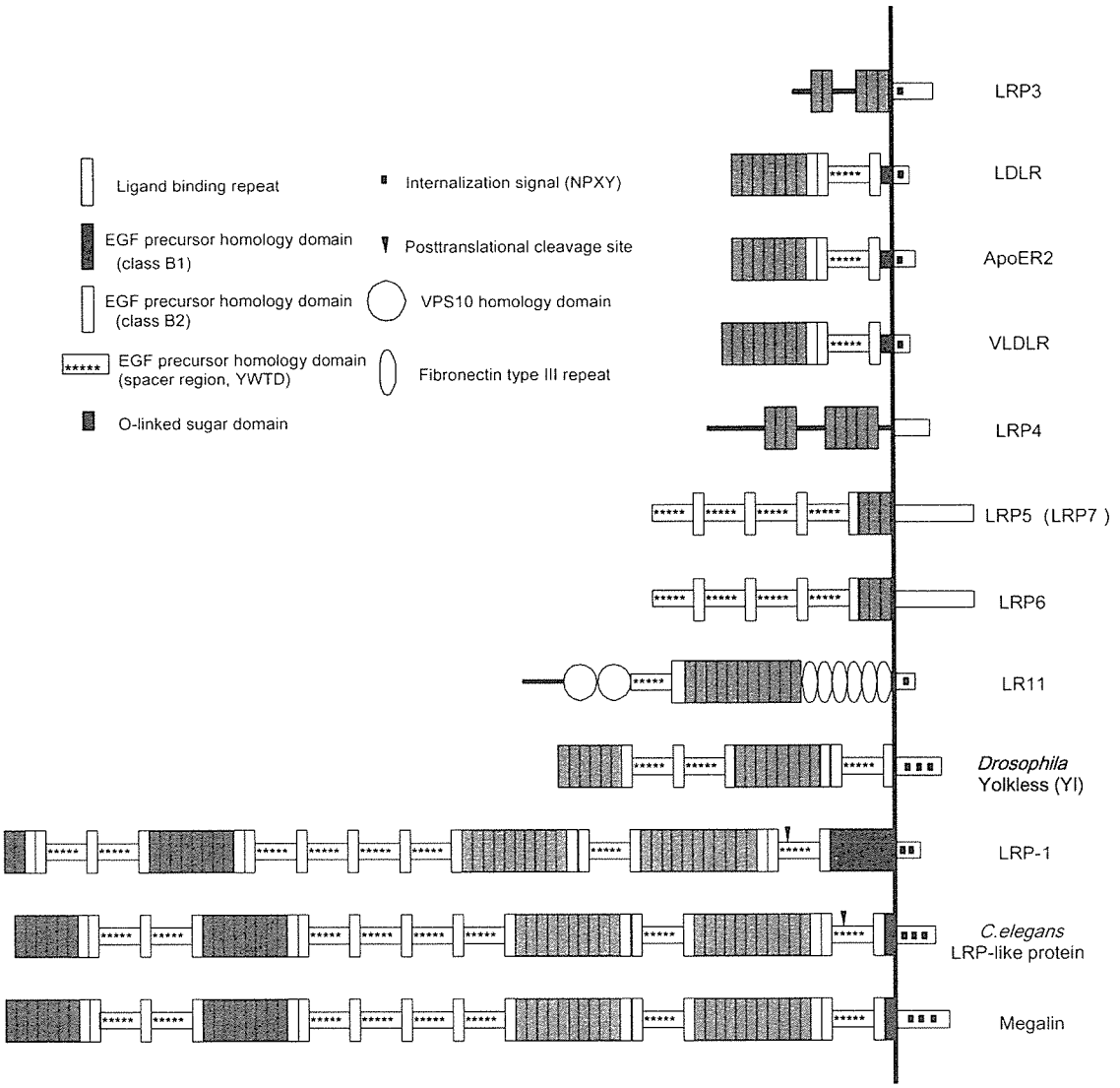

Figure 1. Schematic presentation of the LRs. The common structural modules in most of LRs are: (1) the so-called "LDL receptor ligand binding repeats (type A)," complement-type domains consisting of ≈40 residues displaying a triple-disulfide-bond-stabilized negatively charged surface; (2) epidermal growth factor (EGF) precursor homology repeats (type B1 and B2), also containing 6 cysteins each; (3) EGF precursor homology repeats consisting of ≈50 residues each, most often in groups of 5, with a consensus tetrapeptide, Tyr-Trp-Thr-Asp (YWTD); and (4) in the cytoplasmic region, signals for receptor internalization via coated pits, containing the consensus tetrapeptide Asn-Pro-Xaa-Tyr (NPXY).

is expressed by SMCs of the medial layer and in thickened intimal regions.[23] Thus, changes in the expression of LRs by vascular cells, particularly SMCs, may play a role in the development of atherosclerosis.

The migration and proliferation of SMCs, as well as extracellular matrix (ECM) production and catabolism by these cells, are important events in the development of atherosclerosis and intimal thickening after coronary angioplasty.[24] When thickening of the intima occurs, SMCs migrate from the media into the intima. During migration, SMCs acquire or lose various functions to perform the above-mentioned activities in the intima.[25,26] However, the mechanisms that control the migration of intimal SMCs have not been clarified because of the complex intracellular machinery and the interactions of numerous internal or external factors and signaling pathways. There is conclusive evidence that migration of SMCs from the media into the intima contributes to the formation of stable plaque.[27,28] Here, we focus on the role of LRs in regulating membrane receptor functions related to the migration of SMCs associated with atherosclerosis.

Platelet-Derived Growth Factor–Mediated Migratory Activity of Intimal SMCs

There is a distinct difference in migratory activity between cultured SMCs isolated from the intimal and medial layers of atherosclerotic aortas.[29] Cultured intimal SMCs differ from medial SMCs in many ways, including their morphology, proliferative potential, and gene expression.[29–31] The phenotypic modifications of SMCs that migrate to the intimal layer seem to contribute to an enhanced synthetic capacity, representing a mechanism that influences plaque stability. In fact,

cultured intimal SMCs exhibit a phenotype resembling that of fetal or dedifferentiated SMCs.[25,26] Among the many genes involved in the process of phenotypic modification that occurs in the intima,[32,33] the expression of myosin heavy chain isoforms, such as SM1, SM2, and SMemb/nonmuscle myosic heavy chain-B (NMHC-B), has been well characterized.[25,26,34]

Many factors may contribute to altering the migratory potential of SMCs in the intima, including changes of contact with the ECM and exposure to growth factors. Cultured SMCs tend to mimic these changes because primary cultured cells rapidly lose their differentiation markers and develop a synthetic phenotype. Conversely, SMCs grown in 3D cultures, such as a honeycomb structure, are able to retain the contractile phenotype.[35] Thus, various cell culture models have provided information about factors that influence the migration of intimal SMCs. Among them, sensitivity to growth factors (including platelet-derived growth factor [PDGF]) is known to be important for inducing SMC migration.[25] PDGF-BB–mediated intracellular signals induce migration, which is commonly observed using a migration assay system such as Boyden's chamber. The influence of PDGF-BB on the migration of SMCs is mediated by a specific membrane receptor: PDGF β-receptor.[36] During the process of migration of SMCs from the media into the intima, one of the strongly expressed genes is PDGF β-receptor,[37] which contributes to the migratory capacity of intimal SMCs.[38,39] The PDGF β-receptor is highly expressed even in the media of diabetic models, which show accelerated plaque formation.[40,41] PDGF-BB negatively regulates the transcription of multiple genes in SMCs and thus modulates differentiation.[42] Accordingly, the switch that induces PDGF β-receptor gene expression seems to be closely related to increasing the migratory capacity of intimal SMCs.

Urokinase and Its Receptor System Are Activated During SMC Migration

In addition to chemoattractants, several proteases and their inhibitors are involved in the migration of SMCs through the process of matrix degradation.[24] Local protease activation is important for enhancing the mobility of migrating cells, particularly for SMCs to migrate through the ECM to target sites in plaque or thickened intima. Thus, matrix metalloproteinases (MMPs) are integral for SMC migration into the intima.[24] Conversion of pro-MMPs to active MMPs, as well as MMP-9 expression, is mediated by urokinase-type plasminogen activator (uPA)–generated plasmin.[43,44] The resulting matrix degradation releases growth factors such as fibroblast growth factor-2 and latent transforming growth factor-β, and these chemoattractants further promote the migration of SMCs. Thus, urokinase appears to be necessary for migration of SMCs through the surrounding ECM.

Both tissue-type plasminogen activator and uPA cleave plasminogen to release plasmin. Expression of tissue-type plasminogen activator and uPA is increased in atherosclerotic plaque,[45–47] and a study using knockout mice has revealed a role of uPA in the development of intimal hyperplasia.[48] Accordingly, uPA is thought to play an important role in the target-oriented movement of SMCs because its activation can be localized via binding to its receptor (the uPA receptor) on the cell surface. The receptor-mediated potentiation of protease activity for plasminogen also causes an increase of plasmin activation around cell surface receptors. Subsequent production of plasmin leads to the degradation of ECM components and also has the potential to activate some MMPs. The essential role of this process in enhancing cell mobility has been intensively studied with regard to tumor invasion and neuronal migration.[49,50]

Expression of uPA by medial SMCs increases rapidly and significantly after balloon catheter injury to a vessel, corresponding with the time course of SMC migration.[51] Virally mediated overexpression of uPA by the endothelial cells of the carotid arteries promotes lesion growth in cholesterol-fed rabbits.[52] After arterial injury, intimal thickening is significantly reduced in uPA-deficient mice.[48,53] Thus, uPA itself seems to promote intimal thickening after vascular injury. However, despite the ability of uPA to influence the migration of cultured SMCs,[54–56] intimal formation is unaffected in uPA receptor knockout mice.[57] The specific proteolytic activity of uPA plays a role in the processes of arterial repair after injury, although the details of the mechanism regulating association with its receptor have not been clarified in the setting of atherosclerosis.

In addition to the proteolytic cascade initiated by binding of uPA to its cell surface receptor, uPA possibly facilitates cell migration by inducing intracellular signaling pathways.[58] The uPA receptor is a glycosylphosphatidylinositol-anchored protein, and therefore signaling activity is mediated by its interaction with other membrane molecules. Binding of uPA to its receptor on the cell surface influences the migratory activity through the formation of a complex involving the uPA receptor, vitronectin, and integrin.[50,58] These interactions at the cell membrane stimulate intracellular signaling cascades, as well as uPA receptor–mediated activation of extracellular proteolysis.[50,58] uPA stimulates the migration of SMCs via its receptor signaling cascade containing the Janus kinase, Tyk2, and phosphatidylinositol 3-kinase. Active GTP-bound forms of small GTPases (RhoA and Rac1) are the downstream targets for Tyk2 and phosphatidylinositol 3-kinase activation. Phosphorylation of myosin light chain is one of the end points of the uPA receptor–mediated signaling pathways. Observations suggesting a possible role of uPA (independent of ECM degradation) in cell migration have been reported so the uPA receptor may also modulate migration/invasion in a protease-independent manner. These findings, together with the results obtained in uPA receptor knockout mice,[57] have led to the conception that the uPA receptor modulates SMC migration through cooperation between extracellular proteolysis and intracellular signaling. Proteolysis of the ECM accelerates migration and is coordinated with adhesive and structural changes that promote cell motility, with both processes leading the cells to their targets in the plaques.

LRs Are Novel Modulators of uPA Receptor Function During PDGF-Mediated Migration of SMCs

Functional modulation of the uPA receptor through the pathways with participation of LRs has been established.[59]

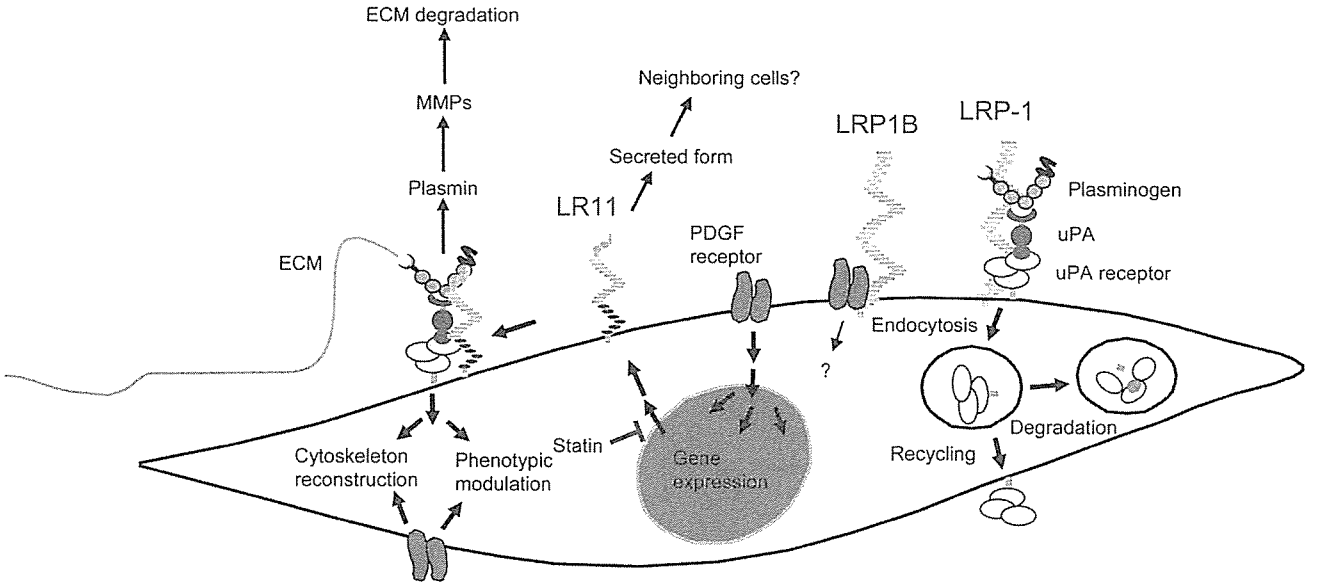

Figure 2. Proposed model for the regulation of SMC migration by LRs through the uPA/uPA receptor system. The uPA/uPA receptor system induces cell migration through both increased degradation of the ECM and receptor-mediated intracellular signaling that promotes motility. uPA receptor expression is regulated by LRs such as LRP-1, VLDL receptor/LR8, and LRP-1B. SMCs in plaques produce LR11, which is localized on the cell surface and also secreted by the cells. LR11 binds to and interacts with the uPA receptor on the cell surface or on neighboring cells. Formation of this complex inhibits internalization of the uPA receptor via other LRs (LRP-1, LRP-1B, etc.) and thereby prevents its degradation and relocation, resulting in the enhanced uPA receptor expression on the cell surface. Finally, SMCs expressing LR11 gain an increased migratory capacity that is mediated by activation of the uPA/uPA receptor system. LR11 gene transcription is induced by PDGF-BB and mediated by the PDGF β-receptor. LRP-1 (and LRP-1B) interacts with the PDGF β-receptor and modulates receptor-mediated intracellular signaling by PDGF-BB, which promotes migratory activity. Thus, LRs possibly regulate the migration of intimal SMCs in atherosclerotic plaques via modulation of PDGF receptor-meditated signaling, which is also linked with the uPA/uPA receptor system. Statins inhibit the migration of intimal SMCs by decreasing uPA receptor expression via the downregulation of LR11 gene expression.

LRs are known to play an integral role in the catabolism of lipoproteins and of complexes between proteinases and their receptors.[2,3] A large member of the family, LRP-1, is involved in the intake of uPA receptors and uPA/uPA receptor complexes by cells for subsequent degradation or recycling.[60] Extensive studies have revealed that other LRs, such as VLDL receptor/LR8[12] and LRP-1B,[61] also have the capacity to catabolize uPA/uPA receptor complexes.

LRP-1 is involved in the internalization of the uPA/uPA receptor complex, in which formation is induced by plasminogen activator inhibitor-1, and this process is dependent on LRP-1.[10,11,62,63] LRP-1 is a large molecule composed of 2 subunits. Two NPXY motifs exist in the intracellular domain of LRP-1, and these motifs are not only important for endocytosis but also for intracellular signaling through molecules such as Shc.[64–66] Inhibition of uPA receptor internalization increases cell surface uPA receptor expression and enhances cell motility.[10,16,63,67]

Deficiency of LRP-1 in SMCs causes atherosclerosis, which is mediated by the modulation of intracellular PDGF signaling.[17] This is attributable to the influence of LRP-1 on PDGF β-receptor signaling or metabolism, possibly because of a molecular interaction at the cell surface.[17,68–70] LRP-1B is the giant family member that is most similar to LRP-1; it also binds to the PDGF β-receptor and modulates receptor-mediated signaling in SMCs.[23] These findings suggest that SMC migration might be regulated by the time-restricted expression of LRs, which determines the outcome of PDGF β-receptor– and uPA receptor–mediated signaling. In accordance with the concept of functional interaction between LRs and membrane signaling receptors, LR11 has been identified by us and others as a negative regulator of protein catabolism for uPA receptor.[71,72] Previous histochemical studies have revealed that LRs are markedly induced during the development of atherosclerotic lesions.[1,18] Altered expression of LRP-1 and the uPA receptor possibly reflects the vascular response to injury. Upregulation of LRP-1 mRNA has been detected in the aortas of rabbits fed a high-cholesterol diet.[1,18] Both LRP-1 mRNA and protein are expressed in normal and atherosclerotic human arteries.[19,20] Increased vascular expression of the uPA receptor is observed in cholesterol-fed rabbits and human atherosclerotic arteries.[73] Because LRs are able to modulate uPA receptor activity and possibly PDGF receptor activity, LRs are expected to regulate the migration of SMCs through the functional modulation of these membrane receptors (Figure 2).

Involvement of LRs in Regulating SMC Migration in the Intima

Recent functional studies using genetically altered animals or cells revealed that LRs are important regulators of the migration of various cells via modulation of cytokine signaling or protease activation.[13,16] SMC-specific inactivation of LRP-1 in mice has revealed a novel role of LRP-1, which forms a complex with the PDGF receptor.[17] LRP-1 ablation results in a decrease of vascular wall integrity and causes marked susceptibility to cholesterol-induced atherosclerosis in mice.[17] In murine embryonic fibroblasts and fibrosarcoma

cells, loss of LRP-1 expression is associated with increased cell surface expression of the uPA receptor and is correlated with increased cell migration in vitro.[10] Similar changes were reported to occur when VLDL receptor/LR8 activity was neutralized in cultured breast cancer cells.[12] LR-mediated regulation of cell migration appears to depend partly on modulation of the uPA/uPA receptor system involved in the degradation of the ECM or modulation of uPA receptor-mediated intracellular signaling through activation of extracellular signal-regulated kinase and Rac1.

A negative regulator of receptor catabolism, LR11, controls uPA receptor localization on the plasma membrane because both the membrane-spanning and secreted forms of LR11 bind to and colocalize with the uPA receptor on the cell surface.[21,74] Expression of LR11 is induced by stimulation of PDGF-BB in SMCs and is observed in intimal SMCs localized at the intima/media border in the atherosclerotic plaques of experimental animals.[21] Overexpression of LR11 by SMCs enhances their migration by elevating uPA receptor expression.[21] Contrarily, neutralization of LR11 reduces the intimal thickening after cuff injury in mice.[21]

Modulation of the LR11/uPAR Pathway for Prevention of Atherosclerosis

Statins are potent inhibitors of the 3-hydroxy-3-methylglutaryl–coenzyme A reductase that are known to be effective for preventing atherosclerosis. Statins have recently been shown to perform a multitude of activities that are involved in the functional modulation of vascular cells such as influences on cell proliferation and secretion.[75,76] One of the major effects of statins on SMCs is modulation of migration. However, the mechanism involved and clinical significance of such inhibition of migration, which has been observed in vitro, have not been elucidated. PDGF-induced migration of SMCs is suppressed by statins in vitro.[77,78] Statins reduce protease expression in atheromatous plaques, and hydrophilic statins decrease SMC numbers and collagen gene expression in vivo.[79] However, phenotypic modulation of intimal SMCs by statins has not yet been investigated. LR11 plays an important role in the induction of migration after enhancement by PDGF-BB in vitro. A potent 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitor, pitavastatin, reduced the expression of both LR11 and SMemb/NMHC-B in atherosclerotic plaques (unpublished data, 2006). In fact, the enhanced expression of LR11, uPA receptor, and SMemb/NMHC-B by cultured intimal SMCs is reduced by pitavastatin to the levels seen in cells from the media. When expression of the uPA receptor, SMemb/NMHC-B, and endogenous LR11 is increased by PDGF-BB, the enhanced migratory activity of SMCs is blocked by pitavastatin via suppression of endogenous LR11 production. Thus, modulation of the LR11/uPA receptor system plays a role in PDGF-induced migration of intimal SMCs (Figure 2).

It has not yet been clarified whether inhibition of the migration of intimal SMCs leads to the regression of atherosclerotic plaque or prevents restenosis after coronary angioplasty. Activation of pathways mediated by the uPA receptor and the PDGF receptor that increase the migration of intimal SMCs is thought to be essential for the formation of mature plaque after endothelial injury leads to the initiation of atherosclerosis. Unregulated expression of these membrane receptors may reduce the stability of plaque because the programmed migration of SMCs from the media to target regions in the intima would be disturbed. LRs are a possible candidate for modulating SMC migration to control the process of atherosclerosis. Selective modification of the LRs/uPA receptor/PDGF receptor system in SMCs, associated with the change to a dedifferentiated phenotype, appears to be important for the occurrence of intimal thickening after angioplasty as well as plaque formation in atherosclerosis.

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Original Article

Distinct Effects of Pravastatin, Atorvastatin, and Simvastatin on Insulin Secretion from a β-cell Line, MIN6 Cells

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In addition to the prevention of cardiovascular diseases by lowering plasma LDL cholesterol, recent studies suggest that statins could have some impact on insulin action. To estimate the direct effects of statins on insulin secretion from pancreatic β-cells, MIN6 cells were treated with pravastatin, simvastatin, or atorvastatin. Basal insulin secretion at low glucose concentration was unexpectedly increased at very high doses of simvastatin or atorvastatin after 24- and 48-hour incubation. Insulin secretion at high glucose was not significantly changed, and thus, net glucose-stimulated insulin secretion was apparently decreased by these lipophilic statins. The changes in insulin secretion were highly associated with increased endogenous SREBP activities in response to HMG-CoA inhibition as estimated by SRE-luciferase assays, and finally after 48-hour incubation, accompanied by impaired cell viability as estimated by MTT assays. In contrast, these changes were much less prominent by the addition of pravastatin. Meanwhile, glucose-stimulated insulin secretion of islets isolated from C57BL/6 mice was not significantly changed by any of the statins. Overall, taken up by β-cells, statins can affect insulin secretion through either HMG-CoA inhibition or cytotoxicity, as observed by the addition of extraordinary high doses of lipophilic statins, but not hydrophilic statins, to the medium.

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Key words; SREBP, Cholesterol, HMG-CoA reductase, Lipotoxicity

Introduction

Statins are specific and potent competitive inhibitors of 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) reductase and are widely used as plasma cholesterol-lowering drugs to prevent the development and progression of atherosclerosis. Evidence for reduction of the incidence of cardiovascular diseases by statins has now been fully established[1-6]. Diabetes is a major risk for cardiovascular diseases, and statins are also used for patients with diabetes[7,8]; however, the effects of statins on glucose insulin metabolism have been implicated, but not fully understood. Statins could have some beneficial impact on peripheral insulin resistance, which can potentially contribute to a potential protective role of statins against the development of diabetes[9]. We recently found that atorvastatin ameliorates insulin resistance in experimental animals[10]; however, it has been reported that statins might have some inhibitory effects on glucose-stimulated insulin secretion[11,12]. These diverse effects of statins to potentially modify the pathology of type 2 diabetes warrant studies on the effects of various statins on β-cell functions, especially insulin secretion in various conditions. In this study, we investigate effects of pravastatin, atorvastatin, and simvastatin on insulin secretion from a β-cell line, MIN6 cells.

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Materials and Methods

Reagents

Simvasatin, atorvastatin, and pravastatin were purchased from Wako (Osaka, Japan).

Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA).

Cell Culture and Treatment

MIN6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/L glucose supplemented with 15% fetal bovine serum (FBS), β-mercaptoethanol (5 μL/L), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37℃ in an atmosphere of 95% air/5% CO_2. The cells were seeded onto 24-well plates at a density 3×10^5 cells/mL and grown overnight to 80% confluency. The cells were washed with phosphate-buffered saline (PBS) and incubated for 24 hours or 48 hours at 37℃ in 0.5 mL of medium containing the indicated concentrations (×IC50 for HepG2) of pravastatin, atorvastatin, or simvastatin. The IC50 of pravastatin, atorvastatin, and simvastatin estimated by HMG-CoA reductase inhibition in HepG2 cells was 9.4 nM, 1.9 nM, and 2.7 nM, respectively[13].

DMSO was used as a solvent of the agents, and the final concentrations did not exceed 0.1% (v/v).

Preparation of Murine Pancreatic Islets

All the animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulations for Animal Experiments, and permitted by the Animal Experiment Committee, University of Tsukuba. All procedures of animal experiments were performed in accordance with Tsukuba University's Animal Studies Committee. Isolation of islets from C57BL/6 mice (Charles River Laboratories) was carried according to the Ficoll-Conray protocol[14]. After isolation, the islets were incubated for 2 hours at 37℃ in an atmosphere of 95% air/5% CO_2 in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin.

Measurement of Insulin Secretion

Prior to stimulation by glucose, MIN6 cells or ten pancreatic isolated islets were preincubated for 30 min in 2.8mM glucose in 0.5% BSA KRBH buffer (129.4 mM NaCl, 5.2 mM KCL, 2.7 mM $CaCl_2$, 1.3 mM KH_2PO_4, 1.3 mM $MgSO_4$, 24.8 mM $NaHCO_3$, and 10 mM HEPES at pH 7.4). The cells were incubated with either 2.8 mM (low) or 20 mM (high) glucose in KRBH buffer for 1 hour. The insulin content of the medium was measured using a mouse insulin ELISA kit (Shibayagi Co., Ltd., Gunma, Japan).

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide) Assays

As another set of the statin experiments, MIN6 cells were supplemented with MTT and incubated for 4 hours. The medium was switched to 0.5 mL of 0.04 N HCl/isopropanol for extraction by shaking for 5 min. MTT values were measured by absorbance.

Transfection and Luciferase Assays

MIN6 cells were seeded onto 24-well plates and cultured overnight. The cells were transfected with a SRE-luciferase reporter plasmid (500 ng) and a pRL-SV40 plasmid (50 ng, Promega) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions as previously decribed[15]. After 4-hour incubation, the cells were subjected to statin treatment as described above. After 24- or 48-hour incubation, the amount of firefly luciferase activity in transfectants was measured and normalized to the amount of renilla luciferase activity.

Statistical Analysis

Data are presented as the mean ± s.e.m. Statistical significance was tested with an unpaired two-tailed Student *t*-test.

Results

MIN6 cells are a well-established murine β-cell line and maintain glucose-stimulated insulin secretion as a physiologic feature of pancreatic β-cells (control data of **Fig. 1** and **2**). The cells were treated with pravastatin, simvastatin, or atorvastatin. Preliminary data demonstrated that the addition of these statins at the respective IC50 doses reported to inhibit HMG-CoA reductase activity in HepG2 cells did not significantly change insulin secretions at low glucose (2.8 mM, basal) or high glucose (20 mM) concentrations. Therefore, we used ×100, ×1000, and ×10000 concentrations of the IC50 for each statin. When cells were treated with these high doses for 24 and 48 hours, simvastatin and atorvastatin increased basal insulin secretion dose-dependently starting at ×100 and at ×1000, respectively (**Figs. 1** and **2**). Pravastatin exhibited this effect only at the highest dose (×10000). In contrast, insulin secretion at high glucose exhibited a trend to increase with each statin in a pattern similar to basal insulin secretion, but was not significant. Thus, glucose stimulation as calculated by the fold-change of high glucose vs. low glucose was reduced at the high doses of simvastatin and atorvastatin, respectively, but not

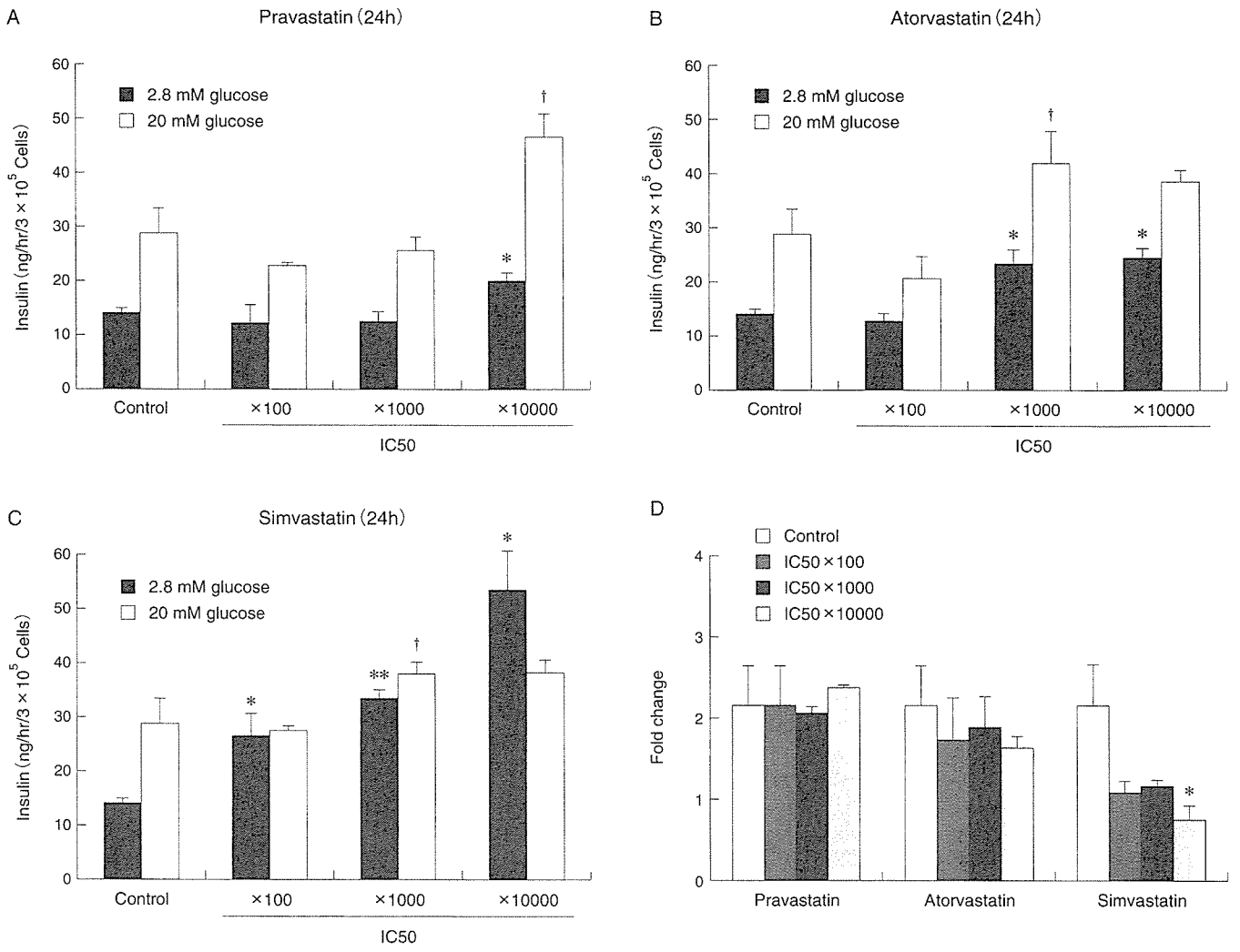

Fig. 1. Effect of incubation with pravastatin (A), atorvastatin (B), or simvastatin (C) for 24 hours on insulin secretion measured at low (black column, 2.8 mM) and high (white column, 20 mM) glucose concentrations in MIN6 cells. A pancreatic β-cell line, MIN6 cells are incubated with the indicated statins at the indicated concentrations (IC50 estimated by HMG-CoA reductase inhibition was 9.4 nM, 1.9 nM, and 2.7 nM for pravastatin, atorvastatin, and simvastatin, respectively.) for 24 hours. Fold change of insulin secretion at high glucose level vs. low glucose level was indicated (D).

All values are the means ± SE. $^{\dagger}p<0.05$, compared with the control with high glucose. $^{*}p<0.05$ and $^{**}p<0.01$, compared with the control with low glucose (A, B, and C). $^{*}p<0.05$, compared with the control (D).

pravastatin. This apparent effect was enhanced when the incubation was extended to 48 hours (**Fig. 2**). Collectively, the time-course and dose-dependent impairment of insulin induction by high glucose was observed in simvastatin and atorvastatin, but not pravastatin.

The primary action of statin is the inhibition of HMG-CoA reductase that catalyzes a rate-limiting step for cholesterol biosynthesis. This inhibition is eventually compensated by adaptive up-regulation of the endogenous pathway at the transcription level through the activation of SREBPs, primarily by SREBP-2[16]. For the estimation of HMG-CoA inhibition states in statin-treated MIN6 cells, we measured endogenous SREBP activities by SRE-luciferase reporter gene assays[17]. Atorvastatin and simavastain dose-dependently induced SREBP activities at 24-hour and 48-hour incubation (**Fig. 3**). The dose-dependency of these statins to cause SREBP activation was roughly similar to that of the effect on insulin secretion. In contrast, pravastatin treatment showed a trend, but did not significantly activate SREBPs. These data indicated that only high dose of lipophilic statins added to the medium exhibited HMG-CoA inhibition in MIN6 cells.

MTT assays as a marker for cell viability and

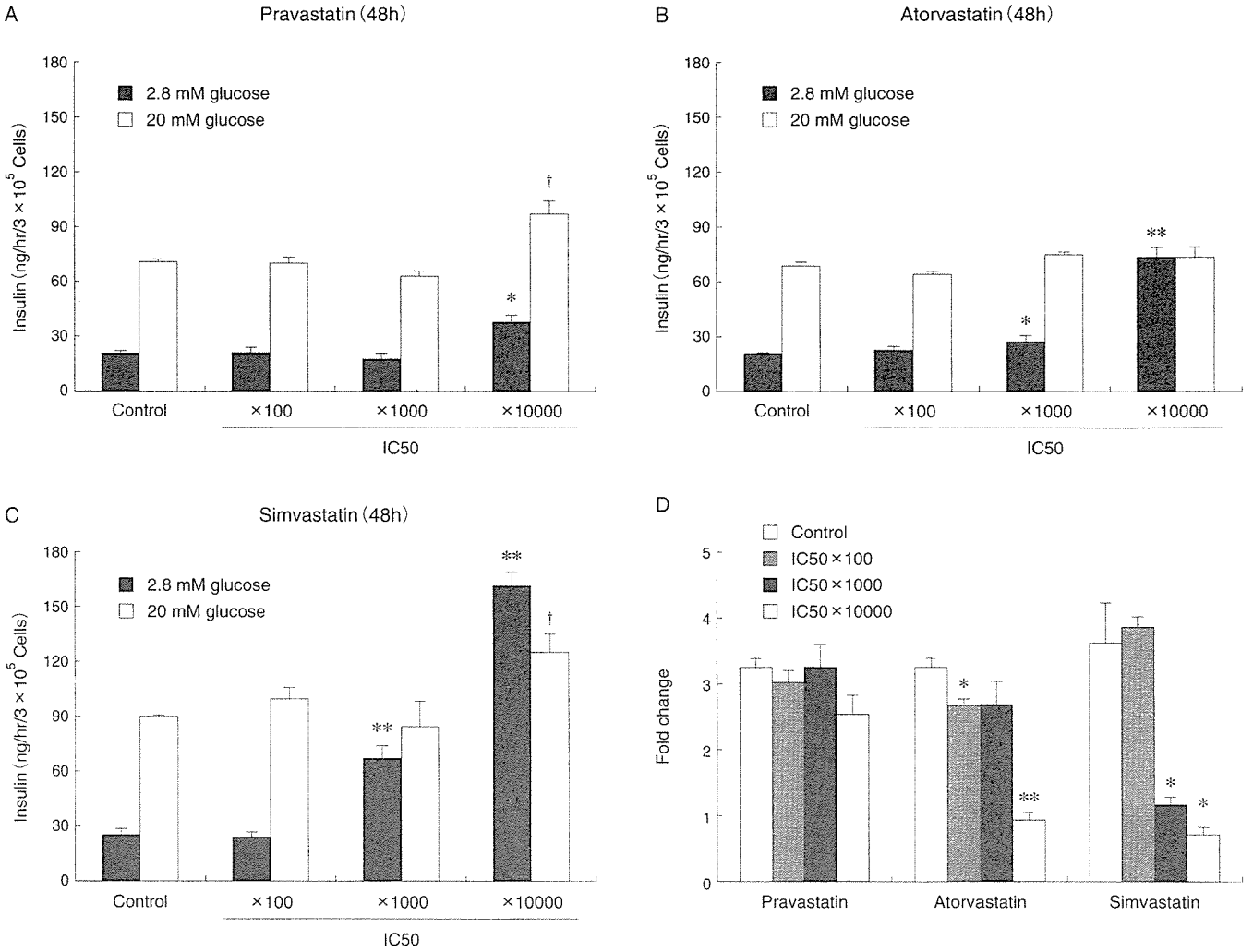

Fig. 2. Effect of incubation with pravastatin (A), atorvastatin (B), or simvasatin (C) for 48 hours on insulin secretion measured at low (black column, 2.8 mM) and high (white column, 20 mM) glucose concentrations in MIN 6 cells. Fold change of insulin secretion at high glucose level vs. low glucose level was indicated (D). Experimental procedures were described in the legend under Fig. 1 except 48-hour incubation with statins.

All values are the means ± SE. $^{\dagger}p<0.05$, compared with the control with high glucose. $^{*}p<0.05$ and $^{**}p<0.01$, compared with the control with low glucose (A, B, and C). $^{*}p<0.05$ and $^{**}p<0.01$, compared with the control (D).

growth demonstrated no effects by any statins after 24-hour incubation; however, extension of the statin treatment to 48 hours significantly affected cell viability at high doses of simvastatin, atorvastatin, and pravastatin in a pattern similar to the effects of these agents on insulin secretion (**Fig. 4**).

Finally, pancreatic islets were isolated from C57BL/6 mice and subjected to treatment with statins. As shown in **Fig. 5**, the islets exhibited significant glucose-stimulated insulin secretion. However, the administration of statins to islets at the concentrations used for MIN6 cells did not evoke significant changes in glucose-stimulated insulin secretion in pancreatic islets.

Discussion

Our data demonstrated that high doses of lipophilic statins such as simvastatin and atorvastatin significantly increased basal insulin secretion in MIN6 cells. Since insulin seretion at high glucose was not significantly increased by these statins, the net glucose-stimulation as estimated by the fold-change of insulin secretion at high glucose vs. low glucose was apparently decreased. The mechanism for these unexpected results is currently unknown. It is highly plausible that the cytotoxicity by high concentrations of lipophylic statins leads to leakage of insulin from insulin-con-

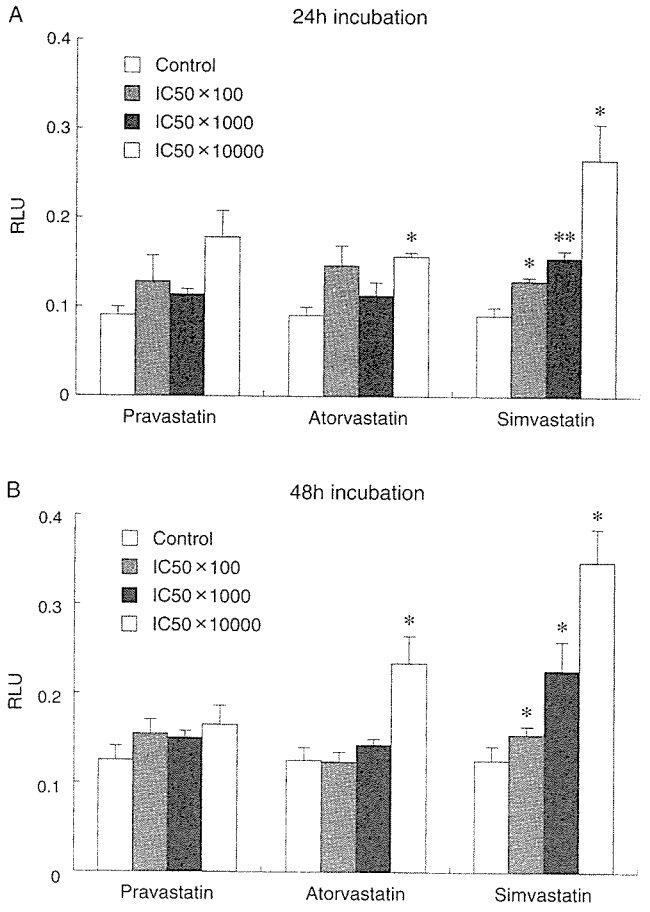

Fig. 3. Endogenous SREBP activities in MIN6 cells after incubation for 24 hours (A) or 48 hours (B) with pravastatin, atorvastatin, or simvasatin as estimated by transfection with an SRE-luciferase reporter gene in MIN6 cells. MIN6 cells were transfected with SRE-luciferase reporter gene and the SV40 expression vector of renilla luciferase as a reference, followed by 4-hour incubation. The cells were treated with statins as described in the legend to Fig. 1 for 24 hours. Finally, the cells were subjected to reporter assays for measurement of firefly luciferase activity normalized to the amount of renilla luciferase activity.

All values are the means ± SE. $^{*}p<0.05$ and $^{**}p<0.01$, compared with the control.

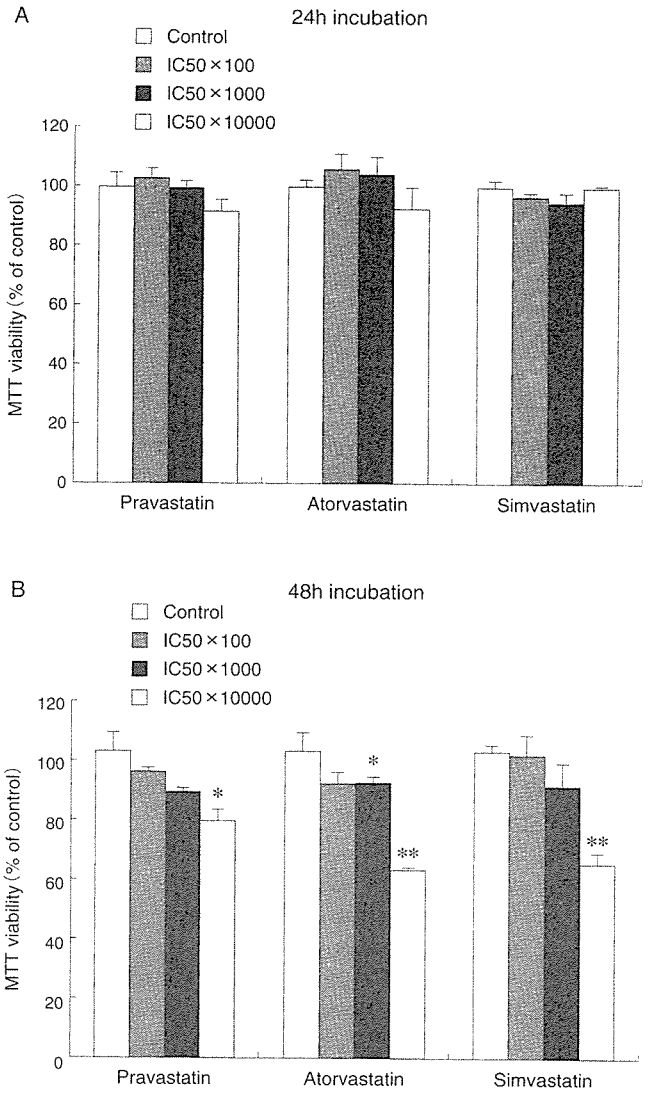

Fig. 4. Effect of incubation with pravastatin, atorvastatin, or simvasatin for 24 hours (A) or 48 hours (B) on MTT assays in MIN6 cells. Cell culture conditions were described in the legend to Fig. 1. After harvesting the cells, MTT assays were performed.

All values the are means ± SE. $^{*}p<0.05$ and $^{**}p<0.01$, compared with the control.

taining granules. Supportively, MTT assays indicate that cell growth and viability are impaired by lipophilic statins. Cytotoxicity could impair ATP production, leading to decreased insulin secretion in response to glucose. Meanwhile, before impaired cell viability was observed after 48-hour incubation, both increased endogenous SREBP activities and changes in insulin secretion were already observed after 24-hour incubation. Thus, HMG-CoA reductase inhibition could be directly involved in the effects on insulin secretion by lipophilic statins. Supportively, mevalonate rescue essentially abolished the statin effect on insulin secretion as well as SREBP activation (data not shown). It is well known that, depending upon its concentration, cellular cholesterol could be both indispensable and cytotoxic for the cell and it is conceivable to speculate that disturbed cholesterol metabolism could be a cause of impaired β-cell function as observed by the addition of oxidized LDL[18]. It is also possible that the activation of SREBPs by statins directly impairs insulin

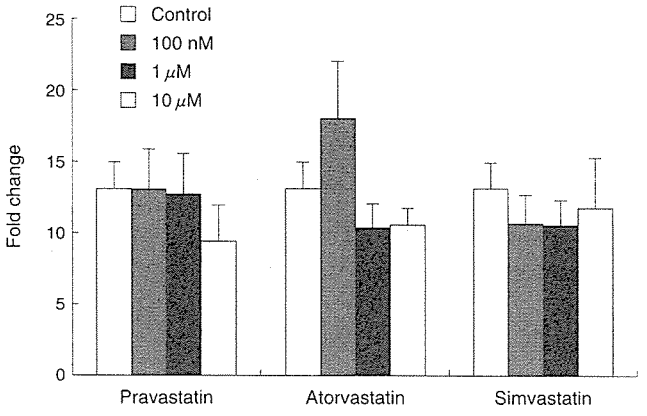

Fig. 5. Effect of incubation for 24 hours with pravastatin, atorvastatin, or simvasatin on insulin secretion at low and high glucose concentrations in isolated islets from C57BL/6 mice. Pancreatic islets were prepared from C57BL/6 mice and incubated with the indicated statins at the indicated concentrations for 24 hours. Insulin secretion at low (2.8 mM) and high (20 mM) glucose concentrations for 1 hour was measured. All values are the means ± SE.

secretion as reported in β-cell-specific SREBP-1c transgenic mice[19] and SREBP-2 transgenic mice (paper in preparation, Ishikawa M).

Whether the effects of lipophylic statins on insulin secretion of MIN6 cells are due to a cytotoxic effect, HMG-CoA reductase inhibition, or both, pravastatin, a hydrophilic statin, exhibits only a minimal trend to this effect. The distinction of SREBP activation among statins suggests that uptake of statins in the medium by MIN6 cells was much less efficient in hydrophilic statins than in lipophilic statins, which also explains the difference in the potential cytotoxicity indicated by MTT assays.

A previous work demonstrated that simvastatin, but not pravastatin, disturbed glucose-stimulated insulin secretion through blockage of an L-type Ca channel in rat isolated single β-cells[12]. In our experimental settings, glucose-stimulated insulin secretion in mouse islets was not inhibited by statins. In contrast to our data on the complex statin effects on MIN6 cells, other statins clearly decreased insulin secretion in HIT cells, a hamster β-cell line, (unpublished data, Okajima F and Oikawa S). Thus, the actions of statins on pancreatic β-cells vary and should be carefully estimated by various cell lines in different conditions. Current data indicate that supra-physiological concentrations of statins are required to see the inhibition of insulin secretion, suggesting that regular doses of statins used for hypercholestemic patients are unlikely to cause deteriorated effects on insulin action, especially in the case of pravastatin. Although isolated islets seem to exhibit less sensitivity to statins than MIN6 cells through the route of addition to the medium, there may be still another mechanism for the uptake of both lipophilic and hydrophilic statins by pancreatic β-cells *in vivo*. Further *in vivo* studies are needed to clarify the cytotoxicity of statins, and more importantly, the physiological roles of cellular cholesterol synthesis in insulin secretion.

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Granuphilin is activated by SREBP-1c and involved in impaired insulin secretion in diabetic mice

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Summary

Granuphilin is a crucial component of the docking machinery of insulin-containing vesicles to the plasma membrane. Here, we show that the granuphilin promoter is a target of SREBP-1c, a transcription factor that controls fatty acid synthesis, and MafA, a β cell differentiation factor. Potassium-stimulated insulin secretion (KSIS) was suppressed in islets with adenoviral-mediated overexpression of granuphilin and enhanced in islets with knockdown of granuphilin (in which granuphilin had been knocked down). SREBP-1c and granuphilin were activated in islets from β cell-specific SREBP-1c transgenic mice, as well as in several diabetic mouse models and normal islets treated with palmitate, accompanied by a corresponding reduction in insulin secretion. Knockdown- or knockout-mediated ablation of granuphilin or SREBP-1c restored KSIS in these islets. Collectively, our data provide evidence that activation of the SREBP-1c/granuphilin pathway is a potential mechanism for impaired insulin secretion in diabetes, contributing to β cell lipotoxicity.

Introduction

Insulin secretion by pancreatic β cells involves sequential intracellular events. Initially, glucose oxidation results in ATP production as controlled by intracellular energy-sensing mechanisms (Bratanova-Tochkova et al., 2002). This is followed by closure of ATP-dependent K^+ channels leading to a change in membrane voltage (depolarization), Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and vesicular transport of insulin-containing granules, culminating in their exocytosis and release of insulin (Rizzoli and Betz, 2005; Rorsman and Renstrom, 2003). Theoretically, impaired insulin secretion could be caused by disturbances in any stage of this highly regulated process (Weir and Bonner-Weir, 2004). Current literature supports the concept that disturbances of energy metabolism in β cells leading to impaired ATP production are the primary pathological cause of impaired insulin secretion following peripheral insulin resistance in type 2 diabetes mellitus (Kahn, 2003). This pathophysiologic process can be observed in isolated islets from diabetic animal models and is characterized by impairment of glucose-stimulated insulin secretion (GSIS) (Diani et al., 2004). Meanwhile, potential perturbations in insulin secretion at steps following ATP production in islets from the diabetic models as estimated by K^+-stimulated insulin secretion (KSIS) have not been fully investigated.

Studies to unveil the molecular basis for exocytosis of secretory granules have been focused on the interactions between components of the fusion machinery (Burgoyne and Morgan, 2003; Rizzoli and Betz, 2005; Rorsman and Renstrom, 2003), including soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs: Vamp2/synaptobrevin, Syntaxin-1a, and SNAP25) and SNARE-associated proteins (Munc13-1 and Munc18-1) on the plasma membrane, and small Rab GTPase family members, Rab3 and Rab27a, on the vesicles. Granuphilin is a Rab27a effecter specific to pancreatic β cells and the pituitary gland. It has two isoforms, granuphilin-a and -b, which derive from alterative splicing (Coppola et al., 2002; Wang et al., 1999). Granuphilin directly binds to Rab27a, and also to syntaxin-1a and Munc18-1, assisting in the docking of insulin granules to the plasma membrane (Coppola et al., 2002; Torii et al., 2002, 2004; Yi et al., 2002). Contrary to expectations, overexpression of granuphilin in a β cell line inhibited insulin secretion (Coppola et al., 2002; Torii et al., 2002). In addition, granuphilin-null islets exhibited inhibition of vesicle docking, yet insulin secretion was increased (Gomi et al., 2005). These gain- and loss-of-function experiments implicated granuphilin as playing a regulatory role in the exocytosis of insulin granules. To date, the regulation of these vesicle proteins and the potential role of granuphilin in β cell lipotoxicity and diabetes are largely unknown.

SREBP-1c is a transcription factor established as a regulator for biosynthesis of fatty acids and triglycerides (Shimano et al., 1997a, 1997b). In addition to nutritional regulation of lipogenic enzymes (Horton et al., 1998; Hasty et al., 2000; Matsuzaka et al., 2004), SREBP-1c is found to play a role in insulin signaling

by inhibiting IRS-2, the major insulin-signaling mediator in the liver and in β cells (Ide et al., 2004; Takahashi et al., 2005). As a model for lipotoxicity by endogenous fatty acids in pancreatic β cells, we previously developed transgenic mice overexpressing the active form of SREBP-1c under the insulin promoter. These mice exhibited impaired insulin secretion in vivo due to ATP depletion caused by enhanced lipogenesis and direct UCP2 induction. In addition, these mice demonstrated loss of β cell mass, presumably due to suppression of IRS-2 and PDX-1 in islets from the transgenic mice (Takahashi et al., 2005). In addition to inhibition of GSIS, SREBP-1c-overexpressing islets exhibited decreased KSIS, which could indicate dysfunction in insulin secretion at a phase following ATP production. Our current study demonstrates that granuphilin is an SREBP-1c target exhibiting a molecular link between β cell lipotoxicity and impaired insulin secretion in the pathogenesis of diabetes development. These data provide a rational to study granuphilin as a potential therapeutic target for treatment of diabetes.

Results

Granuphilin is activated by SREBP-1c in islets

We have previously demonstrated that insulin secretion is blunted in islets isolated from nuclear SREBP-1c transgenic mice under the control of rat insulin promoter I, whereas SREBP-1-null islets demonstrated enhanced insulin secretion compared to wild-type controls (Takahashi et al., 2005). Expression of the transgene was barely detectable in the hypothalamus and was essentially β cell-specific in this transgenic line (Figure S1A in the Supplemental Data available with this article online). In the current study, we found that the negative effect of SREBP-1c on insulin secretion encompassed not only GSIS but also KSIS and induction by arginine (Figure 1A), suggesting that insulin secretion defects caused by SREBP-1c occurred post-ATP production. In an attempt to determine the molecular mechanisms responsible, we explored the expression of genes involved in vesicular transport of insulin granules in islets from these mice. Among many different vesicle factors involved in exocytosis of insulin granules, granuphilin was singly upregulated by overexpression of SREBP-1c and suppressed in the absence of SREBP-1c, as evidenced by real-time PCR (Figure 1B). Immunoblot analysis of islet proteins demonstrated that wild-type islets mainly express granuphilin-a (Figure 1C), and the ratio of granuphilin-a/-b mRNA was roughly 8 (data not shown). Consistent with changes at the mRNA level, both granuphilin-a and -b proteins were robustly induced by overexpression of SREBP-1c and reduced in SREBP-1c-null islets (Figure 1C). SREBP-1c slightly reduced Syntaxin-1a and Munc18-1 proteins but did not affect other related vesicle proteins such as Rab27a, Rab3, Vamp2, and SNAP25 (Figure 1C). Induction of granuphilin mRNA and proteins by acute overexpression of adenoviral SREBP-1c was also observed in a murine insulinoma cell line Min6 (Figure 1D) and islets (Figure 1E), respectively.

Granuphilin promoter is an SREBP target

We next sought to determine whether the granuphilin promoter is a potential direct target of SREBP. Analysis of the GeneBank database revealed that the promoter region of the murine granuphilin gene contains two potential binding sites for SREBP (SREs) and a single site for MafA, which was recently established as an important factor for β cell-differentiation and function (MARE) (Hagman et al., 2005; Kajihara et al., 2003) (Figure S2). Activation of the granuphilin promoter by transcription factors such as SREBPs, MafA, and others was tested in Min6 cells using a luciferase reporter gene fused to the 1.2 kb promoter region of mouse granuphilin (Granu-1200-Luc) (Figure 1F). SREBP-1a and -1c robustly and SREBP-2 moderately stimulated granuphilin promoter activity. MafA was found to be the strongest activator of the granuphilin promoter. A similar pattern of transactivation by these factors was also observed in non-β cells (HEK 293 cells), suggesting that SREBP activation does not require other β cell-specific factors (data not shown). Human granuphilin promoter (0.5 kb) luciferase reporter was also activated by SREBPs and MafA (data not shown).

This region of the mouse granuphilin promoter (Figure 2A) was further analyzed as an SREBP target. Activation of the granuphilin promoter was completely attenuated when SREBPs were mutated (YR mutants) to abolish their binding abilities to authentic SREs (Figure 2B). Deletion studies suggested that the proximal SRE is responsible for SREBP activation of granuphilin promoter (Figure 2C). EMSA assays demonstrated direct and specific binding of SREBPs to this SRE-1 (hereafter referred to as Granuphilin SRE) and not SRE-2 (Figure 2E). Mutation of Granuphilin SRE (Figure 2A) completely abolished the SREBP activation of Granuphilin-Luc (Figure 2D). To determine the physiological relevance of SREBP regulation of granuphilin, MIN6 cells were treated with delipidated serum (DLS) and 25-hydroxycholesterol (25-OH) to activate and suppress endogenous SREBPs, respectively, via modulation of the sterol-regulated SERBP cleavage system. The cells were also treated with T0901317, an LXR agonist, to induce endogenous SREBP-1c. Granu-1200-Luc activity was consistently up- and downregulated by these manipulations, respectively, demonstrating that endogenous SREBPs can also regulate granuphilin promoter activity (Figures 2F and 2G). Chromatin immunoprecipitation assays using MIN6 cells confirmed direct binding of SREBP-1c to Granuphilin SRE in vivo. The signal was increased by incubation with DLS and T0901317 (Figure 2H). Collectively, these data demonstrate that the granuphilin promoter is an SREBP target and that SREBPs regulate granuphilin expression.

Granuphilin promoter is a MafA target

Next, we focused the MARE downstream of the Granuphilin SRE in the granuphilin promoter as diagramed in Figure 3A. Activation of the granuphilin promoter by MafA was completely abolished by deletion of the acidic domain of MafA (Figure 3B). Deletion or mutation of the MARE dramatically suppressed the promoter activity (Figures 3C and 3D). Direct, specific, and in vivo binding of MafA to this MARE was confirmed by EMSA and ChIP assay (Figures 3E and 3F). Considering the two neighboring sites, potential synergism between SREBP-1c and MafA was estimated. Both factors exhibited dose-dependent activation of granuphilin promoter, although MafA has a curve slightly steeper and more saturable than SREBP-1c. Cotransfection of both SREBP-1c and MafA caused only additive activation of the granuphilin promoter (Figures 3G and 3H). In immunoprecipitation experiments, no significant protein-protein interactions between SREBP-1c and MafA were detected (data not shown).

Granuphilin negatively regulates insulin secretion

To determine whether granuphilin could be directly involved in insulin secretion, its expression was manipulated in islets

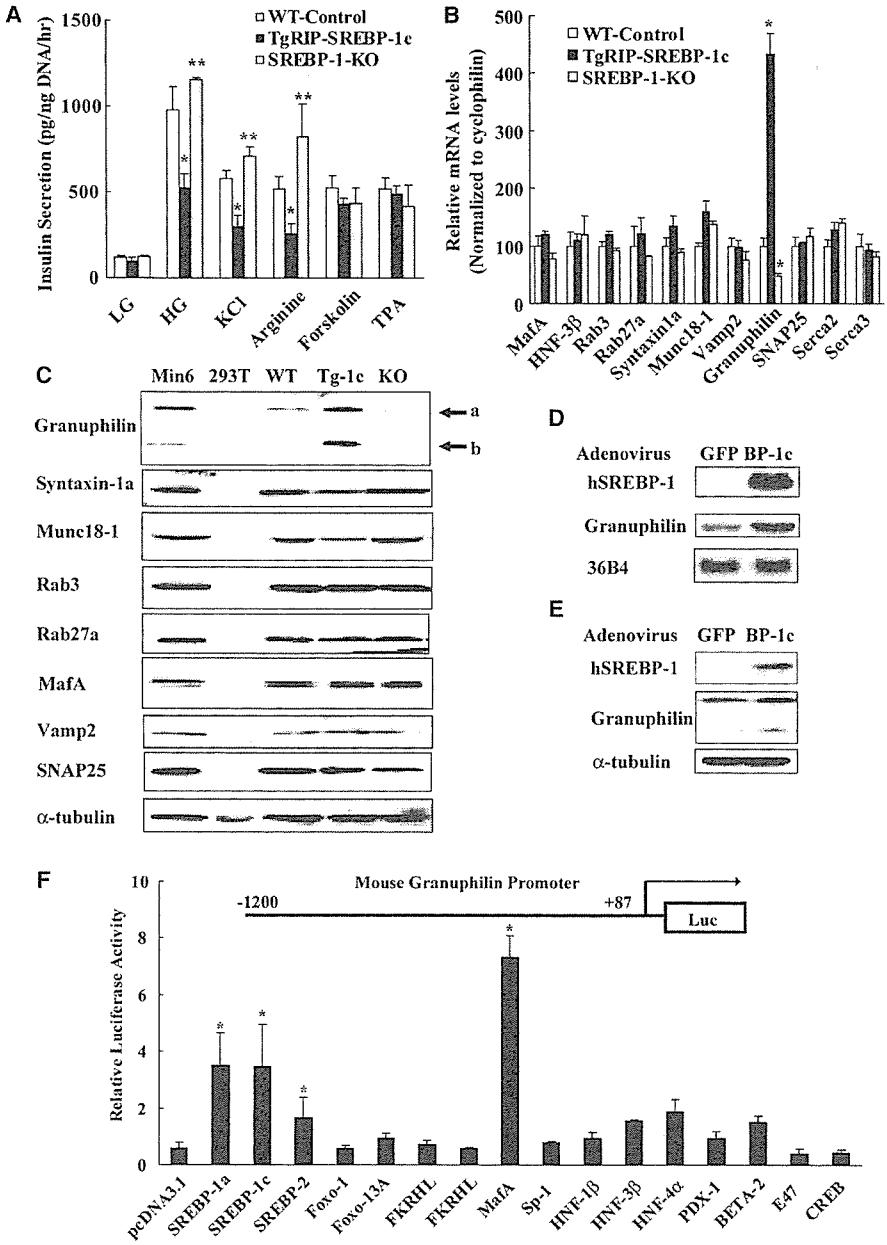

Figure 1. Influence of SREBP-1c on granuphilin expression

Granuphilin and other fusion machinery proteins and insulin secretion were estimated in islets from wild-type (WT-Control), β cell-specific nuclear SREBP-1c transgenic (TgRIP-SREBP-1c), and SREBP-1-null mice (SREBP-1-KO) mice.

A) Basal (low glucose LG; 2.8 mM), high glucose (HG; 20 mM), KCl- (30 mM), arginine- (3 mM), forskolin- (10 μM), or TPA (0.5 μM)-stimulated insulin secretion in isolated islets from age-matched (14- to 17-week-old) male WT-Control (white bars), TgRIP-SREBP-1c (black bars), and SREBP-1-KO (gray bars) mice. Results were normalized to cellular DNA content. *$p < 0.01$ and **$p < 0.05$ (versus WT-Control).

B) mRNA levels of the fusion machinery for exocytosis of insulin granules in the indicated islets as quantified by real-time PCR. Values were normalized to the cyclophilin expression level. *$p < 0.01$ (versus WT-Control).

C) Immunoblot analysis on total proteins (50 μg/lane) of the fusion machinery for exocytosis of insulin granules in the islets from indicated mice.

D) Northern blot analysis of granuphilin and hSREBP-1 gene expression in mouse insulinoma cell line, Min6. Cells were infected by adenoviral-GFP or -SREBP-1c (500 MOI) for 48 hr. Total RNA (15 μg/lane) was isolated and subjected to blot hybridization with the indicated ^{32}P-labeled probe.

E) Immunoblot analysis on nuclear or total proteins of SREBP-1 and mouse granuphilin in the islets from C57BL/6 mice with α-tubulin as a loading control. Islets were infected with adenoviral-GFP or -SREBP-1c (500 MOI) for 48 hr.

F) Activation of the granuphilin promoter by SREBPs and MafA. The mouse granuphilin promoter region (1.2 kb) was fused to a luciferase reporter gene (Granu-1200-Luc). Min6 cells were cotransfected with Granu-1200-Luc as a reporter gene, pSV-40-*Renilla* as a reference, and the indicated expression plasmids or an empty vector. The luciferase activity was normalized to *Renilla* luciferase activity and assays were performed in triplicate. *$p < 0.01$ (versus pcDNA3.1 group).

Results are expressed by mean ± SEM. Studies were performed in triplicate with sets of islets pooled from 3–4 mice per replicate (**A–C** and **E**).

isolated from C57BL/6 mice using adenoviral overexpression and RNA interference (Figures S3A and S3B). Adenoviral overexpression of granuphilin-a suppressed GSIS substantially and KSIS completely but not basal secretion at low glucose concentration, consistent with previous report in a β cell line (Coppola et al., 2002; Torii et al., 2002) (Figure S3C). Adenoviral RNAi (RNAi-784) completely inhibited expression of both granuphilin-a and-b. This knockdown of granuphilin resulted in a trend toward increased GSIS, and significantly increased KSIS, which is also consistent with a recent report describing granuphilin-null mice (Gomi et al., 2005) (Figure S3C). Granuphilin-a overexpression and knockdown did not significantly change either insulin content or ATP/ADP ratio in infected islets, demonstrating that the modulation of insulin secretion by granuphilin occurred in the process post-ATP production (Figures S3D and S3E).

Granuphilin mediates SREBP-1 inhibition of KSIS

We also evaluated the effect of granuphilin knockdown by RNA interference in SREBP-1c-overexpressing islets (Figures 4 and S4). Granuphilin mRNA and protein levels were upregulated by SREBP-1c in these islets in the basal state; however, expression was completely abolished by granuphilin RNAi (Figures 4A and 4B). This suppression of granuphilin did not affect the protein level of endogenous membrane SREBP-1c, transgenic nuclear SREBP-1c, or MafA (Figure 4B). Other vesicle and related molecules were also unchanged at the protein and mRNA levels (Figures 4C and S4A). However, the granuphilin knockdown substantially ameliorated the decreased GSIS caused by SREBP-1c overexpression and completely restored the suppressed KSIS without changes in decreased ATP/ADP ratio and increased triglyceride content (Figures 4D–4F). These data

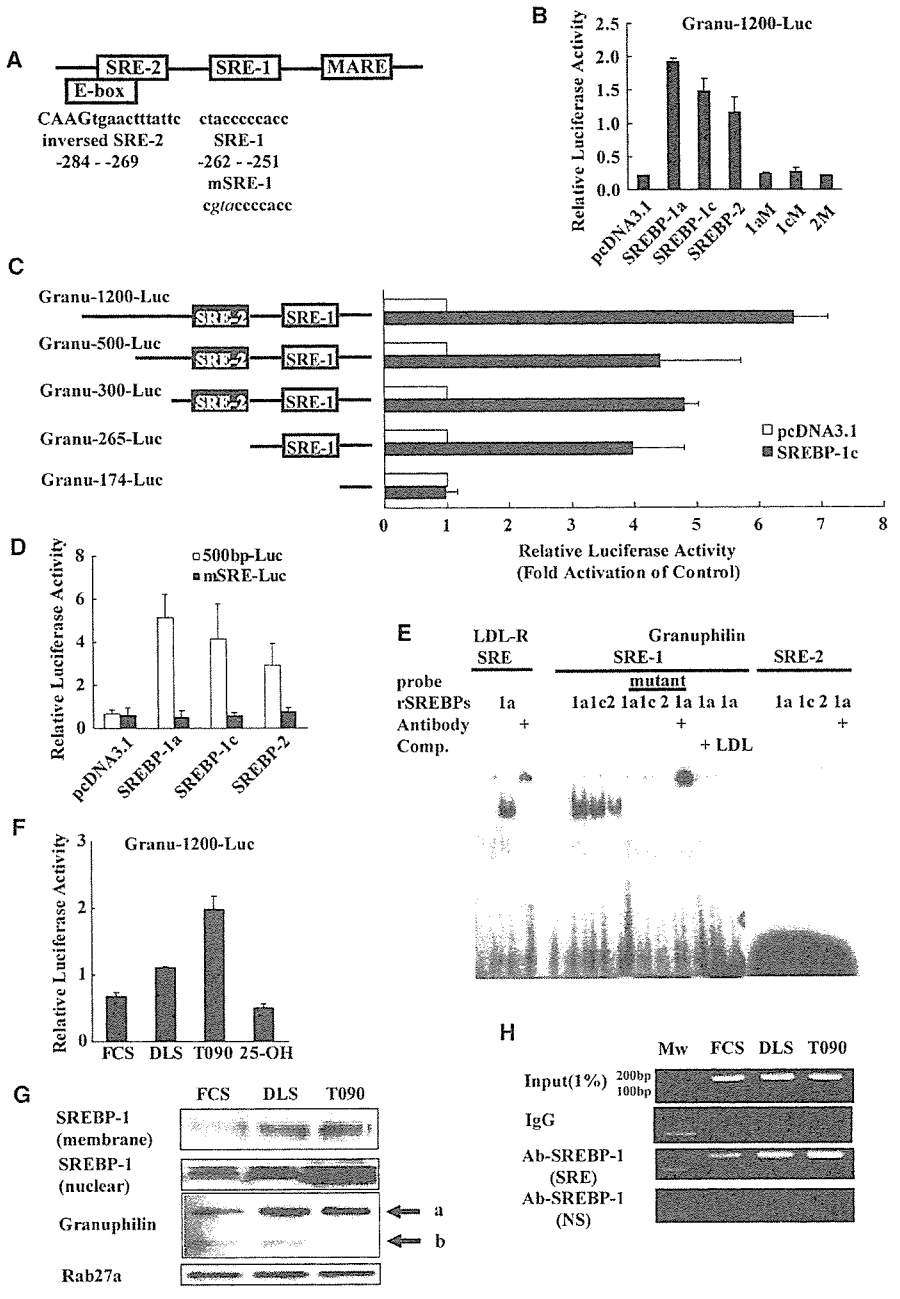

Figure 2. Mouse granuphilin promoter analysis as an SREBP target

A) Schematic representation of the mouse granuphilin promoter with the sequence and location of potential SREBP binding sites (SRE-1 and SRE-2). The SRE-2 element has an inverted consensus sequence and partially overlaps with an E-box.

B) Transcriptional activities of nuclear SREBP-1a, -1c, and -2 and their respective Tyr-Arg mutants on Granu-1200-Luc in Min6 cells.

C) Effects of deletion of SREBP-1c activation in the granuphilin promoter. Sequentially deleted luciferase constructs of the granuphilin promoter, as indicated, were used for transfection studies in Min6 cells.

D) Effects of a mutation in SRE-1 on SREBP activation of granuphilin promoter. Native (Granu-500-Luc) or mutant-SRE-1 (mSRE-Luc) reporter gene plasmids were cotransfected into Min6 cells with pSV40-*Renilla* and expression plasmids for SREBPs.

E) EMSA with radio-labeled probes for the authentic human LDL receptor SRE and granuphilin SRE-1 and SRE-2. In vitro translated SREBP-1a, -1c, and -2 proteins were incubated with these labeled probes. Specific binding was confirmed by super-shift using their respective antibody.

F) Effect of endogenous SREBPs in Min6 cells on Granu-1200-Luc. Cells were incubated with DMEM in 7.5% FCS, in 7.5% DLS, an LXR agonist: T0901317 (1 μM), and 25-hydroxycholesterol (25-OH, 0.1 μg/ml).

G) Immunoblot analysis of endogenous SREBP-1 and granuphilin proteins in Min6 cells. Cells were incubated with DMEM in 7.5% FCS, in 7.5% DLS, and T0901317 (1 μM) for 48 hr.

H) In vivo binding of SREBP-1 to Granuphilin-SRE. Chromatin prepared from the Min6 cells were subjected to the ChIP assay with anti-mouse SREBP-1 antibody and rabbit IgG as a negative control. Immunoprecipitated samples were subjected to PCR using primers to amplify the SRE-element in the granuphilin promoter and for the nonspecific-element (NS). Mw, molecular weight.

The luciferase activity was normalized to *Renilla* luciferase activity, and assays were performed in triplicate (**B–D** and **F**). Results are expressed as mean ± SEM.

indicate that SREBP-1c-mediated inhibition of insulin secretion can be accounted for, in part, by upregulation of granuphilin.

Activation of SREBP-1c/granuphilin inhibits KSIS in islets from diabetic mice

The inhibitory effect of the SREBP-1c/granuphilin pathway on insulin secretion was tested in islets from murine models of diabetes (Figure 5A). Along with consistent upregulation of SREBP-1c, both granuphilin mRNA and protein levels were increased in islets from genetically obese diabetic mice such as ob/ob, KK-Ay, and db/db, as compared to control C57BL/6 mice. In addition, mice made obese with high-fat or high-fat/high-sucrose diets also displayed increased SREBP-1c and granuphilin expression. We next sought to determine the effect of granuphilin knockdown on gene expression in islets from KK-Ay mice (Figures 5B–5E). Comparison of LacZi-Ad-infected islets from C57BL/6 and KK-Ay mice also allowed us to determine basal difference in gene expression under control and diabetic conditions. mRNA levels of SREBP-1c and granuphilin were elevated in the KK-Ay mice compared to C57BL/6 controls. Granuphilin knockdown resulted in essential abolishment of both granuphilin mRNA and protein levels without impacting SREBP-1c levels (Figure 5B). Islets from KK-Ay mice had higher basal nuclear SREBP-1 protein with concomitant increases in SREBP-1c target genes FAS and UCP-2 with significant increases in triglyceride content (Figures 5E and S4B). Protein levels of Syntaxin-1a and Munc18-1 were slightly decreased; however, levels of other vesicle proteins remained unchanged by either the increase in

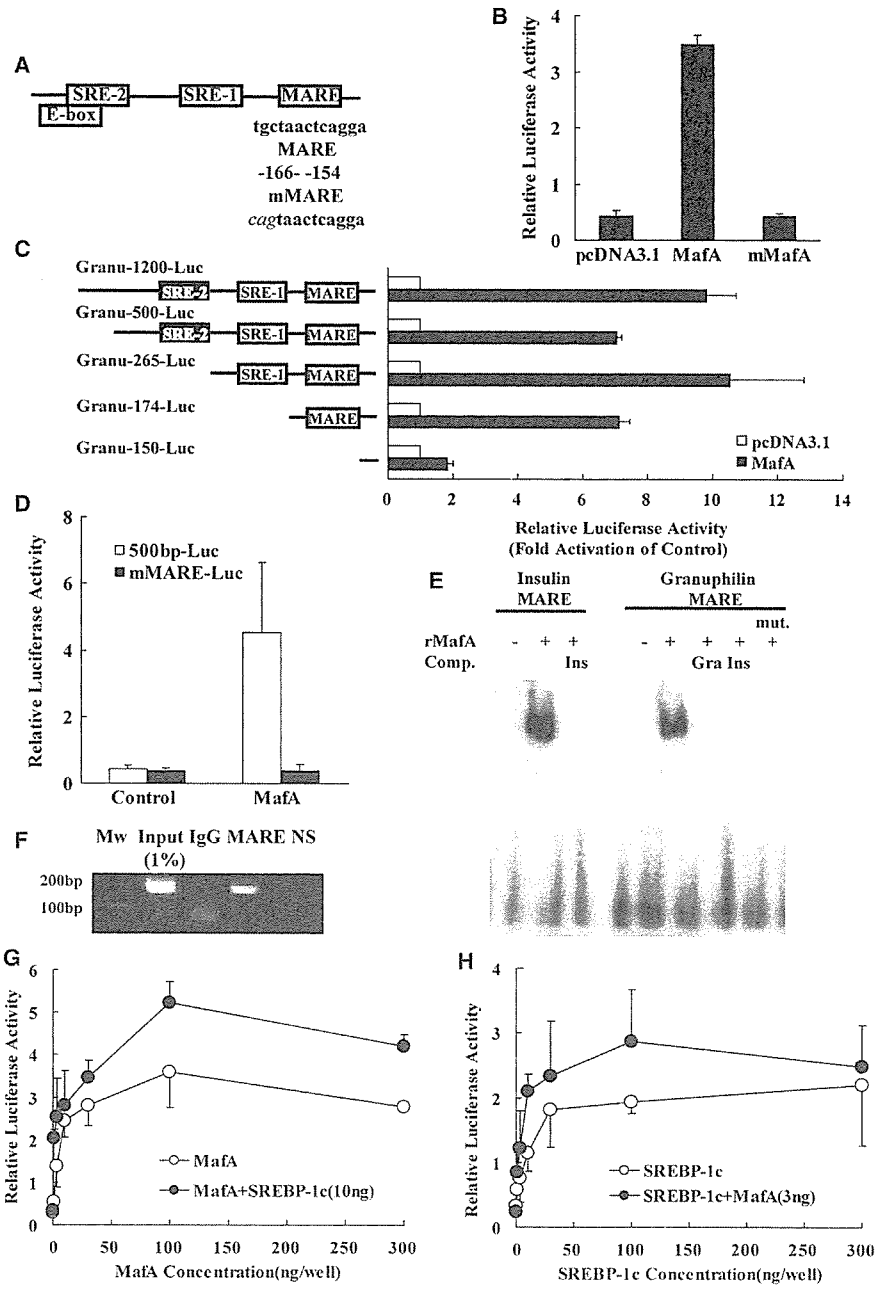

Figure 3. Mouse granuphilin promoter analysis as a MafA target

A) Schematic representation of the granuphilin promoter with sequence and location of a potential MafA binding site (MARE).

B) Transcriptional activities of wild-type MafA and mutant MafA (deletion of transactivation domain, amino acid 229–359) on Granu-1200-Luc in Min6 cells.

C) Deletion studies in MafA activation of granuphilin promoter.

D) Effect of mutation in the MARE on MafA activation of granuphilin. Native (Granu-500-Luc) or mutant-MARE (mMARE-Luc) reporter gene plasmids were cotransfected into Min6 cells with pSV40-*Renilla* and MafA.

E) EMSA with radio-labeled probes for rat insulin II MARE and granuphilin MARE-elements. In vitro translated MafA protein was incubated with these labeled probes. Specific binding was confirmed by an excess of nonlabeled probe.

F) In vivo binding to Granuphilin-MARE. Chromatin prepared from the Min6 cells were subjected to the ChIP assay with anti-rabbit MafA antibody and rabbit IgG as a negative control. Immunoprecipitated samples were subjected to PCR using primers to amplify the granuphilin promoter containing the MARE-element and of a nonspecific-element (NS). Mw, molecular weight.

G) Dose-dependent effects of MafA with cotransfection of SREBP-1c on Granu-Luc-1200 in Min6 cells.

H) Dose-dependent effects of SREBP-1c with cotransfection of MafA on Granu-Luc-1200 in Min6 cells.

The luciferase activity was normalized to *Renilla* luciferase activity and performed in triplicate (**B–D**, **G**, and **H**). Results are expressed as mean ± SEM.

SREBP-1c in the diabetic KK-Ay mice or by knockdown of granuphilin (data not shown). Thus, knockdown of granuphilin does not appear to alter mRNA or protein levels of any other proteins involved in insulin-containing granule transport in islets. Both GSIS and KSIS were reduced in KK-Ay islets with a reduction in the ATP/ADP ratio and an increase in the content of triglycerides (Figures 5C–5E). Knockdown of granuphilin completely restored KSIS but not GSIS without modulating ATP/ADP ratio or triglyceride content. In addition, restoration of decreased KSIS by granuphilin knockdown was also observed in islets from ob/ob and diet-induced obese (DIO) mice (Figures 5F, 5G, S4C, and S4D).

Knockdown of SREBP-1c by adenoviral RNAi was performed in islets from KK-Ay and ob/ob mice that exhibit upregulation of SREBP-1c and granuphilin (Figure S5). Although acute inhibition of SREBP-1c was not complete in these islets, reduction of granuphilin and restoration of decreased GSIS and KSIS were partially but consistently observed to support the concept of the contribution of the SREBP-1c/granuphilin pathway to impaired insulin secretion. Combined, these data implicate the specific involvement of granuphilin in impaired insulin secretion in obesity-related diabetic mice.

Palmitate lipotoxicity involves inhibition of KSIS through granuphilin

Saturated fatty acids are known to impair insulin secretion, supporting the theory that lipotoxicity plays a role in impaired β-cell

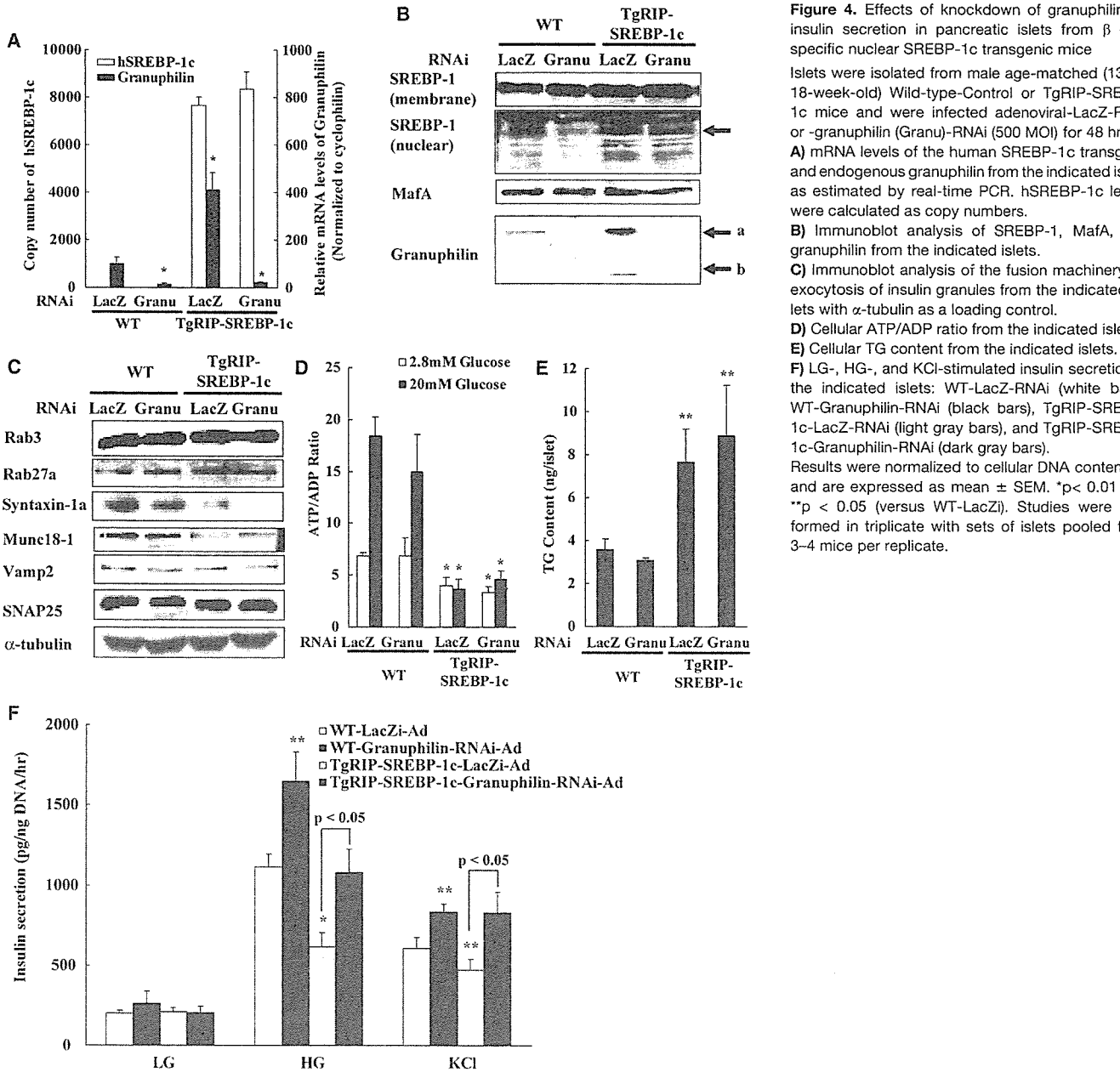

Figure 4. Effects of knockdown of granuphilin on insulin secretion in pancreatic islets from β cell-specific nuclear SREBP-1c transgenic mice

Islets were isolated from male age-matched (13- to 18-week-old) Wild-type-Control or TgRIP-SREBP-1c mice and were infected adenoviral-LacZ-RNAi or -granuphilin (Granu)-RNAi (500 MOI) for 48 hr.

A) mRNA levels of the human SREBP-1c transgene and endogenous granuphilin from the indicated islets as estimated by real-time PCR. hSREBP-1c levels were calculated as copy numbers.

B) Immunoblot analysis of SREBP-1, MafA, and granuphilin from the indicated islets.

C) Immunoblot analysis of the fusion machinery for exocytosis of insulin granules from the indicated islets with α-tubulin as a loading control.

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

E) Cellular TG content from the indicated islets.

F) LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: WT-LacZ-RNAi (white bars), WT-Granuphilin-RNAi (black bars), TgRIP-SREBP-1c-LacZ-RNAi (light gray bars), and TgRIP-SREBP-1c-Granuphilin-RNAi (dark gray bars).

Results were normalized to cellular DNA content (**F**) and are expressed as mean ± SEM. *$p < 0.01$ and **$p < 0.05$ (versus WT-LacZi). Studies were performed in triplicate with sets of islets pooled from 3–4 mice per replicate.

function (Dubois et al., 2004; Joseph et al., 2004). To test whether granuphilin expression might contribute to the pathophysiologic consequences of lipotoxicity, we incubated isolated islets with palmitate (PA) for 48 hr. PA induced both SREBP-1c and granuphilin in normal islets at both mRNA and protein levels (Figures 6A, 6B, and S4E). These inductions were blunted in the absence of SREBP-1c, indicating that SREBP-1c plays a dominant role in PA-induced granuphilin expression. PA reduced KSIS as well as GSIS in SREBP-1c-normal mice; however, the PA-suppressed GSIS and KSIS were almost completely restored in SREBP-1-null mice (Figure 6C). These results suggested that PA-induced insulin secretion impairment is mediated through SREBP-1c. Furthermore, we used adenoviral-granuphilin RNAi treatment to completely suppress granuphilin expression in control and PA-treated islets (Figures 6D and S4F). The absence of granuphilin significantly ameliorated the suppression of both GSIS and KSIS induced by PA (Figure 6E), indicating that granuphilin upregulation is involved in PA-induced impairment of insulin secretion. PA induction of SREBP-1c/granuphilin was investigated at the transcriptional level in MIN6 cells. Both Granu-1200-Luc (Figure 6F) and SRE-Luc (data not shown) were activated by incubation with PA, indicating that induction of nuclear forms of endogenous SREBPs and granuphilin was at the transcriptional level. Knockdown of SREBP-1c led to a strong reduction in PA induction of the granuphilin promoter, but dominant-negative mutant MafA did not. These data demonstrated that incubation of isolated islets with PA, a model for lipotoxicity in β cells, caused impaired insulin secretion at least partially through induction of SREBP-1c and granuphilin.

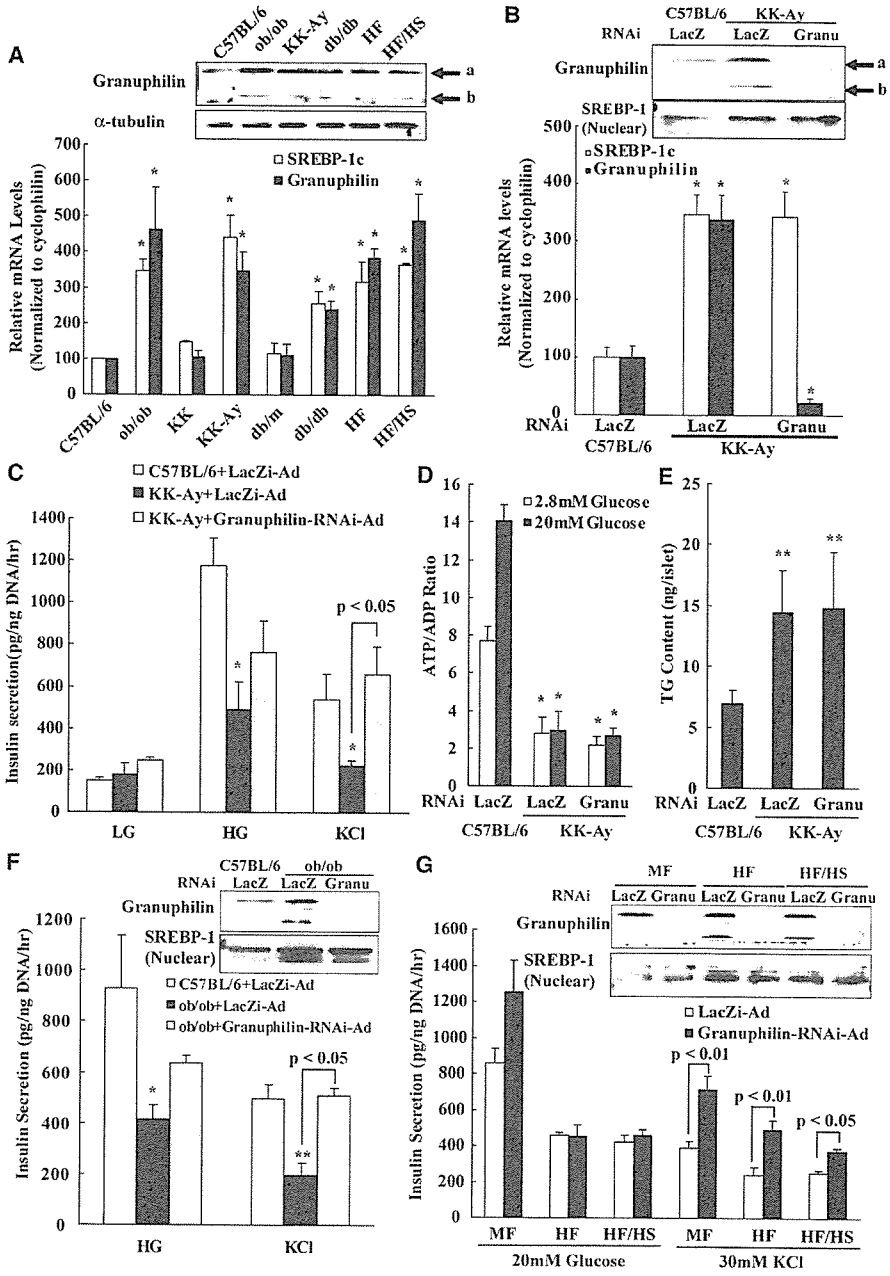

Figure 5. Activation of SREBP-1c and granuphilin in pancreatic islets from different diabetic model mice and effects of granuphilin knockdown on insulin secretion

Islets were isolated from male C57BL/6, ob/ob, KK, KK-Ay (10 weeks old), db/misty, and db/db mice (6 weeks old). C57BL/6, KK-Ay, and ob/ob mice islets were infected adenoviral-LacZ-RNAi or -granuphilin(Granu)-RNAi (500 MOI) for 48 hr.

A) Upper panel: immunoblot analysis of granuphilin from the indicated mice with α-tubulin as a loading control. Lower panel: mRNA levels of SREBP-1c (white bars) and granuphilin (black bars) in the islets from the indicated mice as estimated by real-time PCR. HF: high-fat diet, HF/HS: high-fat and high-sucrose diet. *$p < 0.01$ (versus C57BL/6).

B) Upper panel: immunoblot analysis of granuphilin and SREBP-1 from the indicated islets. Lower panel: mRNA levels of SREBP-1c (white bars) and granuphilin (black bars), from the indicated islets.

C) LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: C57BL/6-LacZ-RNAi (white bars), KK-Ay-LacZ-RNAi (black bars), and KK-Ay-Granuphilin-RNAi (gray bars).

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

E) Cellular TG content from the indicated islets.

F) Upper panel: immunoblot analysis of granuphilin and SREBP-1 from the indicated islets. Lower panel: LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: C57BL/6-LacZ-RNAi (white bars), ob/ob-LacZ-RNAi (black bars), and ob/ob-Granuphilin-RNAi (gray bars).

G) Upper panel: immunoblot analysis of granuphilin and SREBP-1 from indicated islets. Lower panel: LG-, HG-, and KCl-stimulated insulin secretion in the indicated islets: LacZ-RNAi (white bars), Granuphilin-RNAi (black bars).

Results were normalized to cellular DNA content (**C**, **F**, and **G**) and are expressed as mean ± SEM. *$p < 0.01$ and **$p < 0.05$ (versus C57BL/6-LacZ, B-E). Studies were performed in triplicate with sets of islets pooled from 3–4 mice per replicate.

SREBP-1c inhibition of KSIS is abolished in granuphilin KO islets

Finally, the SREBP-1c effect on insulin secretion was investigated in islets from granuphilin-null mice (Figure 7). Adenoviral nuclear SREBP-1c caused changes in its target genes, ATP/ADP ratio, and triglyceride content in both wild-type and granuphilin KO islets in a similar manner to those in SREBP-1c transgenic islets (Figures 7A–7E). As compared to wild-type islets, both GSIS and KSIS were increased in the absence of granuphilin. Suppression of KSIS by SREBP-1c overexpression observed in granuphilin-normal islets was cancelled in granuphilin KO islets. Meanwhile, SREBP-1c-mediated inhibition of GSIS was partially ameliorated, but remained significantly reduced compared to controls (Figure 7F). Taken together with the data from granuphilin knockdown in SREBP-1c transgenic islets, these data demonstrate that granuphilin contributes to SREBP-1c-mediated KSIS inhibition.

Discussion

Granuphilin is a target gene of SREBP and MafA

Our current study clearly shows that mouse granuphilin is an SREBP target. Because SREBP-1c expression is highly regulated by nutrition (Horton et al., 1998; Hasty et al., 2000; Matsuzaka et al., 2004) and is upregulated in pathological states related to over-nutrition and insulin resistance (Biddinger et al., 2005), we propose that SREBP-1-induced granuphilin activation may contribute to β cell lipotoxicity and diabetes that can