Diabetes mellitus is associated with a variety of complications, including alterations in the central nervous system (CNS). We have recently shown that diabetes results in a reduction of cholesterol synthesis in the brain due to decreased insulin stimulation of SREBP2-mediated cholesterol synthesis in neuronal and glial cells. In the present study, we explored the effects of the decrease in cholesterol on neuronal cell function using GT1-7 hypothalamic cells subjected to cholesterol depletion in vitro using three independent methods: 1) exposure to methyl-β-cyclodextrin, 2) treatment with the HMG-CoA reductase inhibitor simvastatin, and 3) shRNA-mediated knockdown of SREBP2. All three methods produced 20–31% reductions in cellular cholesterol content, similar to the decrease in cholesterol synthesis observed in diabetes. All cholesterol-depleted neuron-derived cells, independent of the method of reduction, exhibited decreased phosphorylation/activation of IRS-1 and AKT following stimulation by insulin, insulin-like growth factor-1, or the neurotrophins (NGF and BDNF). ERK phosphorylation/activation was also decreased after methyl-β-cyclodextrin and statin treatment but increased in cells following SREBP2 knockdown. In addition, apoptosis in the presence of amyloid-β was increased. Reduction in cellular cholesterol also resulted in increased basal autophagy and impairment of induction of autophagy by glucose deprivation. Together, these data indicate that a reduction in neuron-derived cholesterol content, similar to that observed in diabetic brain, creates a state of insulin and growth factor resistance that could contribute to CNS-related complications of diabetes, including increased risk of neurodegenerative diseases, such as Alzheimer disease.

Diabetes mellitus is associated with a variety of complications. In addition to the classical complications resulting from micro- and macroangiopathy, there are a number of effects of diabetes involving the central nervous system (CNS), including decline in cognitive function (1, 2) and increased incidence of Alzheimer disease (3, 4) and depression (5). Although some changes in CNS function may represent acute or chronic effects of hyper- or hypoglycemia on the brain or be related to underlying vascular disease, the mechanisms underlying many CNS disorders are still unclear.

Recently, we observed that insulin-deficient diabetes in mice can lead to a reduction in brain cholesterol synthesis, which occurs through a change in expression of cholesterol synthesis enzymes and their upstream regulators SREBP2 and SCAP1 (6, 7). Because cholesterol-containing lipoproteins do not cross the blood-brain barrier, the brain depends on its own cholesterol synthesis for normal cell function (8, 9). Thus, the decrease in brain cholesterol synthesis results in a decline in cholesterol content of synaptosomal membranes, and a similar reduction of cholesterol synthesis in neurons in vitro results in a decrease in synapse formation (6). Cholesterol is also known to be enriched in exocytic domains of plasma membrane (10), and exocytosis has been shown to be decreased in cultured neuron-derived cells after chemical cholesterol depletion with methyl-β-cyclodextrin (11, 12). Thus, a decrease in cholesterol synthesis in diabetes could contribute to neuronal dysfunction in patients with diabetes.

Insulin plays an essential role in glucose homeostasis by regulating glucose production in liver and glucose uptake in muscle and fat. Although neurons were once regarded as insulin-independent tissues, it is now clear that the brain is an insulin-responsive tissue and that neurons express insulin receptors and many components of their downstream signaling pathways (13, 14). Studies using intracerebroventricular injection of insulin and techniques to knock down or knock out insulin signaling proteins have shown that insulin action in the CNS plays an important role in energy homeostasis (15), learning and memory (16), and peripheral glucose metabolism (17). Insulin also has a neurotrophic function (18, 19), and insulin signaling in the brain has been shown to be altered in Alzheimer disease (20, 21). Conversely, it has been reported that nasal insulin admin-
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Methyl-β-cyclodextrin (MBCD) treatment can improve cognitive function in patients with early Alzheimer disease (22, 23). These data suggest that insulin deficiency or insulin resistance may affect cognitive function and the pathophysiology of CNS disease.

Despite a number of reports of impaired neuronal function in diabetic brain and of reports on the role of diabetes in brain cholesterol synthesis, the relationship between a reduction in cholesterol synthesis and neuronal cell function has not been defined. In this study, we explored the role of decreased cholesterol in neuronal cell function by mimicking the decrease in cholesterol observed in diabetes in vitro using chemical depletion, treatment with a statin, and knockdown of SREBP2. In all three cases, neuron-derived GT1-7 cells in which cholesterol had been decreased to a level similar to that seen in diabetes showed impaired insulin and IGF-1 signaling as well as impaired neurotrophin signaling. Cholesterol reduction in GT1-7 cells also resulted in altered autophagy and increased apoptosis. These data indicate that a decrease in cholesterol content in neurons in diabetes could contribute to significant changes in the function of neurons in the brain in this disorder.

Experimental Procedures

Cell Culture—GT1-7 cells (kindly provided by Dr. Pamela Mellon, University of California, San Diego) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM glucose. Cells were cultured at 37 °C under 95% air and 5% CO2 before and during any treatment.

Cholesterol Depletion—GT1-7 cells were grown for 2–3 days before cholesterol depletion. For depletion with methyl-β-cyclodextrin (MBCD), GT1-7 cells were washed with PBS two times and then incubated with DMEM (without FBS) containing 2 mM MBCD for 30 min. For depletion with simvastatin, cells were incubated in growth medium containing 200 nM simvastatin for 48 h. Cells were lysed, and cholesterol concentration of whole cell lysate was measured with the Amplex® Red Cholesterol Assay kit (Invitrogen).

Generation of SREBP2 Knockdown GT1-7 Cells—Lentiviral vector plasmids for murine SREBP2 shRNA (GIPZ Lentiviral shRNAmir, Open Biosystems, Huntsville, AL) and control nonsilencing shRNA were packaged by co-transfection with packaging plasmids in HEK293T cells (Trans-Lentiviral Packaging System, Open Biosystems). Viral particles were concentrated by ultracentrifugation. GT1-7 cells were infected with lentivirus vectors for 48 h followed by a replacement with fresh growth medium containing puromycin (5 μg/ml). Knockdown cell lines were maintained in growth medium containing the same concentration of puromycin. Knockdown was validated by Western blotting for total and cleaved SREBP2 (catalog number LS-B1609, Lifespan Biosciences, Seattle, WA).

Insulin, IGF-1, and Neurotrophin Signaling—Cells were treated for cholesterol depletion as above. Cells were incubated with DMEM containing 25 mM glucose without FBS for 3 h at 37 °C before stimulation. Then medium was replaced by DMEM containing 100 nM insulin, 10 nM IGF-1, 100 nM nerve growth factor (NGF), or 50 ng/ml brain-derived neurotrophic factor (BDNF). After the stimulation, cells were washed with 4 °C PBS three times and lysed in radioimmune precipitation assay buffer containing protease inhibitor mixture and phosphatase inhibitor mixture (Sigma).

Ras Prenylation Measurements—GT1-7 cells were incubated in growth medium containing simvastatin for 48 h. GT1-7 cells treated with simvastatin or SREBP2 knockdown cells were lysed in lysis buffer (150 mM NaCl, 5 mM MgCl2, 1 mM sodium phosphate, 1% Triton X-100, 0.05% SDS, 50 mM HEPES, pH 7.4) containing protease and phosphatase inhibitors. Protein concentration was measured using the bicinchoninic acid assay, and all samples were adjusted to the same concentration. As described previously (24), an equal volume of 4% Triton X-114 was added, vortexed, and incubated at 37 °C for 5 min. Solutions were kept still at room temperature until the phases had separated. The lower phase (lipid phase, which was enriched for membrane proteins) was analyzed by SDS-PAGE and immunoblotting for Ras.

Ras Activation Assay—As described previously (25) to measure Ras activation, a pulldown assay using Raf-1 was performed with a Ras activation assay kit (Millipore Corp., Billerica, MA). GT1-7 cells were treated with simvastatin for 48 h and then lysed in medium containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA, and 2% glycerol. The lysates were incubated with the Raf-1 Ras binding domain-agarose conjugate at 4 °C for 1 h with gentle rocking. The beads were washed and then boiled in LDS sample buffer, and Western blotting was performed to measure the pulled down Ras.

Annexin V Flow Cytometry Assay for Amyloid β-induced Apoptosis—Translocation of phosphatidylserine to the membrane is seen in the early phase of apoptosis and can be detected by binding of annexin V. Amyloid β fragment 25–35 (Abcam, Cambridge, MA) was prepared as described previously (26). GT1-7 cells were grown on 6-well plates for 1 day followed by simvastatin or dimethyl sulfoxide treatment for 48 h. Then growth medium was replaced with fresh growth medium with or without the 25–35 fragment of amyloid β (6 μM) and incubated at 37 °C for 12 h. Cells floating in the medium were collected as a single PBS wash of the wells. The cells were briefly treated with 0.25% trypsin and then combined with the collected floating cells. After spinning the samples for 5 min at 400 × g to remove the medium, cells were incubated for 15 min in 100 μl of cell binding buffer (0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM CaCl2) containing 5 μl of allophycocyanin-annexin V (catalog number 550474, BD Biosciences) and 5 μl of 7-aminoactinomycin D (7-AAD) to detect apoptosis and necrosis. 100,000 cells were sorted per sample using a BD FACS Aria Special Order Research Product.

Caspase 3 Cleavage Assay of Apoptosis—Apoptosis was also detected by cleavage of caspase 3. GT1-7 cells were treated with simvastatin or dimethyl sulfoxide for 48 h. Cells were then incubated in DMEM containing 5.5 mM glucose without FBS for 12 h and lysed in radioimmune precipitation assay buffer containing protease inhibitors. Apoptosis was evaluated by Western blotting of cleaved caspase 3 (catalog number 9661, Cell
Signaling Technology, Danvers, MA) normalized for total caspase 3 (catalog number 9662, Cell Signaling Technology).

Autophagy—GT1-7 cells were treated with simvastatin for 48 h, and SREBP2 knockdown cells were grown for 48 h before the experiment. Then cells were incubated in DMEM with high (25 mM) glucose or low (5.5 mM) glucose containing the lysosomal inhibitor bafilomycin A1 (120 nM; Santa Cruz Biotechnology, Dallas, TX) for 4 h. Cells were lysed in radioimmune precipitation assay buffer containing protease and phosphatase inhibitors. Autophagy was measured by Western blotting for LC3A/B and p62 (catalog numbers 12741 and 5114, Cell Signaling Technology), and autophagy signaling was evaluated by Western blotting of phosphorylated ULK-1 normalized to total ULK1 (catalog numbers 5869 and 8054, Cell Signaling Technology).

Statistical Analysis—All results are expressed as the mean ± S.D. Statistical significance was calculated using an unpaired Student’s t test for comparison between two groups or a two-way analysis of variance with Tukey’s post hoc analysis when two treatment conditions were used. A p value of <0.05 was considered statistically significant.

Results

Modeling the Effect of Diabetes on Neurons by Cholesterol Depletion in Vitro—We have previously shown that there is an ∼25% decrease in expression of multiple genes in the cholesterol synthesis pathway in the brains of mice with insulin-deficient diabetes due to a decrease in activity of the SREBP2/SCAP1 pathway (6, 7). This is associated with a parallel decrease in cholesterol content of synaptosomal membranes and a decrease in synapse formation in vitro (6). To better understand the impact of this change in cholesterol synthesis on cell signaling and cell viability, we used three different methods to induce moderate levels of cholesterol depletion in vitro and characterized their effects on insulin, IGF-1, and neuronal growth factor signaling pathways. These methods were the acute depletion of membrane cholesterol by treatment of cells with MBCD, a more intermediate model of depletion by treatment with the HMG-CoA reductase inhibitor simvastatin, and a chronic reduction in cholesterol synthesis by knockdown of the master regulator of cholesterol biosynthesis SREBP2.

MBCD depletes cholesterol from plasma membranes by sequestering cholesterol in its hydrophobic pocket (27). This effect is rapid. Thus, when GT1-7 hypothalamic GnRH neuron-derived cells were incubated in DMEM containing 2 mM MBCD, there was a decline in total cellular cholesterol of 22.3 ± 5.6% within 10 min, and this remained stable for up to 60 min of exposure to the chemical (Fig. 1A). MBCD depletion of cholesterol was also concentration-dependent. Total cholesterol content of cells was decreased by 29.3% by 2 mM MBCD and by 52.1% by 10 mM MBCD (Fig. 1B).

Statins are widely used for the treatment of hypercholesterolemia; they suppress cholesterol synthesis by competitively inhibiting HMG-CoA reductase, the rate-limiting step in the cholesterol synthesis pathway. In contrast to the acute effect of MBCD, when cells were incubated in the medium containing 200 nM simvastatin, there was a decline in cholesterol that became significant by 48 h (Fig. 1C). Treatment with simvasta-
cholesterol depletion on the responsiveness to insulin of neuronal cells, GT1-7 cells that had been subjected to cholesterol depletion were stimulated with insulin, and downstream protein phosphorylation was assessed. This revealed a reduction in insulin action with a 25.3% decrease in insulin-stimulated phosphorylation of IRS-1 following treatment with MBCD, a 59.0% decrease after simvastatin treatment, and a 69.5% decrease after knockdown of SREBP2. Likewise, insulin-stimulated AKT phosphorylation was decreased in the cholesterol-depleted cells by 34.5, 33.0, and 72.4%, respectively. Insulin-stimulated ERK phosphorylation was even more markedly decreased following treatment with MBCD (63.5% decrease) or simvastatin (82.1% decrease), whereas in SREBP2 knockdown GT1-7 cells, ERK phosphorylation after insulin stimulation was increased by 33% (Fig. 2). Stimulation of the cholesterol-depleted GT1-7 cells by IGF-1 showed a similar pattern with reductions in IRS-1 and AKT phosphorylation in all three models and a decrease in ERK phosphorylation following MBCD and simvastatin treatment but an increase in ERK phosphorylation by ~200% following SREBP2 knockdown (Fig. 3).

Neurotrophin signaling pathways, such as those stimulated by NGF and BDNF, also lead to phosphorylation of AKT and ERK in neurons and play an important role in proliferation of these cells (28). As with insulin and IGF-1 signaling, NGF (Fig. 4A) and BDNF (Fig. 4B) stimulation of AKT phosphorylation was decreased in GT1-7 cells following all three methods of cholesterol depletion. Likewise, NGF- and BDNF-stimulated ERK phosphorylation was decreased in cells treated with MBCD or simvastatin, whereas ERK phosphorylation was increased in the SREBP2 knockdown GT1-7 cells, mimicking the alterations observed in insulin and IGF-1 signaling.

**ERK Phosphorylation Is Enhanced in SREBP2 Knockdown GT1-7 Cells**—As shown in Figs. 2 and 3, whereas the AKT and ERK pathways of insulin/IGF-1 signaling were coordinately impaired following cholesterol depletion with MBCD and simvastatin, phosphorylation of AKT was decreased and phosphorylation of ERK was enhanced in the SREBP2 knockdown cells. To further investigate the mechanism of this differential response to cholesterol depletion, we examined the activation...
and expression of upstream components of the mitogen-activated protein kinase pathway.

MEK is the immediate upstream kinase for ERK (29). Insulin stimulation phosphorylates and activates MEK, which in turn activates ERK. Insulin-stimulated MEK phosphorylation was decreased in cells treated with simvastatin (Fig. 5A) or MBCD (data not shown), consistent with the change in phosphorylation of ERK. Interestingly, in contrast to the increase in ERK phosphorylation, SREBP2 knockdown cells also exhibited decreased phosphorylation of MEK, consistent with decreased upstream insulin signaling (Fig. 5A). One potential mechanism for this differential effect could be the activity of the dual specificity phosphatases (DUSPs) that dephosphorylate ERK. The phosphatases are kept in proximity to ERK by the cholesterol-regulated scaffolding protein oxysterol-binding protein (OSBP) (30). Assessment of Dusp1, Dusp6, Dusp9, and OSBP mRNA revealed no changes in expression between simvastatin-treated cells and control. Furthermore, Dusp6, Dusp9, and OSBP expression was increased, rather than decreased, in SREBP2 knockdown GT1-7 cells, suggesting that changes in expression of these dual specificity phosphatases and OSBP do not account for the difference in ERK activation (data not shown).

Upstream of MEK is the Ras-Raf cascade. Ras is prenylated, and this hydrophobic side chain enhances attachment to membranes and is essential for activation of the MAPK pathway (24). Isoprenoid chains, such as geranylgeranyl diphosphate and farnesyl diphosphate, are products of the HMG-CoA reductase pathway (31). Statins inhibit this pathway and have been reported to influence the activation of the MAPK pathway (32). To determine the effect of cholesterol reduction on Ras function, we examined Ras prenylation using Triton X-114 extraction to isolate the membrane fraction followed by Western blotting. As shown in Fig. 5B, the hydrophobic membrane was rich in prenylated Ras (upper panels), whereas unprenylated Ras was extracted in the aqueous cytosolic fraction (lower panels). As expected from its pharmacological effect, prenylated Ras was decreased in simvastatin-treated Ras, whereas unprenylated Ras was extracted in the aqueous cytosolic fraction (lower panels). As expected from its pharmacological effect, prenylated Ras was decreased in simvastatin-treated cells. SREBP2 knockdown cells also exhibited a reduction in prenylated Ras, whereas total Ras protein levels were not changed by either method of cholesterol depletion. Assessment of Ras activation...
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was measured by its binding to Raf using a pulldown assay with whole cell lysates followed by blotting with anti-Ras antibody. This also revealed a decrease in activated Ras in the Raf pull-down of simvastatin-treated GT1-7 cells, whereas there was no significant change in the total amount of Ras protein in the total cell lysates (Fig. 5C).

Apoptosis Is Enhanced in Cholesterol-depleted Neuron-derived Cells—Apoptosis of neurons is increased in neurodegenerative disease, including Alzheimer disease. Amyloid β, one of the pathogenic components of Alzheimer disease, interacts with membrane lipids in the initial stage of the neurodegenerative change (33, 34). To determine whether cholesterol depletion affected apoptosis, we assessed annexin V-allophycocyanin binding (35) and 7-AAD uptake following exposure of the cells to amyloid β using flow cytometry. Treatment with amyloid β caused an increase in cell death (7-AAD staining) and a trend toward increased early (annexin V staining) and late (annexin V and 7-AAD co-staining) apoptosis in control cells. Simvastatin alone caused an increase in early and late apoptosis with a trend toward increased cell death. When simvastatin was combined with amyloid β, significantly more early and late apoptosis and a trend toward more cell death than in control cells treated with amyloid β were observed (Fig. 6A). Cleaved caspase also showed a tendency to increase in simvastatin-treated cells in the basal state and showed a more marked increase after apoptosis induction by serum starvation in these cells (Fig. 6B), consistent with enhanced apoptosis in neuron-derived cells after cholesterol depletion.

Autophagy Is Altered by Cholesterol Depletion—Neuronal cells contain abundant lysosomes (36). Autophagy is a lysosome-directed vesicular trafficking pathway and a major degradation pathway of intracellular components that is essential for survival and adaptation to nutrient elimination (37). Autophagy also plays a role in maintenance of neuronal cells and has been implicated in clearance of pathogens and antigen presentation (38), including the clearance of amyloid β protein from neuronal cells (39). LC3I is converted to LC3II by lipidation during autophagy. To determine whether there was a change in autophagy in cholesterol-depleted cells, we examined the expression of LC3II under different nutrient conditions. As expected, the autophagosomal protein LC3II was increased in untreated cells when exposed to low glucose (5.5 mM) conditions as compared with high glucose (25 mM) medium (Fig. 7A, top and middle panels). In contrast, SREBP2 knockdown cells exhibited a trend toward increased expression of LC3II in the basal state and impaired elevation of LC3II in response to low glucose levels (Fig. 7A, top panel). Similarly, the simvastatin-treated cells showed a trend toward higher LC3II protein and did not further increase LC3II in response to low glucose (Fig. 7A, middle panel). The protein p62, which helps target ubiquitinated proteins to the autophagosome, was also increased with simvastatin treatment under high glucose conditions and was further increased in low glucose conditions (Fig. 7A, bottom panel).

ULK-1 plays a regulatory role in autophagy. ULK-1 is dephosphorylated at serine 555 and inactivated when nutrients are plentiful and phosphorylated/activated when nutrients are limited (40). Ser-555 phosphorylation of ULK-1 was increased in simvastatin-treated GT1-7 cells in the basal state. Although Ser-555 phosphorylation of ULK-1 was significantly increased in low glucose in control cells, there was no increase when the simvastatin-treated cells were exposed to low glucose (Fig. 7B). These results suggest that the autophagy response to nutrient limitation is impaired in cholesterol-depleted neuronal cells.

Discussion

Diabetes causes micro- and macrovascular complications in a variety of organs. In addition, alterations in metabolism may play a role in complications through protein glycosylation, oxidative damage, and impairment of clearance or protein turnover (41). Recently, we found that diabetes impairs cholesterol synthesis in the brains of diabetic mice, suggesting that changes in cellular cholesterol may contribute to neuronal dysfunction in the brain in diabetes (6).

In this study, we used three methods to produce a reduction of cholesterol content in cultured hypothalamic cells in vitro to mimic the change exhibited in the brain of diabetic mice. In each method, we obtained a 20–30% reduction of cholesterol, equivalent to the cholesterol depletion observed in synaptosomes of diabetic mice (6). A previous report described regulation of insulin signaling by membrane cholesterol (42). In that study of retinal neurons, MBCD was used to deplete membrane cholesterol. Using much higher concentrations than used in
In this study, they showed that after a 60% reduction in cholesterol insulin signaling could be completely blocked. In the present study, in addition to using lower concentrations of MBCD, we used siRNA and simvastatin to deplete whole cell cholesterol, not just membrane cholesterol, but only by about 20–30%, similar to the reduction in cholesterol synthesis found in streptozotocin diabetic mice (6). Thus, our study shows different aspects of cholesterol depletion and should be a closer model for cholesterol depletion that occurs in diabetic neuronal cells.

We found that in cholesterol-depleted neuron-derived cells the insulin and IGF-1 signal cascades were impaired both at the level of IRS-1 phosphorylation and downstream in the AKT and mitogen-activated protein kinase pathways, leading to a variety of changes in cellular function, including altered autophagy and apoptosis. Although cholesterol depletion by treatment with MBCD, simvastatin, or SREBP2 knockdown resulted in a down-regulation of insulin signaling in the AKT pathway, signaling through the MAPK pathway was more complex. Thus, all three methods of cholesterol reduction resulted in a decrease in insulin stimulation of MEK, but only MBCD and simvastatin treatment resulted in a reduction of ERK phosphorylation, whereas insulin-stimulated ERK phosphorylation was up-regulated in SREBP2 knockdown cells, indicating a further alteration between MEK and ERK activation in these knockdown cells.

ERK phosphorylation is inactivated by a family of MAPK/DUSPs. Changes in DUSPs did not explain the paradoxical increase in ERK phosphorylation in SREBP2 knockdown cells because in these cells expression of Dusp6 and Dusp9 was increased, not decreased. Scaffolding proteins regulate ERK proximity to the DUSPs. One such protein is OSBP. This scaffolding protein is sensitive to cholesterol levels in the membrane and releases ERK in response to low cholesterol, resulting in an increase in phosphorylated ERK (30). It may be that this cell line requires the chronic depletion of cholesterol generated by the SREBP2 knockdown to disrupt the ERK-DUSP-OSBP complex. Further investigation will be needed to determine the exact mechanism accounting for the increased ERK activation in the SREBP2 knockdown cells and define the difference of DUSP activation between SREBP2 knockdown and other treatments.
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Alzheimer disease is characterized by the accumulation of amyloid β forming amyloid plaques and neurofibrillary tangles (34). A number of studies have suggested a relation between cholesterol and amyloid β and have shown that cholesterol content affects transport, proteolytic cleavage, aggregation, and toxicity of amyloid β (43). Excess brain cholesterol has been associated with increased formation and deposition of amyloid β peptide from amyloid precursor protein (44). Although some studies have suggested statins have adverse effects on short term memory, others have suggested a beneficial effect on Alzheimer disease, in both cases, the effect of statins on the concentration of amyloid β in blood and cerebrospinal fluid remains unclear (45). What is clear from this study is that cholesterol depletion from neuron-derived cells by any method, including simvastatin treatment, impaired insulin signaling.

Recently, two groups have reported the association of insulin resistance of brain neuronal cells and Alzheimer disease (20, 21). In those studies, insulin resistance was associated with cognitive decline of the patients with Alzheimer disease, and antidiabetes agent treatment improved cognitive function in Alzheimer disease model mice. Our finding of impaired insulin signaling following cholesterol depletion could be a part of the mechanism of the higher risk of Alzheimer disease in diabetic patients. Furthermore, cholesterol-depleted cells exhibit enhanced apoptosis when exposed to amyloid β. This increased vulnerability for apoptosis might contribute to an increased risk of Alzheimer disease in diabetes.

Insulin receptors are widely expressed in the brain. Direct injection of insulin into the brain reduces food intake and body weight (15). By contrast, brain-specific insulin receptor knock-out mice exhibit hyperphagia and obesity (46). Insulin signaling also controls peripheral glucose metabolism and insulin sensitivity by regulating hepatic glucose production (17). Although the exact nature of insulin resistance in the CNS is not clear, our finding of insulin signal impairment in cholesterol-depleted neuron-derived cells suggests another mechanism by which insulin deficiency in the CNS can augment the effect of diabetes on brain dysfunction.

Although autophagy is essential for adapting to the fasting condition, constitutive autophagy is important for the maintenance of cellular metabolic turnover, homeostasis, and protein clearance. We found that activation of autophagy was impaired with little increase in nutrient deprivation-induced autophagy in cholesterol-depleted cells. This impairment of autophagy may contribute to the neuronal damage in diabetic complications. It has been reported that the expression of beclin-1, an essential regulator for initiating the autophagic process, is decreased in Alzheimer disease patients, and its depletion caused accumulation of amyloid β (47). It has also been reported that amyloid β and its precursor can co-localize to LC3-positive autophagosomes (48, 49). These findings demonstrate the role of autophagy in clearance of amyloid β protein and indicate how impaired autophagy in cholesterol-depleted...
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cells could affect the clearance of amyloid β and promote the pathological changes of Alzheimer disease in the diabetic brain. In neuron-derived cells in which cholesterol levels were reduced, autophagy was also enhanced in the basal state. This enhancement of autophagy could further contribute to altered neuronal cell viability and function.

In summary, we have demonstrated that cholesterol depletion in neuron-derived cells causes insulin signaling deficiency, alters autophagy, and enhances apoptosis induced by cytotoxic stress. These results suggest that reduction in cholesterol could contribute to insulin resistance in neurons in diabetes and be an additional pathological mechanism that could contribute to the increased risk of diabetic patients for development of neurodegenerative disease, including Alzheimer disease.

Author Contributions—K. F. designed, performed, and analyzed parts of experiments shown in Figs. 1–7 and wrote the original paper. H. A. F. designed, performed, and analyzed parts of experiments in Figs. 1, 4, 6, and 7 and performed all revisions to the manuscript. C. R. K. conceived the study and contributed to data analysis and revision of the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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