However, the mechanism may not be so simple. Unexpectedly, the UPR, represented by ER chaperone expression, was not significantly reduced (Fig. 9a, ESM Figs 2 and 3) to the extent that we expected from the remarkable level of diabetes prevention. Because the UPR is a protective response against ER stress, an apoptotic pathway may have been preferentially suppressed under these conditions. Several pathways are reportedly involved in ER stress-induced apoptosis, including the C/EBP-homologous protein (CHOP), the IRE1–TNF receptor-associated factor 2 (TRAF2)–apoptosis signal-regulating kinase 1 (ASK1), and the caspase 12 pathways [40]. The precise mechanism whereby pioglitazone prevented apoptosis in beta cells awaits determination. The beta cell protection exerted by thiazolidinediones including pioglitazone has been demonstrated in other rodent models [41, 42] and also in humans [43]. Although the precise mechanisms are not fully understood, one possibility is an indirect action through improvements in systemic glucose and lipid metabolism. Another mechanism involves direct actions on pancreatic beta cells [44]. Recent reports have demonstrated that thiazolidinediones directly improve beta cell function [45], ameliorate lipotoxicity [46] and prevent beta cell apoptosis [47, 48]. In $Wfs1^{-/-}$ A^{y}/a mice, direct protective effects, as well as indirect effects, are likely to be exerted. Elucidation of the mechanism whereby pioglitazone directly protects beta cells against apoptosis in $Wfs1^{-/-}$ A^{y}/a mice would provide insights into the mechanism of beta cell death in patients with Wolfram syndrome, as well as the function of WFS1 protein in beta cells.

This is one of a few good models showing that one genetic defect predisposes beta cells to profound failure upon ER stress induced by systemic insulin resistance [30, 49]. Our findings are important for the understanding of the molecular pathophysiology of Wolfram syndrome. In addition, a common process may be involved in conventional type 2 diabetes patients, whose beta cells decrease very slowly but progressively. In this context, a recent report has confirmed that common variants in the *WFS1* gene confer risk of type 2 diabetes [50]. Therefore knowledge from this model would help us to understand the mechanisms of, and to develop a way of preventing beta cell loss in patients with conventional type 2 diabetes mellitus.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Impact of lipid phosphatases SHIP2 and PTEN on the time- and Akt-isoform-specific amelioration of TNF-α-induced insulin resistance in 3T3-L1 adipocytes

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[1]*Department of Internal Medicine and* [2]*Department of Clinical Pharmacology, University of Toyama;* [3]*Sainou South Hospital, Toyama; and* [4]*Division of Molecular Medical Science, Hiroshima University, Hiroshima, Japan*

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Ikubo M, Wada T, Fukui K, Ishiki M, Ishihara H, Asano T, Tsuneki H, Sasaoka T. Impact of lipid phosphatases SHIP2 and PTEN on the time- and Akt-isoform-specific amelioration of TNF-α-induced insulin resistance in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 296: E157–E164, 2009. First published November 11, 2008; doi:10.1152/ajpendo.90581.2008.—TNF-α is a major contributor to the pathogenesis of insulin resistance associated with obesity and inflammation by serine phosphorylating and degrading insulin receptor substrate-1. Presently, we further found that pretreatment with TNF-α inhibited insulin-induced phosphorylation of Akt2 greater than Akt1. Since lipid phosphatases SH2-containing inositol 5′-phoshatase 2 (SHIP2) and phosphatase and tensin homologs deleted on chromosome 10 (PTEN) are negative regulators of insulin's metabolic signaling at the step downstream of phosphatidylinositol 3-kinase, we investigated the Akt isoform-specific properties of these phosphatases in the negative regulation after short- and long-term insulin treatment and examined the influence of inhibition on the amelioration of insulin resistance caused by TNF-α in 3T3-L1 adipocytes. Adenovirus-mediated overexpression of WT-SHIP2 decreased the phosphorylation of Akt2 greater than Akt1 after insulin stimulation up to 15 min. Expression of a dominant-negative ΔIP-SHIP2 enhanced the phosphorylation of Akt2 up to 120 min. On the other hand, overexpression of WT-PTEN inhibited the phosphorylation of both Akt1 and Akt2 after short- but not long-term insulin treatment. The expression of ΔIP-PTEN enhanced the phosphorylation of Akt1 at 120 min and that of Akt2 at 2 min. Interestingly, the expression of ΔIP-SHIP2, but not ΔIP-PTEN, protected against the TNF-α inhibition of insulin-induced phosphorylation of Akt2, GSK3, and AS160, whereas both improved the TNF-α inhibition of insulin-induced 2-deoxyglucose uptake. The results indicate that these lipid phosphatases possess different characteristics according to the time and preference of Akt isoform-dependent signaling in the negative regulation of the metabolic actions of insulin, whereas both inhibitions are effective in the amelioration of insulin resistance caused by TNF-α.

insulin signaling; SH2-containing inositol 5′-phoshatase 2; phosphatase and tensin homologs deleted on chromosome 10

THE ACTIVATED INSULIN RECEPTOR phosphorylates insulin receptor substrates (IRS) at tyrosine residues (17, 26, 28). The tyrosine phosphorylated IRS binds to the regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase), which in turn activates the p110 catalytic subunit (3, 32, 36). The activation of PI3-kinase is known to be important for the various metabolic actions of insulin (3, 33, 37). PI3-kinase functions as a lipid kinase to produce $PI(3,4,5)P_3$ from $PI(4,5)P_2$ in vivo (32). $PI(3,4,5)P_3$ acts as a key lipid second messenger in insulin signaling to further downstream molecules, including Akt (30, 38). Lipid phosphatases were identified to hydrolyze $PI(3,4,5)P_3$ in the negative regulation of insulin signaling (12, 20, 25). SH2-containing inositol 5′-phoshatase 2 (SHIP2) functions as a lipid phosphatase possessing 5′-phosphatase activity to hydrolyze $PI(3,4,5)P_3$ to $PI(3,4)P_2$ (38). Phosphatase and tensin homologs deleted on chromosome 10 (PTEN) act as a 3′-lipid phosphatase hydrolyzing $PI(3,4,5)P_3$ to $PI(4,5)P_2$ (20). Targeted disruption of the SHIP2 gene in mice resulted in increased insulin sensitivity and conferred protection against obesity induced by a high-fat diet (4, 33). Since homozygous disruption of the PTEN gene in mice results in embryonic lethality, the tissue-specific role of PTEN was investigated (7). Liver, skeletal muscle, or adipose tissue-specific disruption of PTEN ameliorated glucose metabolism in the animal model of diabetes (18, 34, 39); therefore, lipid phosphatases, both SHIP2 and PTEN, appear to be implicated in glucose metabolism.

Adipocytes are important target tissues of insulin, and 3T3-L1 cells are well-characterized adipocytes (22, 23, 38). Overexpression of SHIP2 and PTEN is reported to inhibit insulin-induced phosphorylation of Akt and glucose uptake (22, 23, 38); however, the effect of SHIP2 and PTEN expression has been examined only after short-term insulin treatment with controversial results (23, 35). In addition, the role of these lipid phosphatases in the regulation of metabolic signaling after long-term insulin treatment is unknown; therefore, it would be of particular importance to clarify the possible differences in characteristics and properties among these lipid phosphatases to further understand the molecular mechanism of the negative regulation of insulin signaling.

Akt is one of the downstream target molecules of PI3-kinase important for glucose metabolism (5, 6, 38). Akt1 and Akt2 are the isoforms mainly expressed in adipocytes (31, 38). Studies (5, 6) with Akt1 and Akt2 knockout mice revealed that Akt2 is preferentially implicated in glucose metabolism, whereas Akt1 is mainly involved in cell growth. The comparative effect of SHIP2 and PTEN on short- and long-term insulin-induced phosphorylation of Akt1 and Akt2 is uncertain in 3T3-L1 adipocytes. In addition, TNF-α is an important cytokine implicated in the development of insulin resistance in type 2 diabetes (14, 27, 39); therefore, investigation of the ameliorative effect by inhibition of these endogenous lipid phosphatases

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on TNF-α-induced insulin resistance is important to clarify the therapeutic value in type 2 diabetes.

In the present study, we directly compared the role of lipid phosphatases SHIP2 and PTEN in short- and long-term insulin-induced phosphorylation of Akt1 and Akt2 in 3T3-L1 adipocytes. Furthermore, we investigated whether the inhibition of endogenous SHIP2 and PTEN by phosphatase-defective mutant expression protects against the impairment of insulin-induced phosphorylation of Akt, GSK3, and Akt substrate 160 (AS160), and glucose uptake by pretreatment with TNF-α.

MATERIALS AND METHODS

Materials. Human crystal insulin was provided by Novo Nordisk Pharmaceutical (Copenhagen, Denmark). 2-[^{3}H]deoxyglucose (2DG; 3,330 GBq/mM) was purchased from NEN Life Science Products (Boston, MA). Human recombinant TNF-α was obtained from Pepro Tech (Rocky Hill, NJ). The two polyclonal anti-SHIP2 antibodies were described previously (12). A monoclonal anti-phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories (Lexington, KY). A polyclonal anti-Thr$^{308/309}$ phospho-specific Akt antibody and a polyclonal anti-Ser$^{473/474}$ phospho-specific Akt antibody, a polyclonal anti-Akt antibody, a polyclonal anti-Akt2 antibody, a polyclonal anti-Ser$^{21/9}$ phospho-specific GSK3α/β antibody, a polyclonal anti-GSK3α/β antibody, an a polyclonal anti-Ser/Thr-phospho-specific Akt substrate antibody, and a polyclonal anti-AS160 antibody were from Cell Signaling (Beverly, MA). A monoclonal anti-Akt1 antibody and a monoclonal anti-PTEN antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal anti-IRS-1 antibody was from Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence reagents were from GE Healthcare Bio-Science (Tokyo, Japan). DMEM was from GIBCO-BRL Japan (Tokyo, Japan). All other reagents were of analytical grade and purchased from Sigma Chemical (St. Louis, MO) or Wako Pure Chemical Industries (Osaka, Japan).

Adenoviral vectors. Adenoviral vectors encoding wild-type SHIP2 (WT-SHIP2), a phosphatase-defective mutant SHIP2 (ΔIP-SHIP2) containing Pro^{687} to Ala, Asp^{691} to Ala, and Arg^{692} to Gly changes (38), wild-type PTEN (WT-PTEN), and a phosphatase-defective mutant PTEN (ΔIP-PTEN) containing Cys^{124} to Ser change (23) were described previously.

Cell culture and infections with adenovirus. 3T3-L1 fibroblasts were grown and passaged in DMEM supplemented with 10% donor calf serum. Cells at 2–3 days postconfluence were used for differentiation. The differentiation medium contained 10% FBS, 250 nM dexamethazone, 0.5 mM IBMX, and 500 nM insulin. After 3 days, the differentiation medium was replaced with postdifferentiation medium containing 10% FBS and 500 nM insulin. After 3 more days, the postdifferentiation medium was replaced with DMEM including 25 mM glucose supplemented with 10% FBS. SHIP2 and PTEN were transiently expressed in differentiated 3T3-L1 adipocytes by means of adenovirus-mediated gene transfer. A multiplicity of infection (MOI) of 10–40 plaque-forming units (PFU)/cell was used to infect 3T3-L1 adipocytes in DMEM containing 2% FBS, with the virus being left on the cells for 16 h before removal. Subsequent experiments were conducted 24–48 h after initial addition of the virus (38). The efficiency of adenovirus-mediated gene transfer of SHIP2 and PTEN was ~95%.

Insulin and TNF-α treatment. 3T3-L1 adipocytes grown in six-well multiplates were incubated with DMEM containing 0.1% FBS without or with 17 nM insulin at 37°C for 2- to 120-min periods. For experiments with TNF-α treatment, 20 nM TNF-α were added for 16 h and then treated with 17 nM insulin for 10 or 120 min.

Immunoprecipitation and Western blotting. The cells were lysed in a buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium deoxycholate, 1 mM β-glycerophosphate, 1% NP40, 1 mM PMSF, 1 mM Na_3VO_4, 50 mM sodium fluoride, 10 μg/ml of aprotinin, and 10 μM leupeptin, pH 7.4, for 30 min at 4°C. The lysates were centrifuged to remove insoluble materials. The supernatants (100 μg of protein) were immunoprecipitated with antibodies for 2 h at 4°C. The precipitates or the lysates were then separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes using a Bio-Rad Transblot apparatus. The membranes were blocked in a buffer containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 2.5% BSA or 5% nonfat milk, pH 7.5, for 2 h at 20°C. They were then probed with antibodies for 2 h at 20°C or for 16 h at 4°C. After the membranes were washed in a buffer containing 50 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.5, the blots were incubated with a horseradish peroxidase-linked secondary antibody and subjected to enhanced chemiluminescence detection using ECL reagent according to the manufacturer's instructions (GE Health Science Bio-Science; Ref. 38). Densitometric analysis was conducted directly from the blotted membrane by utilizing LAS-4000 lumino-image analyzer system (Fujifilm, Tokyo, Japan). The relative phosphorylation level of each protein was calculated as the ratio of phosphorylated to total protein level.

Measurement of 2-deoxyglucose uptake. 3T3-L1 adipocytes grown in six-well multiplates were pretreated with TNF-α and serum starved for 2 h. The cells were washed twice with PBS and incubated with Krebs-Ringer phosphate-HEPES buffer, 10 nM HEPES, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 2.5 mM NaH_2PO_4, containing 1% BSA, pH 7.4, for 1 h at 37°C. The cells were subsequently stimulated with various concentrations of insulin. After a 15-min insulin treatment, 3.7 kBq of 2-[^{3}H]deoxyglucose (2-DG) were added for 4 min. The reaction was stopped by the addition of 10 μnol/l cytochalasin B. The cells were washed three times with PBS and solubilized with 0.2 mM SDS-0.2 N NaOH (38). The radioactivity incorporated into the cells was measured by liquid scintillation counting.

Statistical analysis. Data are expressed as means ± SE. P values were determined by one-way ANOVA with Bonferroni's correction test, and $P < 0.05$ was considered significant.

RESULTS

Expression of lipid phosphatases in 3T3-L1 adipocytes. SHIP2 (140-kDa) is a 5′-lipid phosphatase and PTEN (54-kDa) is a 3′-lipid phosphatase, both of which are known to be involved in the negative regulation of insulin signaling (22, 23, 30, 38). Endogenous SHIP2 and PTEN were clearly observed in control 3T3-L1 adipocytes. Consensus amino acids located within the catalytic domain of lipid phosphatases were mutated to generate phosphatase-defective SHIP2 (ΔIP-SHIP2) and PTEN (ΔIP-PTEN) (23, 38). Wild-type and phosphatase-defective lipid phosphatases were transiently expressed in 3T3-L1 adipocytes by means of adenovirus-mediated gene transfer. By transfecting with these lipid phosphatase genes at an MOI of 10 PFU/cell (Fig. 1, *A* and *B*) and 40 PFU/cell (Fig. 1, *C* and *D*), we observed a 2.5- and 5-fold increase in expression levels of SHIP2 and a 4- and 7-fold increase in expression levels of PTEN, respectively, over the endogenous level in 3T3-L1 adipocytes. Since the obtained results with expression at an MOI of 10 and 40 PFU/cell were similar, the after analyses were shown at an MOI of 40 PFU/cell.

Effect of expression of wild-type lipid phosphatases on insulin-induced phosphorylation of Akt. Akt is one of the downstream target molecules of PI3-kinase and has been shown to mediate the metabolic actions of insulin (5, 38). We compared the effect of WT-SHIP2 and WT-PTEN expressions on the phosphorylation of Akt1 and Akt2 after insulin stimulation for up to 120 min in 3T3-L1 adipocytes. Overexpression

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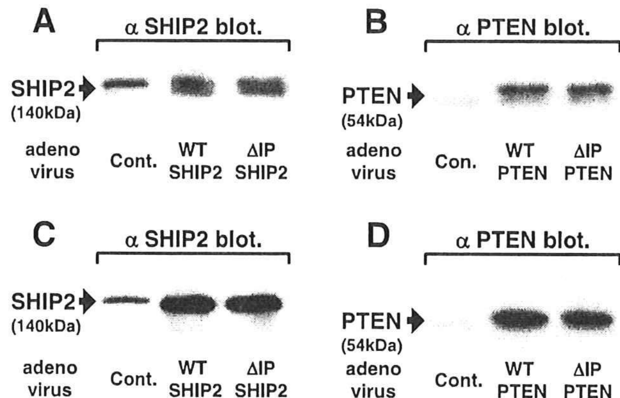

Fig. 1. Expression of lipid phosphatases in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with LacZ, wild-type SH2-containing inositol 5′-phosphatase 2 (WT-SHIP2), phosphatase-defective mutant SHIP2 (ΔIP-SHIP2), wild-type phosphatase and tensin homologs deleted on chromosome 10 (WT-PTEN), and ΔIP-PTEN at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU)/cells (*A* and *B*) and 40 PFU/cells (*C* and *D*). After infection, cells were lysed and subjected to immunoblot analysis with anti-SHIP2 antibody (*A* and *C*) and anti-PTEN antibody (*B* and *D*). Results represent 3 separate experiments.

of WT-SHIP2 mildly inhibited the phosphorylation of Akt1 at Thr[308] after insulin stimulation for up to 120 min (13.0% reduction at 5 min; Fig. 2*A*). On the other hand, overexpression of WT-SHIP2 greatly inhibited Akt2 phosphorylation at Thr[309] after insulin treatment for up to 15 min (30.9% reduction at 5 min; Fig. 2*C*). Overexpression of WT-PTEN significantly inhibited insulin-induced Akt1 phosphorylation at 2 and 5 min by 26.6 and 16.5%, respectively (Fig. 2*B*). Insulin-induced phosphorylation of Akt2 at 2 and 5 min was also inhibited by 29.1 and 20.0%, respectively, by the overexpression of WT-PTEN (Fig. 2*D*). Similar results were obtained concerning Akt phosphorylation at Ser[473/474] residue and GSK3 phosphorylation at Ser[21/9] residue (data not shown).

Effect of expression of phosphatase-defective (ΔIP) lipid phosphatases on insulin-induced phosphorylation of Akt. Expression of ΔIP-SHIP2 enhanced Akt1 phosphorylation at Thr[308], and phosphorylation was significantly enhanced by 70.4% after 120 min of insulin treatment (Fig. 3*A*). The enhancing effect was more apparent in Akt2 phosphorylation at Thr[309]. Phosphorylation of Akt2 at 2, 5, and 120 min of insulin treatment was significantly augmented by 20.7, 16.6, and 93.7%, respectively, by the expression of ΔIP-SHIP2 (Fig. 3*C*). Expression of ΔIP-PTEN enhanced long-term insulin-stimulated Akt1 phosphorylation, which was significantly augmented by 43.9% after 120 min of insulin treatment (Fig. 3*B*). Phosphorylation of Akt2 at only 2 min of insulin stimulation was slightly enhanced by 17.7% by the expression of ΔIP-PTEN (Fig. 3*D*). Similar results were again obtained concerning the phosphorylation of Akt at Ser[473/474] residue and GSK3 at Ser[21/9] residue (data not shown).

Expression of phosphatase-defective lipid phosphatases ameliorates insulin-induced phosphorylation of Akt after TNF-α treatment. We next examined the ameliorative effect of the expression of phosphatase-defective lipid phosphatases on the decreased insulin-induced phosphorylation of Akt by pre-

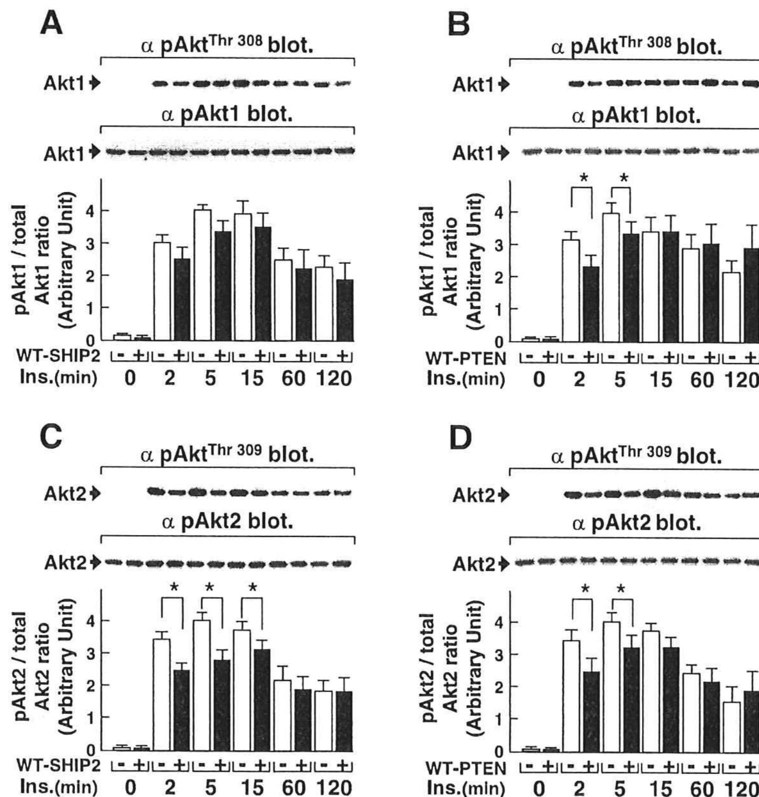

Fig. 2. Effect of wild-type SHIP2 and PTEN overexpression on insulin-induced phosphorylation of Akt. 3T3-L1 adipocytes were transfected with WT-SHIP2 (*A* and *C*) and WT-PTEN (*B* and *D*) at an MOI of 40 PFU/cell. Cells were serum starved for 16 h and subsequently treated with 17 nM insulin at 37°C for indicated times. Cells were immunoprecipitated with anti-Akt1 antibody (*A* and *B*) or anti-Akt2 antibody (*C* and *D*). Precipitates were separated by 7.5% SDS-PAGE and immunoblotted with anti-Thr[308/309]-phospho-specific Akt antibody, anti-Akt1 antibody, or anti-Akt2 antibody. Amount of Akt phosphorylated at Thr[308/309] corrected for total protein level was quantitated by densitometry. Results are means ± SE of 5 separate experiments. *$P < 0.05$ vs. amount of phosphorylated Akt in LacZ-transfected cells with respective insulin treatment.

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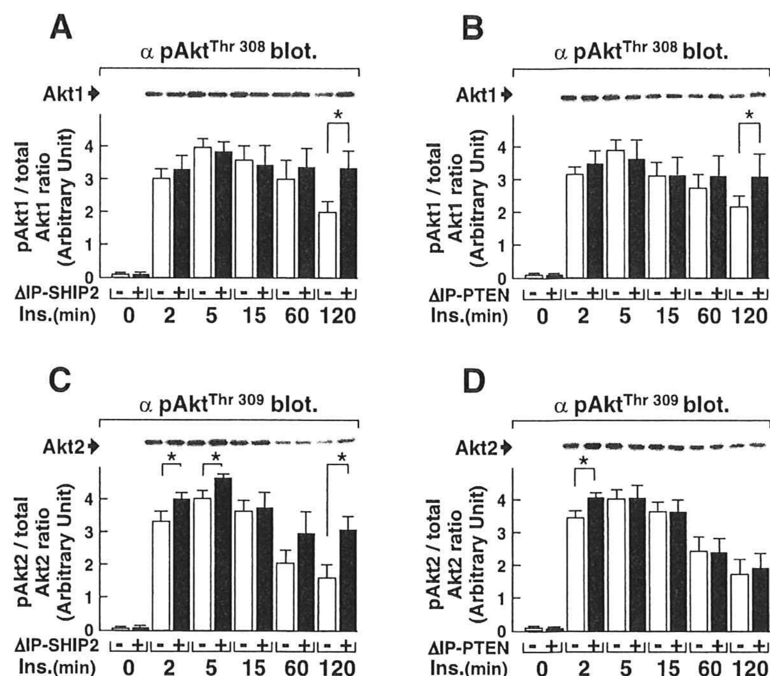

Fig. 3. Effect of phosphatase-defective SHIP2 and PTEN expression on insulin-induced phosphorylation of Akt. 3T3-L1 adipocytes were transfected with ΔIP-SHIP2 (*A* and *C*) and ΔIP-PTEN (*B* and *D*) at an MOI of 40 PFU/cell. Cells were serum starved for 16 h and subsequently treated with 17 nM insulin at 37°C for indicated times. Cells were immunoprecipitated with anti-Akt1 antibody (*A* and *B*) or anti-Akt2 antibody (*C* and *D*). Precipitates were separated by 7.5% SDS-PAGE and immunoblotted with anti-Thr$^{308/309}$-phospho-specific Akt antibody. Amount of Akt phosphorylated at Thr$^{308/309}$ corrected for total protein level was quantitated by densitometry. Results are means ± SE of 5 separate experiments. $*P < 0.05$ vs. amount of phosphorylated Akt in LacZ-transfected cells with respective insulin (Ins.) treatment.

treatment with TNF-α (Fig. 4). Pretreatment with TNF-α decreased the phosphorylation of Akt1 at Thr^{308} after insulin stimulation for 10 min (*lane 5* vs. *7*) but not for 120 min (*lane 9* vs. *11*). The TNF-α-induced decrease in insulin-induced phosphorylation of Akt1 at 10 min was not apparently affected by the expression of either ΔIP-SHIP2 (Fig. 4*A*) or ΔIP-PTEN (Fig. 4*B*). In contrast, the expression of either phosphatase-defective mutant enhanced insulin-induced phosphorylation of Akt1 at 120 min even after pretreatment with TNF-α (Fig. 3, *A* and *B*, and Fig. 4, *A* and *B*). On the other hand, pretreatment with TNF-α markedly inhibited the insulin-induced phosphorylation of Akt2 (Thr^{309} residue) at 10 min (*lane 5* vs. *7*) and also at 120 min (*lane 9* vs. *11*). The decreased phosphorylation of Akt2 by pretreatment with TNF-α was effectively ameliorated by the expression of ΔIP-SHIP2 (Fig. 4*C*) but not by the expression of ΔIP-PTEN (Fig. 4*D*). Similar results were ob-

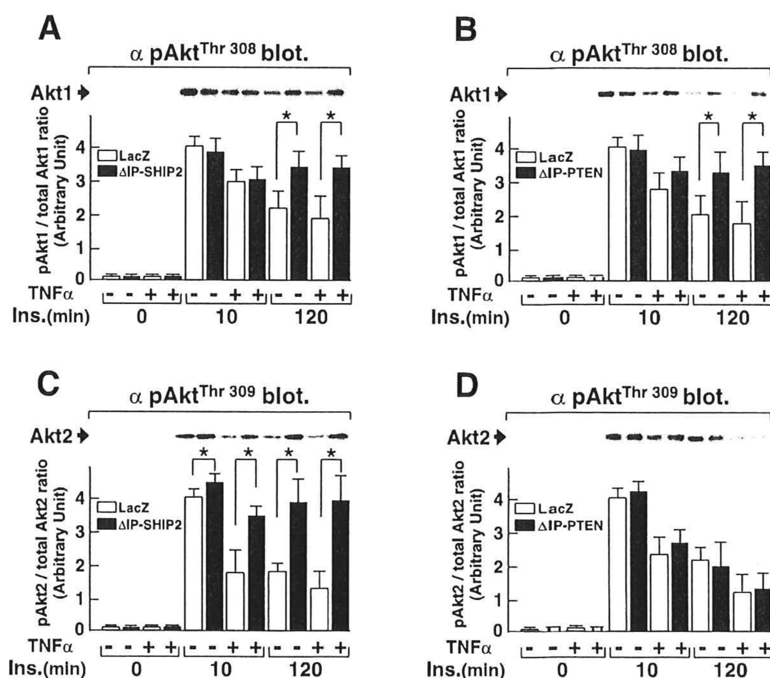

Fig. 4. Effect of phosphatase-defective SHIP2 and PTEN expression on TNF-α-induced decrease in phosphorylation of Akt. 3T3-L1 adipocytes were transfected with ΔIP-SHIP2 (*A* and *C*) and ΔIP-PTEN (*B* and *D*) at an MOI of 40 PFU/cell. Serum-starved transfected cells preincubated with 20 nM TNF-α for 16 h were treated with 17 nM insulin for 10 and 120 min. Cells were immunoprecipitated with anti-Akt1 antibody (*A* and *B*) or anti-Akt2 antibody (*C* and *D*). Precipitates were separated by 7.5% SDS-PAGE and immunoblotted with anti-Thr$^{308/309}$-phospho-specific Akt antibody. Amount of phosphorylated Akt at Thr$^{308/309}$ corrected for total protein level was quantitated by densitometry. Results are means ± SE of 4 separate experiments. $*P < 0.05$ vs. amount of phosphorylated Akt in LacZ-transfected cells with respective insulin treatment.

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tained by the reciprocal immunoblotting studies with anti-Akt1 antibody or anti-Akt2 antibody after immunoprecipitation with anti-Thr$^{308/309}$ phospho-specific Akt antibody (data not shown). In addition, similar findings were again observed concerning the phosphorylation of Akt at Ser$^{473/474}$ residue (data not shown).

Expression of phosphatase-defective lipid phosphatases ameliorates insulin-induced phosphorylation of GSK3 and AS160 after TNF-α treatment. GSK3 and AS160 are important downstream molecules of Akt implicated in the metabolic action of insulin (3, 28, 37). We therefore investigated the ameliorative effect of the expression of phosphatase-defective lipid phosphatases on the decreased insulin-induced phosphorylation of GSK3 and AS160 by pretreatment with TNF-α (Fig. 5). Pretreatment with TNF-α decreased the phosphorylation of GSK3 and AS160 after insulin stimulation for 10 min (*lane 5* vs. *7*) and for 120 min (*lane 9* vs. *11*). TNF-α-induced decrease in insulin-induced phosphorylation of both GSK3 and AS160 was relatively more ameliorated by the expression of ΔIP-SHIP2 (Fig. 5, *A* and *C*) than that of ΔIP-PTEN (Fig. 5, *B* and *D*). These results indicate that the change of GSK3 and AS160 phosphorylations is relatively correlated with the phosphorylation of Akt2 rather than Akt1 by the expression of ΔIP-SHIP2.

Expression of phosphatase-defective lipid phosphatases does not affect insulin-induced degradation and tyrosine phosphorylation of IRS-1 after TNF-α treatment. We further examined whether expression of phosphatase-defective lipid phosphatases affects insulin-induced degradation and tyrosine phosphorylation of IRS-1 after TNF-α treatment, since TNF-α is an important adipokine that causes insulin resistance by facilitating the degradation of IRS-1 in 3T3-L1 adipocytes (14). As a result, insulin-induced tyrosine phosphorylation of IRS-1 is decreased after pretreatment with TNF-α (14). Consistent with these findings, pretreatment with TNF-α caused the degradation of IRS-1, and the subsequent tyrosine phosphorylation of IRS-1 after insulin treatment for 10 and 120 min was decreased. The expression of neither ΔIP-SHIP2 (Fig. 6*A*) nor ΔIP-PTEN (Fig. 6*B*) affected TNF-α- and insulin-induced degradation of IRS-1. Similarly, the suppressing effect of TNF-α on insulin-induced tyrosine phosphorylation of IRS-1 at 10 and 120 min was not affected by the expression of either ΔIP-SHIP2 (Fig. 6*C*) or ΔIP-PTEN (Fig. 6*D*). These results indicate that the expression of ΔIP-SHIP2 and ΔIP-PTEN ameliorates the decreased Akt-dependent signaling without affecting degradation of IRS-1. It is of note that treatment with TNF-α itself also did not affect the expression of endogenous SHIP2 and PTEN (data not shown).

Effect of expression of phosphatase-defective lipid phosphatases on insulin-induced glucose uptake after TNF-α treatment. We finally examined the effect of the expression of phosphatase-defective lipid phosphatases on insulin-induced 2-DG uptake in the presence or absence of TNF-α (Fig. 7). The expression of either ΔIP-SHIP2 or ΔIP-PTEN enhanced insulin-induced 2-DG uptake, although the former augmented it greater than the latter. Treatment with TNF-α inhibited 1.7- and 17-nM insulin-induced 2-DG uptake by 42.7 and 19.0%,

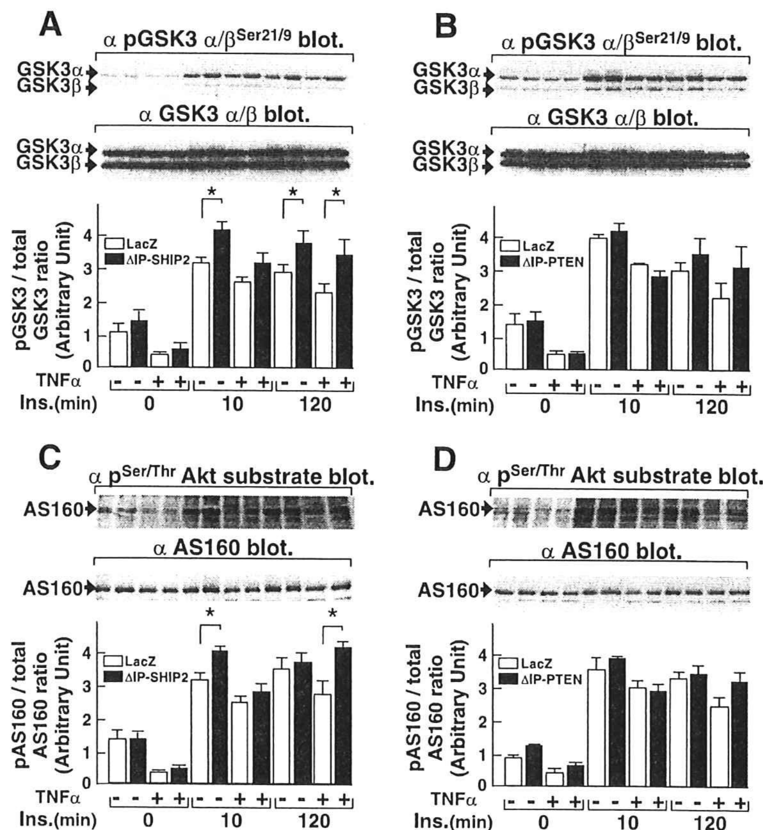

Fig. 5. Effect of phosphatase-defective SHIP2 and PTEN expression on TNF-α-induced decrease in phosphorylation of GSK3 and AS160. 3T3-L1 adipocytes were transfected with ΔIP-SHIP2 (*A* and *C*) and ΔIP-PTEN (*B* and *D*) at an MOI of 40 PFU/cell. Serum-starved transfected cells preincubated with 20 nM TNF-α for 16 h were treated with 17 nM insulin for 10 and 120 min. Total cell lysates were separated by 7.5% SDS-PAGE and immunoblotted with anti-Ser$^{21/9}$-phospho-specific GSK3α/β antibody (*A* and *B*), anti-GSK3α/β antibody (*A* and *B*), anti-Ser/Thr-phospho-specific Akt substrate antibody (*C* and *D*), or anti-AS160 antibody (*C* and *D*). Amount of phosphorylated GSK3 and AS160 corrected for total GSK3 and AS160 levels, respectively, was quantitated by densitometry. Results are expressed as means ± SE of 4 separate experiments. $*P < 0.05$ vs. amount of phosphorylated GSK3 or AS160 in LacZ-transfected cells with respective insulin treatment.

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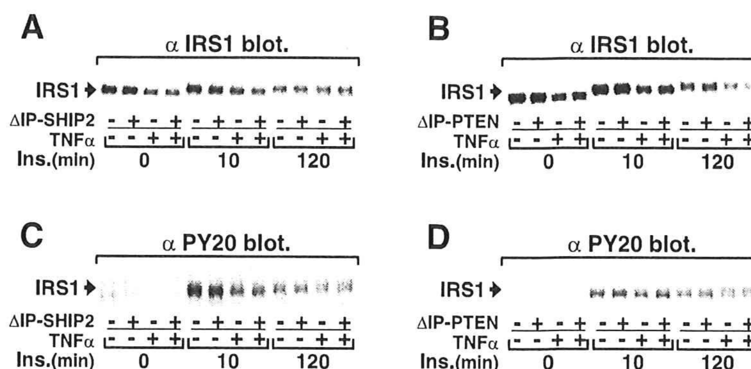

Fig. 6. Effect of phosphatase-defective SHIP2 and PTEN expression on insulin-induced degradation and tyrosine phosphorylation of IRS-1 after pretreatment with TNF-α. 3T3-L1 adipocytes were transfected with ΔIP-SHIP2 (*A* and *C*) and ΔIP-PTEN (*B* and *D*) at an MOI of 40 PFU/cell. Cells were serum starved for 16 h and subsequently pretreated with 20 ng/ml of TNF-α for 16 h. They were then treated with 17 nM insulin at 37°C for 10 and 120 min. Cells were lysed and separated by 7.5% SDS-PAGE and immunoblotted with anti-IRS-1 antibody (*A* and *B*) or anti-phosphotyrosine antibody (*C* and *D*). Amount of IRS protein and tyrosine-phosphorylated IRS-1 was quantitated by densitometry. Results are means ± SE of 4 separate experiments. *$P < 0.05$ vs. amount of IRS in LacZ-transfected cells with respective insulin treatment.

respectively. Interestingly, the expression of either ΔIP-SHIP2 or ΔIP-PTEN ameliorated the reduced 2-DG uptake by pretreatment with TNF-α to the control level. The amount of GLUT4 protein was not altered by treatment with TNF-α or the expression of both ΔIP-SHIP2 and ΔIP-PTEN (data not shown).

DISCUSSION

SHIP2 and PTEN are lipid phosphatases known to be involved in the negative regulation of insulin signaling in vivo and/or in vitro (4, 18, 20, 23, 33–35, 38, 39); however, the possible difference in characteristics and properties among these lipid phosphatases after short- vs. long-term insulin treatment is still unknown. Our previous reports (31) showed that SHIP2 predominantly regulates the phosphorylation of

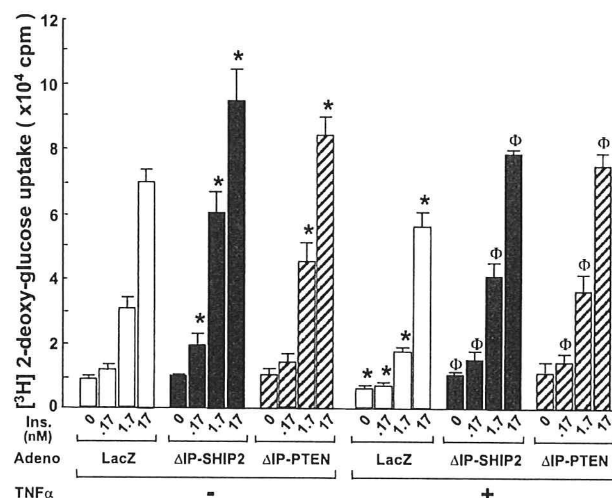

Fig. 7. Effect of phosphatase-defective SHIP2 and PTEN expression on TNF-α-induced inhibition of glucose uptake. 3T3-L1 adipocytes were transfected with ΔIP-SHIP2 or ΔIP-PTEN at an MOI of 40 PFU/cell. Serum-starved transfected cells pretreated with 20 nM TNF-α for 16 h were incubated in glucose-free medium for 30 min. After the cells had been stimulated with 10 nM insulin for 15 min, 3.7 kBq of 2-[^{3}H]deoxyglucose (2-[^{3}H]DG) were added for 4 min. Reaction was stopped by the addition of 10 μM cytochalasin B. Cells were washed 3 times with PBS and solubilized with 0.2 mM SDS-0.2 N NaOH. Radioactivity incorporated into the cells was measured with a liquid scintillation counter. Results are means ± SE of 5 separate experiments. *$P <$ 0.05 vs. 2-[^{3}H]DG uptake in LacZ-transfected control cells with respective insulin treatment. Φ$P < 0.05$ vs. 2-[^{3}H]DG uptake in TNF-α-treated cells with respective insulin treatment.

Akt2, but not Akt1, after short-term insulin treatment. Again, our current results showed that overexpression of WT-SHIP2 inhibited insulin-induced phosphorylation of Akt2 rather than Akt1 only after insulin treatment for up to 15 min. Interestingly, the effect was diminished after 60 and 120 min of insulin stimulation. Furthermore, the expression of ΔIP-SHIP2 enhanced insulin-induced phosphorylation of Akt2 after both short- and long-term insulin treatments, whereas phosphorylation of Akt1 was augmented only after 120 min of insulin treatment by the expression; therefore, the present results further clarified the characteristics of the regulation of insulin signaling by SHIP2, indicating that SHIP2 predominantly regulates the phosphorylation of Akt2 rather than Akt1 in a time-specific manner.

PTEN is a 3′-lipid phosphatase hydrolyzing PI(3,4,5)P_3 to PI(4,5)P_2 (20). It is reported that overexpression of WT-PTEN inhibited short-term insulin-induced activation of Akt, although the isoform-specific difference was not examined (22, 23, 35). On the other hand, the expression of ΔIP-PTEN did not affect short-term insulin-induced metabolic signaling, whereas the amount of PI(3,4,5)P_3 was increased in 3T3-L1 adipocytes (23). In contrast, depletion of PTEN protein by siRNA-mediated gene silencing enhanced short-term insulin-induced phosphorylation of Akt (35). The present studies showed that overexpression of WT-PTEN inhibited the phosphorylation of Akt1 and Akt2 after insulin stimulation for up to 5 min and that the effect was diminished thereafter. Our results clearly indicate that the effect of WT-PTEN expression is not specific to either Akt1 or Akt2 and is seen only after short-term insulin treatment. In addition, the expression of ΔIP-PTEN enhanced Akt2 phosphorylation, and only had a mild impact on augmenting the phosphorylation of Akt1 after 2 min of insulin treatment. Furthermore, ΔIP-PTEN expression enhanced the phosphorylation of Akt1, but not Akt2, after 120 min of insulin treatment. Taken together, PTEN appears to be implicated in the regulation of both short- and long-term insulin treatment-induced phosphorylation of Akt, whereas the Akt isoform-specific difference during regulation is ambiguous. SHIP2 is reported to translocate from cytosol to plasma membrane whereby phosphorylation of Akt2 is preferentially regulated upon insulin treatment (31), whereas the redistribution of PTEN is uncertain in 3T3-L1 adipocytes. The possible difference of subcellular redistribution between SHIP2 and PTEN may be a reason to cause an alteration in the isoform and temporal specific effects of Akt phosphorylation. Alternatively, it is possible that the stronger effect of SHIP2 on Akt2 and the

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equivalent effects of PTEN on Akt1 and Akt2 reflect different properties of the pleckstrin homology (PH) domain of isoforms. Along this line, the PH domain of Akt2 may have higher affinity to PI(3,4,5)P_3 than PI(3,4)P_2, whereas the PH domain of Akt1 has similar affinities to both PI(3,4,5)P_3 and PI(3,4)P_2.

SHIP2 appears to be implicated in insulin resistance as a cause of type 2 diabetes in addition to the control of glucose homeostasis (4, 15, 16, 21, 33). SHIP2 knockout mice demonstrated enhanced phosphorylation of Akt in the skeletal muscle and liver, whereas whole body glucose homeostasis is not altered in mice fed a normal chow diet (33). However, the mice were protected from obesity and insulin resistance caused by a high-fat diet (33). Consistent with the report, the liver-specific inhibition of endogenous SHIP2 via the adenovirus-mediated expression of ΔIP-SHIP2 ameliorated glucose metabolism and insulin resistance in diabetic *db/db* mice and KK-A^y mice (8, 9). In addition, muscle denervation is known to cause insulin resistance characterized by a decrease in the ability of insulin to stimulate glucose uptake and glycogen synthesis in rats (1). A reduction of SHIP2 expression using an antisense oligonucleotide against SHIP2 mRNA ameliorated insulin resistance in rats (1). Furthermore, insulin resistance caused by chronic insulin treatment was effectively ameliorated by the expression of ΔIP-SHIP2 (29). Taken together, inhibition of endogenous SHIP2 appears to be valuable in the amelioration of insulin resistance in type 2 diabetes. Concerning the pathological impact of PTEN in glucose homeostasis (10, 13), heterozygous deletion of the PTEN gene in IRS-2 knockout mice conferred protection from insulin resistance, although homozygous disruption of the PTEN gene in mice resulted in embryonic lethality (19). Antisense oligonucleotide-mediated inhibition of endogenous PTEN expression in the liver led to the amelioration of elevated glucose levels and decreased insulin sensitivity in diabetic *ob/ob* and *db/db* mice (2). Adipose tissue-specific knockout of PTEN is known to protect against streptozotocin-induced diabetes (18). Muscle-specific knockout of PTEN resulted in the amelioration of decreased insulin-induced phosphorylation of Akt in the soleus caused by high-fat feeding (39). Although tissue-specific inhibition of PTEN may also appear to be a therapeutic target in the treatment of type 2 diabetes with insulin resistance, the main role of PTEN is the regulation of cell growth and tumor suppressor (7, 18, 34, 39); therefore, care should be taken when inhibiting PTEN for therapeutic usage because of possible tumor formation. Taken together, it is important to clarify the different molecular mechanisms by which inhibition of these lipid phosphatases ameliorates the state of insulin resistance.

TNF-α is an important adipokine causing insulin resistance by impairing insulin signaling (14, 27, 40). In the present study, we clarified that treatment with TNF-α impaired insulin-induced phosphorylation of Akt2 more profoundly than Akt1 in 3T3-L1 adipocytes. Interestingly, the expression of dominant-negative SHIP2 and PTEN enhanced the phosphorylation of Akt1 induced by insulin treatment for 120 min together with TNF-α. The present results indicate that enhancement of PI3-kinase-dependent insulin signaling by the inhibition of either lipid phosphatase is sufficient to ameliorate insulin-induced phosphorylation of Akt. Interestingly, inhibition of SHIP2, but not PTEN, effectively restored the impaired phosphorylation of Akt2 caused by TNF-α treatment. Similarly, the impaired phosphorylation of GSK3 and AS160 was ameliorated relatively more effectively by inhibition of SHIP2 than that of PTEN. Since Akt2 rather than Akt1 is closely related to the control of glucose metabolism (5), inhibition of SHIP2 rather than PTEN might be a more suitable approach to ameliorate decreased metabolic signaling of insulin in the state of insulin resistance; however, inhibition of either SHIP2 or PTEN ameliorated reduced insulin-induced glucose uptake caused by TNF-α to the same extent. It is uncertain why the extent of amelioration in glucose uptake is similar, whereas inhibition of SHIP2 improved the phosphorylation of Akt2 more than PTEN. Amelioration of Akt1 phosphorylation may be sufficient to improve glucose uptake, at least under our experimental conditions. Alternatively, the PI(3,4,5)P_3-mediated pathway independent of Akt may regulate glucose uptake. It might be necessary to examine the effect of glucose uptake more precisely to dissect the ameliorative role of inhibition between SHIP2 and PTEN.

In summary, the present results indicate that SHIP2 predominantly regulates insulin-induced phosphorylation of Akt2 rather than Akt1 in a time-specific manner and that PTEN regulates insulin-induced phosphorylation of both Akt1 and Akt2, whereas isoform specificity is ambiguous in the regulation in 3T3-L1 adipocytes. In addition, the expression of the dominant-negative SHIP2 effectively ameliorated decreased phosphorylation of Akt2 caused by pretreatment with TNF-α, whereas the reduced phosphorylation of Akt1 was restored by the expression of either of these phosphatase-defective lipid phosphatases without affecting the degradation of IRS-1. Our results further extend the knowledge that inhibition of both SHIP2 and PTEN is an attractive approach to ameliorate the metabolic action of insulin in the state of insulin resistance, whereas inhibition of SHIP2 appears to have more impact than PTEN on the amelioration of Akt2 phosphorylation.

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Regular Article

Neuropathy is associated with depression independently of health-related quality of life in Japanese patients with diabetes

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Objectives: To identify factors independently associated with depression in Japanese patients with diabetes, after controlling for potential confounding factors.

Methods: Among 197 outpatients with diabetes, 129 (type 1: 24, type 2: 105) completed a questionnaire concerning socio-demographic and health-related variables. Depression screening was done using Zung's Self-Rating Depression Scale test, followed by diagnostic interviews by experienced psychiatrists employing the Diagnostic Statistical Manual of Mental Disorders, 4th edition (DSM-IV).

Results: Forty-seven patients (36.4%) had symptomatological depression. A Self-Rating Depression Scale cut-off score of 40 had good sensitivity (100%) and modest specificity (59%) for detecting major depressive episode, in accordance with the DSM-IV. Diabetic patients suffering from depression were more likely to have neuropathy, retinopathy, body pain, a feeling of poor general health, and lack of social support, than the non-depressed patients. However, age, gender, marital status, diabetes type, insulin requirement, duration of diabetes, hemoglobin A1c (HbA1c) and the presence of nephropathy did not differ between the two groups. In multivariate logistic regression analysis, body pain (OR 3.26, 95% CI 1.31–8.08) and the presence of microvascular complications (OR 2.81, 95% CI 1.13–6.98) were independent factors associated with depression. Specifically, diabetic neuropathy (OR 3.10, 95% CI 1.17–8.22) was associated with depression independently of age, gender, marital status, social supports, quality of life, diabetes type, duration of diabetes, HbA1c, and insulin requirement.

Conclusions: A diabetic complication, specifically neuropathy, was independently associated with depression in patients with diabetes. The present findings indicate the need to find a biological base common to both depression in diabetes and diabetic neuropathy.

Key words: depression, diabetes, mental health, neuropathy, quality of life

DEPRESSION IS MORE common among diabetic patients in western countries. Recent studies have documented a doubling of depression rates in individuals with, as compared to those without, diabetes.[1,2] It has been shown that diabetic patients who are depressed have poorer glycemic control,[3] are less physically active,[4] and more obese.[5] Depression appears to be a very important problem in the management of diabetes.

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To date, the socio-demographic factors reported to be associated with depression include: female gender,[2] younger age,[2,6] being unmarried,[2,7,8] lower levels of education[6,8] and lack of social support.[9] Factors concerning health-related quality of life (QOL), such as perception of poor general health[10] and body pain,[11] were also associated with depression. Poor glycemic control,[3,7] types of diabetes treatment,[8] duration of diabetes,[8] presence of diabetic complications,[8,12] diabetic neuropathy[13–15] and retinopathy[9,16] were similarly associated with depression. However, these reports did not adequately control for potential confounding factors. Are there any factors underlying both depression and diabetes?

The prevalence of major depressive episodes in the Japanese diabetic population according to the Diagnostic Statistical Manual of Mental Disorders 4th edition (DSM-IV),[17] has not been investigated. In addition, only a few studies designed to identify factors associated with depression in individuals with diabetes in Asian countries, including Japan, have been reported. We first investigated the prevalence of depression, diagnosed by experienced psychiatrists, to confirm the increased prevalence of major depressive episodes in Japanese diabetic patients. Then we investigated factors possibly associated with depression in diabetic patients using multivariate logistic analysis, after controlling for the potential confounding factors.

METHODS

Subjects

All 197 patients who visited the Diabetes and Metabolism Unit, Tohoku University Hospital in November 2003 were recruited. The diagnosis of type 1 or type 2 diabetes had been made in accordance with the criteria of the American Diabetes Association.[18] One hundred twenty-nine of the 197 patients (65%) completed a questionnaire concerning socio-demographic and health-related variables. This study was conducted under the guidelines of the ethics committee at the Tohoku University. The procedures were fully explained to each subject before the assessments and written informed consent was obtained from each subject.

Measurement

Gender, age, marital status, the number of family members, educational level, and social support were assessed as socio-demographic variables. For social support, one item was selected from among the five items used in previous Japanese studies[19,20] as the strongest association with depression was found in the present samples. Health-related quality of life was assessed using the subscales for pain and perception of general health in the Japanese version of the Short-Form 36 Health Survey questionnaire.[21,22] All scores were dichotomized by the average score of the Japanese general population.

Diabetes type (Types 1 and 2), duration of illness (≧10 and <10 years), body mass index (BMI), medical regimen, anti-hypertensive requirements, hypolipidemic requirements, and blood pressure were obtained from the patient's medical record. Fasting plasma glucose concentration, hemoglobin A1c (HbA1c, ≧7 and <7%), and plasma lipid levels (Triglycerides [TG], total cholesterol [TC], high density lipoprotein cholesterol [HDL], and low density lipoprotein cholesterol [LDL]) were also evaluated as variables representing glycemic control status.

Microvascular complications were defined as the presence of at least one of the following: diabetic nephropathy, neuropathy or retinopathy. Diabetic nephropathy was defined as the presence of persistent proteinuria. Diabetic neuropathy was defined as the presence of symmetric neuropathic symptoms in the lower extremities and/or absence of bilateral Achilles tendon reflexes. Diabetic retinopathy, diagnosed by experienced ophthalmologists, was categorized as: simple, preproliferative or proliferative retinopathy, or none.

Assessment of depression

The Zung Self-Rating Depression Scale (SDS)[23] was used to screen for depression. Patients scoring 40 or more on the SDS were defined as suffering from depression.[24,25] The Japanese version of the SDS has been extensively validated.[26] All patients with a score of 40 or more on the SDS were suggested to undergo a psychiatric evaluation using the DSM-IV to diagnose a possible major depressive episode within the following one-month period. Almost the same number of patients with scores under 40 on the SDS had the same interviews as the controls. None of the subjects who were interviewed were taking any psychoactive drugs during the study.

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Statistical analysis

The χ^2 test or *t*-test was used to compare differences in characteristics between diabetic patients with and without depression. Multivariate logistic regression analysis was used to estimate the association between depression and potential predictor variables by calculating odds ratios (OR) and 95% confidence intervals (95% CI). All statistical analyses were performed using SPSS for Windows, version 11.5J. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Prevalence of depression and major depressive episode

Forty- seven out of 129 subjects (36.4%) had depression. Thirty-one of the 47 depressed subjects underwent diagnostic interviewing by experienced psychiatrists. Seven (type 1: 3, type 2: 4) of these 31 were diagnosed as having a major depressive episode. None of 34 subjects with SDS scores below 40, who had the same interviews, were diagnosed as having a major depressive episode. (Fig. 1 shows the experimental design.) Thus, the prevalence of a current major depressive episode in adult diabetic patients was estimated to be 7.9%. There is no difference of the prevalence between type 1 and type 2 ($P = 0.34$). In this study, an SDS cut-off of 40 yielded good sensitivity (100%) and modest specificity (59%) for detecting a major depressive episode in accordance with the DSM-IV.

Comparison of characteristics of diabetic patients with and without depression

The characteristics of patients with and without depression are presented in Table 1. The depressed

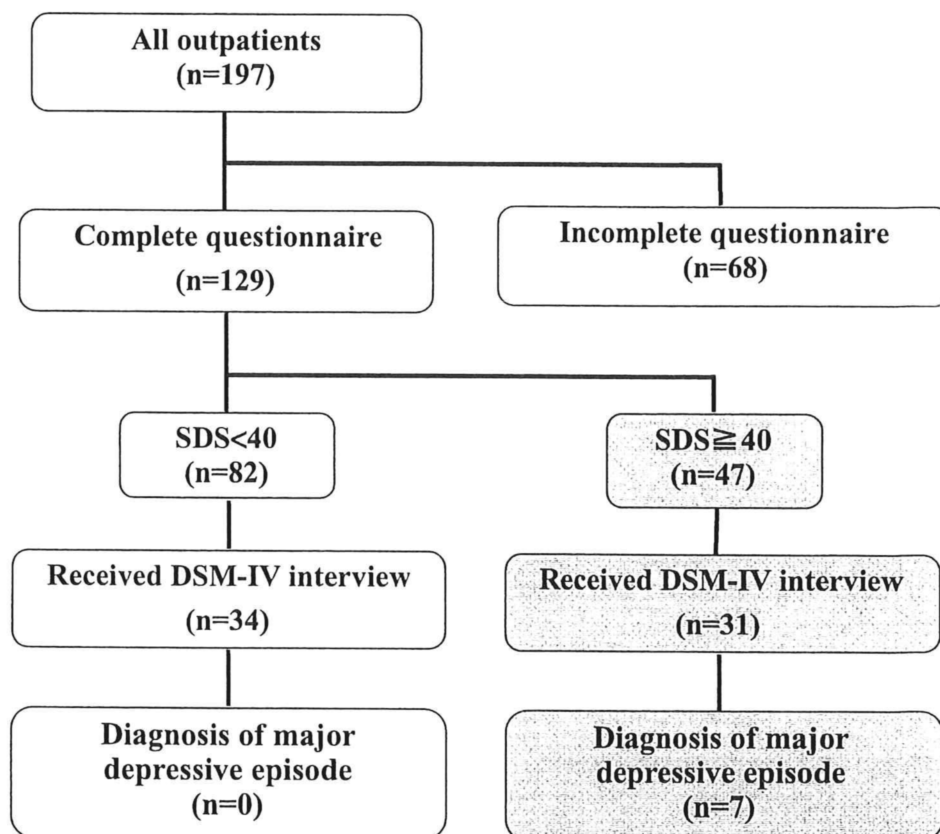

Figure 1. Experimental design. SDS, Zung Self-Rating Depression Scale; DSM-IV, Diagnostic Statistical Manual of Mental Disorders, 4th edition.

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Table 1. Characteristics of patients with and without depression

Variables	SDS < 40 (n = 82)	SDS ≧ 40 (n = 47)
Gender		
Female	35	23
Male	47	24
Age	54.12 ± 10.31	52.72 ± 10.47
Martial status		
Married	66	32
Unmarried	16	15
Number of family members	3.13 ± 1.45	3.32 ± 1.80
Social support		
+	77	38
–	5	9*
SF36: pain	76.72 ± 23.80	63.24 ± 27.00**
SF36: general health	54.82 ± 18.00	40.93 ± 22.91**
Education		
~ junior high school	9	4
~ high school	42	33
~ college (university)	31	10
Diabetes type		
Type 1	13	11
Type 2	69	36
Duration of illness		
<10 years	53	24
≧10 years	29	23
BMI (kg/M^2)	24.07 ± 4.69	24.07 ± 3.74
Insulin		
+	45	27
–	37	20
Anti-hypertensive agent		
+	25	12
–	57	35
Hypolipidemic agent		
+	27	9
–	55	38
Blood pressure (mmHg)	127.6 ± 15.0	126.3 ± 15.8
Diastolic	77.4 ± 10.1	75.0 ± 8.8
Systolic	146.61 ± 38.20	154.04 ± 66.90
Fasting blood glucose (mg/dl)	7.00 ± 1.19	7.12 ± 1.69
HbA1c (%)	112.76 ± 59.93	118.57 ± 73.70
TG (mg/dl)	193.82 ± 28.90	193.60 ± 35.99
TC (mg/dl)	56.40 ± 19.84	52.60 ± 17.25
HDL (mg/dl)	113.57 ± 26.10	117.40 ± 30.83
LDL (mg/dl)		
Microvascular complications		
+	28	28**
–	54	19
Nephropathy		
+	12	5
–	70	42
Neuropathy		
+	14	18**
–	68	29
Retinopathy		
+	19	20*
–	63	27

Data are means ± SD or N. BMI, body mass index; HbA1c, hemoglobin A1c; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; SDS, Zung Self-Rating Depression Scale; SF36, Short-Form 36 Health Survey questionnaires, TC, total cholesterol; TG, triglycerides; persons without pain have higher pain scores, persons who perceive themselves as having good health have higher scores for general health. *$P < 0.05$; **$P < 0.01$ by t-test or χ^2 test.

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patients were more likely to lack social support ($P < 0.05$), have pain (P. 0.01), and feel that their general health was poor ($P < 0.01$), than patients without depression. The prevalence of microvascular complications, that is, diabetic neuropathy and/or retinopathy, was also higher in the patients with than in those without depression ($P < 0.01$). In contrast, age, gender, marital status and educational level did not differ between the two groups. There was also no difference in the diabetes type, duration of diabetes, BMI, insulin requirement, blood pressure, antihypertensive medication requirement, fasting plasma glucose concentration, HbA1c, plasma lipid levels, hypolipidemic medication requirement or the presence of nephropathy between the depressed and nondepressed patients.

Multivariate analysis

Tables 2 and 3 show the factors predictably associated with depression in diabetic patients. As shown in Table 2, the subjective feeling of body pain (OR = 3.26, 95% CI = 1.31–8.08) and the presence of microvascular complications (OR = 2.81, 95% CI = 1.13–6.98) were independently associated with depression. In the analysis focused on neuropathy rather than the presence of microvascular complications in general, the presence of diabetic neuropathy (OR = 3.10, 95% CI = 1.17–8.22) was significantly associated with depression (Table 3). This association was independent of age, gender, marital status, social support, pain, a perception of poor general health, diabetes type, duration of diabetes, HbA1c, and insulin requirement. Neither nephropathy nor retinopathy was independently associated with depression.

Table 2. Factors independently associated with depression: with respect to microvascular complications

Independent variables	OR	95% CI	p
Age	0.99	0.95–1.03	0.76
Gender			
Male	1.00		
Female	1.10	0.47–2.58	0.83
Marital status			
Married	1.00		
Unmarried	1.55	0.60–4.01	0.37
Social support			
+	1.00		
−	2.04	0.68–8.55	0.17
SF 36: pain			
≧76.2	1.00		
<76.2	3.26	1.31–8.08	0.011*
SF 36: general health			
≧65.0	1.00		
<65.0	1.34	0.47–3.80	0.58
Type of diabetes			
Type 2	1.00		
Type 1	2.02	0.68–5.98	0.21
Duration of diabetes (years)			
<10	1.00		
≧10	1.75	0.74–3.92	0.21
HbA1c (%)			
<7.0	1.00		
≧7.0	0.56	0.23–1.37	0.20
Insulin			
−	1.00		
+	0.76	0.28–2.02	0.58
Microvascular complication			
−	1.00		
+	2.81	1.13–6.98	0.026*

*$P < 0.05$ by multivariate logistic regression analysis.
65.0, mean score on the general health subscale of the SF36 in the Japanese population (persons who perceive themselves as having good health have higher scores); 76.2, mean score on the pain subscale of the SF36 in the Japanese population (persons without pain have higher scores); 95% CI, 95% confidence intervals; HbA1c, hemoglobin A1c; OR, odds ratio; SF36: Short-Form 36 Health Survey questionnaire.

DISCUSSION

The prevalence of a DSM-IV major depressive episode in adult diabetic patients was estimate to be 7.9%, that is, higher than that of the general Japanese population (1%; DSM-III)[27] or working population (4%; ICD-10).[28] The prevalence range for current depression, diagnosed by structured diagnostic interviews of diabetic patients, was reported to be 11.0–19.9% in uncontrolled studies.[29] The prevalence determined in this study was lower than those reported previously. It might partially be explained by the fact that 16 out of 47 depressive patients who seemed to have severed depressive symptoms refused to be psychiatrically interviewed. However, our result confirmed an increased prevalence of a major depressive episode in diabetic patients as compared to the general population not only in Western countries but also in Japan.

Our findings on the characteristics of patients with and without depression are partially consistent with the results of earlier studies on the relationship

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Table 3. Factors independently associated with depression: with respect to neuropathy

Independent variables	OR	95% CI	*P*
Age	1.00	0.95–1.04	0.83
Gender			
Male	1.00		
Female	1.04	0.45–2.43	0.93
Marital status			
Married	1.00		
Unmarried	1.74	0.67–4.51	0.26
Social support			
+	1.00		
–	2.64	0.76–9.22	0.13
SF 36: pain			
≧76.2	1.00		
<76.2	3.53	1.42–8.81	0.007**
SF 36: general health			
≧65.0	1.00		
<65.0	1.25	0.44–3.50	0.68
Type of diabetes			
Type 2	1.00		
Type 1	1.96	0.66–5.77	0.23
Duration of diabetes (years)			
≧10	1.71		
<10	1.00	0.75–3.93	0.21
HbA1c (%)			
<7.0	1.00		
≧7.0	0.57	0.23–1.41	0.22
Insulin			
–	1.00		
+	0.78	0.30–2.11	0.64
Neuropathy			
–	1.00		
+	3.10	1.17–8.22	0.023*

*$P < 0.05$; **$P < 0.01$ by multivariate logistic regression analysis.
65.0, mean score on the general health subscale of the SF36 in the Japanese population (persons who perceive themselves as having good health have higher scores); 76.2, mean score on the pain subscale of the SF36 in the Japanese population (persons without pain have higher scores); 95% CI, 95% confidence intervals; HbA1c, hemoglobin A1c; OR, odds ratio; SF36, Short-Form 36 Health Survey questionnaire.

between diabetes and depression. Relationships between lack of social support,[9] perception of poor general health[10] or body pain[11] and depression in diabetic patients have been reported previously. Similarly, the presence of microvascular complications,[8,12] specifically neuropathy[13–15] and retinopathy,[9,16] are related to depression. However, our results do not support a relationship between depression and the following factors; female gender,[2] younger age,[2,6] being unmarried,[2,7] lower levels of education,[6,8] poor glycemic control,[3,7] types of diabetes treatment[8] and duration of diabetes.[8] The discrepancies across studies might be attributable, at least in part, to differences in patient characteristics. The patients in our study had essentially adequate glycemic control (HbA1c: 7.0 ± 1.4, FBS: 149 ± 50) at the start of the study and most had attained a high level of education. Thus, glycemic control status and educational level appear not to be related to depression.

In multivariate logistic regression analysis, the presence of microvascular complications, specifically neuropathy, was associated with depression independently of age, gender, marital status, social support, pain, perception of general health, diabetes type, duration of diabetes, HbA1c, and insulin requirement. Diabetic complications decreased health-related QOL, which is associated with increased risk of psychological impairment.[30,31] One of the subscales for health-related QOL, body pain, was also reported to be strongly associated with depression.[11,13] It was also suggested that individuals with inadequate support are most at risk of becoming depressed when disability related to illness increased.[31] Thus, several previous reports suggested depression in diabetic patients to be secondarily induced by decreased health-related QOL and/or inadequate social support. However, our results demonstrate that depression is not necessarily secondarily induced by pre-existing factors because a diabetic complication, specifically neuropathy, was independently associated with depression regardless of health-related QOL and/or social support.

There is a possibility that diabetic microvascular impairment induces both neuropathy[32] and so-called vascular depression[33] in diabetic patients. However, it seems unlikely that depression in diabetic patients would be vascular in origin because neither nephropathy nor retinopathy was independently associated with depression in this study.

The present findings indicate the need to find a biological base common to both depression in diabetes and diabetic neuropathy.

However, there are limitations to interpreting the results of this study. First, because this analysis is based on cross-sectional data, causality can not be determined. Prospective studies are needed. Second, this study has sample size limitation. Future studies

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enrolling adequate samples of diabetic patients are required.

CONCLUSION

Diagnostic interviews, conducted by experienced psychiatrists, demonstrated a higher prevalence of current major depressive episodes (7.9%) in Japanese diabetic patients than in the general population. Multivariate logistic regression analyses demonstrated neuropathy to be an independent factor associated with depression in diabetic patients.

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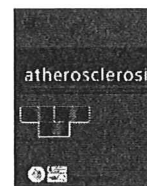

Carotid arterial elasticity is a sensitive atherosclerosis value reflecting visceral fat accumulation in obese subjects

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ABSTRACT

Background: We previously reported the arterial elasticity value we measured to reflect the characteristic features of vessel walls, and to possibly be useful for detecting early stage atherosclerosis in type 2 diabetes. Obesity, especially visceral adiposity, is well known to play a crucial role in the development of metabolic disorders and atherosclerosis. To assess whether arterial elasticity value reflects the effect of obesity on atherosclerosis, we examined the associations of obesity characteristics with atherosclerosis values including arterial elasticity, carotid intima–media thickness (IMT) and pulse wave velocity (PWV).
Methods: Three atherosclerosis values were measured in 78 obese subjects (body mass index ≥30). We investigated the associations of atherosclerosis values with obesity-related parameters including abdominal fat accumulation determined by computed tomography.
Results: Arterial elasticity values were positively related to established atherosclerosis values, carotid IMT and PWV, in obese subjects. Age, systolic blood pressure and hypertension also correlated with these atherosclerosis values. Single regression analysis showed all three atherosclerosis values to correlate significantly with visceral fat area. Intriguingly, visceral fat area is an independent variable affecting arterial elasticity, but not IMT or PWV. Furthermore, multiple regression analysis revealed that arterial elasticity correlates strongly with visceral fat area.
Conclusions: Arterial elasticity value we measure is a new parameter for evaluating atherosclerosis in subjects with visceral adiposity and more sensitive than the currently established atherosclerosis values, carotid IMT and PWV. Measuring arterial elasticity has the potential to reveal minute vascular changes, and may have broad clinical applications for evaluating early stage atherosclerosis.
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Obesity has increased dramatically, becoming a global epidemic in recent decades [1]. Obesity is closely associated with the development of atherosclerosis [2], via rising incidences of metabolic disorders, including diabetes, dyslipidemia, hypertension, inflammation, and the prothrombotic state [3]. Furthermore, obesity is involved in sympathetic nerve activation as well as cardiac structural and functional adaptations [4], and is reportedly an independent cardiovascular risk factor [5]. Recent studies have shown that adipocytokines, such as PAI-1, TNF-α and adiponectin, play crucial roles in the development of metabolic disorders and atherosclerosis, in various tissues including the vasculature [6]. In particular, visceral fat accumulation, rather than the body mass index (BMI) or subcutaneous fat accumulation, was shown to be strongly associated with various obesity-related disorders [7], and is thus considered to be a major risk factor for cardiovascular disease [8]. Many studies have shown that increasing body weight is closely related to the surrogate markers associated with atherosclerosis, such as carotid intima–media thickness (IMT) and pulse wave velocity (PWV). In addition, visceral adiposity is reportedly related to atherosclerosis, which is determined by carotid IMT [9–13], coronary calcification [14] and arterial stiffness [15,16]. In some reports, this relationship persisted after adjustments for multiple linear regression analysis [12–15]. While visceral fat areas were correctly measured by computed tomography (CT) scans in few studies [12,15], abdominal ultrasound was employed in many

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reports [9–11,13,14,16], which is often imprecise to determine visceral fat area. It has been widely recognized that non-invasive methods of evaluating atherosclerosis have limitations, such as slow changes in carotid IMT and the influence of blood pressure on PWV. Thus, an accurate and practical means of evaluating atherosclerosis is needed.

Recently, we developed a novel non-invasive method for measuring a change in thickness of multiple layers preset in arterial wall during a single heartbeat [17,18]. Briefly, multiple points were preset from the luminal surface to the adventitia of the posterior wall along an ultrasonic beam and the displacements at these preset points were estimated by applying the phased tracking method to the received echo. A layer was defined as being between two points. A minute change in thickness of the layer was obtained by subtraction of the displacements at these two points and then, the strain of the layer was obtained by dividing the change in thickness by the original thickness set at the end diastole. By changing the depth and applying the same procedure, the strains at multiple depths in arterial wall were obtained at constant intervals, usually 80 μm. This innovative phased tracking method enables us to evaluate regional characteristics of the artery in detail; during a single heartbeat these sites either deform easily in soft regions or there is little deformation in hard regions. We integrated changes in thickness, which we describe with the term "arterial elasticity". This "arterial elasticity" measurement is a promising approach to evaluating atherosclerosis [19,20]. Therefore, we applied this method to in vivo detection of regional changes in human carotid arterial walls. In a study of subjects with type 2 diabetes, carotid arterial elasticity correlated significantly with currently established values for atherosclerosis, such as carotid artery IMT and PWV. Intriguingly, in subjects with IMT <1.1 mm, who are classified as not having atherosclerosis as defined by IMT criteria, arterial elasticity correlated with the number of risk factors, i.e. diabetes, dyslipidemia, hypertension and smoking, suggesting that arterial elasticity has potential for detecting early stage atherosclerosis. It was also suggested that measuring arterial elasticity would allow evaluation of qualitative changes in the carotid arterial wall [21].

Herein, to assess whether the effect of obesity on atherosclerosis can be evaluated using arterial elasticity, we examined the associations of obesity characteristics with atherosclerosis values including arterial elasticity, carotid IMT and PWV. We further evaluated the impact of fat distribution on atherosclerosis.

1. Methods

1.1. Study subjects

The study subjects were recruited from among patients with BMI over 30 at Tohoku University Hospital. Patients with type 1 diabetes, renal failure (serum creatinine >2.0 mg/dL), severe heart failure (NYHA functional class 2–4), atrial fibrillation and peripheral arterial disease were excluded from the study. The study protocol was approved by the Tohoku University Institutional Review Board. Informed consent was obtained from each patient.

We used the following criteria for the diagnosis of metabolic disorders. Diabetes was defined as fasting blood glucose ≥7.0 mmol/dl (126 mg/dl) and/or hemoglobin A1c ≥6.5%, based on the definition proposed by the Japan Diabetes Society, or taking antidiabetic drugs including insulin. Dyslipidemia was defined as LDL cholesterol ≥3.6 mmol/dl (140 mg/dl) and/or triglyceride ≥1.7 mmol/l (150 mg/dl), based on the definition proposed by the Japan Atherosclerosis Society in 2007, or taking lipid-lowering drugs. The subjects whose systolic blood pressure (BP) ≥140 mmHg and/or diastolic BP ≥90 mmHg (Japanese Society of Hypertension guidelines 2004) or who were taking antihypertensive drugs were defined as having hypertension. The subjects who currently smoked were classified as current smokers.

1.2. Measurement of arterial wall elasticity

Real-time measurement of regional elasticity in the far wall of common carotid artery (CCA) was achieved based on a previously described method [22] with ultrasound diagnostic equipment (prototype system by Panasonic), which was specialized for measuring regional elasticity. With this system, an ultrasound beam sequentially scanned an artery along its length at 32 positions at intervals of 200 μm with a linear type 7.5-MHz probe.

Multiple points were preset from the luminal surface to the adventitia along each beam at constant intervals of 80 μm, and the displacements at these preset points were estimated by applying the phased tracking method to the received echo. A layer was defined as being between two points, where the distance between these two points (i.e. the thickness of the layer) was set at 320 μm. As shown in Fig. 1C, minute changes in thickness of the layer were obtained by subtraction of the displacements at these two points and then, the strain of the layer was obtained by dividing the change in thickness by the original thickness (320 μm) which was set at the end diastole. By changing the depth of the layer at intervals of 80 μm and applying the same procedure, the strains at multiple depths were obtained at intervals of 80 μm.

The elasticity of each layer was obtained from the maximal strain and the pulse pressure measured at the upper arm. The maximal strain is defined by the absolute value of difference between the maximum and minimum of the measured change in thickness, as shown in Fig. 1C, and the maximal strain was determined at each location, independent of time.

Using the above procedure, the elasticity was obtained at intervals of 80 μm in the direction of depth and 200 μm along its length, as shown in Fig. 1B. Regional elasticity values of multiple sites in each layer were displayed as shown in Fig. 1A and a mean regional elasticity value (kPa) of bilateral CCA was used for analysis.

1.3. Measurement of carotid artery intima–media thickness

IMT of the carotid arteries was measured using ultrasound diagnostic equipment (EUB-450, Hitachi Medico, Tokyo, Japan) with an electrical linear transducer (center-frequency of 7.5 MHz). By B-mode ultrasound, CCA, carotid bulb, and portions of the internal and external carotid arteries on both sides were scanned with the subject in the supine position. IMT was measured at a point on the far wall of the CCA, 1 cm proximal to the bifurcation [23], from the longitudinal scan plane that showed the intima–media boundaries most clearly.

1.4. Measurement of PWV

PWV values were measured using an automatic waveform analyzer (BP-203RPE; Colin Co., Komaki, Japan) [24]. Pulse waves were recorded on the right brachial artery and both posterior tibial arteries. The average PWV was calculated by dividing the arm–ankle distance by the pulse wave transmission time between these points on both sides.

1.5. Measurement of abdominal fat area

Abdominal subcutaneous and intra-abdominal fat areas were measured by CT scans with a SOMATOM Definition (Siemens AG., Munich, Germany) at the level of the fourth lumbar vertebra. The border of the intra-abdominal cavity was outlined on the CT image and the total area of visceral fat was measured at an attenuation range of −200 and −50 Hounsfield units [25].