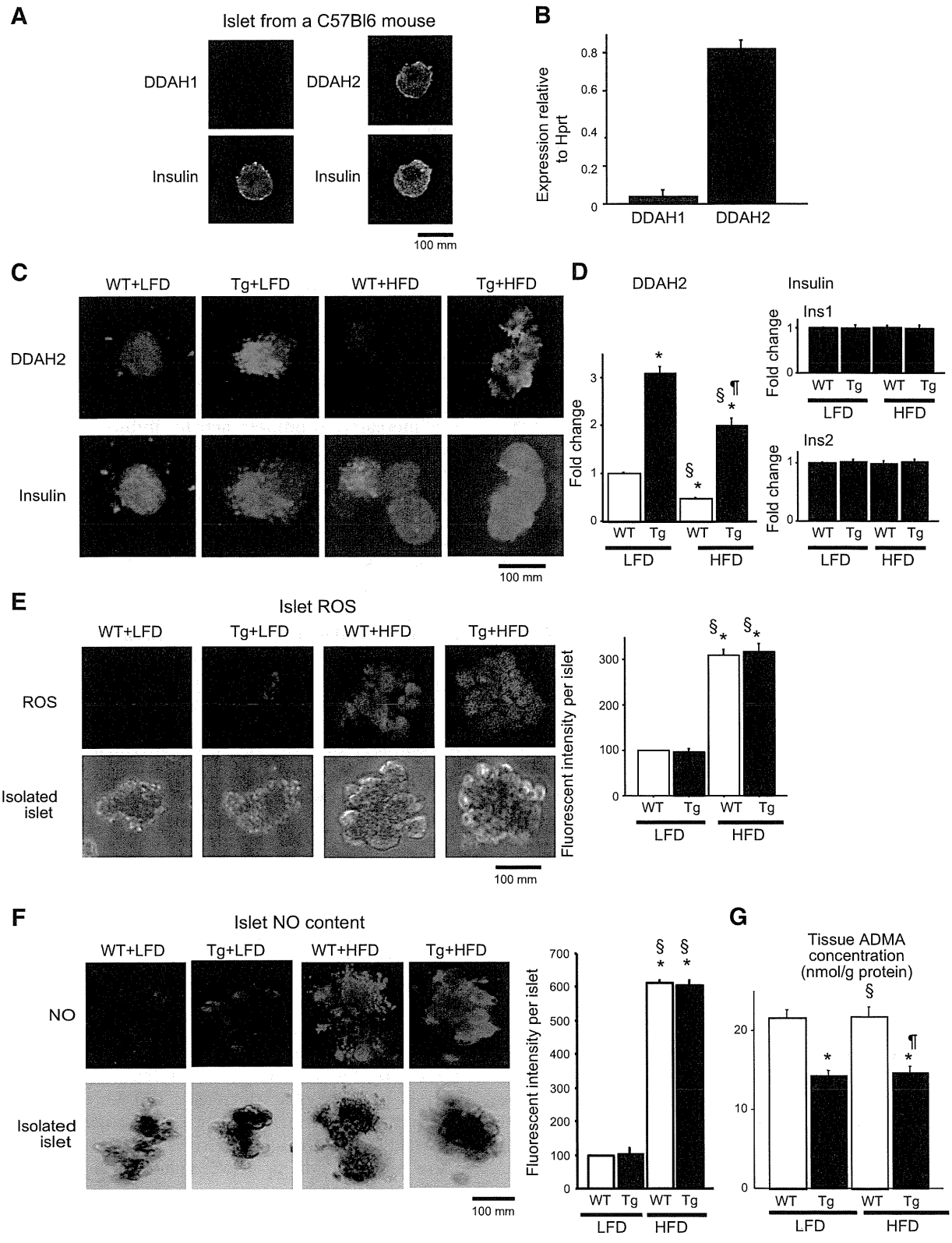

Figure 3. Pancreatic ROS and NO levels, blood pressure, and plasma ADMA levels in DDAH2 Tg mice. *A*) DDAH1 (left panels) and DDAH2 (right panels) immunohistochemistry in islets from C57BL6 mice. Bottom panels: insulin immunohistochemistry. Results are representative of 3 independent experiments. *B*) Quantitative real-time PCR analysis of islet DDAH1 and DDAH2 expression. Experiments were repeated 3 times. *C*) Representative immunofluorescence images of pancreatic sections using antibodies for murine DDAH2 and insulin. *D*) Quantitative real-time PCR analysis of islet DDAH2, insulin 1 (Ins1), and insulin

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revealed a protein-protein interaction between DDAH2 and secretagogin, we focused on secretagogin expression in these mice. We found that islet secretagogin immunofluorescence was increased in LFD-fed Tg mice compared with that in LFD-fed WT mice. Islet secretagogin immunofluorescence was reduced in HFD-fed WT mice, but this reduction was rescued in HFD-fed Tg mice (**Fig. 4*A***). These changes in secretagogin protein expression were consistent with the changes in secretagogin mRNA expression (Fig. 4*B*). These data indicate that, in Tg mice, secretagogin expression is increased *via* a transcriptional regulatory mechanism.

We next examined whether β-cell-specific DDAH2 overexpression directly regulates secretagogin expression in MIN-6 cells, a murine β-cell line with intact glucose-responsive insulin secretion, similar to that in primary pancreatic β cells (24). In DDAH2-overexpressing cells (Fig. 4*C*), the mRNA and protein expression of secretagogin was significantly increased (Fig. 4*D*). GSIS was also significantly increased at 24 and 48 h after transfection (Fig. 4*E*). These data indicate that DDAH2 directly enhances GSIS in β cells. Therefore, we examined whether the effects of DDAH2 on GSIS were dependent on up-regulation of secretagogin by performing knockdown experiments. Secretagogin siRNA significantly reduced secretagogin mRNA expression in MIN-6 cells (Fig. 4*F*, left panel) and markedly blunted DDAH2-enhanced GSIS, indicating that DDAH2 stimulates GSIS by inducing secretagogin expression (Fig. 4*F*, middle and right panels). We further tested whether β-cell-specific secretagogin overexpression directly enhances GSIS in MIN-6 cells. In secretagogin-overexpressing cells (Fig. 4*G*, left panel), GSIS was significantly increased at 24 and 48 h after transfection (Fig. 4*G*, right panel). Overall, these data indicate that DDAH2 overexpression enhances GSIS in pancreatic β cells by up-regulating secretagogin expression.

DDAH2 is a transcriptional regulator of secretagogin

We determined the transcriptional regulation of secretagogin by DDAH2. Sequence analysis using Transcription Element Search System (TESS) software (http://www.cbil.upenn.edu/cgi-bin/tess) revealed putative binding sites for the transcription factors CEBPα, GATA1, Sp1, GR, GCN4, TBP, activator protein (AP)-1, AP-2, and GCF in a 1578-bp region surrounding the transcriptional start site of secretagogin (**Fig. 5*A***). We performed luciferase assays to test whether DDAH2 transfection increases secretagogin promoter activity by measuring the promoter activity of 5′ deletion constructs (−1525 Luc, −680 Luc, −450 Luc, −346 Luc, and −84 Luc). These assays showed that, in DDAH2-transfected MIN-6 cells, secretagogin promoter activity with the promoter region spanning from −1525 and −680 to the transcription start site was increased by 4.7- and 4.9-fold, respectively, as compared with that in control vector-transfected cells (Fig. 5*B*). However, this increase in promoter activity was abolished by deleting the promoter region from −1525 to −450. This indicates that the region spanning −680 to −450 is essential for DDAH2-induced secretagogin expression. TESS analysis also revealed that the DDAH2 response region between −680 and −450 contained two consensus Sp1 sites between −543 and −483 (Fig. 5*A*).

We previously reported that DDAH2 binds directly to PKA, which activates Sp1 and the promoter of the VEGF gene (8). Therefore, we hypothesized that these Sp1 sites in the secretagogin promoter are involved in the increased promoter activity induced by DDAH2 overexpression. Because this region contains 2 Sp1 sites, we examined the role of these sites by establishing three constructs with a mutation in the distal Sp1 site alone (M1), the proximal Sp1 site alone (M2), or both sites (M3) (Fig. 5*C*). Mutations of either the distal or proximal Sp1 sites resulted in a significant decrease in basal promoter activity. However, the proximal Sp1 site seems to be more important than the distal site in the response to DDAH2 because disruption of the proximal Sp1 site caused a greater decrease in DDAH2 responsiveness. Disruption of both Sp1 sites severely impaired the responsiveness to DDAH2. These results confirm that the response to DDAH2 depends on the integrity of both Sp1 sites, although the proximal Sp1 site was more important.

To examine whether nuclear protein extracts from DDAH2-overexpressing cells could interact with the Sp1 sites, EMSAs (Fig. 5*D*, *E*) were performed using a biotin-labeled consensus Sp1 probe and nuclear extracts prepared from MIN-6 cells. Binding to the Sp1 oligonucleotide was increased using nuclear extracts from DDAH2-transfected MIN-6 cells as compared with those from untransfected control cells (Fig. 5*D*, lane 1 *vs.* lane 2). The addition of unlabeled Sp1 oligonucleotide attenuated binding (Fig. 5*D*, lane 3) and an anti-Sp1 antibody abolished binding (Fig. 5*E*, lane 5), indicating that Sp1 is the principal DNA-binding component of this protein-DNA complex.

It is possible that decreased ADMA levels or increased NO levels caused by DDAH2 overexpression might affect secretagogin promoter activity. Pretreatment with L-NAME or ADMA failed to alter the secreta-

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2 (Ins2) expression in WT or Tg mice fed a LFD or an HFD for 12 wk. Experiments were repeated 3 times. *E*) Islet ROS levels. H_2O_2 was visualized by confocal microscopy with the H_2O_2-sensitive fluorescent probe CM-H2DCFDA. Left panels: representative immunofluorescence images. Right panel: quantification of ROS levels (n=3 mice/group). *F*) Islet NO content. NO was visualized by confocal microscopy with the NO-sensitive fluorescent probe DAF-2-DA. Left panels: representative immunofluorescence images. Right panels: quantification of NO levels (n=3 mice/group). *G*) Pancreatic ADMA levels measured by ELISA. Scale bars = 100 μm. All images are representative of 3 independent experiments (n=8 mice/group). $^*P < 0.05$ *vs.* LFD-fed WT (WT+LFD); $^{\S}P < 0.05$ *vs.* LFD-fed Tg (Tg+LFD); $^{\P}P < 0.05$ *vs.* HFD-fed WT (WT+HFD).

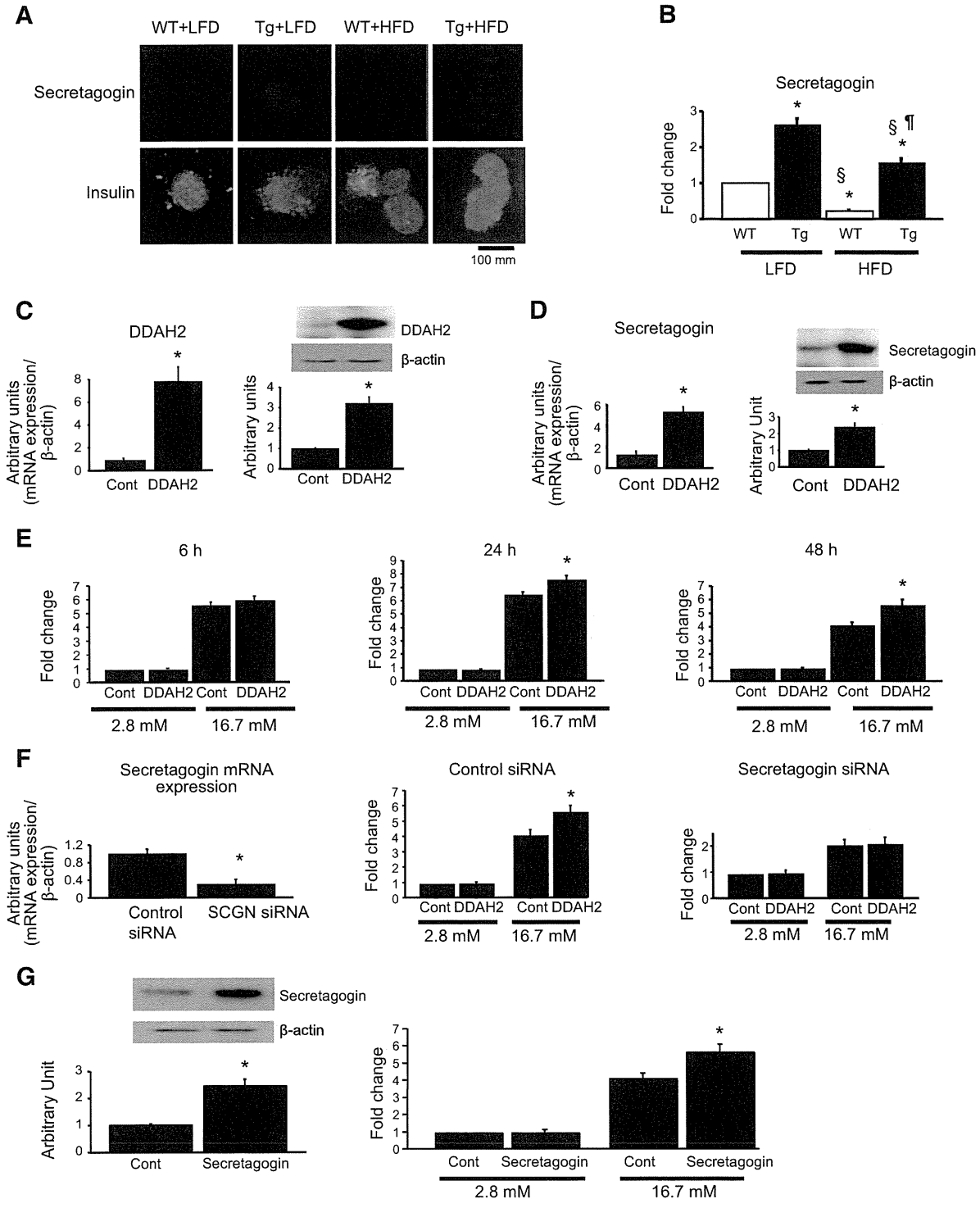

Figure 4. Up-regulation of secretagogin expression in DDAH2 Tg mice. *A*) Pancreatic sections stained for secretagogin and insulin. Scale bar = 100 μm. Results are representative of 3 independent experiments. *B*) Quantitative RT-PCR analysis of secretagogin mRNA expression in islets from WT or Tg mice fed LFD or HFD for 12 wk. $^{*}P < 0.05$ *vs.* WT+LFD; $^{\S}P < 0.05$ *vs.* Tg+LFD; $^{\P}P < 0.05$ *vs.* WT+HFD (n=8 mice/group). *C, D*) RT-PCR analysis and immunoblotting for DDAH2 (*C*) and secretagogin (*D*) in DDAH2- or control (Cont) vector-transfected MIN-6 cells for 24 h. $^{*}P < 0.05$ *vs.* control (n= 5 independent experiments). *E*) GSIS effects in DDAH2-transfected MIN-6 cells. After transfection, cells were cultured for 60 min in KRBB with either 2.8 or 16.7 mM glucose. Supernatant insulin concentrations were measured at 6, 24, and 48 h after glucose stimulation. Data are shown as the fold increase over control at 2.8 mM glucose. $^{*}P < 0.05$ *vs.* control at 16.7 mM glucose (n= 5 independent experiments). *F*) Secretagogin-knockdown effects in MIN-6 cells. Left panel: secretagogin mRNA expression in MIN-6 cells transfected with control or secretagogin siRNAs. $^{*}P < 0.05$ *vs.* control siRNA (n= 5 independent experiments). Center and right

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gogin promoter activity in basal (control) or DDAH2-transfected conditions (Supplemental Fig. S2), suggesting that the ADMA and NOS/NO pathways do not affect the basal secretagogin level or mediate DDAH2-induced up-regulation of secretagogin.

Sirt1 regulates the DDAH2/secretagogin pathway

We next sought to identify the upstream regulator of the DDAH2/secretagogin pathway controlling insulin secretion. We hypothesized that Sirt1, a NAD-dependent deacetylase, up-regulates DDAH2 expression in β cells to ultimately up-regulate secretagogin expression because it was reported that Sirt1 plays a critical role in insulin secretion and GSIS in the pancreas (25) and increases DDAH2 expression in vascular endothelial cells (26). Pancreatic expression of Sirt1, DDAH2, and secretagogin was decreased in HFD-fed WT mice (**Fig. 6*A***). In MIN6 cells, overexpression of Sirt1 up-regulated DDAH2 and secretagogin expression (Fig. 6*B*).

On the basis of these results, we examined the transcriptional regulation of DDAH2 by Sirt1. TESS analysis revealed several putative binding sites for the transcription factors nuclear factor 1, AP-1, CREB, AP-2, and Sp1 in a 1345-bp fragment surrounding the transcriptional start site of DDAH2 (Fig. 6*C*). Therefore, we performed luciferase assays to test whether Sirt1 overexpression increases DDAH2 promoter activity by measuring the promoter activity of 5′ deletion constructs (−1344 Luc, −917 Luc, −648 Luc, −440 Luc, and −109 Luc). These assays showed that in Sirt1-transfected MIN-6 cells, DDAH2 promoter activity for the promoter regions spanning from −1344, −917, and −648 to the transcription start site increased by 4.9-, 4.8-, and 4.6-fold, respectively, compared with that in control vector-transfected cells (Fig. 6*D*). However, this increase in promoter activity was abolished by deleting the promoter region from −1344 to −440. This indicates that the region spanning −648 to −440 is essential for Sirt1-induced DDAH2 expression. TESS analysis revealed that the region between −648 and −440 contained consensus CREB binding sites between −543 to −483 (Fig. 6*C*).

It was recently reported that Sirt1 deacetylates and activates CREB-regulated transcription coactivator 1 (TORC1) by promoting its dephosphorylation and its interaction with CREB (27). Introducing a mutation in the CREB sites resulted in a significant decrease in basal promoter activity and the loss of Sirt1-induced transcription activation of the DDAH2 promoter (Fig. 6*E*). To examine whether nuclear protein extracts from Sirt1-overexpressing cells could interact with the CREB binding sites, EMSAs (Fig. 6*F, G*) were performed using a biotin-labeled consensus CREB probe and nuclear extracts prepared from MIN-6 cells. Binding to the CREB oligonucleotide was increased using nuclear extracts from DDAH2-transfected MIN-6 cells as compared with those from untransfected control cells (Fig. 6*F*, lane 1 *vs.* lane 2). The addition of unlabeled CREB oligonucleotide (Fig. 6*F*, lane 3) or an anti-CREB antibody (Fig. 6*G*, lane 5) attenuated this binding, indicating that CREB is the DNA-binding component of this protein–DNA complex. Collectively, these data indicate that Sirt1 regulates the DDAH2/secretagogin pathway by transcriptionally regulating DDAH2 expression.

DDAH2 overexpression rescues defective GSIS in pancreatic Sirt1 deficiency

Finally, we investigated the effects of pancreatic Sirt1 deficiency. To achieve this, we generated β-cell-specific Sirt1-KO mice using the Cre-*loxP* system (RIP-Sirt1$^{-/-}$ mice) and measured insulin secretion from β cells. These mice contained a null mutation for Sirt1 in β cells, as confirmed by immunofluorescence (**Fig. 7*A***), which is consistent with previous reports using these RIP-Cre transgenic mice (28). DDAH2 and secretagogin protein expression levels were significantly decreased in islets isolated from RIP-Sirt1$^{-/-}$ mice compared with those from RIP-Sirt1$^{+/+}$ mice, confirming the role of Sirt1 as an upstream regulator of the DDAH2/secretagogin pathway (Fig. 7*B*). We also confirmed that Sirt1 deletion was specific to islets (Fig. 7*C*), consistent with earlier reports (29).

To examine whether restoration of pancreatic DDAH2 affects insulin secretion in RIP-Sirt1$^{-/-}$ mice, we crossed RIP-Sirt1$^{-/-}$ mice with Tg mice. We assessed the mRNA expression levels of DDAH2 and secretagogin in islets from WT+RIP-Sirt1$^{+/+}$, Tg+RIP-Sirt1$^{+/+}$, WT+RIP-Sirt1$^{-/-}$, and Tg+RIP-Sirt1$^{-/-}$ mice by real-time PCR. Islet DDAH2 expression was greater in Tg+RIP-Sirt1$^{+/+}$ mice than in WT+RIP-Sirt1$^{+/+}$ mice. By contrast, islet DDAH2 expression was decreased in WT+RIP-Sirt1$^{-/-}$ mice, confirming Sirt1-mediated regulation of DDAH2. This down-regulation was rescued in islets from Tg+RIP-Sirt1$^{-/-}$ mice (Fig. 7*D*). In parallel, secretagogin expression was increased in islets from Tg+RIP-Sirt1$^{+/+}$ mice compared with that in islets from WT+RIP-Sirt1$^{+/+}$ mice. Secretagogin expression was decreased in islets from WT+RIP-Sirt1$^{-/-}$ mice because of down-regulation of DDAH2. This reduction was rescued in islets from Tg+RIP-Sirt1$^{-/-}$ mice (Fig. 7*E*).

Finally, we examined GSIS in β cells isolated from these mice. GSIS was reduced in islets from WT+RIP-Sirt1$^{-/-}$ mice, and this reduction was restored in those

panels: effects of control (center) or secretagogin (right) siRNAs on GSIS. $^*P < 0.05$ *vs.* control at 16.7 mM glucose (n=5 independent experiments). *G*) MIN-6 cells were transfected with secretagogin or control for 24 h. Left panel: secretagogin immunoblotting. Right panel: secretagogin overexpression effects on GSIS. $^*P < 0.05$ *vs.* control at 16.7 mM glucose (n=5 independent experiments).

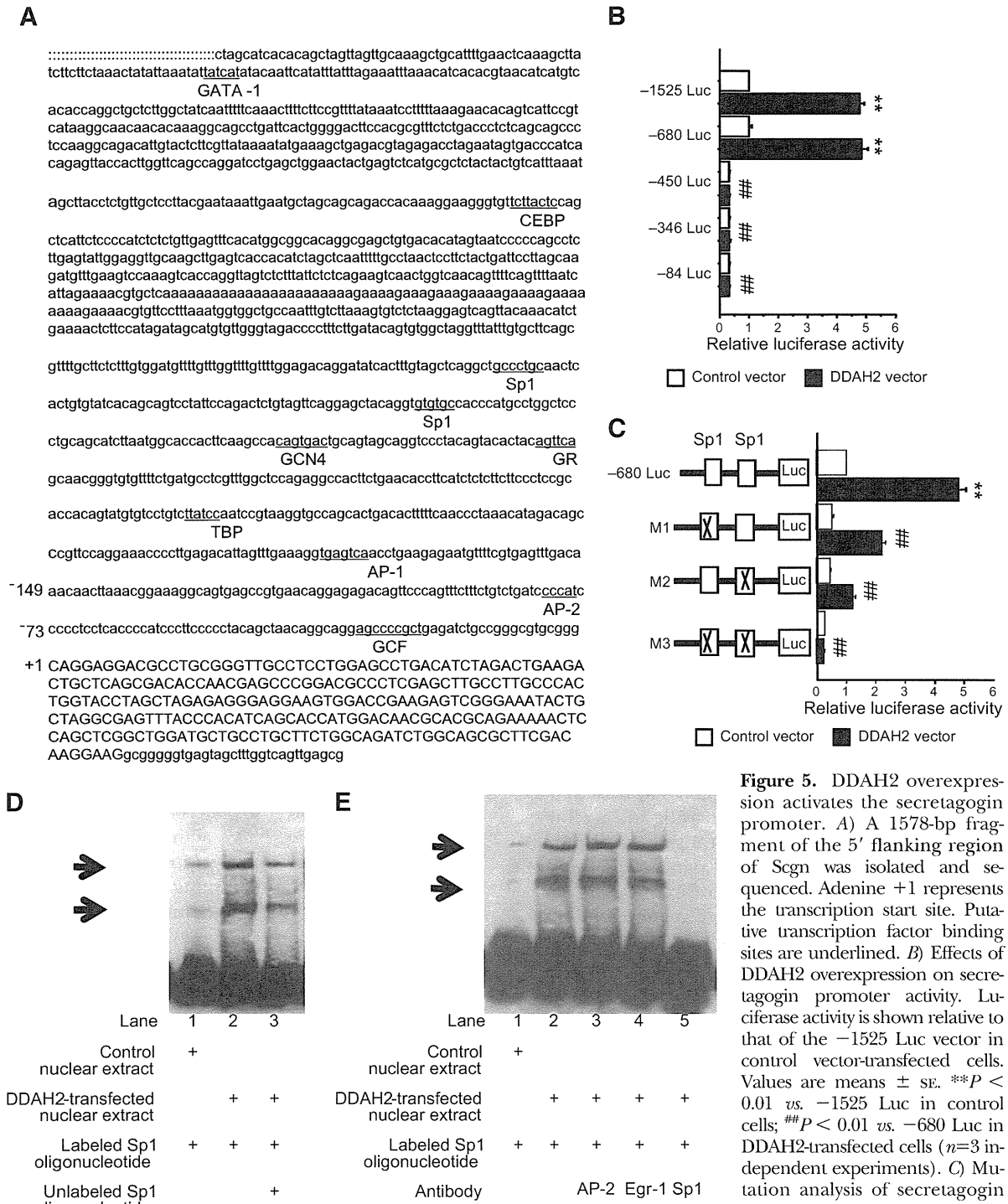

Figure 5. DDAH2 overexpression activates the secretagogin promoter. *A*) A 1578-bp fragment of the 5′ flanking region of Scgn was isolated and sequenced. Adenine +1 represents the transcription start site. Putative transcription factor binding sites are underlined. *B*) Effects of DDAH2 overexpression on secretagogin promoter activity. Luciferase activity is shown relative to that of the −1525 Luc vector in control vector-transfected cells. Values are means ± SE. $^{**}P < 0.01$ *vs.* −1525 Luc in control cells; $^{\#\#}P < 0.01$ *vs.* −680 Luc in DDAH2-transfected cells (n=3 independent experiments). *C*) Mutation analysis of secretagogin promoter activity in MIN-6 cells. −680 Luc, WT secretagogin promoter between −680 and +53; M1, distal Sp1 mutation; M2, proximal Sp1 mutation; M3, mutation in both Sp1 sites. $^{**}P < 0.01$ *vs.* −680 Luc in control cells; $^{\#\#}P < 0.01$ *vs.* −680 Luc in DDAH2-transfected cells (n=3 independent experiments). *D*) EMSAs of 25 μg of nuclear extracts from DDAH2-transfected (lanes 2 and 3) or untransfected (lane 1) MIN-6 cells using biotin-labeled probes. Arrow indicates the band for the putative protein–DNA complex of the Sp1 protein and oligonucleotide. Competition EMSAs were performed with excess unlabeled probe (lane 3). Results are representative of 3 independent experiments. *E*) Nuclear extracts were incubated with AP-2, Egr-1, or Sp1 antibodies before adding the probe. DNA–protein interaction was inhibited by anti-Sp1 antibodies (lane 5). Results are representative of 3 independent experiments.

in Tg+RIP-Sirt1$^{-/-}$ mice. Since the expression of DDAH2 and secretagogin was reduced in WT+RIP-Sirt1$^{-/-}$ mice, these changes resulted in impairments in GSIS. DDAH2 overexpression restored GSIS in islets from Tg+RIP-Sirt1$^{-/-}$ compared with islets from WT+RIP-Sirt1$^{-/-}$ mice, indicating a pivotal role of the Sirt1/DDAH2/secretagogin pathway in β-cell insulin secretion (Fig. 7*F*).

DISCUSSION

In the present study, we first elucidated the localization of DDAH2 in β cells and found that its expression was decreased in HFD-fed mice. DDAH2 Tg mice showed improvements in glucose tolerance and insulin secretion compared with WT mice. DDAH2 overexpression improved GSIS by increasing secretagogin transcription *via* Sp1-dependent promoter activity. In addition, using β-cell-specific Sirt1-KO mice, we showed that Sirt1 plays a crucial role in the DDAH/secretagogin pathway. Finally, we revealed a novel function of DDAH2 in regulating insulin secretion through an ADMA- and NO-independent pathway.

HFD-induced impaired GSIS is a major characteristic of T2DM, and several molecules have been implicated in this process. Uncoupling protein 2 (UCP2), for example, may negatively regulate β-cell function, as mice lacking UCP2 expression have an increased β-cell mass and retained insulin secretion capacity during conditions of glucolipotoxicity (30). Ramsey *et al.* (25) reported that GSIS was restored in HFD-fed β-cell-specific Sirt1-overexpressing (BESTO) mice with the suppression of UCP2 expression. Sirt1 expression was decreased in HFD-fed mice by decreasing intracellular-type nicotinamide phosphoribosyltransferase (iNampt) expression, which leads to reduced pancreatic levels of nicotinamide mononucleotide and Sirt1 (31). Therefore, the HFD may down-regulate pancreatic Sirt1 expression, resulting in increased UCP2 expression and reduced insulin secretory capacity. However, down-regulation of UCP2 was also reported to increase ROS production and increase the risk for β-cell damage, especially in diabetic conditions (32). In addition, UCP2 in β cells exerts relatively minor effects on mitochondrial function, raising questions over the effects of moderately elevated UCP2 levels in β cells, as seen in T2DM (33). Our findings imply that pancreatic Sirt1 enhances GSIS *via* DDAH2-mediated up-regulation of secretagogin. Thus, our current findings suggest that the Sirt1/DDAH2/secretagogin pathway is involved in HFD-induced impairments of GSIS and that down-regulation of Sirt1 by the HFD may be a critical event in this pathway (Supplemental Fig. S3*A*, *B*). This mechanism could also explain the improvements in glucose intolerance in BESTO mice and Sirt1-mediated improvements in insulin secretion (Supplemental Fig. S3*C*). Accordingly, our data support our proposed mechanism as one of the main pathways linking pancreatic DDAH2 to insulin secretion, although other mechanisms or signaling pathways might have a greater influence on GSIS in HFD-fed animals. For example, as we previously reported (11), DDAH2 binds to PKA, which increases Sp1 phosphorylation and consequently up-regulates VEGF. Binding of DDAH2 to PKA might improve GSIS in Tg mice because the PKA pathway is one of the major pathways that regulates GSIS (34). Further studies are needed to examine the involvement of other mechanisms in this process.

Another explanation for reduced DDAH2 expression in HFD is that the HFD induces islet interleukin (IL)-1β expression, which activates the expression of iNOS, resulting in excessive NO production (35). Excessive production of NO, interferes with electron transfer, up-regulates ROS generation, inhibits mitochondrial ATP synthesis, and increases the expression of proinflammatory genes (36). Treatment with an IL-1 receptor antagonist was reported to protect against HFD-induced apoptosis of β cells and induced β-cell proliferation, thus improving GSIS (37). On the other hand, local ROS generation decreases DDAH2 gene and protein expression (38). It was also reported that lipopolysaccharide (39) and high glucose (40) down-regulate DDAH2 expression in endothelial cells by increasing ROS levels. Therefore, we measured tissue ROS levels and found that ROS generation was enhanced in islets from HFD-fed WT mice (Fig. 3*E*), which may reduce DDAH2 expression.

Although the HFD reduced DDAH2 expression, it did not affect plasma or islet ADMA levels. This may be explained by changes in the expression and/or activity of other enzymes involved in the production, degradation, or efflux of ADMA from cells.

ADMA alters the balance between NO and superoxide production, and thus increases ROS generation (41) by uncoupling NOS activity (42). Therefore, DDAH2 overexpression may support NO bioavailability by preventing NOS uncoupling and reducing local ROS. However, islet ROS generation was not stopped in Tg mice, despite reduced tissue ADMA levels (Fig. 3*G*). Our results indicate that ADMA hardly affected HFD-induced ROS generation in pancreatic β cells. It is possible that HFD-induced iNOS induction and NO production play significant roles in NO metabolism in β cells, whereas DDAH2 does not.

Secretagogin is a member of the EF-hand CBP superfamily (43), members of which act as either signaling proteins (Ca^{2+} sensors) or buffering proteins. Our present results suggest that secretagogin is a Ca^{2+} sensor because DDAH2-induced secretagogin expression did not affect Ca^{2+} concentrations. A recent study (44) revealed that HFD feeding reduced the number of docked insulin granules without affecting intracellular Ca^{2+} or ATP concentrations. As secretagogin improved GSIS independently of Ca^{2+}, the underlying mechanism could involve protein–protein interactions between secretagogin and 25-kDa synaptosome-associated protein (SNAP-25). In the central nervous system, secretagogin interacts with SNAP-25 and enhances the formation of neurotransmitter vesicles (39). SNAP-25

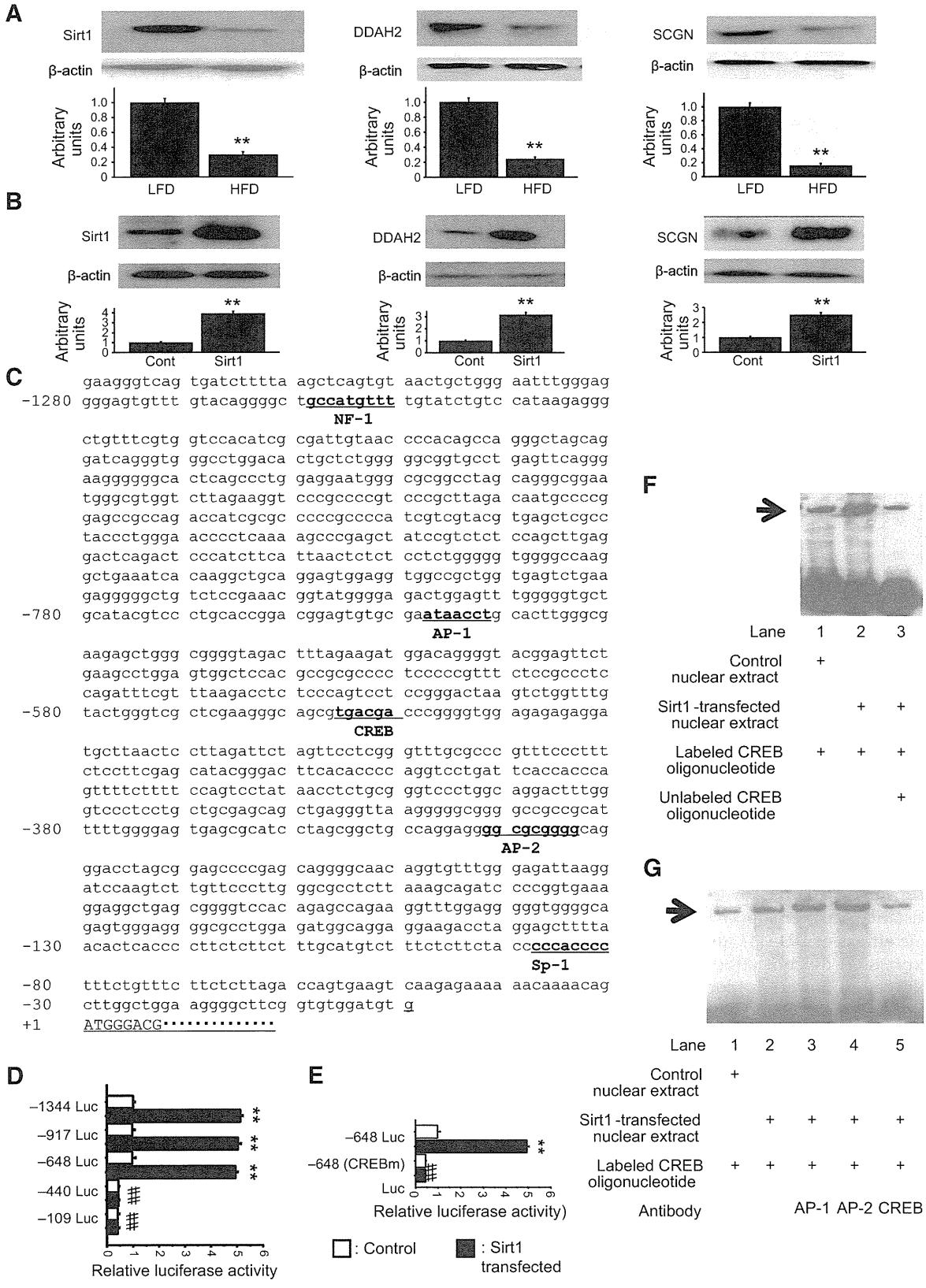

Figure 6. Sirt1 regulates DDAH2 expression by activating its promoter. *A*) Pancreatic Sirt1, DDAH2, and SCGN expression in LFD- and HFD-fed WT mice. $^{**}P < 0.01$ *vs.* LFD-fed WT mice ($n=3$ mice/group). *B*) Sirt1, DDAH2, and SCGN expression in MIN-6 cells overexpressing Sirt1. Bottom panels: immunoblot quantifications. $^{**}P < 0.01$ *vs.* control ($n=3$ independent

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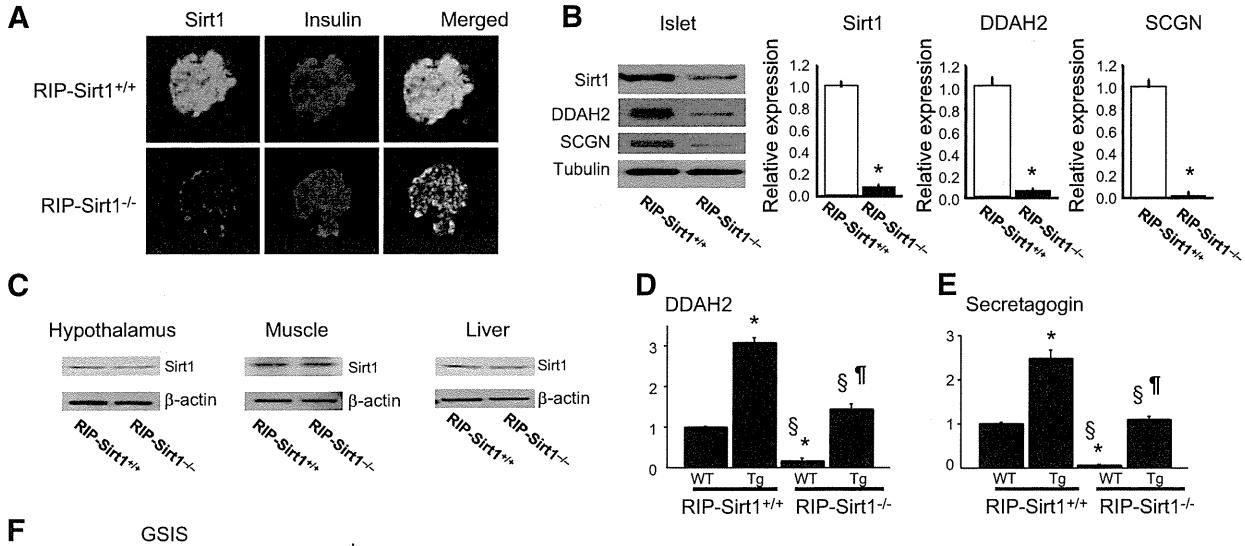

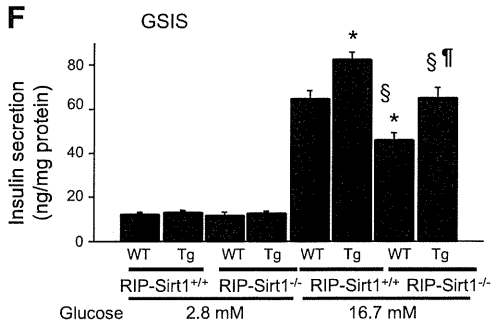

Figure 7. Effects of pancreas-specific Sirt1 deletion. *A*) Sirt1 and insulin immunofluorescence in pancreatic sections of RIP-Sirt1$^{-/-}$ and RIP-Sirt1$^{+/+}$ mice (view: ×20). Results are representative of 3 independent experiments. *B*) Representative Western blots (left panel) and quantification (right panels) of Sirt1 expression in isolated islets. RIP-Sirt1$^{-/-}$ and RIP-Sirt1$^{+/+}$ mice were crossed with DDAH2 Tg mice or WT mice (n=3 independent experiments). *C*) Sirt1 expression in the hypothalamus, muscle, and liver from RIP-Sirt1$^{+/+}$ mice (n=3 independent experiments). *D, E*) Quantitative real-time PCR analysis of DDAH2 (*D*) and secretagogin (*E*) mRNA expression in islets from WT+RIP-Sirt1$^{+/+}$, Tg+RIP-Sirt1$^{+/+}$, WT+RIP-Sirt1$^{-/-}$, and Tg+RIP-Sirt1$^{-/-}$ mice. $^{*}P <$ 0.05 *vs.* WT+RIP-Sirt1$^{+/+}$; $^{\S}P < 0.05$ *vs.* Tg+RIP-Sirt1$^{+/+}$; $^{\P}P < 0.05$ *vs.* WT+RIP-Sirt1$^{-/-}$ (n=8 mice/group). *F*) GSIS in isolated islets (n=8 mice/group).

also plays a significant role in the exocytosis of docked insulin vesicles (45). In RIN5-F cells, secretagogin binds to SNAP-25 and ATP synthase (20). Accordingly, secretagogin may interact with SNAP-25 to enhance GSIS as a Ca^{2+}-signaling protein.

Although DDAH2 overexpression did not affect insulin sensitivity, DDAH1 overexpression improves insulin resistance, further supporting the differences in the systemic effects of DDAH1 and DDAH2. While DDAH2 overexpression did not affect blood pressure, DDAH1 overexpression lowered blood pressure with an increase in systemic NO and a decrease in systemic ADMA levels (9). Accordingly, the vasodilatory effects of DDAH1 overexpression in insulin-sensitive organs could have favorable effects on glucose metabolism through increased blood supply. Gene silencing studies (46) revealed that DDAH1 siRNA increased BP with changes in plasma ADMA levels, whereas DDAH2 siRNA did not. The different roles of DDAH1 and DDAH2 in the vasculature seem to be responsible for their different effects on systemic insulin sensitivity.

In summary, we have demonstrated that DDAH2 overexpression in islets may improve GSIS by transcriptionally activating secretagogin expression *via* Sp1 in 2 animal models with impaired GSIS. We also showed that Sirt1 transcriptionally regulates the DDAH2/secretagogin pathway. Our results suggest that DDAH2 offers a novel therapeutic target for T2DM by reversing HFD-induced impairments in GSIS.

experiments). *C*) A 1345-bp fragment of the 5′-flanking region of *Ddah2* was isolated and sequenced. Adenine +1 represents the transcription start site. Putative transcription factor binding sites are underlined. *D*) Effects of Sirt1 overexpression on DDAH2 promoter activity. Luciferase activity is shown relative to that of the −1344 Luc vector in control cells. Values are expressed as means ± SE. $^{**}P < 0.01$ *vs.* −1344 Luc in control cells; $^{\#\#}P < 0.01$ *vs.* −648 Luc in Sirt1-transfected cells (n=3 independent experiments) *E*) Mutation analysis of Sirt1 promoter activity in MIN-6 cells. −648 Luc, WT secretagogin promoter between −648 and +1; −648(CREBm) Luc, mutation in CREB-binding site. $^{**}P < 0.01$ *vs.* −680 Luc in control cells; $^{\#\#}P < 0.01$ *vs.* −680 Luc in Sirt1-transfected cells (n=3 independent experiments). *F*) EMSAs of 25 μg of nuclear extracts from Sirt1-transfected (lanes 2 and 3) or untransfected (lane 1) MIN-6 cells using biotin-labeled probes. Arrow indicates the band for the putative protein–DNA complex of CREB and oligonucleotide. Competition EMSAs were performed with excess unlabeled probe (lane 3). Results are representative of 3 independent experiments. *G*) Nuclear extracts were incubated with AP-1, AP-2, or CREB antibodies before adding the probe. DNA–protein interaction was inhibited by anti-CREB antibodies (lane 5). Results are representative of 3 independent experiments.

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ORIGINAL

Lower beta cell function relates to sustained higher glycated albumin to glycated hemoglobin ratio in Japanese patients with type 2 diabetes

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Abstract. The aim of this study was to clarify the relationship between baseline beta cell function and future glycated albumin (GA) to glycated hemoglobin ratio (GA/HbA1c) in patients with type 2 diabetes. In our retrospective cohort, 210 type 2 diabetic patients who had been admitted to our hospital and in whom HbA1c and GA had been measured at baseline and 2 years after admission were included in this study. Baseline beta cell function was assessed by postprandial C-peptide immunoreactivity index (PCPRI) during admission. With intensification of treatment during admission, HbA1c and GA were significantly decreased 1 year and 2 years after admission. While baseline HbA1c was not significantly correlated with HbA1c after 2 years, baseline GA/HbA1c was strongly correlated with GA/HbA1c after 2 years ($r = 0.575$, $P < 0.001$). When the patients were divided into two groups according to median PCPRI, patients with low PCPRI showed higher GA/HbA1c both at baseline and after 2 years compared to those with high PCPRI. There was a significant negative correlation between PCPRI and GA/HbA1c after 2 years ($r = -0.379$, $P < 0.001$). Multiple regression analysis revealed that PCPRI was an independent predictor of GA/HbA1c after 2 years. In conclusion, our findings suggest that lower beta cell function is associated with sustained higher GA/HbA1c ratio in patients with type 2 diabetes.

Key words: Glycated albumin, Glycated hemoglobin, Type 2 diabetes, Beta cell function, C-peptide

TYPE 2 DIABETES is characterized by impaired beta cell function to maintain normoglycemia [1, 2]. Beta cell function has been reported to be an important predictor of treatment failure in patients with type 2 diabetes [3, 4]. We have previously reported that lower beta cell function was associated with higher HbA1c after 2 years in patients with type 2 diabetes [5]. However, the association of beta cell function with other glycemic indices remains established.

Glycated albumin (GA) is another glycemic index, which reflects average blood glucose level for the past 1 to 2 weeks, and it has recently been reported that GA more sensitively reflects glycemic excursion than does HbA1c [6-10]. Previous studies have suggested an association between postprandial glucose excursion and cardiovascular disease [11-15]. Since it has been reported that GA or the ratio of GA to HbA1c, but not HbA1c, was associated with the progression of carotid artery intima-media thickness [16], GA or GA/HbA1c ratio is also expected to be a superior predictor of cardiovascular disease compared with HbA1c.

We and others have reported that lower beta cell function is associated with higher GA and GA/HbA1c ratio in patients with type 2 diabetes, at least partly reflecting the association between lower beta cell function and greater glycemic excursion [8, 17, 18]. However, those studies were cross-sectional, and the association between beta cell function and future GA/HbA1c level, especially after intensification of treatment, remains uncertain. Therefore, in this study, using a retrospective, longitudinal cohort of patients who had been admitted to our hospital for poor glycemic control, we

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Abbreviations: GA, glycated albumin; CPR, C-peptide immunoreactivity; JDS, Japan Diabetes Society; JNS, Japan Nephrology Society; NGSP, National Glycohemoglobin Standardization Program; FPG, fasting plasma glucose; PPG, postprandial plasma glucose; PCPRI, postprandial CPR index; GFR, glomerular filtration rate; CV, coefficient of variance; CI, confidence interval; DCCT, Diabetes Control and Complications Trial

aimed to clarify 1) whether GA/HbA1c changes after intensive treatment during and after admission and 2) whether beta cell function predicts future GA/HbA1c level in patients with type 2 diabetes.

Methods

Subjects

The details of our retrospective cohort have been reported elsewhere [5, 8]. This retrospective cohort consisted of subjects with type 2 diabetes who had been admitted to our hospital between 2000 and 2007. We excluded subjects with renal failure defined as serum creatinine level ≥ 2 mg/dL (corresponding to glomerular filtration rate (GFR) < ~30 mL/min/1.73m^2), subjects with nephrotic syndrome and subjects with liver cirrhosis, which affect GA/HbA1c ratio [19]. Since it has been reported that the beta cell response to glucose is blunted above a plasma glucose level of 180 mg/dL [20], subjects with fasting plasma glucose (FPG) level ≥ 200 mg/dL on the day of CPR measurement were also excluded. Among the entire cohort (n = 689), glycated albumin levels were available in 316 subjects [8]. Of those, 210 subjects (66%) in whom glycated albumin level 2 years after admission was available (137 men and 73 women, age 63 ± 12 years (mean ± SD), duration of diabetes 12 ± 10 years, BMI 24.7 ± 4.2 kg/m^2, Table 1) were included in this study. There was no significant difference in clinical characteristics such as age, sex, duration of diabetes, BMI and HbA1c between

Table 1 Baseline characteristics according to baseline PCPRI level.

	Total (n = 210)	Low PCPRI (n = 105)	High PCPRI (n = 105)	*P* value
Sex (male/female)	137/73	65/40	72/33	0.39
Age (years)	63 ± 12	64 ± 13	63 ± 12	0.41
Duration of diabetes (years)	12 ± 10	15 ± 11	10 ± 8	0.001
Family history of diabetes (%)	50.0	52.4	47.6	0.58
BMI (kg/m^2)	24.7 ± 4.2	23.4 ± 3.6	26.0 ± 4.3	<0.001
eGFR (mL/min/1.73m^2)	68.6 ± 19.3	69.4 ± 20.4	67.8 ± 18.2	0.53
HbA1c (%)	9.8 ± 1.7	10.2 ± 1.8	9.5 ± 1.5	0.002
GA (%)	27.9 ± 6.9	30.8 ± 7.0	25.1 ± 5.6	<0.001
GA/HbA1c	2.83 ± 0.48	3.02 ± 0.47	2.65 ± 0.41	<0.001
CPR indices				
FPG (mg/dL)	146 ± 28	149 ± 30	142 ± 25	0.07
FCPR (ng/mL)	1.72 ± 0.97	1.21 ± 0.60	2.23 ± 1.01	<0.001
FCPRI	1.20 ± 0.68	0.81 ± 0.35	1.59 ± 0.70	<0.001
PPG (mg/dL)	242 ± 64	264 ± 69	221 ± 51	<0.001
PCPR (ng/mL)	4.44 ± 2.42	2.78 ± 1.15	6.10 ± 2.20	<0.001
PCPRI	1.94 ± 1.15	1.05 ± 0.33	2.82 ± 0.99	<0.001
UCPR (μg/day)	66.9 ± 49.0	46.8 ± 35.8	86.8 ± 52.3	<0.001
Medication before admission				
SU (%)	51.4	49.5	53.3	0.68
Glinide (%)	1.9	1.0	2.9	0.37
BG (%)	12.4	8.6	16.2	0.14
TZD (%)	13.3	13.3	13.5	1.00
α-GI (%)	31.4	31.4	31.4	1.00
Insulin (%)	16.7	27.6	5.7	<0.001
Medication at discharge				
SU (%)	21.0	9.5	32.4	<0.001
Glinide (%)	10.0	7.6	12.4	0.36
BG (%)	12.4	5.7	19.1	0.006
TZD (%)	1.0	0.0	1.9	0.50
α-GI (%)	31.0	31.4	30.5	1.00
Number of OHA	0.75 ± 0.80	0.54 ± 0.77	0.96 ± 0.78	<0.001
Insulin (%)	64.8	86.7	42.9	<0.001

Low and high PCPRI groups were divided by the median of PCPRI (1.63).
eGFR, estimated glomerular filtration rate; GA, glycated albumin; FPG, fasting plasma glucose; FCPR, fasting C-peptide immunoreactivity; FCPRI, FCPR index; PPG, postprandial plasma glucose; PCPR, postprandial CPR; PCPRI, PCPR index; UCPR, urinary CPR; SU, sulfonylurea; BG, biguanide; TZD, thiazolidinedione; α-GI, α-glucosidase inhibitor; OHA, oral hypoglycemic agent. Data are expressed as mean ± SD or percentage. *P* value; low *vs.* high PCPRI.

the patients included in this study and those excluded from this study, most of whom were not able to be followed for 2 years because of referral to other hospitals or clinics (N = 106, all $P > 0.05$, data not shown).

Before admission, 108 subjects (51.4%) were treated with a sulfonylurea, 4 (1.9%) with a glinide, 26 (12.4%) with a biguanide, 28 (13.3%) with a thiazolidinedione, 66 (31.4%) with an α-glucosidase inhibitor and 35 (16.7%) with insulin. Sixty-two subjects (29.5%) were receiving no medication. Most patients had been admitted to hospital for one to two weeks because of poor glycemic control, and received basal-bolus insulin therapy using regular and NPH insulin during admission. At discharge, 44 subjects (21.0%) were treated with a sulfonylurea, 21 (10.0%) with a glinide, 26 (12.4%) with a biguanide, 2 (1.0%) with a thiazolidinedione, 65 (31.0%) with an α-glucosidase inhibitor and 136 (64.8%) with insulin. Among the subjects treated with insulin, nine (6.6%) were treated with once daily injection, 71 (52.2%) with twice daily injections and 50 (36.8%) with multiple daily injections (basal-bolus therapy). Seven subjects (3.3%) were receiving no medication. All patients were treated by diabetes specialists according to the guidelines of the Japan Diabetes Society (JDS) [21]. This study was conducted according to the principles expressed in the Declaration of Helsinki, and was approved by the ethics review committee of Keio University School of Medicine, Tokyo, Japan.

Measurements

All measurements were conducted during admission, and assayed by the Department of Laboratory Medicine, Keio University School of Medicine as previously described [5, 8]. Plasma glucose and serum CPR levels were measured after overnight fasting and 2 h after breakfast during admission, usually within a few days after admission under basal-bolus insulin therapy. All patients received the ideal dietary caloric intake calculated from their ideal body weight (*i.e.* height $(m)^2$ x 22 x 25 kcal/kg) when blood samples were obtained. Plasma glucose was measured by glucose oxidase method, and CPR was measured by enzyme immunoassay (ST AIA-PACK C-Peptide, TOSOH, Tokyo, Japan). CV of the within-run and between-day precision of CPR was 2.39% and 2.97%, respectively. Fasting and postprandial CPR indices were calculated as previously reported [5, 8]; *i.e.*, fasting or postprandial serum CPR (ng/mL) / fasting or postprandial plasma glucose (mg/dL) x 100, respectively. In addition, 24 h urinary CPR was also measured within a few days after admission. HbA1c and GA were measured at the time of admission, one year and two years after admission. HbA1c was measured by HPLC and expressed as the National Glycohemoglobin Standardization Program (NGSP) value, as defined by JDS [22]. GA was measured by enzymatic method. Coefficient of variance (CV) of within-run and between-day precision were 0.4-1.0% and 0.5-0.9% for HbA1c, and 0.9-1.2% and 0.6-0.7% for GA, respectively. Estimated glomerular filtration rate (eGFR: mL/min/1.73 m^2) was calculated according to the Statement of the Japan Nephrology Society (JNS) as follows: 194 x serum creatinine $(mg/dL)^{-1.094}$ x age $(years)^{-0.287}$ (x 0.739 for women) [23].

Statistical analysis

Descriptive statistics were calculated for the baseline characteristics. Homogeneity of distributions between the two groups was examined with Student's *t* test or Fisher's exact test. Paired *t* test was used to assess the changes in glycemic parameters. The association between two variables was estimated with Pearson's correlation coefficient. Univariate regression analysis was performed, followed by stepwise multivariate regression analysis including confounders to evaluate the robustness of the results. These analyses were performed with the Statistical Package for the Social Sciences (version 19.0; SPSS, Chicago, IL). Data were expressed as mean ± S.D. in the text and tables, and mean ± S.E. in the figures. Values of $P < 0.05$ were considered statistically significant.

Results

Changes in glycemic indices after admission

In the total subjects, HbA1c and GA were significantly decreased at 1 year and 2 years after admission with intensification of treatment during and after admission (HbA1c: 9.8 ± 1.7, 7.8 ± 1.3 and 7.9 ± 1.3%, GA: 27.9 ± 6.9, 21.7 ± 4.3 and 21.9 ± 5.3% at baseline, 1 year and 2 years, respectively, all $P < 0.001$ *vs.* baseline). GA/HbA1c ratio also decreased after admission, while the statistical significance was marginal (2.83 ± 0.48, 2.79 ± 0.37 and 2.77 ± 0.42 at baseline, 1 year and 2 years, $P = 0.04$ and 0.02 *vs.* baseline, respectively). There was a significant correlation between baseline GA and GA after 2 years ($r = 0.244$, $P < 0.001$, Fig. 1), whereas the correlation between baseline HbA1c and

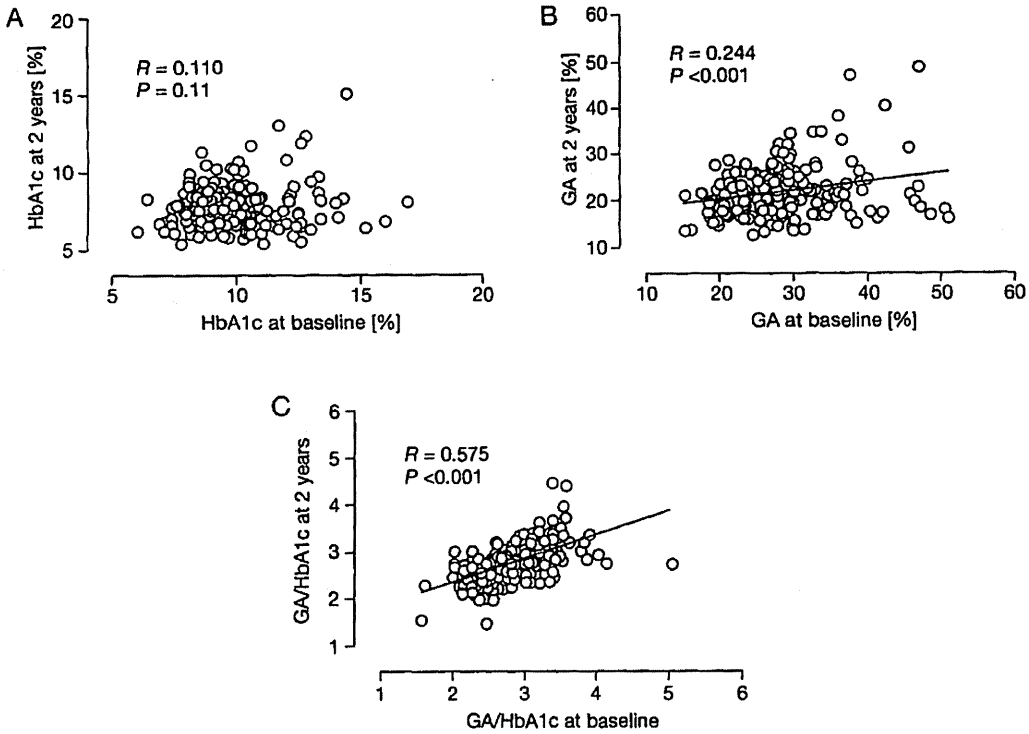

Fig. 1 Correlations between baseline values and values 2 years after admission of HbA1c (A), GA (B) and GA/HbA1c ratio (C).

HbA1c after 2 years was not significant (r = 0.110, P = 0.11). Baseline GA/HbA1c was strongly correlated with GA/HbA1c after 2 years (r = 0.575, P <0.001).

Association between postprandial CPR index level and GA/HbA1c ratio after 2 years

To elucidate the correlation between postprandial CPR index (PCPRI) and future GA/HbA1c ratio, we classified the subjects into two groups according to the median of PCPRI (1.63). Comparison of baseline characteristics between the two groups is shown in Table 1. Subjects with low PCPRI showed longer duration of diabetes, lower BMI, higher HbA1c, higher GA and higher GA/HbA1c, as previously reported [8]. All CPR indices were significantly lower in patients with low PCPRI than in those with high PCPRI. Most of the subjects with low PCPRI had started insulin therapy at discharge.

Changes in glycemic indices after admission are shown in Fig. 2. In both groups, HbA1c and GA were significantly ameliorated 1 year and 2 years after admission (Fig. 2A and B), although GA/HbA1c ratio significantly decreased only in the low PCPRI group (Fig. 2C). HbA1c and GA remained significantly higher in the low PCPRI group compared with the high PCPRI group at 1 year and 2 years after admission, and GA/HbA1c ratio also remained significantly higher at 1 year and 2 years after admission in the low PCPRI group (Fig. 2). There were significant negative correlations between PCPRI and HbA1c, GA and GA/HbA1c ratio after 2 years (r = -0.182, P = 0.008, r = -0.365, P <0.001, and r = -0.379, P <0.001, Fig. 3), although the correlation was stronger for GA and GA/HbA1c ratio than for HbA1c.

Baseline parameters relating to GA/HbA1c after 2 years

Univariate regression analysis showed that age, duration of diabetes, BMI, baseline GA, eGFR, use of BG and TZD, as well as PCPRI were associated with GA/HbA1c ratio after 2 years (Table 2). Stepwise multiple regression analysis revealed that age, BMI, baseline HbA1c, baseline GA, eGFR, PCPRI and use of insulin were independent variables predicting GA/HbA1c ratio 2 years after admission (Table 2).

Since multivariate analysis suggested an independent effect of insulin therapy on GA/HbA1c ratio, the effect

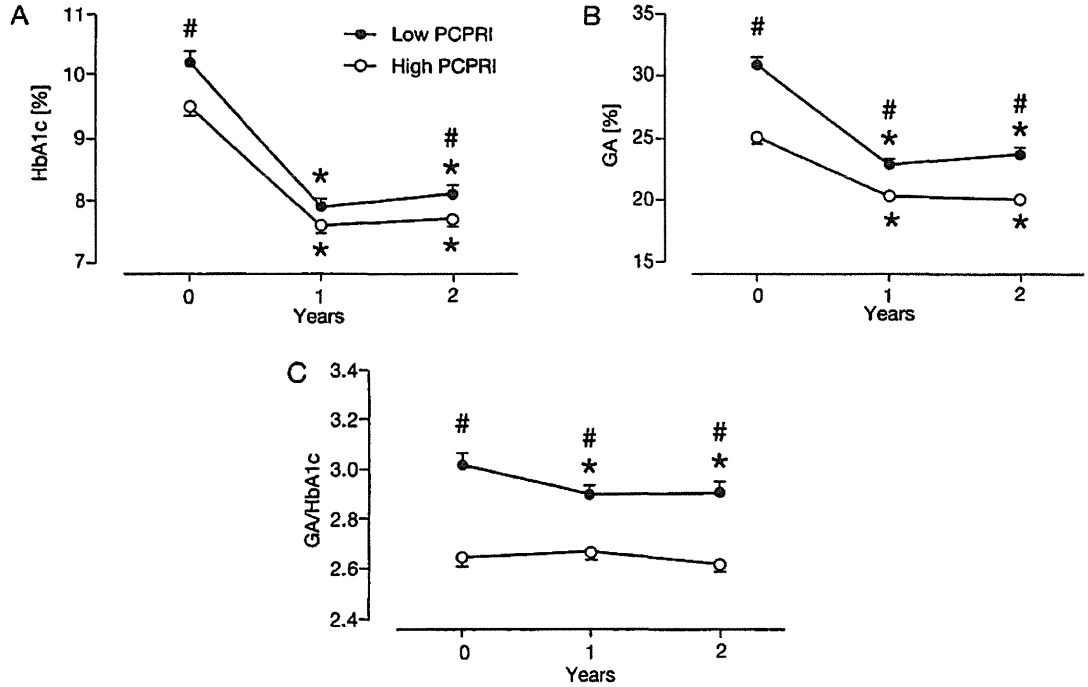

Fig. 2 Changes in HbA1c (A), GA (B) and GA/HbA1c ratio (C) during 2 years after admission. Closed circles; low postprandial CPR index (PCPRI) group, open circles; high PCPRI group. * P <0.05 *vs.* 0 year. # P <0.05 *vs.* high PCPRI group.

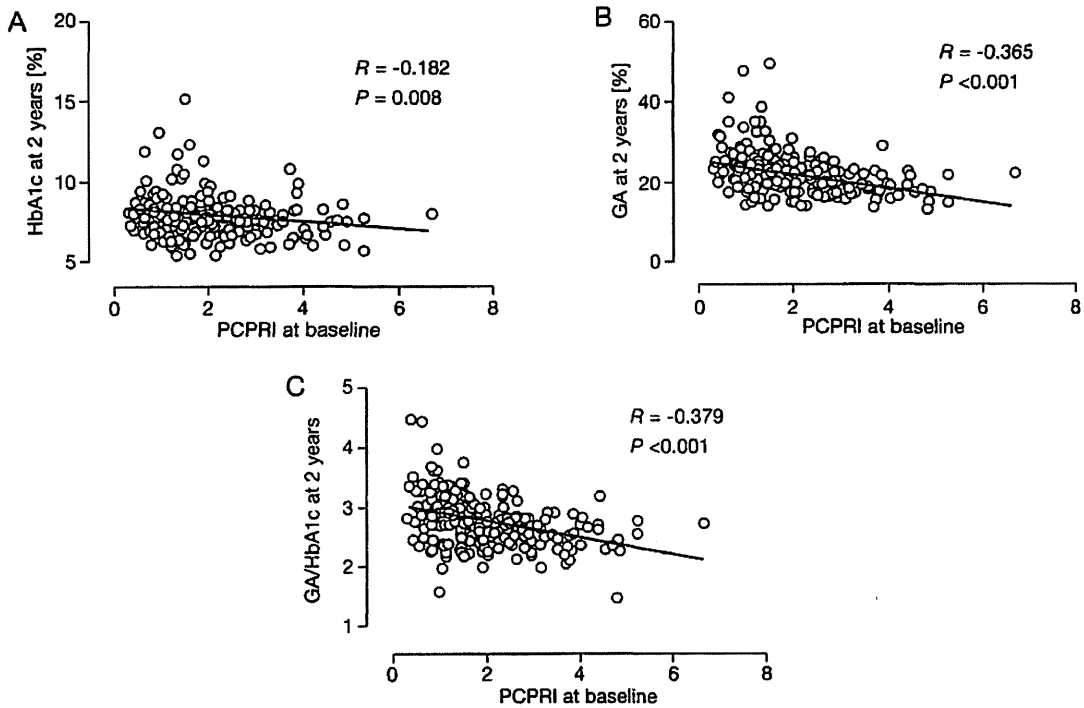

Fig. 3 Correlations between baseline postprandial CPR index (PCPRI) and HbA1c (A), GA (B) and GA/HbA1c ratio (C) 2 years after admission.

Table 2 Univariate and stepwise multivariate regression analysis of baseline parameters for association with GA/HbA1c ratio after 2 years.

Variables	Univariate regression		Stepwise multivariate regression (adjusted R^2 = 0.439)	
	Odds ratio (95%CI)	*P* value	Odds ratio (95%CI)	*P* value
Sex (male = 1, female = 0)	0.99 (0.88 – 1.12)	0.93	-	-
Age (years)	1.01 (1.01 – 1.02)	<0.001	1.01 (1.00 – 1.01)	0.04
Duration of diabetes (years)	1.01 (1.01 – 1.02)	<0.001	-	-
Family history of diabetes (yes = 1, no = 0)	0.98 (0.87 – 1.10)	0.71	-	-
BMI (kg/m^2)	0.96 (0.95 – 0.97)	<0.001	0.98 (0.97 – 0.99)	0.006
HbA1c (%)	0.98 (0.95 – 1.02)	0.31	0.90 (0.86 – 0.93)	<0.001
GA (%)	1.02 (1.01 – 1.03)	<0.001	1.04 (1.03 – 1.05)	<0.001
eGFR (mL/min/1.73m^2)	0.96 (0.96 – 0.96)	0.003	0.99 (0.99 – 0.99)	0.03
PCPRI	0.87 (0.83 – 0.91)	<0.001	0.91 (0.87 – 0.96)	<0.001
Use of SU (yes = 1, no = 0)	0.95 (0.83 – 1.09)	0.48	-	-
Use of glinide (yes = 1, no = 0)	1.18 (0.98 – 1.43)	0.08	-	-
Use of BG (yes = 1, no = 0)	0.83 (0.70 – 0.99)	0.04	-	-
Use of TZD (yes = 1, no = 0)	0.53 (0.30 – 0.95)	0.04	-	-
Use of α-GI (yes = 1, no = 0)	1.04 (0.92 – 1.17)	0.55	-	-
Number of OHAs	0.98 (0.92 – 1.06)	0.64	-	-
Use of insulin (yes = 1, no = 0)	1.09 (0.97 – 1.23)	0.15	0.87 (0.78 – 0.97)	0.01

CI, confidence interval; eGFR, estimated glomerular filtration rate; GA, glycated albumin; PCPRI, postprandial CPR index; SU, sulfonylurea; BG, biguanide; TZD, thiazolidinedione; α-GI, α-glucosidase inhibitor; OHA, oral hypoglycemic agent. Medication at discharge was included in the analysis. All variables listed in this table were included in stepwise multiple regression analysis. Adjusted R^2; a measure for the model fit.

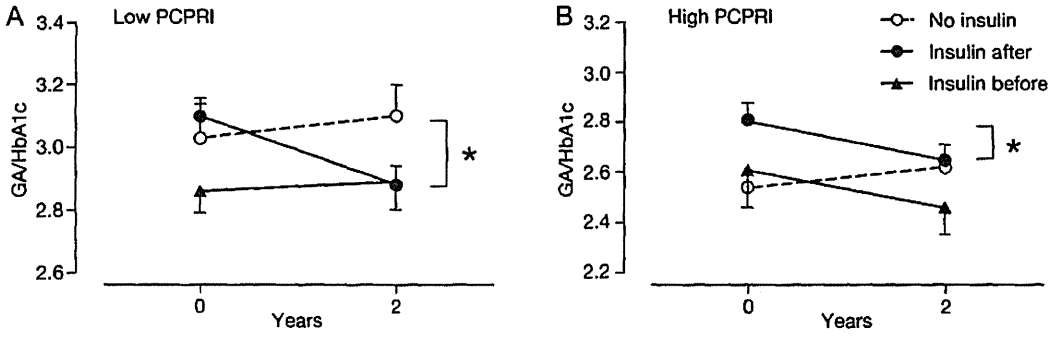

Fig. 4 Effects of insulin therapy on change in GA/HbA1c ratio 2 years after admission in low postprandial CPR index (PCPRI) (A) and high PCPRI groups (B). Open circles and dotted line; no insulin use after admission (N = 14 and 60 in low PCPRI group and high PCPRI group, respectively), closed circles; insulin use after admission (N = 62 and 39), closed triangles; continuous insulin use before and after admission (N = 29 and 6). * P <0.05 *vs.* 0 year.

of insulin therapy on the change in GA/HbA1c ratio after 2 years was also assessed. Starting insulin therapy after admission was associated with a significant reduction in GA/HbA1c ratio after 2 years (N = 101, 2.99 ± 0.49 *vs.* 2.79 ± 0.44, P <0.001), whereas no reduction in GA/HbA1c ratio after 2 years was observed in subjects who either did not start insulin therapy after admission (N = 74, 2.64 ± 0.42 *vs.* 2.71 ± 0.36, P = 0.04) or had already started insulin therapy before admission (N = 35, 2.81 ± 0.39 *vs.* 2.81 ± 0.47, P = 0.98). A significant reduction in GA/HbA1c was observed in both subjects treated with once or twice daily insulin injections (N = 62, 2.99 ± 0.42 *vs.* 2.85 ± 0.50, P = 0.005) and subjects with multiple daily injections (N = 34, 2.98 ± 0.59 *vs.* 2.72 ± 0.32, P = 0.01). The same effect of insulin therapy on GA/HbA1c was observed in both the low PCPRI and high PCPRI groups (Fig. 4).

Discussion

In this study, we report that 1) baseline GA/HbA1c ratio was significantly correlated with GA/HbA1c after 2 years even though the HbA1c level was ameliorated with intensification of treatment during this period, and 2) lower baseline PCPRI was independently related to higher GA/HbA1c ratio after 2 years.

Epidemiological studies have shown that postprandial hyperglycemia rather than fasting hyperglycemia predicts future development of cardiovascular disease [11, 12]. It has been shown that GA and GA/HbA1c are more sensitively correlated with postprandial glucose excursion than is HbA1c [6, 7, 8, 9]. Thus, not only HbA1c but also GA and GA/HbA1c are expected to be important markers for the development of diabetic complications [24]. However, the longitudinal change in GA or GA/HbA1c in clinical settings, especially after intensification of treatment, remains uncertain.

The strong association between baseline GA/HbA1c and GA/HbA1c after 2 years observed in this study suggests that GA/HbA1c remains relatively constant over the years. Therefore, it is of interest to explore the factors associated with future GA/HbA1c. It has been reported that various factors including age and BMI affected GA/HbA1c in cross-sectional studies [8, 25]. We were able to confirm the association between these factors and future GA/HbA1c in univariate and multivariate regression analyses. In addition, we also demonstrated that low beta cell function related to not only baseline GA/HbA1c ratio but also GA/HbA1c after 2 years.

Beta cell dysfunction is a hallmark of type 2 diabetes and progressively declines with disease duration [26, 27]. The association between lower beta cell function and higher GA/HbA1c after 2 years observed in this study suggests that patients with lower beta cell function are exposed to sustained greater glucose excursion even after improvement of HbA1c by intensification of treatment. These findings indicate the importance of beta cell function for reducing glycemic variability. It has also been reported that GA or GA/HbA1c, but not HbA1c, was associated with the severity of coronary atherosclerosis or progression of carotid artery intima-media thickness [16, 28, 29]. Thus, it could be speculated that lower beta cell function may contribute to the progression of atherosclerosis through sustained glycemic instability. On the other hand, recent analysis comparing the predictive ability between GA and HbA1c in a subpopulation of the Diabetes Control and Complications Trial (DCCT) cohort showed a significant association between cardiovascular disease and HbA1c but not GA in patients with type 1 diabetes [30]. Thus, the association between GA, GA/HbA1c ratio and cardiovascular disease remains unclear and further investigations are needed to clarify this hypothesis.

Multivariate regression analysis in this study revealed that other than PCPRI, age, BMI, baseline GA, baseline HbA1c, eGFR and insulin therapy were independently correlated with future GA/HbA1c, which is consistent with the findings of previous cross-sectional studies [8, 25]. We observed a significant reduction in GA/HbA1c of ~0.2 after 2 years in subjects who started insulin therapy after admission, in both the low PCPRI and high PCPRI groups. Therefore, the introduction of insulin therapy might have resulted in improvement of GA/HbA1c during the study, especially in patients with low beta cell function, in most of whom insulin was introduced during admission, suggesting the efficacy of insulin therapy to reduce glycemic variability in these subjects. Recent studies have shown that introduction of insulin therapy improved beta cell function [31, 32]. Thus, the reduction of GA/HbA1c after introducing insulin therapy may partly reflect improvement of beta cell function. Unfortunately we were not able to confirm this possibility in the present study since we did not evaluate PCPRI during the follow-up period.

It was, however, noted that even after introducing insulin therapy, GA/HbA1c level in the patients with low beta cell function remained significantly higher than that in those with high beta cell function. Recent studies suggest that the effect of treatment strategy on glycemic variability is relatively small [9, 10, 33, 34]. Whether this was due to insufficient control of glycemic variability with current therapy or to other factors affecting GA/HbA1c should be addressed in future prospective studies.

There are limitations of this study. Although we excluded patients with renal failure, nephrotic syndrome or liver cirrhosis, which affect GA/HbA1c ratio, anemia or thyroid disease in patients also might affect GA/HbA1c ratio [19]. Second, incretin therapy was not available during this study period. Since incretin therapy has been reported to improve beta cell function and reduce glycemic variability in patients with type 2 diabetes [35], the effects of incretin therapy on GA/HbA1c need to be investigated in future studies.

In conclusion, our study showed that lower beta cell function was associated with a sustained higher GA/

HbA1c ratio even after amelioration of HbA1c with intensive treatment in patients with type 2 diabetes. These findings emphasize the importance of beta cell function in controlling glycemic variability.

Acknowledgement

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nature medicine

Renal tubular Sirt1 attenuates diabetic albuminuria by epigenetically suppressing Claudin-1 overexpression in podocytes

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Sirtuin 1 (Sirt1), a NAD$^+$-regulated deacetylase with numerous known positive effects on cellular and whole-body metabolism, is expressed in the renal cortex and medulla. It is known to have protective effects against age-related disease, including diabetes. Here we investigated the protective role of Sirt1 in diabetic renal damage. We found that Sirt1 in proximal tubules (PTs) was downregulated before albuminuria occurred in streptozotocin-induced or obese (*db/db*) diabetic mice. PT-specific *SIRT1* transgenic and *Sirt1* knockout mice showed prevention and aggravation of the glomerular changes that occur in diabetes, respectively, and nondiabetic knockout mice exhibited albuminuria, suggesting that Sirt1 in PTs affects glomerular function. Downregulation of Sirt1 and upregulation of the tight junction protein Claudin-1 by SIRT1-mediated epigenetic regulation in podocytes contributed to albuminuria. We did not observe these phenomena in 5/6 nephrectomized mice. We also demonstrated retrograde interplay from PTs to glomeruli using nicotinamide mononucleotide (NMN) from conditioned medium, measurement of the autofluorescence of photoactivatable NMN and injection of fluorescence-labeled NMN. In human subjects with diabetes, the levels of SIRT1 and Claudin-1 were correlated with proteinuria levels. These results suggest that Sirt1 in PTs protects against albuminuria in diabetes by maintaining NMN concentrations around glomeruli, thus influencing podocyte function.

Diabetic nephropathy is the most common cause of end-stage renal disease, affecting about one-third of subjects with diabetes mellitus[1]. Early diabetic nephropathy is characterized by mesangial hypertrophy and glomerular hyperfiltration with microalbuminuria. Sirtuin 1 (Sir2, also called Sirt1) is a NAD$^+$-dependent protein deacetylase and a member of the sirtuin class of proteins. Sirt1, a mammalian ortholog of Sir2, deacetylates histones and various transcription factors, protecting against acute and chronic stress[2,3]. Sirt1 mitigates diabetes by attenuating hepatic insulin resistance[4,5] and enhancing pancreatic insulin secretion[6]. Recently, the renal protective effects of Sirt1 (refs. 7,8) and a pathogenic role for Sirt1 in diabetic nephropathy[9–12] have been reported, although the relationship between renal Sirt1 and the pathogenesis of kidney damage in diabetes has not been investigated.

Our group recently produced transgenic (TG) mice overexpressing SIRT1 specifically in the PTs and reported that SIRT1 alleviated acute kidney injury[13,14]. In diabetic nephropathy, PT changes are evident even in the early stages[15]. PT changes are reportedly closely linked to loss of renal function and predict the progression of diabetic nephropathy more accurately than glomerular changes[15]. Therefore, our TG mice are good models for exploring the PT-specific role of Sirt1 in diabetic nephropathy. We used these mice, as well as PT-specific *Sirt1* knockout (KO) mice, to investigate the protective role of Sirt1 in diabetes-induced albuminuria.

RESULTS

Sirt1 in diabetes and the effects of SIRT1 overexpression

Downregulation of Sirt1 expression in a diabetic milieu or high-glucose condition has been reported in various cells[16,17]. We examined Sirt1 expression in PTs and glomeruli after streptozotocin (STZ) treatment of male C57BL/6J mice. By immunostaining, we detected Sirt1 expression in both PTs and glomeruli before STZ treatment. Eight weeks after STZ treatment, Sirt1 levels were decreased in PTs but remained unchanged in glomeruli. Twenty-four weeks after STZ treatment, Sirt1 levels in glomeruli were also decreased (**Fig. 1a**). Laser microdissection followed by RT-PCR revealed that *Sirt1* mRNA expression decreased in PTs before it decreased in glomeruli in diabetic mice (**Fig. 1b** and **Supplementary Fig. 1a,b**). These observations indicate that the molecular alterations in PTs occur at a very early stage in diabetes before the increase in albuminuria, as has been documented in previous reports[18–20].

To delineate the importance of this change in PTs before a change in glomeruli, we examined the effects of Sirt1 overexpression in PT-specific Flag-tagged *SIRT1* TG mice. First we confirmed that TG mice

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