherwise noted) polyoxyethylene-nonylphenylether, 3 mM ATP, 3 mM $MgCl_2$, 1.5 mM $CaCl_2$, monoacylglycerol-specific lipase, glycerol kinase, glycerol-3-phosphate oxidase, 0.075% *N,N*-bis-(4-sulfobutyl)-3-methylaniline-2 Na, peroxidase, ascorbic acid oxidase. Reaction mixture R-2 contained 50 mM Tris-HCl (pH9.5), 0.15% 4-aminoantypirine. Automated assay for activity was performed with a Model 7080 Hitachi analyzer. In the lipase assay, 160 μl of R-1 was incubated at 37°C with 3 μl of samples for 5 min, and 80 μl of R-2 was added. Within-run coefficient of variations was 0.9–1.0%. Calibration curve of lipase activity was linear ($r = 0.999$) between 0 and 320 U/l. Analytical recoveries of purified HL added to plasma were 96.6–99.8%. HL activity in postheparin plasma measured in this method had a closer correlation with HL mass by a sandwich ELISA ($r = 0.888$, $P < 0.0001$) than those in the conventional method using [^{14}C-]triolein ($r = 0.730$, $P < 0.0001$). This assay method for HL activity can be applied to an automatic clinical analyzer.—Imamura, S., J. Kobayashi, S. Sakasegawa, A. Nohara, K. Nakajima, M. Kawashiri, A. Inazu, M. Yamagishi, J. Koizumi, and H. Mabuchi. **A novel method for measuring human hepatic lipase activity in postheparin plasma.** *J. Lipid Res.* **2007.** 48: 453–457.

Supplementary key words dioleoylglycerol • quinonediimine dye • automatic clinical analyzer

Hepatic lipase (HL), a lipolytic enzyme that is a secreted glycoprotein, is synthesized by hepatocytes and bound to heparin sulfate proteoglycans at the surface of liver sinusoidal capillaries. HL plays a major role in lipoprotein metabolism as a lipolytic enzyme that hydrolyzes triglycerides (TGs) and phospholipids in chylomicron remnants, intermediate-density lipoproteins (IDLs), and high-density lipoproteins (HDLs). Patients with HL deficiency present with hypercholesterolemia or hypertriglyceridemia and accumulate β-very-low-density lipoproteins (VLDLs), chylomicron remnants, IDLs, TG-rich low-density lipoproteins (LDLs), and HDLs (1–7).

To date, the only available methods for measuring HL activity in postheparin plasma (PHP) have used ^{3}H- or ^{14}C-labeled trioleoyl glycerol as substrates in the presence of 1 M NaCl, because lipoprotein lipase (LPL) activity is known to be completely inhibited and remaining activities are considered to correspond to HL activity under these conditions. This assay procedure is complicated and does not appear to be suitable for routine work (8, 9). Several years ago, a method for measuring HL protein mass by ELISA in PHP was developed (10).

Still, to diagnose HL deficiency, demonstrating the lack of HL activity is considered to be essential. In the present study, therefore, we have developed and established, using dioleoylglycerol as a substrate, a novel and simple assay system for measuring HL activity in PHP that can be applied to an automatic clinical analyzer.

MATERIALS AND METHODS

Subjects

Seventy-four hyperlipidemic subjects (men/women 45/29) were recruited for this study (**Table 1**). The statement of institutional approval of the study was in accordance with the Declara-

Manuscript received 2 June 2006 and in revised form 21 September 2006.

Published, JLR Papers in Press, November 7, 2006.
DOI 10.1194/jlr.D600022-JLR200

Abbreviations: BMI, body mass index; EL, endothelial lipase; IDL, intermediate-density lipoprotein; LPL, lipoprotein lipase; PHP, postheparin plasma; TG, triglyceride.

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TABLE 1. Clinical profile of the study subjects

	Total (n = 74)	Men (n = 45)	Women (n = 29)
Age, years	53 ± 16	52 ± 16	54 ± 16
BMI, kg/m^2	23 ± 3	23 ± 2	22 ± 4
Total cholesterol, mg/dl	259 ± 92	246 ± 90	278 ± 95
TGs, mg/dl	137 ± 87	146 ± 66	105 ± 40
HDL-C, mg/dl	51 ± 17	47 ± 19	60 ± 12
Fasting plasma glucose, mg/dl	96 ± 8.3 (n = 64)	96 ± 7.4 (n = 40)	102 ± 34 (n = 24)
HbA1c, %	5.1 ± 0.5 (n = 53)	5.1 ± 0.5 (n = 32)	5.4 ± 1.1 (n = 21)

BMI, body mass index; HDL, high-density lipoprotein, TG, triglyceride.

tion of Helsinki, and informed consent was obtained from all participants. Exclusion criteria included: age >75 years, body mass index (BMI) >30 kg/m^2, diabetes mellitus, abnormal liver or muscle enzymes, creatinemia, use of antioxidants and lipid regulators, habitual alcohol intake >3 standard drinks/day, or endocrinological disorder. Blood samples were obtained following a fast of ≥12 h. PHP was obtained 10 min after 30 U/kg heparin injection. The monoclonal anti-LPL antibody 5D2 (MAb 5D2) was kindly provided by Dr. John Brunzell of the University of Washington, Seattle.

Method for measuring lipase activity in partially purified HL and LPL fractions

HL and LPL were prepared from PHP using heparin-Sepharose column chromatography (11). HL and LPL were eluted with 0.8 M NaCl and 1.6 M NaCl, respectively. Lipase activity was assayed measuring the increase in absorbance at 546 nm (subwave length; 660 nm) due to the production of quinonediimine dye based on the previously described assay procedure (12, 13). Reaction mixture R-1 contained 50 mM Tris-HCl (pH 9.5), 0.5 mM dioleoylglycerol, 0.4% polyoxyethylene-nonylphenylether, 3 mM ATP, 3 mM $MgCl_2$, 1.5 mM $CaCl_2$, monoacylglycerol-specific lipase (0.75 U/ml), glycerol kinase (0.75 U/ml), glycerol-3-phosphate oxidase (37.5 U/ml), 0.075% *N,N*-bis-(4-sulfobutyl)-3-methylaniline-2 Na, peroxidase (10 U/ml), and ascorbic acid oxidase (10 U/ml). Reaction mixture R-2 contained 50 mM Tris-HCl (pH 9.5), and 0.15% 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one. Automated assay of lipase activity was performed with a Model 7080 Hitachi automatic clinical analyzer. In the lipase assay, 160 μl of R-1 was incubated at 37°C with 3 μl of samples for 5 min, and then 80 μl of R-2 was added. This reaction produced a violet quinonediimine dye with a peak absorbance at 546 nm. HL activity generating 1 μmol of monoglyceride from diglyceride per minute was defined as 1 unit.

The method for measuring HL activity in PHP

We measured HL activity in PHP using the above-mentioned method either in the presence (HL activity) or absence (total lipase activity) of 1 M NaCl.

Separation of HL and LPL by heparin-Sepharose CL-6B

PHP (5 ml) was dialyzed against 1 l of 1.6 M NaCl containing 10 mM PIPES-NaOH (pH 7.5) at 4°C for 18 h. The dialysate was dialyzed against 500 ml of 0.3 M NaCl containing 10 mM PIPES-NaOH (pH 7.5) at 4°C for 18 h.

The dialysate was applied to a heparin-Sepharose CL-6B column (1.6 × 3 cm). The column was washed with 30 ml of 0.3 M NaCl containing 0.1 mM EDTA, 10% sucrose, 0.025% BSA, and 10 mM PIPES-NaOH (pH 7.5).

HL and LPL were eluted with 20 ml of 0.8 M NaCl containing 0.1 mM EDTA, 10% sucrose, 0.025% BSA, 10 mM PIPES-NaOH (pH 7.5), 20 ml of 1.6 M NaCl containing 0.1 mM EDTA, 10% sucrose, 0.025% BSA, and 10 mM PIPES-NaOH (pH 7.5) respectively. These HL and LPL fractions were concentrated with an Amicon Ultra membrane (molecular cut; 30 kDa), reconstituted with 2 ml of 10 mM PIPES-NaOH (pH 7.5) containing 10% sucrose and 0.1 mM EDTA. The concentrated HL and LPL was stored at −20°C.

Effects of MAb 5D2 on lipase activity in PHP- or NaCl-eluted fraction from heparin-Sepharose

To evaluate the specificities of the lipase activity determined by the present method, we investigated the effects of the addition of MAb 5D2 in the reaction mixture against the enzyme activity in either a PHP- or an 0.8 M NaCl-eluted fraction from heparin-Sepharose.

The conventional method for measuring HL activity in PHP

In the conventional method for measuring HL activity in PHP, total lipase activity was measured using Triton X-100-emulsified [^{14}C]triolein, based on the previously reported method (8, 9). The remaining activity in the presence of 1 M NaCl was defined as HL activity.

The method for measuring HL protein mass in PHP

HL protein mass in PHP was detected using a sandwich ELISA following the previously described method (10).

Statistical analysis

Statistical evaluation was performed using StatView-J 5.0 software (SAS Institute, Cary NC, on a Macintosh Computer). Pearson's correlation coefficients analysis was carried out. Results were expressed as mean ± SD, and the significance levels were set at $P < 0.05$.

RESULTS

Effect on lipase activity of addition of polyoxyethylene-nonylphenylether in the reaction mixture

Lipase activity detected in our method increased in a dose-dependent manner with increasing concentrations of polyoxyethylene-nonylphenylether, a nonionic detergent, in the reaction mixture (**Fig. 1**). Lipase activity detected the fractions eluted in 0.3 M, 0.8 M, and 1.6 M NaCl using reaction mixtures containing 0.5 mM dioleoylglycerol (**Table 2**). Lipase activity was detected only in the fractions of heparin-Sepharose chromatography eluted in 0.8 M NaCl, suggesting that this activity corresponds to HL. In

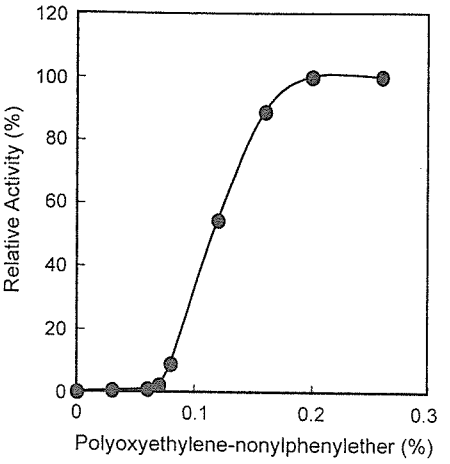

Fig. 1. Effect of increasing concentrations of polyoxyethylene-nonylphenylether on lipase activity in the reaction mixture for HL activity assay. Methods were described in detail in the Materials and Methods section, except that the effect of various concentrations of polyoxyethylene-nonylphenylether on HL activity in the reaction mixture was investigated in this particular experiment. The horizontal line shows the final concentration of polyoxyethylene-nonylphenylether in the reaction mixture.

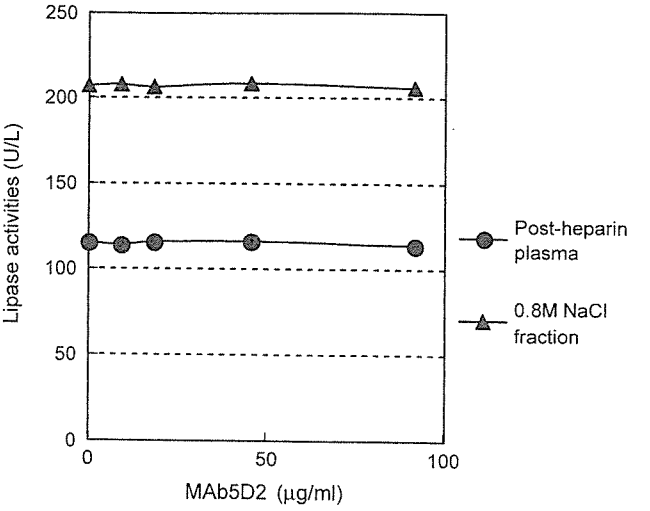

Fig. 2. Effect of anti-lipoprotein lipase monoclonal antibody 5D2 on lipase activity in postheparin plasma (PHP)- or 0.8 NaCl-eluted fraction from heparin-Sepharose. The reaction condition for the measurement of lipase activity was described in Materials and Methods, with the exception that increasing amounts of 5D2 were added in the reaction mixture. PHP was obtained from a normal volunteer 10 min after 30 U/kg heparin injection.

contrast, the LPL fraction (1.6 M NaCl) of heparin-Sepharose chromatography showed no lipase activity, even when apolipoprotein C-II (apoC-II) was added as LPL-specific activator.

Effect of MAb 5D2 on lipase activity in the PHP- or NaCl-eluted fraction

To determine whether the activity measured in the present method is affected by anti-LPL MAb, we studied the additive effect of the MAb 5D2 on lipase activity in the PHP- or 0.8 M NaCl-eluted fraction from heparin-Sepharose (**Fig. 2**). MAb 5D2 did not affect lipase activity at all in either in the PHP- or the 0.8 M NaCl-eluted fraction, whereas lipase activity in PHP was inhibited by 40% in the presence of the MAb 5D2 in an assay system for LPL activity (data not shown).

Correlation of HL activity with HL mass in PHP

HL activity in PHP measured in the present method was highly correlated with HL mass by a sandwich ELISA in 74 hyperlipidemic Japanese subjects (**Fig. 3**). By comparison, HL activity measured in the conventional method using Triton X-100-emulsified [^{14}C]triolein as a substrate [conventional HL (cHL) activity] had a weaker correlation with HL mass.

TABLE 2. Lipase activity in the fractions eluted in 0.3 M, 0.8 M, and 1.6 M NaCl using dioleoylglycerol as substrate

Fractions	Activity (U/l)	Total Activity (mU)
Plasma	252	1,260
0.3 M NaCl	0	0
0.8 M NaCl eluate	38	760
0.8 M NaCl concentrate	320	640
1.6 M NaCl concentrate	0	0

We also analyzed the correlation of HL activity with HL mass in PHP in men (n = 45). HL activity in PHP measured in the present method was highly correlated with HL mass by a sandwich ELISA ($r = 0.886$, $P < 0.001$). By comparison, cHL activity had a weaker correlation with HL mass ($r = 0.676$, $P < 0.001$).

Furthermore, we analyzed correlation of HL activity with HL mass in PHP in women (n = 29). HL activity in PHP measured in the present method was highly correlated with HL mass by a sandwich ELISA ($r = 0.901$, $P < 0.001$). By comparison, cHL activity had a slightly weaker correlation with HL mass ($r = 0.852$, $P < 0.001$).

HL activity in PHP measured in the presence or absence of NaCl

The remaining activity in the presence of 1 M NaCl using Triton X-100-emulsified [^{14}C]triolein as a substrate is usually interpreted as HL activity. Unexpectedly, the correlation coefficient of HL activity in the absence of 1 M NaCl with HL mass was almost equal to that in the presence of 1 M NaCl with HL mass (0.892 vs. 0.901 in women; 0.895 vs. 0.886 in men) (**Table 3**).

Correlation of HL activity with age and BMI

HL activity did not show a significant correlation with age or BMI in the study subjects (Table 3). Even measured separately for each gender, no correlation existed (data not shown).

Correlation of HL activity with lipids and lipoproteins

HL activity in the present method did not have significant associations with total cholesterol or TG, and had

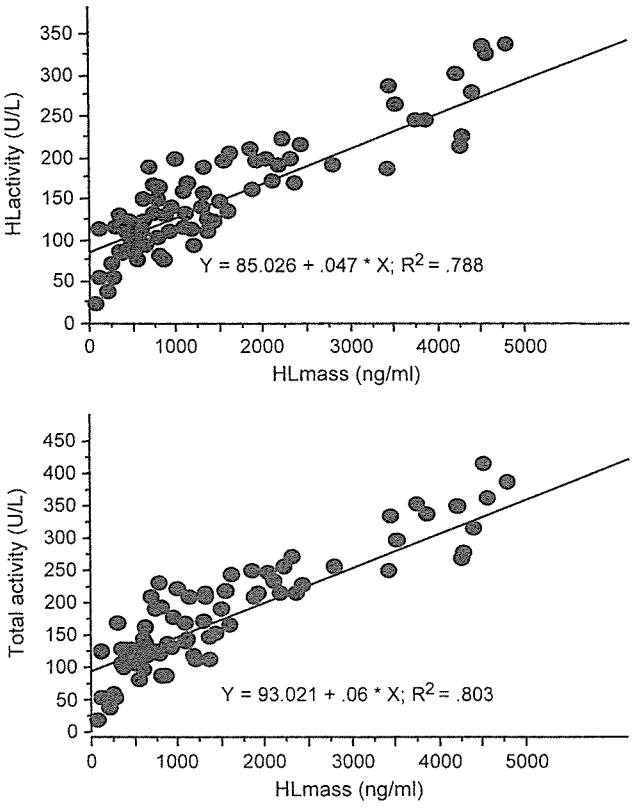

Fig. 3. Relationship of HL mass to HL activity and total lipase activity from PHP in 74 hyperlipidemic Japanese subjects (men/women 45/29). Lipase activity in PHP was measured using the method described in Materials and Methods in either the presence (HL activity) or absence (total lipase activity) of 1 M NaCl. HL activity generating 1 μmol of monoglyceride from diglyceride per minute was defined as 1 unit. HL mass is shown in ng/ml.

an inverse association with HDL-C and HDL_2-C (Table 3). Measured separately for each gender, the observed associations between HL activity and HDL-C did not reach statistical significance, partly because of the small sample size (data not shown).

Other findings

The within-run (n = 20) coefficient of variation was 0.9–1.0%. The calibration curve of lipase activity was linear ($r = 0.999$) between 0 and 500 U/l. Analytical recoveries of purified HL added to plasma were 96.6–99.8%. This method was free of interference by bilirubin C, bilirubin F, ascorbic acid, and intra-lipid. Weak interference by hemoglobin was observed. High activity of human pancreatic lipase (1,000 U/l) showed no lipase activity.

DISCUSSION

In the present study, we have developed a novel method for measuring HL activity in PHP. Instead of radioisotope-labeled substrate, we have used 0.2 mM dioleoylglycerol as a substrate for this new lipase assay.

TABLE 3. Correlation of HL activity and mass with several metabolic parameters in men and women combined (N = 74)

	T-Lipase Activity	HL Activity	HL Mass	cHL Activity
BMI	−0.035	−0.059	0.024	−0.057
Total cholesterol	−0.075	−0.042	−0.047	0.240[a]
TGs	0.144	0.167	0.290[a]	0.119
HDL-C	−0.330[b]	−0.310[b]	−0.356[b]	−0.183
HDL_2-C	−0.317[b]	−0.300[a]	−0.348[b]	−0.231
HDL_3-C	−0.070	−0.051	−0.031	0.159
T-lipase activity	na	0.978[c]	0.896[c]	0.743[c]
HL activity	0.978[c]	na	0.888[c]	0.747[c]
HL mass	0.896[c]	0.888[c]	na	0.730[c]
cHL activity	0743[c]	0.747[c]	0.730[c]	na

T-lipase, total lipase; HL, hepatic lipase; na, not available; cHL activity, HL activity measured in conventional method using Triton X-100-emulsified [^{14}C]triolein.

Lipase activities measured in the absence and the presence of 1 M NaCl were referred to as T-lipase activity and HL activity, respectively.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

The finding shown in Fig. 1 suggests that the existence of polyoxyethylene-nonylphenylether, a nonionic detergent, in the reaction mixture appeared to be a critical factor in the expression of HL enzyme activity, although at this time, the precise mechanisms have not been elucidated. Glycerol-1,2-dioleate became clear by adding nonionic detergent, suggesting that glycerol-1,2-dioleate came to exist in the reaction mixture as water-soluble mixed micelles with this nonionic detergent.

The result shown in Table 2 that lipase activity was detected only in the 0.8 M NaCl-eluted fraction of the heparin-Sepharose indicated that lipase activity detected in the present method corresponds to that of HL. Also, the finding that the MAb 5D2 did not affect lipase activity in the 0.8 M NaCl-eluted fraction and PHP confirmed that this method specifically detected HL activity (Fig. 2). HL activity obtained using this method showed stronger associations with HL mass by sandwich ELISA than did cHL activity measured using Triton X-100-emulsified [^{14}C]triolein (8, 9). This suggests that the present method could be more reliable for measuring HL activity in PHP than the conventional method using Triton X-100-emulsified [^{14}C]triolein as a substrate.

A previous report has shown extremely high correlation between HL mass and activity in PHP (10). However, their method for measuring HL activity requires ^{3}H-labeled triolein and is labor intensive and time consuming. HL is a lipolytic enzyme catalyzing the hydrolysis of TG and PL in IDL and HDL_2. Whether HL is atherogenic or antiatherogenic is still the subject of debate (14–17). Because HL lowers plasma concentrations of the pro-atherogenic apoB-containing lipoproteins as well as the anti-atherogenic HDL, the net effect of these HL-induced alterations in plasma lipoproteins on coronary artery disease is not easily predictable. Patients with HL deficiency present with hypercholesterolemia or hypertriglyceridemia and accumulate β-VLDLs, chylomicron remnants, IDLs, TG-rich LDLs, and HDLs (1–7). The clinical profile of this lipid disorder could be quite similar to that of type III hyperlipidemia,

which makes it difficult to identify this lipid disorder. To identify patients with HL deficiency, it is essential to demonstrate the lack of HL activity in PHP. In contrast, LPL deficiency is relatively easily identified, because patients with this lipid disorder usually have drastic hyperlipidemia due to marked accumulation of chylomicrons in the serum, causing acute pancreatitis (7). To date, as mentioned above, the available method for measuring HL activity in PHP has used ^{3}H- or ^{14}C-labeled trioleoyl glycerol as substrates in the presence of 1 M NaCl (8, 9). However, these assay procedures are complicated and require the use of radioisotopes. In the present study, therefore, we have developed and established a novel assay system for measuring HL activity in PHP using dioleoylglycerol as a substrate, without requiring radioisotope labeling. The results presented in Table 3, showing that total lipase activity (measured in the absence of 1 M NaCl) was similar to HL activity (measured in the presence of 1 M NaCl) in terms of correlation coefficients with HL mass, suggested that this assay could be suitable for measuring HL activity in the presence or absence 1 M NaCl.

With regard to the correlation of HL activity with lipid and lipoproteins, there were inverse correlations with HDL-C (HDL_2-C) in the whole subjects, but there was no correlation with BMI, total cholesterol, TGs, or HDL_3-C. These findings might be compatible with the fact that HL is involved in catalyzing hydrolysis of TG in HDL_2-C (7, 18).

In addition to LPL and HL, in the past decade, considerable attention has been paid to the physiological role of endothelial lipase (EL) (19), which is known to have higher phospholipase activity and lower activity in TG hydrolysis, compared with the other two lipases (20). To our knowledge, however, there has been no report on what proportion of lipase activity in PHP is accounted for by EL. Despite this, we presume that there is little or no possibility that our present method for measuring HL activity overlapped the activity of EL, in view of their high positive correlation to HL mass.

In summary, we have developed a novel and simple method for the assay of HL activity in PHP, which is suitable for application to an automatic clinical analyzer.

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Letter to the Editor

The distribution of fasting and non-fasting serum triglyceride levels in Japanese population

Dear editor,

In recent years, accumulating evidence shows that non-fasting serum triglycerides (TG) are important marker for assessing the risk of cardiovascular disease [1–4]. Several clinical studies have shown that delayed elimination of postprandial TG-rich lipoprotein as well as higher TG levels is associated with coronary artery disease (CAD) [5,6]. It has been reported that the presence of CAD is associated with higher postprandial TG concentrations in plasma compared with healthy controls, even after correction for higher levels of fasting TG in CAD group [6–8]. To date, much of the knowledge about the relationship between lipid and lipoprotein metabolism and the development of atherosclerosis and cardiovascular disease is based on measurements in the fasting state. However, human spends their majority of time in a non-fasting state.

In the guideline proposed by the Japanese Atherosclerosis Society [9], the decision point of serum TG is simply put as <1.69 mmol/l in fasting state across all series of subjects no matter what the degree of risks individuals have. In addition, it is known that serum TG levels change considerably before and after meals. In this circumstance, we investigated how TG values in fasting and non-fasting distributed in Japanese individuals who received annual medical checkup consisting of 8223 men and 16,154 women, among whom 12,990 (M/F 4676/8314) and 11387 (3547/7840) subjects were analyzed for fasting and non-fasting TG levels, respectively. Subjects who fasted for at least 12 h after the last meal were defined as fasting. In non-fasting individuals, the distribution time since last meal was 5.1% < 1 h, 25.4% for <2 h, 35.6% for <3 h, 21.2% for <4 h, and 12.8% >4 h. Serum TG values for individuals in these 5 categories were 1.38±0.83, 1.45±0.91, 1.48±0.98, 1.44±1.00 and 1.39±0.90 (mean±S.D. in mmol/l), respectively. The study subjects were given questionnaire on their habit whether or not they had a regular habit of drinking and/or smoking to fill in. In fasting and non-fasting TG subjects, 37% and 39% men, respectively, were smokers, whereas 6.3% and 5.7% women, respectively, were smokers. In fasting and non-fasting TG subjects, 66% and 66% men, respectively, were drinkers whereas 18% and 18% women, respectively were drinkers. Statistical evaluation was performed with StatView 5.0. Results were shown as mean±S.D. Pearson's correlation coefficients analysis was carried out. Statement of institutional approval of the study in accordance with the Declaration of Helsinki and informed consent were obtained from all of the participants in this study. A $p<0.05$ were considered significant. In individuals analyzed for fasting TG, age, body mass index (BMI), plasma glucose (PG), HbA1c, total cholesterol (TC), TG and high-density lipoprotein-cholesterol (HDL-C) were 57±13 years, 22.9±3.2 kg/m^2, 5.38±1.05 mmol/l, 5.3±0.9% (n=6132), 5.26±0.91, 1.19±0.79 and 1.61±

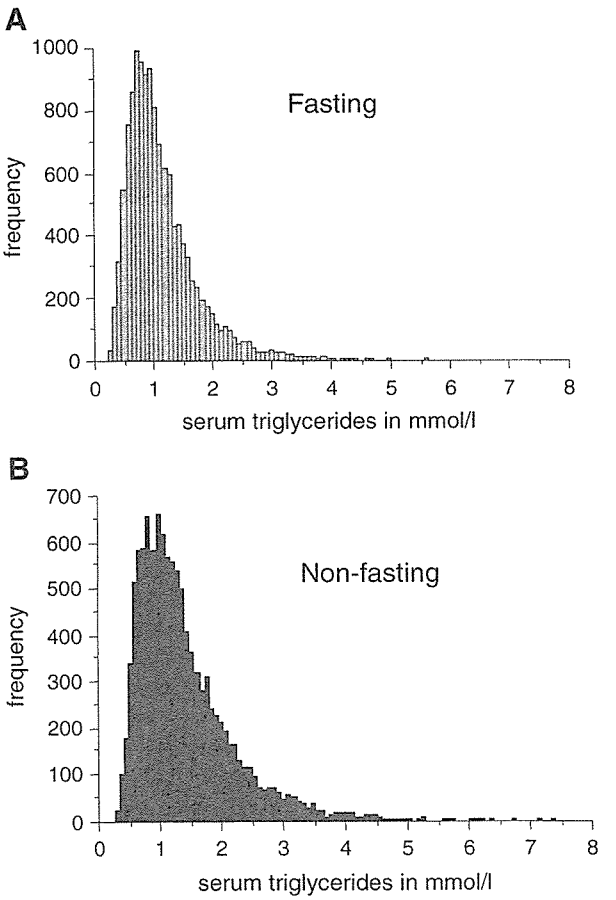

Fig. 1. Distribution of number of subjects for fasting (n=12,990) (A) and non-fasting (n=11,387) (B) serum TG levels in the study population. The median, the 1st and the 3rd quartiles of fasting TG levels were 1.01, 0.73 and 1.41 mmol/l, respectively. With regards to non-fasting TG levels, the median, the 1st and the 3rd quartile of them were 1.22, 0.86 and 1.75 mmol/l, respectively.

0009-8981/$ - see front matter
doi:10.1016/j.cca.2006.06.032

Table 1
Correlations of fasting or non-fasting serum triglycerides versus other metabolic parameters

		Age	BMI	TC	HDL-C	PG	HbAlc
Fasting TG	Men N=4676	−0.100	0.307	0.314	−0.434	0.126	0.100
	Women N=8314	0.306	0.329	0.356	−0.431	0.161	0.236
Non-fasting TG	Men N=3547	−0.135	0.315	0.360	−0.375	0.092	0.095
	Women N=7840	0.316	0.339	0.388	−0.433	0.200	0.253

All of the correlation coefficients showed $p<0.0001$.
TG, triglycerides; BMI, body mass index; TC, total cholesterol; PG, plasma glucose.

0.39 mmol/l, respectively. In those for non-fasting TG, age, BMI, PG, HbA1c, TC, TG and HDL-C were 58±15 years, 22.9±3.3 kg/m^2, 5.99±1.89 mmol/l, 5.3±0.9% (n=5483), 5.08±0.91, 1.45±0.95 and 1.50±0.39 mmol/l, respectively.

Since TG levels, unlike cholesterol levels, do not distribute normally, we measured median, the first and the third quartiles and quartile deviations of TG values in fasting and non-fasting states. The distribution of fasting TG values are shown in Fig. 1A. In men and women combined, the median, the 1st and the 3rd quartile of fasting TG levels were 1.01, 0.73 and 1.41 mmol/l, respectively. In men alone, the median, the first and the third quartile of fasting TG levels were 1.13, 0.82 and 1.62 mmol/l, respectively, whereas in women those values were 0.95, 0.70 and 1.30 mmol/l, respectively. The distribution of non-fasting TG values are shown in Fig. 1B. In men and women combined, the median, the first and the third quartile of non-fasting TG levels were 1.22, 0.86 and 1.76 mmol/l, respectively. In men alone, the median, the first and the third quartile of non-fasting TG levels were 1.36,0.95 and 1.99 mmol/l respectively, whereas in women those values were 1.16, 0.82 and 1.65 mmol/l, respectively.

In men and women combined, median plus twice the quartile deviation for fasting TG value was calculated to be 1.68 mmol/l, being almost equal to the decision point of fasting TG levels of 1.69 mmol/l. Similarly, in men and women combined, median plus twice the quartile deviation for non-fasting TG was calculated to be 2.12 mmol/l, which could be used as decision point of non-fasting serum TG levels.

Iso et al. [1] conducted a 15.5-year prospective study ending in 1997 of 11,068 Japanese aged 40–69 years and found that relative risk of coronary heart disease adjusting for coronary risk factors and time since last meal associated with a 1 mmol/l increase in TG was 1.29 for men and 1.42 for women. They also showed the baseline data for TG distribution in men and women in non-fasting states. On the other hand, since TG levels for Japanese especially for men has increased drastically during the past decade [10], updated data might be more desirable to determine how TG distributes in Japanese population currently.

In this study, we also analyzed the relationships of TG with other metabolic parameters (Table 1). Overall, between fasting and non-fasting states, TG values showed similar associations with age, BMI, TC, HDL-C, PG and HbA1c. When men and women were separately considered, age had positive relations with TG levels both in fasting and non-fasting states in women, whereas not in men. Also, the associations of TG with parameters for glucose metabolism were weaker in men than in women. In conclusion, we have shown the distribution of fasting and non-fasting TG levels in a large number of individuals who underwent annual medical checkup in Ishikawa prefecture Japan.

Acknowledgement

The authors express their appreciation to Sankyo, Banyu, Pfizer, Astellas, Kissei, Kaken, Shionogi, Astrazeneca for their financial support for conducting this work.

We do not have any conflict of interest.

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22 May 2006

Original Article

The Relationship of Percent Body Fat by Bioelectrical Impedance Analysis with Blood Pressure, and Glucose and Lipid Parameters

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The objective of this study was to clarify the clinical significance and usefulness of measuring percent body fat (PBF) when compared with body mass index (BMI) in the Japanese population. A total of 2,483 Japanese individuals (1,380 men and 1,103 women) who underwent a medical checkup from 1999-2002 were employed. PBF was determined using bioelectrical impedance analysis (BIA). Relationships of age, BMI and PBF with several metabolic parameters, including blood pressure, lipids and plasma glucose levels were assessed in both genders separately. In men, PBF was a stronger determinant of total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C) and triglycerides (TG) compared with age and BMI, whereas in women, age was the strongest determinant of TC and LDL-C. In both genders, BMI was the strongest determinant of serum HDL-C among age, PBF and BMI. Based on these data, we suggest that measuring PBF by BIA is superior to BMI for predicting TC, LDL-C and TG in Japanese men.

J Atheroscler Thromb, 2006; 13:221-226.

Key words; Percent body fat, Bioelectrical impedance analysis, Body mass index, Plasma lipids

Introduction

Obesity is determined based on an individual's BMI, which is defined as body weight (kg) divided by squared body height (m^2). The use of bioelectrical impedance analysis (BIA) for determining percent body fat (PBF) is widely accepted as a safe, rapid, low cost and reliable technique[1-3]. This method, as is the case in BMI, does not provide information on body fat distribution, which is in contrast to, such as CT[4,5], MRI[6] and ultrasonography[7]. Nevertheless, PBF determined by BIA, as in the case with BMI, is widely used in Japan for screening an individual's body fat mass in medical checkups because of its simpleness; however, the clinical significance of measuring PBF has not been well studied in detail.

With this background, the aim of this study was to clarify the usefulness and clinical significance of measuring PBF by analyzing the relationship of PBF with several metabolic parameters, including blood pressure, plasma glucose, and plasma lipid levels in 2,483 Japanese individuals (1,380 men and 1,103 women) who underwent medical checkups from 1999-2002 in our department.

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Received: February 3, 2006
Accepted for publication: July 20, 2006

Materials and Methods

Table 1 shows the clinical profiles of the study subjects.

For individuals who underwent a medical checkup more than once during this period, the latest data

Table 1. Profile of the study subjects

	men	women	*p*
n	1,380	1,103	
age, yr	52.4 ± 9.7	52.4 ± 9.5	ns
body mass index, kg/m^2	23.7 ± 3.0	22.8 ± 3.1	< 0.0001
percent body fat, %	23.1 ± 5.4	29.4 ± 6.2	< 0.0001
systolic BP, mmHg	123.7 ± 16.5	119 ± 17.6	< 0.0001
diastolic BP, mmHg	80.6 ± 10.8	75.2 ± 23.6	< 0.0001
fasting plasma glucose, mg/dL	99.8 ± 11.1	94.5 ± 9.8	< 0.0001
HbA1c, %	4.98 ± 0.37	4.94 ± 0.35	< 0.001
total cholesterol, mg/dL	200 ± 33.5	208 ± 34.3	< 0.0001
triglycerides, mg/dL	129 ± 66.3	94.3 ± 57.1	< 0.0001
HDL-cholesterol, mg/dL	55.4 ± 14.6	67.1 ± 16.2	< 0.0001
LDL-cholesterol, mg/dL	120 ± 31.2	122 ± 30.9	ns
white blood cell, $\times 10^3$	5.9 ± 1.7	5.1 ± 1.4	< 0.0001
red blood cell, $\times 10^6$	4.83 ± 0.39	4.4 ± 0.3	< 0.0001
Hb, g/dL	15.1 ± 1.1	13 ± 1.2	< 0.0001

BP, blood pressure; Hb, hemoglobin
Values are shown as the mean ± sd.

were used for this study. Individuals with AST > 100, ALT > 100, Cre > 1.5, HbA1c > 6.5%, TG > 400 mg/dL were excluded from this study. Subjects taking anti-hypertensive, oral-hypoglycemic agents, insulin treatment, or lipid-lowering medications were also excluded. BMI was obtained by body weight (kg) divided by squared body height (m^2). PBF was determined from bioelectrical impedance analyses (BIA) using TANITA TBF-215 (TANITA Corporation, Tokyo, Japan). This method measures the flow of electrical signals as they pass through fat and lean water in the body. When the amount of fat and lean matter or water changes, so do the signals, giving a reliable and accurate measurement of the amount of each of these components that make up the total weight of the person. The measurements were performed in a standing position, with electrodes in contact with the soles and heels of both feet. Serum total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL)-C levels were determined by standard enzymatic methods. LDL-C levels were calculated with the Friedewald formula. A statement of institutional approval of the study in accordance with the Declaration of Helsinki and informed consent were obtained from all of the participants in this study.

Statistical Analysis

Statistical evaluation was performed using StatView-J 5.0 software (SAS Institute, Cary NC on a Macintosh Computer).

The results were expressed as the mean ± SD and the significance level was set at $p < 0.05$. Pearson correlation coefficients were used to evaluate the relationship of age, BMI, PBF with metabolic parameters.

Results

Relationship of PBF with BMI

PBF had a strong correlation with BMI both in men ($r = 0.813$, $p < 0.0001$) and women ($r = 0.888$, $p < 0.0001$).

Relationship of PBF with Age

PBF showed a weak inverse relationship with age in men ($r = -0.149$, $p < 0.0001$), but had no significant relation with age in women ($r = 0.055$, $p = 0.054$).

Relationships of Age, BMI and PBF with Several Metabolic Parameters in Men (Table 2)

Age showed a positive relationship with sBP, while its relation with dBP was subtle. Both BMI and PBF showed positive relationships with sBP and dBP. Age, BMI and PBF showed a weak association with FPG and HbA1C. There was no association of age with any of the lipid and lipoprotein parameters except for its subtle relation with Log TG. In contrast, both BMI and PBF were positively associated with TC, LDL-C and, to a higher degree, with log TG, while being inversely associated with HDL-C levels.

Table 2. Correlation coefficient of age, BMI and PBF with metabolic parameters in men

	age	BMI	PBF
sBP	0.235***	0.263***	0.21***
dBP	0.097**	0.303***	0.271***
fasting plasma glucose	0.14***	0.178***	0.177***
HbA1c	0.22***	0.159***	0.152***
total cholesterol	−0.002	0.171***	0.242***
LDL-cholesterol	0.033	0.201***	0.25***
log-triglycerides	−0.082*	0.337***	0.371***
HDL-cholesterol	0.009	−0.312***	−0.283***

sBP, systolic blood pressure; dBP, diastolic blood pressure
***$p < 0.0001$; **$p < 0.001$, *$p < 0.01$

Table 3. Correlation coefficient of age, BMI and PBF with metabolic parameters in women

	age	BMI	PBF
sBP	0.379***	0.277***	0.254***
dBP	0.133***	0.159***	0.148***
fasting plasma glucose	0.289***	0.237***	0.223***
HbA1c	0.351***	0.21***	0.162***
total cholesterol	0.292***	0.102**	0.158***
LDL-cholesterol	0.269***	0.179***	0.227***
log-triglycerides	0.244***	0.243***	0.258***
HDL-cholesterol	−0.034	−0.287***	−0.266***

sBP, systolic blood pressure; dBP, diastolic blood pressure
***$p < 0.0001$; **$p < 0.001$, *$p < 0.01$

Table 4. Multiple regression analysis on the relationship of age, BMI and PBF with metabolic parameters in men

variables	age			BMI			PBF		
	β	t	p	β	t	p	β	t	p
sBP	0.264	11.25	< 0.0001	0.227	5.725	< 0.0001	0.067	1.667	0.0957
dBP	0.139	5.839	< 0.0001	0.231	5.74	< 0.0001	0.098	2.399	0.0165
FPG	0.17	6.953	< 0.0001	0.078	1.873	0.0613	0.137	3.289	0.001
HbA1c	0.256	10.62	< 0.0001	0.101	2.464	0.0138	0.091	2.198	0.0281
total cholesterol	0.037	1.529	0.1264	−0.049	−1.197	0.2314	0.3	7.185	< 0.0001
LDL-cholesterol	0.01	0.401	0.6884	0.014	0.33	0.7413	0.13	3.002	0.0027
Log triglycerides	−0.046	−1.989	0.0468	0.135	3.421	0.0006	0.261	6.558	< 0.0001
HDL-cholesterol	−0.015	−0.638	0.5239	−0.235	−5.804	< 0.0001	−0.1	−2.457	0.0141

FPG, fasting plasma glucose

Relationships of Age, BMI and PBF with Several Metabolic Parameters in Women (Table 3)

Age, BMI and PBF showed positive associations with sBP and, to a lesser degree, with dBP. In women, the association of age, BMI and PBF with FPG were higher and likewise those with HbA1c were higher than in men. Unlike in men, age had significant associations with serum TC, Log TG and LDL-C levels, but not with HDL-C levels. BMI showed a positive association with Log TG and, to a lesser degree, with TC and LDL-C, while showing an inverse relationship with serum HDL-C. Similarly, PBF showed a positive association with Log TG and an inverse one with HDL-C, and the associations of PBF with TC or LDL-C were relatively weak compared with those in men.

Multiple Regression Analysis on the Relationships of Age, BMI and PBF with Several Metabolic Parameters in Men (Table 4)

Multiple regression analysis with BMI, age and PBF as independent variables and with several metabolic parameters as dependent variables showed that the relation of PBF with blood pressure was not as strong as age or BMI.

The relation of PBF with FPG and HbA1c was not as strong as age. Of note, the relation of PBF with TC and TG was more pronounced than BMI and age with these lipid parameters. In contrast, BMI was more strongly associated with serum HDL-C levels than PBF and age.

Multiple Regression Analysis on the Relationships of Age, BMI and PBF with Several Metabolic Parameters in Women (Table 5)

The relationship between blood pressure and PBF did not persist after adjustment for BMI and age. Similarly, the relationship between glucose metabolism parameters and PBF did not persist or almost disappeared after adjustment for other co-variants. In contrast, the relationship between PBF and TC, TG and LDL-C was stronger than those between BMI

Table 5. Multiple regression analysis on the relationship of age, BMI and PBF with metabolic parameters in women

variables	age			BMI			PBF		
	β	t	p	β	t	p	β	t	p
sBP	0.367	13.96	<0.0001	0.188	3.358	0.0008	0.062	1.104	0.2698
dBP	0.118	4.204	<0.0001	0.129	2.094	0.0364	0.033	0.535	0.5926
FPG	0.264	9.792	<0.0001	0.086	1.464	0.1436	0.116	1.976	0.0484
HbA1c	0.326	12.337	<0.0001	0.212	3.664	0.0003	−0.036	−0.617	0.5375
total cholesterol	0.285	10.523	<0.0001	−0.213	−3.582	0.0004	0.329	5.558	<0.0001
LDL-cholesterol	0.259	9.539	<0.0001	−0.12	−2.011	0.0445	0.311	5.23	<0.0001
Log triglycerides	0.229	8.54	<0.0001	0.017	0.287	0.774	0.248	4.245	<0.0001
HDL-cholesterol	−0.015	−0.547	0.5844	−0.221	−3.676	0.0002	−0.078	−1.305	0.1921

FPG, fasting plasma glucose

Table 6. Pearson's correlation coefficients between serum lipids vs. body mass index or percent body fat by gender and age

age (y)	n	body mass index				percent body fat				PBF vs BM
		TC	log TG	HDL-C	cLDL-C	TC	log TG	HDL-C	cLDL-C	
men										
30-39	122	0.146	0.387***	−0.43***	0.246**	0.25**	0.417***	−0.383***	0.323***	0.858***
40-49	438	0.153**	0.356***	−0.334***	0.182***	0.236***	0.348***	−0.247***	0.238***	0.817***
50-59	477	0.209**	0.309***	−0.289***	0.225***	0.269***	0.396***	−0.314***	0.265***	0.822***
60-69	276	0.166**	0.304***	−0.279***	0.206***	0.246***	0.333***	−0.276***	0.283***	0.792***
70-79	67	0.136	0.327**	−0.144	0.125	0.133	0.296*	−0.165	0.148	0.699***
women										
30-39	86	−0.04	0.317**	−0.414***	0.07	0.056	0.342***	−0.344**	0.148	0.917***
40-49	355	0.134*	0.273***	−0.306***	0.228***	0.211**	0.321***	−0.278***	0.279***	0.876***
50-59	408	0.081	0.212***	−0.263***	0.141**	0.178**	0.238***	−0.236***	0.23***	0.885***
60-69	208	0.072	0.147*	−0.249***	0.165*	0.086	0.141*	−0.247***	0.183**	0.902***
70-79	46	−0.058	0.27	−0.213	−0.038	−0.023	0.299*	−0.3*	0.04	0.883***

*$p<0.05$, **$p<0.01$, ***$p<0.001$
TC, total cholesterol; TG, triglycerides; PBF, percent body fat; BMI, body mass index

and these lipid parameters. However, the association between PBF and HDL-C did not persist after adjustment for BMI and age, while in contrast, BMI was the independent determinant of HDL-C levels among age, BMI and PBF.

Relationships of BMI or PBF with Several Metabolic Parameters After Subclassifying the Subjects According to Age Group (Table 6)

In men, PBF had better associations with LDL-C across all ages and with TG in middle age than did BMI. In women, PBF had better associations with LDL-C and TG than did BMI across the age. In contrast, PBF had weaker associations than BMI with HDL-C in young to middle aged men and women. Also, the relation between PBF and BMI was weaker in the elderly population.

Discussion

In this study, we analyzed the relationships of PBF by BIA with several metabolic parameters in 1,380 men and 1,103 women who underwent medical check-ups from 1999-2002. Our findings are consistent with a previous study that compared with BMI. PBF by BIA was more strongly correlated with serum lipids except for HDL-C in Japanese population[8], although unlike our study they did not present data on PG and BP. We also subclassified the subjects according to age and measured Pearson's correlation coefficients between lipids and BMI or PBF by gender and age.

Despite PBF measured by BIA being a simple,

convenient and popular way of assessing body fat in daily clinical practice[1-3], to our knowledge its clinical significance has not been well clarified. This is in stark contrast to the BMI, which has long been recognized as a predictor of morbidity and mortality due to numerous chronic diseases, including type 2 diabetes, cardiovascular disease and stroke[9, 10].

Obesity is determined based on an individual's BMI, which just like PBF, does not provide any information on an individual's body fat distribution but is the widely standardized parameter for defining obesity. Indeed, in this study, we found that this parameter was a good predictor of BP, especially for men. PBF, on the other hand, was not likely to be a good predictor of BP in both genders. Likewise, it was not a good predictor of an individual's fasting glucose and HbA1c levels in both genders. In contrast, it was found to be the strongest predictor of TC, LDL-C and TG among age, BMI and PBF in men. Although the mechanism of TG's association with PBF has not been elucidated, we presume that it is highly related to the fact that adipose tissue is the main store of TG in the body. TG in adipose tissue undergoes hydrolysis by a hormone-sensitive lipase to form free fatty acids. The liver in turn takes up free fatty acids from the circulation and cause the formation and secretion of VLDL[11]. VLDL is subsequently metabolized into IDL, leading to the formation of LDL. Among the lipid parameters we investigated, unlike TC, TG or LDL-C, HDL-C was much more closely associated with BMI than was PBF. We speculate the potential reasons for this as follows: HDL-C is first secreted by the liver and intestine as small, lipid poor, apo A-1 particles termed nascent HDL[12]. These HDL particles interact with the ATP-binding cassette transport protein A1 (ABC A1) on peripheral cells such as arterial wall macrophages to drive cholesterol efflux[13, 14]. This step is followed by the maturation of HDL particles by the function of lecithin-cholesterol acyltransferase (LCAT)[15, 16]. Cholesteryl ester transfer protein, which is produced in the liver, spleen, skeletal muscle and adipocytes, is involved in cholesteryl ester transfer from HDL particles to apoB-containing particles, including very low density lipoprotein (VLDL) and LDL[17, 18]. Hepatic lipase (HL), which is mainly produced in the liver, also plays a key role in the metabolism of HDL particles[19, 20]. Specifically, HL hydrolyzes TG in HDL, generating small-modified HDL particles, which are taken up primarily by the scavenger receptor class B type I on the surface of hepatocytes[21, 22]. These lots of steps for determining serum HDL concentration might account for the relatively weak association of HDL-C with PBF.

In both sexes, but especially in men, young subjects had closer associations between BMI and PBF than did elder individuals, and these associations slightly declined with age, which may be due to increased in inter-individual variations of body composition with age[23], since body composition may be affected by many age-related changes, such as decreases in bone, muscle, and body water, and distribution of fat[24, 25]. Also, it has been shown that PBF measured by BIA is less precise and less accurate in the elderly than in young individuals[26].

Indeed, in our study, the degree of the association of lipids to BMI or PBF also changed according to age.

Besides the method studied in this study, recently, an excellent and sophisticated technique by BIA was introduced[27], which appears to be able to provide us with information on visceral fat mass without using CT. Given that the importance of measuring visceral fat, not whole body fat, has been increasingly emphasized recently[28], we hope that this method will prevail in the near future.

Recently, more and more attention has been given to the measurement of waist circumference, because it has now become the essential component for diagnosing metabolic syndrome. Our previous study has shown that PBF versus BMI was highly related to waist circumference in middle-aged men ([$r = 0.893$, $p < 0.0001$] versus [$r = 0.876$, $p < 0.0001$]) in the Kanazawa district[29].

In conclusion, based on our findings of 1,380 male and 1,103 female Japanese healthy subjects, we suggest that measuring percent body fat by bioelectrical impedance analyses (BIA) may be superior to BMI for predicting an individual's serum lipids except for HDL-C in middle-aged men and women.

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Original Article

Effect of Walking with a Pedometer on Serum Lipid and Adiponectin Levels in Japanese Middle-aged Men

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Objective: To clarify the effects of walking with a pedometer on metabolic parameters, including adiponectin (APN).
Methods: We recruited 44 male Japanese volunteers (age, 37 ± 9 yrs; body mass index (BMI), 24.2 ± 2.9 kg/m^2; fasting plasma glucose (FPG), 96 ± 11 mg/dL; total cholesterol (TC) 190 ± 26 mg/dL; triglycerides (TG) 119 ± 80 mg/dL; HDL-C56 ± 14 mg/dL). Subjects were instructed to walk with a pedometer and record the number of steps they walked every day for 50 days. Serum adiponectin (APN) levels were measured by enzyme immunoassay. Treatment effects were examined by Wilcoxon's rank test.
Results: The average number of steps was 8211 ± 2084 per day. There were significant reductions in BMI, sBP, TG and TNF-α levels after 50 days, but no changes in adiponectin levels. We then divided the subjects into 2 groups according to the steps walked per day, namely, more than 8000 steps (MT group, n=22) and less than 8000 steps (LT group, n=22) and found that the reduction in TG and BP was observed only in the MT group.
Conclusions: Walking with a pedometer is effective for improving metabolic parameters, such as TG and blood pressure, but is not sufficient to increase adiponectin levels in Japanese men.

J Atheroscler Thromb, 2006; 13:197-201.

Key words; Waist circumference, Percent body fat, Triglycerides, HOMA-R, Adiponectin

Introduction

To prevent lifestyle-related disease, such as dyslipidemia, hypertension, type 2 diabetes (T2DM) and cardiovascular disease, it is important to exercise daily. Walking with a pedometer is easy and can be incorporated in to daily life even for individuals with a busy schedule. It is generally accepted that regular exercise, including walking, is beneficial for preventing life-related disease, such as type 2 diabetes, hypertension and hyperlipidemia[1-3].

Adiponectin (APN), an adipocytokine, is a plasma protein expressed exclusively in adipose tissue[4-6], the plasma levels of which are linked to insulin sensitivity[7-13]. APN mRNA and its plasma concentrations are known to be reduced in T2DM and atherosclerotic disease[14, 15]. Several studies in humans, monkeys and rodents have shown that APN is an insulin-sensitizing cytokine and exhibited anti-atherogenic moieties[16].

Several studies have been conducted on the effects of exercise using a bicycle ergometer and treadmill walking on plasma APN levels in obese or overweight subjects[17-19], and they showed that exercise did not change APN levels. However, to our knowledge, no study has investigated the effects of walking with a pedometer, a practical form of daily exercise, on APN levels in individuals of normal body weight.

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Received: February 22, 2006
Accepted for publication: May 24, 2006