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SAD-A Potentiates Glucose-Stimulated Insulin Secretion as a Mediator of Glucagon-Like Peptide 1 Response in Pancreatic β Cells

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Type 2 diabetes is characterized by defective glucose-stimulated insulin secretion (GSIS) from pancreatic β cells, which can be restored by glucagon-like peptide 1 (GLP-1), an incretin hormone commonly used for the treatment of type 2 diabetes. However, molecular mechanisms by which GLP-1 affects glucose responsiveness in islet β cells remain poorly understood. Here we investigated a role of SAD-A, an AMP-activated protein kinase (AMPK)-related kinase, in regulating GSIS in mice with conditional SAD-A deletion. We show that selective deletion of SAD-A in pancreas impaired incretin's effect on GSIS, leading to glucose intolerance. Conversely, overexpression of SAD-A significantly enhanced GSIS and further potentiated GLP-1's effect on GSIS from isolated mouse islets. In support of SAD-A as a mediator of incretin response, SAD-A is expressed exclusively in pancreas and brain, the primary targeting tissues of GLP-1 action. Additionally, SAD-A kinase is activated in response to stimulation by GLP-1 through cyclic AMP (cAMP)/Ca^{2+}-dependent signaling pathways in islet β cells. Furthermore, we identified Thr443 as a key autoinhibitory phosphorylation site which mediates SAD-A's effect on incretin response in islet β cells. Consequently, ablation of Thr443 significantly enhanced GLP-1's effect on GSIS from isolated mouse islets. Together, these findings identified SAD-A kinase as a pancreas-specific mediator of incretin response in islet β cells.

Glucose responsiveness is a unique metabolic feature of islet β cells that controls the rate of glucose-stimulated insulin secretion (GSIS) (1). Although both glucose transport and phosphorylation control key steps in glucose responsiveness by pancreatic β cells, cyclic AMP (cAMP) generated from glucose metabolism and from the activation of incretin receptors is also absolutely required for normal β cell responsiveness to glucose (2, 3). Consequently, a loss of glucose responsiveness of pancreatic β cells is a key hallmark of type 2 diabetes mellitus (T2DM), as supported by a recent report that a loss of the first phase of insulin secretion is the only defect commonly associated with a combined risk allele score of eight T2DM genes (4). Accordingly, treatment of T2DM patients with glucagon-like peptide 1 (GLP-1), an incretin hormone, normalizes blood glucose levels by improving glucose responsiveness of islet β cells (5). The clinical importance of the incretin effect is further underscored by the high remission rate of T2DM after bariatric surgery, which restores glucose sensing in islet β cells predominantly by stimulating GLP-1 secretion (6). Finally, recent development in the field has also implicated exciting roles of GLP-1 in islet β cell differentiation, growth, and survival (2).

The term "incretin effect" was coined from early observations that oral administration of glucose enhances insulin secretion to a greater extent than that seen with isoglycemic intraperitoneal administration (7), which led to the identification of GLP-1 and glucose-dependent insulinotropic peptide (GIP) as incretin hormones. GLP-1 is secreted in response to oral ingestion of nutrient and strongly enhances GSIS through activation of the adenylate cyclase coupled with incretin receptor, leading to increased production of cAMP (2, 8). The increase in intracellular cAMP ($[cAMP]_i$) exerts its powerful potentiating effect on GSIS through activation of both protein kinase A (PKA)-dependent and PKA-independent signaling pathways, with the latter involving activation of Epac2 (cAMP-GEFII) pathways (9, 10). Hence, defective glucose-induced cAMP production is associated with onset of T2DM, which can also be restored by GLP-1 treatment (11). In contrast to the proximal signaling events on the GLP-1 effect in islet β cells, which are well elucidated in recent years, the signaling pathways distal to PKA and Epac activation remain poorly defined.

We have found that SAD-A, a member of the AMP-activated protein kinase (AMPK) subfamily that is activated by stimuli that evoke GSIS (cAMP and Ca^{2+}) (12, 13), is expressed exclusively in pancreas and brain, the primary targeting tissues of GLP-1 action. SAD-A is also regulated by the tumor suppressor LKB1 (14), which has recently been implicated in the regulation of islet morphology, β cell size and polarity, and GSIS (15–17). SAD-A and the related kinase SAD-B play important roles in neuronal polarization, cell cycle, centrosome duplication, and neurotransmitter release (18–22), but little is known about whether these kinases have functions in the pancreatic β cells. Our recent work shows that SAD-A is required for stimulus-secretion coupling of GSIS through activation of p21-activated kinase (PAK1) and cytoskeletal remodeling (23). In the present study, we investigated a key role of SAD-A in regulating islet β cell function as a mediator of incretin response in mice with conditional deletion of SAD-A in

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the pancreas. We show that SAD-A is activated by glucose and GLP-1 stimulation in islet β cells, leading to great enhancement of glucose sensing by pancreatic β cells. In the process, we also identified Thr443, a novel autoinhibitory phosphorylation site that modulates SAD-A's effect on incretin response in islet β cells.

MATERIALS AND METHODS

Animals. A SAD-A conditional mutant line was generated by flanking the first exon with *loxP* sites ($SAD^{loxP/loxP}$). Pdx-Cre mice were obtained from Jackson Laboratory as previously described (24). All animal experiments were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine in compliance with approved institutional animal care and use protocols according to NIH guidelines (25).

OGTT and ITT. An oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were performed in overnight food-deprived mice ($n = 10$). Glucose was delivered by oral gavage at 2.5 g/kg of body weight after initial measurement of fasting blood glucose. Insulin was delivered by intraperitoneal injection (0.75 U/kg of body weight; Novolin, Novo Nordisk). Blood glucose was determined 0, 30, 60, 90, and 120 min after the glucose or insulin load by a OneTouch Ultra 2 glucometer (Lifescan, Milpitas, California).

Plasmid constructs and reagents. Antibodies used in the present studies include anti-Flag antibodies, anti-β-actin antibodies, and anti-Flag M2 affinity resin from Sigma (St. Louis, MO) and anti-phospho-PAK1 (Thr423) from Cell Signaling (Boston, MA) and PhosphoSolutions (Aurora, CO). The SAM peptide substrate used for the SAD-A kinase assay was purchased from Peptide 2.0, Inc. (Chantilly, VA). P81 phosphocellulose paper was from Millipore (Billerica, MA).

Kinase assays. To evaluate SAD-A activity, INS-1 or MIN-6 islet β cells were transfected with various plasmid expression vectors for SAD-A and its mutants. The MIN-6 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 15% fetal calf serum (FCS), 25 mM glucose, 100 μM β-mercaptoethanol, and 100 units/ml of penicillin/streptomycin. After 48 h of transfection, MIN-6 cells were cultured at low glucose for 2 h and then stimulated with indicated insulin secretagogues for 30 min. The cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Equal protein was immunoprecipitated with 2.5 μg of anti-Flag antibodies at 4°C overnight. The resins were washed twice in lysis buffer and twice in kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 2 mM $MnCl_2$) and were used for a kinase assay in 30-μl reaction mixtures with 25 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 16 μg of SAM peptide substrate. After incubation at 30°C for 30 min, the reactions were stopped. Incorporation of [^{32}P]phosphate into the peptide substrate was determined by applying the reaction mixture to P81 phosphocellulose paper and scintillation counting after washing in 0.75% phosphoric acid.

Islet isolation and insulin secretion assay. Islets were isolated from 8-week-old C57BL/6 mice or 3-month-old SAD-A-null mice and their wild-type (WT) control mice by collagenase XI (Sigma, St. Louis, MO) perfusion and Histopaque (Sigma) separation from acinar and ductal tissue. Islets were then handpicked and cultured for 8 h in RPMI 1640 plus 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 before assay for adenoviral infection and insulin secretion. For recombinant adenovirus-mediated overexpression, islets were infected at a multiplicity of infection (MOI) of 100 with either control adenoviruses or recombinant adenoviruses overexpressing SAD-A kinase and its mutants. Insulin secretion studies were performed 48 h postinfection and were analyzed in six repeated samples with 10 islets each. Islets were washed and preincubated in Krebs-Ringer bicarbonate HEPES buffer (KRBH) (114 mM NaCl, 4.7 mM KCl, 1.16 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 20 mM HEPES, 0.2% bovine serum albumin [BSA]) with 2.8 mM glucose for 1 h, followed by 1 h of incubation with 600 μl of KRBH buffer containing indicated concentrations of glucose or secretagogues. Insulin levels were determined by an insulin radioimmunoassay (RIA) kit (Millipore; catalog number RI-13K) and were normalized to total cellular protein levels.

For perifusion studies, cultured islets (100 islets) were placed on a nylon filter in a plastic perifusion chamber (Millipore; catalog number SX001300) and were perifused at a flow rate of 1 ml per min. The perifusion apparatus consisted of a Rainin Dynamax peristaltic pump model RP-1, a water bath (37°C), and a fraction collector (ISCO Retriever II). The perifusate was a Krebs buffer (pH 7.4) containing 114 mmol/liter NaCl, 5 mmol/liter KCl, 1 mmol/liter $MgCl_2$, 1 mmol/liter NaH_2PO_4, 2.2 mmol/liter $CaCl_2$, 24 mmol/liter $NaHCO_3$, 10 mmol/liter HEPES (pH 7.4, adjusted with NaOH), and 1% BSA (Millipore; catalog number 82-002-7) equilibrated with 95% O_2 and 5% CO_2.

Statistical analysis. Results are shown as averages and standard errors of the mean (SEM). Student's *t* test, a nonparametric Mann-Whitney U test, or analysis of variance (ANOVA) was used to calculate differences between groups where appropriate. *P* values of <0.05, <0.01, and <0.001 were considered statistically significant.

RESULTS

SAD-A is activated by cAMP- and [Ca^{2+}]-dependent pathways in islet β cells. SAD-A is a kinase under the control of LKB1, which is implicated in islet β cell morphology and glucose homeostasis, but the functional importance of SAD-A in these aspects has not been studied. To explore the potential role of SAD-A in metabolism, we profiled SAD-A expression in various metabolic tissues, including pancreas, which plays a key role in glucose homeostasis, by a Northern blot analysis. Surprisingly, we found that SAD-A was most abundantly expressed in the pancreas, in addition to the previously reported expression in the brain (Fig. 1A). Consistent with the finding, we further showed that SAD-A protein was also abundantly expressed in the pancreatic islets (Fig. 1B). Importantly, SAD-A expression in islets, but not in exocrine pancreas, was significantly upregulated in response to glucose stimulation (Fig. 1B), implying a key role of SAD-A in glucose and energy homeostasis.

The selective expression in pancreas prompted us to investigate a role of the SAD-A kinase in regulating GSIS. We first determined whether the SAD-A kinase activity in islet β cells is regulated by glucose and forskolin, a surrogate of GLP-1 action by increasing $[cAMP]_i$ levels, by immunoprecipitation (IP) kinase assay. To facilitate the study, we generated several MIN-6 β cell lines that stably express Flag-tagged SAD-A to bypass the need of SAD-A antibodies for the IP kinase assay, since none of the SAD-A antibodies from commercial sources or from our labs recognized the endogenous SAD-A protein for IP kinase assay or immunohistochemistry. In support of a potential role of SAD-A in GSIS, SAD-A kinase activity was significantly upregulated in response to the onset of GSIS in MIN-6 β cells, as shown by the results from IP kinase analysis (Fig. 1C). It should be noted that kinase activity of the vector control was from contamination, since no SAD-A was immunoprecipitated using anti-Flag antibodies. Consistent with previous reports that SAD-A is activated by PKA and CaMKK1 (12, 13), SAD-A kinase activity was also significantly stimulated by forskolin and was potently inhibited by verapamil and H89, inhibitors of calcium channel and PKA, respectively.

Overexpression of SAD-A greatly enhances GLP-1's potentiating effect on GSIS. The finding that SAD-A kinase activity was potently stimulated by cAMP prompted us to investigate a role of SAD-A in regulating glucose responsiveness in isolated mouse islets, since GLP-1 improves glucose sensing in islet β cells through

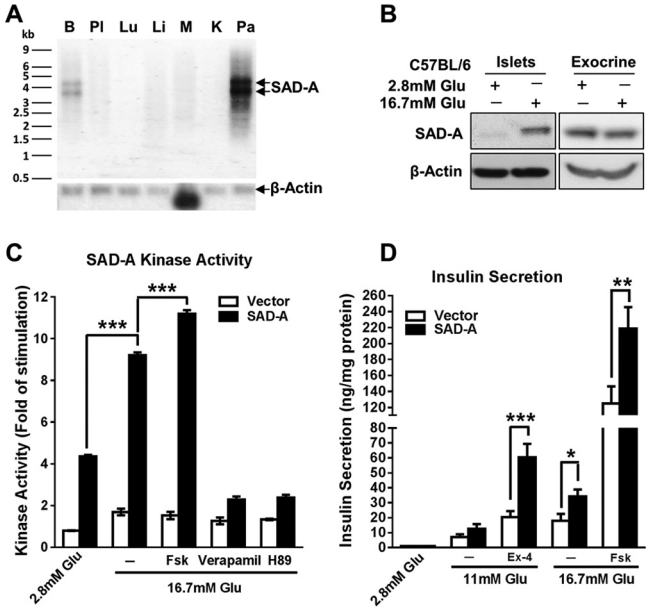

FIG 1 cAMP-dependent activation of SAD-A potentiates GSIS from pancreatic β cells. (A) Human SAD-A mRNA is expressed exclusively in pancreas and brain, the primary targeting tissues of incretin action, as demonstrated by a Northern blot analysis. (B) Glucose potently stimulated SAD-A translation in endocrine islets but not in exocrine pancreas in response to treatment with high glucose for 30 min, as analyzed by Western blot analyses using anti-SAD-A antibodies. (C) SAD-A was activated in response to the onset of GSIS in MIN-6 β cells. MIN-6 β cells stably expressing Flag-SAD-A or empty vector (negative control) were stimulated for 30 min with 16.7 mM glucose or 16.7 mM glucose plus various reagents, including 10 μM forskolin (Fsk; an activator of PKA), 5 mM verapamil (an L-type calcium channel blocker), and 10 μM H89 (an inhibitor for PKA). The recombinant SAD-A was immunoprecipitated from the treated MIN-6 cells using anti-Flag antibodies and analyzed for SAD-A kinase activity. (D) Adenoviral overexpression of SAD-A in isolated mouse islets significantly enhanced GSIS in response to treatment with 11 mM glucose, 11 mM glucose plus 10 nM exendin-4 (Ex-4), 16.7 mM glucose, and 16.7 mM glucose plus 10 μM forskolin, respectively. $n = 3$ to 6. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$ (all compared to the vector control).

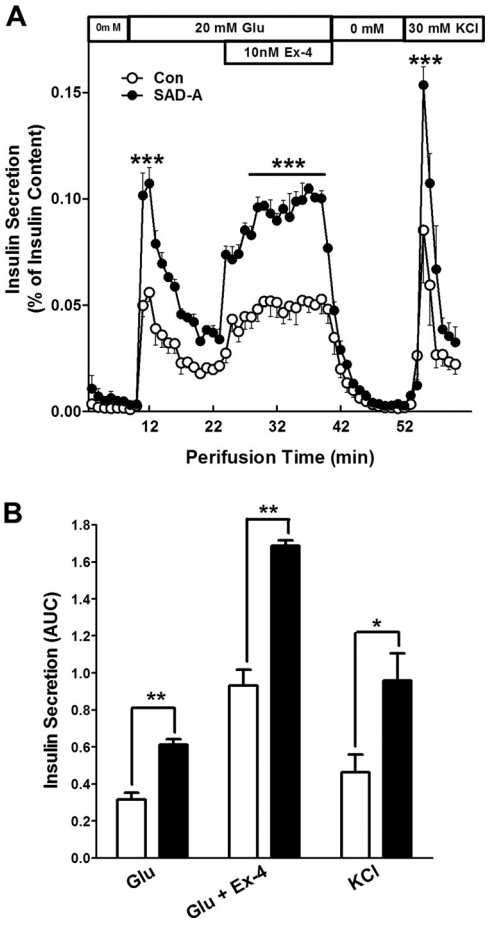

FIG 2 Overexpression of SAD-A dramatically enhanced GLP-1's effect on GSIS in perifused mouse islets. (A) Isolated islets from pSADKO mice and the WT controls were infected with recombinant adenoviruses expressing SAD-A or vector control. After 48 h of infection, the infected islets were perifused sequentially with a perifusion medium which contains 20 mM glucose (Glu), 20 mM glucose plus 10 nM exendin-4 (Ex-4), and 30 mM KCl, followed by analysis of insulin secretion by radioimmunoassay. (B) Analysis of the area under the curve (AUC) of insulin secretion levels in panel A. $n = 3$ to 6. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$ (all compared to the vector control).

activation of cAMP-dependent signaling pathways. To examine the role of SAD-A overexpression in incretin effects on GSIS, we developed recombinant adenoviruses overexpressing Flag-tagged SAD-A as a vehicle to deliver a high level of SAD-A expression in isolated primary islets. Using the recombinant adenoviruses overexpressing Flag-tagged SAD-A or the vector control, we next analyzed the effect of SAD-A overexpression on GSIS and GLP-1 action in isolated mouse islets. As shown by Fig. 1D, SAD-A overexpression significantly enhanced GSIS. More importantly, SAD-A overexpression greatly potentiated the effect of forskolin (Fsk) and exendin-4 (Ex-4), a therapeutic long-acting analogue of GLP-1, on GSIS. In contrast, overexpression of SAD-A did not affect basal insulin secretion at 2.8 mM glucose, which is consistent with the glucose-dependent manner of incretin response.

Using islet perifusion analysis, we next profiled the effect of SAD-A on insulin secretion in response to stimulation with glucose, glucose plus exendin-4, and KCl. As shown in Fig. 2A and quantified in Fig. 2B, overexpression of SAD-A did not affect basal insulin secretion but greatly enhanced GSIS. Strikingly, overexpression of SAD-A greatly enhanced the potentiating effect of exendin-4 on GSIS. Furthermore, SAD-A overexpression also enhanced KCl-stimulated insulin secretion, a finding which is consistent with SAD kinase as a downstream target of $[Ca^{2+}]_i$-dependent signaling pathways (12). These findings are consistent with a previous report that GLP-1 enhances insulin secretion through mechanisms involving the regulation of ion channels, including ATP-sensitive K^+ channels and voltage-dependent Ca^{2+} channels (8).

Targeted deletion of SAD-A in pancreas causes glucose intolerance by impairing the GLP-1 response. To link the *in vitro* findings to a physiological relevance, we next determined the role of SAD-A in regulating GSIS and incretin responses *in vivo* using a newly generated mouse line with a floxed SAD-A allele ($SAD^{loxP/loxP}$) (Fig. 3A). The $SAD^{loxP/loxP}$ mice were crossed with transgenic mice expressing the Cre recombinase directed by a Pdx1 promoter (24) to generate mice with selective deletion of SAD-A in the pancreas (pSADKO). The pSADKO mice were compared with $SAD^{loxP/loxP}$ mice, which are referred to as wild-type controls (Con) in the present study. The pSADKO mice were born at the predicted Mendelian ratio and survived to adulthood without any obvious physiological abnormality. Western blot

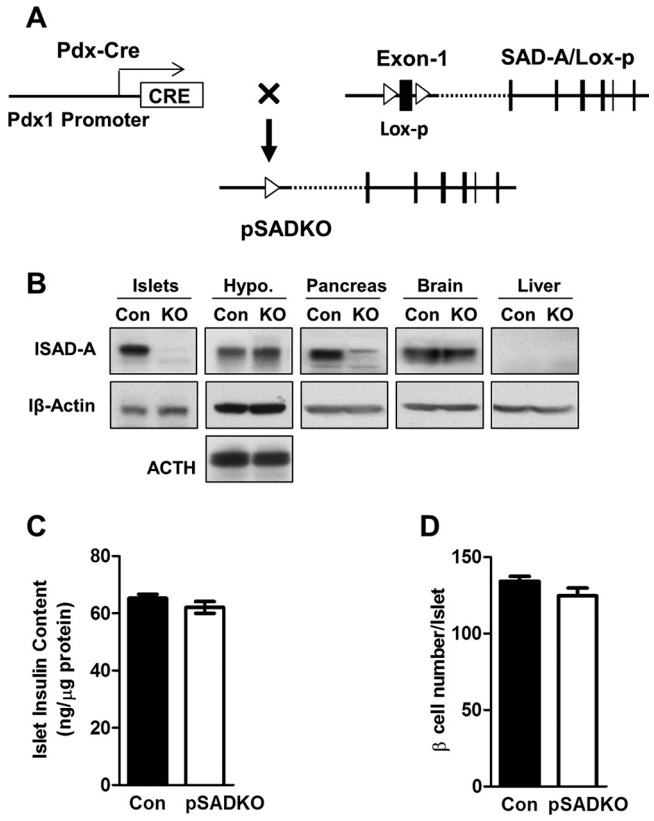

FIG 3 Generation of mice with conditional deletion of SAD-A in the pancreas. (A) Schematic diagram depicting the strategy used for the generation of mice with targeted deletion of SAD-A in the pancreas (pSADKO). (B) Western blot analysis of SAD-A protein expression in various tissues isolated from pSADKO mice (KO) and WT controls (Con), including islets, hypothalamus (Hypo.), pancreas, brain, and liver using anti-SAD-A antibody and β-actin as an internal control for protein loading. Additionally, adrenocorticotropin (ACTH) was used as a tissue-specific marker for hypothalamus. (C) pSADKO mice and the wild-type control mice were analyzed for islet insulin content by radioimmunoassay. $n = 5$. (D) Analysis of islet β cell numbers by morphometric analysis. The results were obtained from 3 pSADKO mice and WT controls, with 25% of each pancreas assessed from evenly sectioned samples across the entire pancreas.

analysis indicates that SAD-A expression was virtually absent in pancreatic islets and nearly depleted in pancreas in pSADKO mice but was unaffected in nonpancreatic tissues, including brain and liver (Fig. 3B). Specifically, targeted deletion of SAD-A in the pancreas did not affect the expression of SAD-A in the hypothalamus, which plays a key role in energy homeostasis. The results are consistent with previously reported specificity of the Pdx1-Cre transgene (24).

To identify a role of SAD-A in maintaining glucose homeostasis, we first analyzed the effect of SAD-A deficiency on glucose excursion in pSADKO mice relative to the WT controls by an oral glucose tolerance test (OGTT). As shown in Fig. 4A, SAD-A deficiency did not affect the blood glucose level in the fasting state compared with WT controls (Con). In contrast, pSADKO mice exhibited significantly higher blood glucose levels than the WT controls during OGTT. Remarkably, such a difference was totally abolished during an intraperitoneal glucose tolerance test (IPGTT), which bypasses incretin effects on GSIS (Fig. 4B). In support of the observations, pSADKO mice exhibited a level of hyperglycemia similar to that of WT controls during IPGTT that matches the blood glucose levels observed in the pSADKO mice during OGTT. The results suggest a defective GLP-1 response in pSADKO mice, which is supported by the results that the pSADKO mice exhibited significantly lower serum insulin levels than WT controls during OGTT (Fig. 4C). In contrast, such differences were completely abolished during IPGTT (Fig. 4D). The defective incretin response is unlikely to be caused by a developmental defect of islet β cells in pSADKO mice, since there were no significant differences between pSADKO mice and WT controls in islet insulin content and β cell numbers (Fig. 3C and D).

In addition to the pancreas, the Pdx-Cre transgene was recently reported to also be expressed in the hypothalamus (26), a key organ in regulating energy homeostasis, raising the possibility of a compounded effect of SAD-A deficiency in the brain on GSIS. To address this issue, we next analyzed the effect of SAD-A deficiency on body weight and insulin sensitivity. Consistent with a lack of any detectable effect of the Pdx-Cre transgene on SAD-A expression in the hypothalamus (Fig. 3B), pSADKO mice exhibited a weight gain similar to that of the WT controls when fed a regular diet (Fig. 4E). Furthermore, SAD-A deficiency did not significantly affect insulin sensitivity, as evidenced by similar glucose excursion between pSADKO mice and WT controls during an insulin tolerance test (Fig. 4F). The results suggest that the impaired GSIS in pSADKO mice is caused primarily by a defect in incretin response on GSIS in islet β cells.

cAMP-dependent dephosphorylation of SAD-A at Thr443 regulates incretin's effect on GSIS. To determine the molecular mechanisms by which SAD-A kinase mediates GLP-1's effect in islet β cells, we next analyzed potential SAD-A phosphorylation sites which are regulated by cAMP-mediated signaling pathways. As shown in Fig. 5A, the human SAD-A protein is organized by a serine/threonine kinase domain, a ubiquitin-associated (UBA) domain, and a proline-rich domain (PRD). In search of a potential regulatory phosphorylation site of SAD-A, we identified a consensus sequence motif (SXXX**T**XXGTP [the phosphorylation site is boldfaced]) embedded in the PRD domain that shares a similar sequence motif with the major phosphorylation site of p21-associated kinase (PAK1) at Thr423 (Fig. 5B) (27). Based on this sequence similarity, we used the anti-pPAKThr423 antibody to probe Western blots and found strong reactivity with SAD-A. The identity of the epitope was further confirmed by showing that the antibody did not recognize a SAD-A mutant in which Thr443 was replaced by Ala (T443A). This result demonstrates that Thr443 is a novel phosphorylation site of SAD-A (Fig. 5C). Indeed, the Thr443 phosphorylation was not affected by individual mutation which abolishes previously identified regulatory sites, including the ATP binding site (K48M), the phosphorylation site of LKB1 and CaMKK1 (T174A) (12, 19), and the phosphorylation site of PKA (T260A) (13). In further support of Thr443 as a major regulatory phosphorylation site of SAD-A, the T443A mutation also changed the migration pattern of SAD-A protein bands on SDS-PAGE. In contrast to WT SAD-A, which exhibited doublet bands on SDS-PAGE, the T443A mutant exhibited only the lower band (Fig. 5C). In further support of SAD-A as a mediator in GLP-1 response, SAD-A is dephosphorylated in response to stimulation with forskolin in 293T cells (Fig. 5C) and exendin-4 (Ex-4) in MIN-6 β cells (Fig. 5D). These results suggest that Thr443 is a novel autoinhibitory phosphorylation site of SAD-A.

We next examined whether phosphorylation of SAD-A at

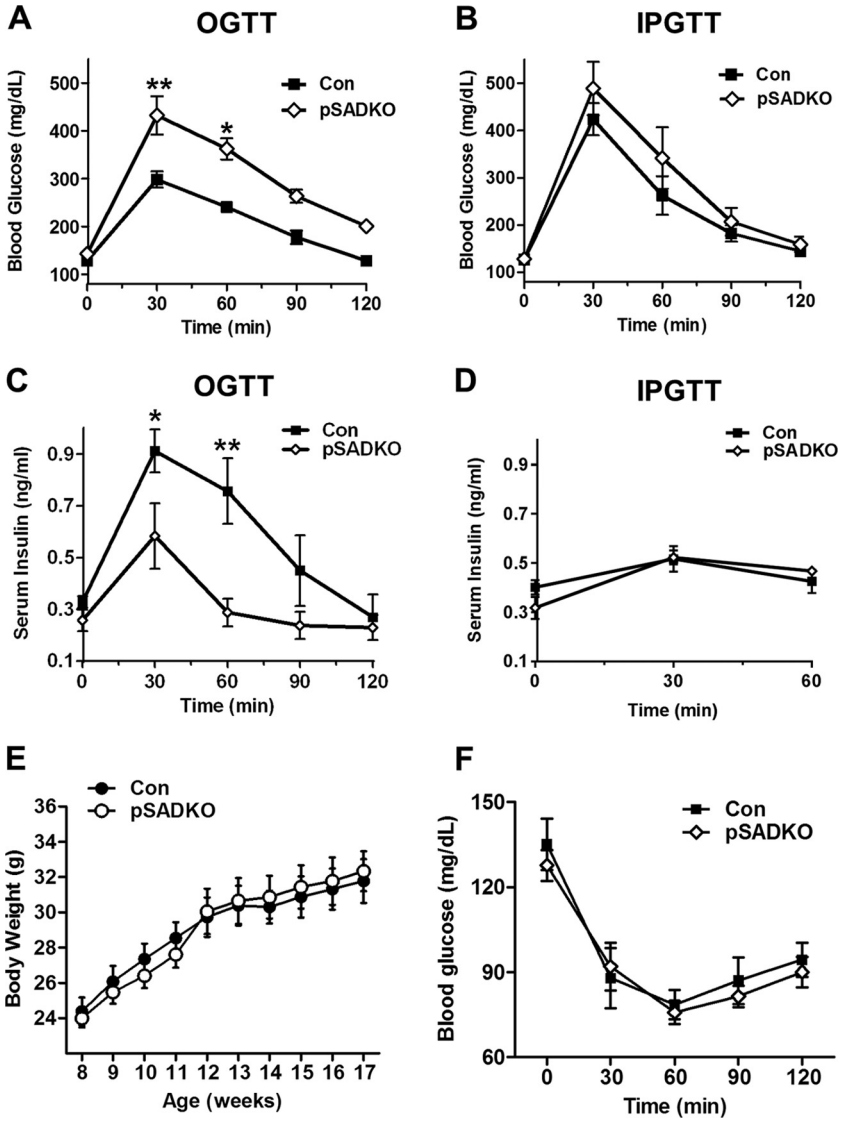

FIG 4 Conditional deletion of SAD-A in pancreases causes glucose intolerance by impairing incretin response. (A) pSADKO mice exhibited glucose intolerance relative to the WT controls during an oral glucose tolerance test (OGTT) after overnight fasting. (B) pSADKO mice exhibited glucose excursion similar to that of the WT controls during an intraperitoneal glucose tolerance test (IPGTT) after overnight fasting. (C and D) pSADKO mice exhibited impaired insulin secretion *in vivo* relative to WT controls during OGTT (C) but not during IPGTT (D). (E) pSADKO mice exhibited a growth rate and weight gain similar to those of the WT controls when fed a regular chow diet. (F) pSADKO mice exhibited insulin sensitivity similar to that of WT controls. Insulin tolerance tests were carried out in pSADKO mice and WT controls after overnight fasting. The mice were subjected to intraperitoneal injection of insulin at 0.75 U/kg of body weight, followed by measurement of blood glucose levels at indicated time intervals after the injection. $n = 10$. *, $P < 0.05$; **, $P < 0.01$ (all compared to the WT controls).

Thr443 would play a key role in regulating SAD-A kinase activity, GSIS, and incretin response. We first examined the effect of a T443A mutation on SAD-A kinase activity in islet β cells by IP kinase assay. Consistent with T443 as a major autoinhibitory site, the T443A SAD-A mutant exhibited significantly higher kinase activity in INS-1 β cells than the WT SAD-A when cultured at 2.8 mM glucose (Fig. 6A). In contrast, SAD-A, but not the kinase-dead mutant (K48M), was dramatically activated in response to glucose stimulation in INS-1 β cells (Fig. 6A). Intriguingly, T443A was also activated by high glucose, but to a much less extent than WT SAD-A, which was likely mediated by other activation sites, such as Thr260 by PKA and Thr174 by CaMKK1. We next analyzed the effect of the T443A mutation on GSIS. In further support of Thr443 as an autoinhibitory phosphorylation site of SAD-A, overexpression of the T443A mutant SAD-A significantly enhanced GSIS. Remarkably, overexpression of the T443A mutant SAD-A also greatly potentiated the effect of GLP-1 on GSIS in isolated mouse islets compared with that for WT SAD-A (Fig. 6B). Although the T443A mutant SAD-A is constitutively activated, overexpression of the T443A mutant did not affect basal insulin secretion at 2.8 mM glucose, which further supports our hypothesis that SAD-A mediates incretin response, since GLP-1 does not potentiate GSIS at low glucose. It should be noted that the level of SAD-A kinase activity measured *in vitro* did not fully correlate with GSIS *in vivo* at high glucose. Together, these results suggest that cAMP-mediated dephosphorylation of Thr443 plays a major

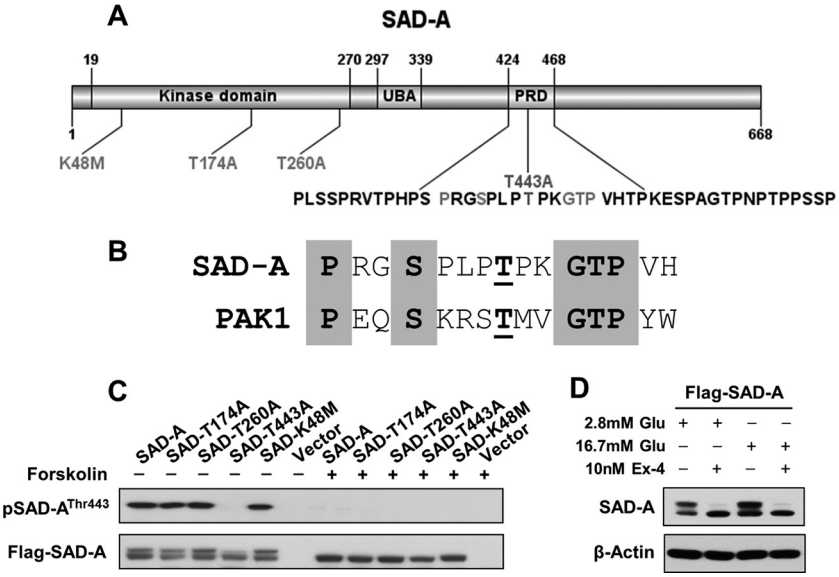

FIG 5 Identification of Thr443 as a novel autoinhibitory phosphorylation site of SAD-A. (A) The human SAD-A protein consists of a Ser/Thr kinase domain, a ubiquitin-associated domain (UBA), and a proline-rich domain (PRD) where the Thr443 phosphorylation site is located. Highlighted are mutations of important regulatory sites of the human SAD-A kinase, including an ATP binding site (K48M), an LKB1 and CaMKK1 phosphorylation site (T174A), a PKA phosphorylation site (T260A), and Thr443 (T443A). (B) Consensus sequence between the phosphorylation site of PAK1 at Thr423 and SAD-A at Thr443. (C) 293T cells transiently transfected with expression vector for Flag-tagged SAD-A and various point mutants as detailed in panel A. At 48 h after the transfection, the transfected cells were stimulated with or without 10 μM forskolin and analyzed for phosphorylation of Thr443 by Western blot analysis using anti-pSAD-A^{Thr443} antibody. The expression levels of Flag-tagged SAD-A were verified by Western blot analysis using anti-Flag antibodies. (D) Western blot analysis of Thr443 phosphorylation of SAD-A transiently expressed in INS-1 cells in response to stimulation with 10 nM exendin-4 (Ex-4) at low and high glucose levels.

role in regulating SAD-A's effect on glucose sensing in islet β cells as a mediator of GLP-1 response.

DISCUSSION

Loss of glucose sensing in pancreatic β cells is a key hallmark of T2DM, which can be restored by treatment with GLP-1 (28). The current studies investigate a role of SAD-A in regulating glucose responsiveness in islet β cells as a pancreas-specific mediator of incretin response. Using newly generated mice with conditional deletion of SAD-A in the pancreas, we demonstrate that SAD-A deficiency impairs GSIS by abrogating the GLP-1 effect, leading to glucose intolerance. Likewise, overexpression of SAD-A in isolated mouse islets significantly enhanced GSIS and GLP-1 response. Although a recent study suggested that the Pdx-Cre transgene was also expressed in the hypothalamus (26), which may potentially complicate the phenotype of the pSADKO mice, our results show that the SAD-A expression in hypothalamus was not significantly affected in pSADKO mice. Consistent with this finding, SAD-A deficiency in the pancreas did not affect weight gain or insulin sensitivity in pSADKO mice. A key role of SAD-A in GSIS is further supported by our findings that SAD-A is expressed exclusively in pancreas and brain, the primary targeting tissues of GLP-1 action. Additionally, SAD-A expression in pancreatic islets, but not in exocrine pancreas, was significantly upregulated by the onset of GSIS. Furthermore, SAD-A kinase is significantly activated in islet β cell responses to the onset of GSIS and stimulation with GLP-1. Finally, the importance of SAD-A in regulating glucose homeostasis is further underscored by its genetic location. SAD-A, but not SAD-B, is localized to the *IDDM2* diabetic locus in the vicinity of the insulin gene on human chromosome 11p15.5 (29).

SAD-A is a member of the AMPK-related family of protein kinases under the control of the LKB1 kinase (30, 31). Among the 12 members of the AMPK-related family of protein kinases, SAD-A shares the highest sequence homology with AMPK. AMPK is a master regulator of energy homeostasis, being activated in response to an increase in the AMP/ATP ratio under low nutrient conditions (32). These functions are partly mediated by its regulatory role in glucose sensing of hypothalamic neurons (33). However, it remains questionable whether AMPK regulates glucose sensing by pancreatic β cells (34). In addition to AMPK, other members in this family have also been implicated in energy homeostasis (35–37). Finally, consistent with our findings in this study, targeted deletion of LKB1 in pancreatic β cells significantly increased β cell mass and insulin secretion (15, 16). Therefore, this study has filled an important gap by providing key insights into SAD-A as a key regulator of glucose sensing in islet β cells as a downstream target of LKB1 signaling.

Major progress has been made in recent years in understanding the proximal signaling events involved in incretin action, including the activation of cAMP- and [Ca^{2+}]-mediated signal transduction pathways (9, 38). In contrast, little is known about molecular details of how incretin hormones regulate the "amplifying phase" of GSIS in β cells, although both PKA and CaMKK1 signaling pathways are implicated as distal amplifiers for stimulus-secretion coupling signals originating from fuel metabolism and from incretins (39). In this study, we identified SAD-A as a pancreas-specific regulator of glucose responsiveness that mediates the GLP-1 effect in islet β cells, a finding which is supported by multiple lines of evidence. We show that SAD-A is activated in islet β cells in response to treatment with glucose, GLP-1, and forskolin, which is completely abolished by a PKA inhibitor and a

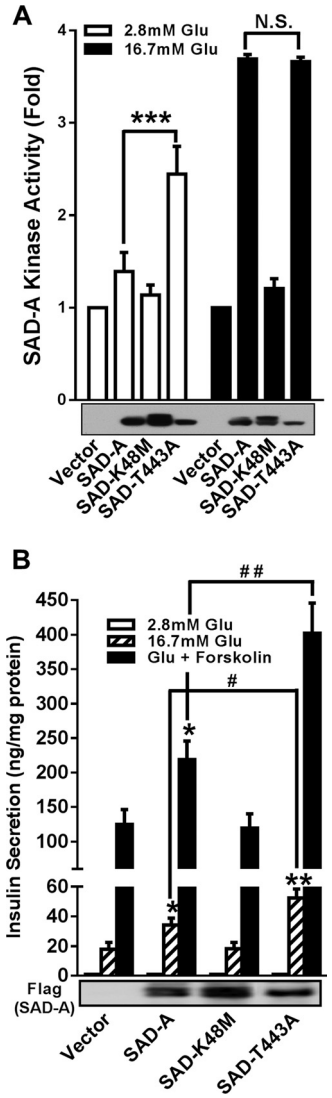

FIG 6 Ablation of Thr443 significantly stimulates SAD-A kinase activity and GSIS from isolated mouse islets. (A) INS-1 β cells were transiently transfected with the expression vectors for Flag-SAD-A or the indicated SAD-A point mutants and then stimulated with 16.7 mM glucose for 30 min, followed by an immunoprecipitation (IP) kinase assay using anti-Flag antibodies. Expression levels of Flag-SAD-A and its mutants were verified by Western blot analysis (lower panel). (B) Overexpression of the T443A mutant SAD-A significantly enhanced insulin secretion from isolated mouse islets in response to stimulation with glucose (Glu) and glucose plus forskolin compared to that for WT SAD-A or its kinase-dead mutant (K48M). The expression level of the recombinant SAD-A was verified by Western blot analysis using anti-Flag antibodies (lower panel). $n = 6$. *, $P < 0.05$ compared to the vector control; **, $P < 0.01$ compared to the vector control; #, $P < 0.05$ compared to the WT SAD-A; ##, $P < 0.001$ compared to the WT SAD-A.

calcium channel blocker. Our results are consistent with previous reports that SAD kinase is activated by PKA- and CaMKK1-mediated signaling pathways (12, 13). Additionally, we identified Thr443 as a novel autoinhibitory phosphorylation site of SAD-A, which is regulated by cAMP- and GLP-1-mediated signaling pathways. The Thr443 site is embedded in the PRD domain of SAD-A but is absent in the SAD-B isoform, which is expressed exclusively in brain. Accordingly, we show that Thr443 is dephosphorylated in response to treatment with GLP-1. Ablation of Thr443 significantly enhanced SAD-A activity in pancreatic β cells, leading to a great enhancement in GSIS and incretin effect in isolated mouse islets. Although the protein phosphatase that regulates the dephosphorylation of SAD-A at T443 remains elusive, we speculate that protein phosphatase 2A (PP2A) may be involved in the process, a theory which is supported by a previous observation that PP2A is activated by PKA through direct phosphorylation (40). Finally, we show that the Thr443 phosphorylation site shares a conserved sequence motif with the major phosphorylation site of PAK1 at Thr423. Our recent work shows that SAD-A phosphorylates PAK1 at Thr423, which is required for the onset of GSIS (23). In addition to SAD-A, PAK1 is also phosphorylated at Thr423 by other kinase regulators (41). Therefore, it can be envisaged that the same kinase regulator which phosphorylates PAK1 at Thr423 may coordinately regulate SAD-A kinase activity through phosphorylation of Thr443 during the onset of GSIS.

Together, the current studies identified a key role of SAD-A in regulating glucose homeostasis as the first tissue-specific kinase mediator of incretin response in islet β cells. More importantly, our findings suggest that targeting SAD-A with small-molecule activators may provide a novel treatment for T2DM by mimicking the therapeutic action of incretin hormones. In contrast to the previously identified kinase regulators of GSIS, such as PKA and Epac2, which are ubiquitously expressed, SAD-A kinase is expressed exclusively in pancreas and brain, thus providing a more desirable drug target for T2DM without potential side effects on other tissues.

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