Published in final edited form as:

Sci Signal. ; 5(216): ra23. doi:10.1126/scisignal.2002519.

Cannabinoids Induce Pancreatic β -Cell Death by Directly Inhibiting Insulin Receptor Activation

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Abstract

Cannabinoid 1 (CB1) receptors have been previously detected in pancreatic β cells, where they influence insulin action. We now report that CB1 receptors form a heteromeric complex with insulin receptors and $G\alpha_i$, which inhibits insulin receptor kinase activity in β cells by directly binding to the activation loop in the tyrosine kinase domain of the insulin receptor. Consequently, phosphorylation of pro-apoptotic protein Bad was reduced, leading to activation of Bad and induction of β -cell death. Pharmacological blockade or genetic deficiency of CB1 receptors led to reduced blood glucose and increased β -cell survival after injury due to enhanced insulin receptor signaling and reduced activation of Bad. These findings provide direct evidence of physical and functional interactions between CB1 and insulin receptors and provide a mechanism whereby peripherally acting CB1 receptor antagonists improve insulin action in insulin-sensitive tissues independent of the other metabolic effects of CB1 receptors.

INTRODUCTION

Insulin secreted from pancreatic β cells activates a number of intracellular signaling pathways in virtually all mammalian cells, including β cells, that regulates not only energy

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SUPPLEMENTARY MATERIALS

Fig. S1. CB1 receptor abundance in pancreatic β -cell lines.

Fig. S2. Effects of ACEA depend on the CB1 receptor.

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Fig. S4. Effects of $G\alpha_{i3}$ on insulin receptor signalling.

Fig. S5. Improved β -cell mass due to enhanced β -cell survival in STZ-treated *CB1R*^{-/-} mice.

Fig. S6. Enhanced insulin signaling in β cells of STZ-treated *CB1R*^{-/-} mice.

Fig. S7. Increased phosphorylation of p27 at Ser¹⁰ and Thr¹⁵⁷ in islets of STZ-treated mice by CB1 receptor blockade.

Fig. S8. Increased abundance of PDX-1, GLUT2, and glucokinase in β cells of STZ-treated *CB1R*^{-/-} mice.

Table S1. Details of the antibodies used for immunoblotting, immunoprecipitation, and immunofluorescence studies.

Author contribution: W.K. designed and performed experiments, wrote/reviewed and edited the manuscript. Q.L. designed and performed some experiments and provided advice and reagents. Y-K.S. and O.D.C. performed some experiments and analyzed data. E.K.L. contributed to the design of some experiments, the interpretation of data, and the discussion. M.G. provided advice and reviewed the manuscript. R.N.K. provided advice and reagents, and reviewed the manuscript. J.M.E. designed and performed experiments, and wrote, reviewed, and edited the manuscript.

Competing interests: Use of the Wild-type (β IRWT) and insulin receptor-deficient (β IRKO) β -cell lines requires a material transfer agreement (MTA) from the Joslin Diabetes Center (R.N.K.).

homeostasis, but also cellular proliferation and apoptosis. The actions of insulin are mediated by the insulin receptor, which is broadly distributed in normal tissues. The insulin receptor is composed of two extracellular α -chains involved in ligand binding and two intracellular β -chains that include the tyrosine kinase domain (1, 2). Insulin binding to the α -chains induces a structural change that places the phosphorylation sites of one β -chain within reach of the active site of the other β -chain and facilitates autophosphorylation at Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³ in the activation loop of the β -chains (3). Mutation of these tyrosine residues reduces insulin-stimulated autophosphorylation and kinase activity, and results in a parallel loss of biological function (4, 5). The receptor also undergoes autophosphorylation at other tyrosine residues in the juxtamembrane region and the C-terminal tail (6, 7). Tyrosine phosphorylation increases the catalytic activity of the receptor and also serves as docking sites for downstream signaling proteins such as the insulin receptor substrates (IRS) (8). A well-characterized signaling cascade that is activated by insulin is the IRS-phosphoinositide 3-kinase (PI3K)-AKT cascade, in which AKT is a critical mediator of insulin responses such as gene expression, protein synthesis, cell growth and survival, and glucose metabolism (8). AKT promotes cell survival and growth by phosphorylating the pro-apoptotic protein Bad (which results in inactivation of Bad) (9, 10), the transcriptional regulator FoxO (which results in activation of FoxO) (11, 12), and the cyclin-dependent kinase inhibitor p27 (which results in activation of p27) (13–15). This is also true for pancreatic β cells, because targeted mutations of genes in β cells that encode the insulin receptor and its downstream molecules such as IRS2, AKT, FoxO1, and p27 reduce β -cell growth or survival, resulting in age-dependent diabetes mellitus (16–20). In addition, AKT-mediated phosphorylation of FoxO1 positively regulates *insulin* transcription, insulin secretion, and β -cell growth and survival by increasing the abundance of the pancreatic transcription factor pancreas/duodenum homeobox-1 (PDX-1) (16, 18).

The presence of cannabinoid 1 (CB1) receptors and the necessary enzymes for catalysing biosynthesis and degradation of endogenous cannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA) in β cells of human and mouse islets has been demonstrated by several groups (21–24). We also confirmed in our previous report (25) that CB1 receptors are present in β cells and that β cells synthesize the endogenous cannabinoids in a glucose-dependent manner. The CB1 receptor is a G protein-coupled receptor that is activated by endogenous cannabinoids, which are lipid transmitters synthesized ‘on demand’ by Ca²⁺-dependent enzymes in the brain and the periphery (25–27). Tetrahydrocannabinol, the main psychoactive compound in cannabis, is an exogenous ligand of CB1 receptors, which are distributed in several brain areas as well as hepatocytes (28) and muscle (29). Cannabinoids induce cell cycle arrest and apoptosis by inhibiting the PI3K-AKT cascade in various cancer cells (30–32). We and others have reported that endogenous cannabinoids influence insulin action through regulation of insulin receptor signaling in insulin-sensitive tissues such as muscle, liver, and islets of Langerhans (25, 28, 33, 34). We now provide in depth mechanistic insight into how the blockade of the CB1 receptor inhibits apoptosis in β cells. Previous research using CB1 receptor antagonists in animals had indicated that the resulting improvement in insulin action was due to weight loss (35), but we now provide evidence for direct crosstalk between CB1 and insulin receptors.

RESULTS

Activation of CB1 receptors induces β -cell death in an insulin receptor-dependent manner

Activation of the CB1 receptor by the synthetic full agonist WIN55,212-2 or by endogenous cannabinoids (AEA or 2-AG) decreased the viability (Fig. 1A), increased the cytotoxicity (Fig. 1B), and activated caspase-3 (Fig. 1C) in mouse insulinoma MIN6 cells in a dose-dependent manner. A similar, but less pronounced response was seen in α TC1 glucagonoma cells, likely because CB1 receptors are less abundant in these cells than in β -cell lines (fig.

S1). The CB1 receptor-mediated decrease in viability of MIN6 cells was reduced by Ac-DNLD-CHO, an inhibitor of caspase-3 and -7 (Fig. 1D).

Because insulin receptor signaling is a key regulator of β -cell survival (16–20) and because CB1 receptor agonism and antagonism influence insulin action (25, 33, 34), we next investigated the potential role of insulin receptors as mediators of CB1 receptor-controlled β -cell survival using β cells established from wild-type or β -cell-specific insulin receptor knockout mice (17, 36, 37). The effects of WIN55,212-2 on caspase-3 activation (Fig. 1E) and cell viability (Fig. 1F) were reduced in β cells from knockout mice compared those from wild-type mice. WIN55,212-2 decreased, in a dose-dependent manner, the phosphorylation of IRS1/2 at Tyr⁶¹² and AKT at Ser⁴⁷³ in β cells from wild-type mice, but not in those from knock-out mice (Fig. 1G). Lack of insulin receptor did not alter the abundance of the CB1 receptor (Fig. 1G). Consistently, the viability of β cells from wild-type mice, but not that of β cells from knock-out mice, was increased by the CB1 receptor antagonists tested (AM251, rimonabant and SLV-319) (Fig. 1H), and the effect of rimonabant was prevented by ACEA, a selective CB1 receptor agonist (fig. S2A).

Activation of the CB1 receptor diminishes insulin-induced phosphorylation of Bad by inhibiting insulin receptor kinase activity

To test the effect of CB1 receptor activation on insulin-stimulated autophosphorylation of insulin receptor, MIN6 cells were pretreated with ACEA before addition of exogenous insulin. Consistent with our previous report (25), ACEA diminished exogenous insulin-stimulated autophosphorylation of the insulin receptor at Tyr¹¹⁶² and Tyr¹¹⁶³ and phosphorylation of IRS1/2 and AKT (Fig. 2A). Knockdown of CB1 receptors by siRNA (fig. S2B) abolished the ability of ACEA to inhibit insulin-stimulated phosphorylation of insulin receptor and AKT (fig. S2C). To further confirm whether the CB1 receptor interferes with insulin-stimulated autophosphorylation and kinase activity of insulin receptor, we carried out in vitro kinase assays with the recombinant p30 fragment of IRS1, which harbors the Tyr⁶¹² residue, and immune complexes of the β -subunit of the insulin receptor after addition of exogenous insulin with vehicle or ACEA to β cells from wild-type mice. We observed that activation of CB1 receptors inhibited insulin-stimulated autophosphorylation of insulin receptors, resulting in reduced kinase activity (Fig. 2B).

Bad is a pro-apoptotic member of the Bcl-2 family that promotes cell death by displacing Bax from binding to Bcl-xL and Bad activity is directly inhibited by AKT-mediated phosphorylation at Ser¹³⁶ (9), which promotes binding of Bad to 14-3-3 protein instead of to Bcl-xL (Fig. 2C) (9, 10, 38). Thus, we examined the effect of CB1 receptor agonists on Bad activity in β cells from wild-type mice. ACEA prevented the insulin-stimulated phosphorylation of Bad at Ser¹³⁶, decreased the amount of Bad bound to 14-3-3, and increased the amount of Bad bound to Bcl-xL (Fig. 2D). To further confirm the inhibitory effects of the CB1 receptor on insulin-stimulated phosphorylation of Bad, we transfected Flag-tagged wild-type insulin receptor or an insulin receptor mutant with Ala substitutions for Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³ residues, into β cells from knock-out mice (Fig. 2E). Bad phosphorylation was detected in β -cells from knock-out mice reconstituted with wild-type insulin receptor, presumably due to endogenous insulin secretion, and treatment with ACEA reduced the phosphorylation of Bad in these cells. In contrast, phosphorylation of Bad was not altered in β cells from knock-out mice reconstituted with the tyrosine-phosphorylation deficient insulin receptor mutant (Fig. 2E). Knockdown of Bad by siRNA (Fig. 2F) abolished the ability of WIN55,212-2 to activate caspase-3 (Fig. 2G), increased β -cell viability (Fig. 2H) and prevented the inhibitory actions of WIN55,212-2 on β -cell viability (Fig. 2I). Insulin receptor signaling was also higher in livers from CB1 receptor-null (CB1R^{-/-}) mice (39) than in those from wild-type littermates, and WIN55,212-2 treatment decreased phosphorylation of insulin receptors in human hepatocarcinoma HepG2 cells (fig.

S3, A and B). WIN55,212-2 treatment also increased cleaved caspase-3 and Bad activity and reduced cell viability (fig. S3, B–D). Similarly, viability of primary human hepatocytes was decreased by WIN55,212-2 treatment (fig. S3E). In sum, our results suggest that CB1 receptor signaling functions to inhibit insulin receptor signaling, including in non-insulin-secreting cells.

The CB1 receptor forms a heteromeric complex with the β subunit of the insulin receptor and $G\alpha_i$

The CB1 receptor associated with the β -subunit of the insulin receptor (IR β) in β cells from wild-type mice (Fig. 3A). This association was increased by treatment with ACEA (Fig. 3A) and decreased by treatment with exogenous insulin, an effect that was reversed by ACEA (Fig. 3B). In reconstituted β cells from knock-out mice, the CB1 receptor showed greater association with the tyrosine phosphorylation-deficient mutant than with the wild-type receptor, an interaction that was increased by ACEA treatment (Fig. 3C). This result is consistent with the finding that exogenous insulin decreased IR β association with CB1 receptor (Fig. 3B). Using a series of IR β deletion mutants (Fig. 3D), we determined that the CB1 receptor primarily bound to the activation loop in the tyrosine kinase domain of IR β (Fig. 3E).

Given that CB1 receptor-mediated activation in β -cell lines increased the activity of $G\alpha_i$, which mediates the inhibitory effect of CB1 receptor activation on insulin-stimulated β -cell proliferation by its association with insulin receptor (25), we examined whether $G\alpha_i$ mediates the association of IR β with CB1 receptor in β cells from wild-type mice. We found that the CB1 receptor formed a heteromeric complex with IR β and $G\alpha_{i3}$, and siRNA-mediated silencing of $G\alpha_{i3}$ reduced the association (Fig. 3F), suggesting that the CB1 receptor associates with IR β through $G\alpha_i$.

$G\alpha_i$ mediates the inhibitory effects of CB1 receptors on insulin receptor kinase activity by direct binding to IR β

Of the three subtypes of $G\alpha_i$ proteins, $G\alpha_{i1}$ and $G\alpha_{i3}$ were present mainly in β cells of both human and mouse, whereas $G\alpha_{i2}$ was distributed mainly in α cells (fig. S4A). Thus, we examined the role of $G\alpha_{i1}$ and $G\alpha_{i3}$ on CB1 receptor-mediated inhibition of insulin receptor signaling. $G\alpha_{i3}$ co-localized with insulin receptor at the cell membrane (Fig. 4A) and bound to the activation loop of IR β (Fig. 4B). Using an in vitro binding assay, we found that GST-IR β pulled down $G\alpha_{i1}$ and more IR β associated with active GTP-bound $G\alpha_{i1}$ compared with inactive GDP-bound $G\alpha_{i1}$ (Fig. 4C), indicative of a direct and specific association of $G\alpha_i$ with IR β . Because activation of CB1 receptors increased the activity of $G\alpha_i$ in β -cell lines (25), these results also suggest that $G\alpha_i$ mediates the inhibitory effect of CB1 receptor activation on insulin receptor kinase activity by its association with IR β . Indeed, autophosphorylation and kinase activity of the insulin receptor were reduced by binding of recombinant GTP-bound $G\alpha_{i1}$ in vitro (Fig. 4D). Overexpression of $G\alpha_{i3}$ led to decreased tyrosine phosphorylation of the insulin receptor in β cells from knock-out mice reconstituted with wild-type insulin receptor, but not those reconstituted with the tyrosine phosphorylation-deficient mutant (fig. S4B). Conversely, knockdown of $G\alpha_{i3}$ by siRNA increased phosphorylation of the insulin receptor, AKT, and Bad in β cells from knock-out mice reconstituted with wild-type insulin receptor (fig. S4C), and abolished the ability of ACEA to inhibit insulin-stimulated phosphorylation of IRS1/2 and AKT in β cells from wild-type mice (Fig. 4E). Moreover, knockdown of $G\alpha_{i3}$ in β cells from wild-type mice resulted in increased β -cell viability (Fig. 4F) and partially attenuated some of the inhibitory actions of ACEA (Fig. 4G).

CB1 receptor blockade improves β -cell growth and survival in streptozotocin-treated mice

We next investigated if CB1 receptor modulation might be beneficial to β -cell survival after injury. To examine the ability of β cells to regenerate after multiple injections of low-dose streptozotocin (STZ) in young adult animals, we injected the CB1 receptor antagonist AM251 beginning one day after terminating STZ treatment of 2-month-old CD1 mice (Fig. 5A). Multiple injections of low dose of STZ cause selective β -cell destruction that in turn induces immune reactions against islets and, over time, the remaining β cells attempt to survive and proliferate (40, 41). Mice treated with DMSO for 3-weeks after terminating STZ treatment (STZ-DMSO) had high blood glucose concentrations (>300 mg/dl) due to low insulin concentrations, rendering the mice overtly diabetic, whereas blood glucose and insulin concentrations in the STZ-treated counterparts given AM251 (STZ-AM251) were less affected (Fig. 5, B and C). Islet architecture in STZ-DMSO mice was also disrupted, and was accompanied by reduced insulin staining density (Fig. 5D, upper panel) and β -cell mass (Fig. 5D, lower panel) compared to non-STZ-treated mice. In contrast, the islet architecture of STZ-AM251 mice had a close to normal appearance and the β -cell mass was close to that of non-STZ-treated mice (Fig. 5D). As previously reported (42, 43), multiple injections of low dose of STZ induced caspase-3 activation in the STZ-treated mice, and the STZ-AM251 mice had less caspase-3 activity compared to their DMSO-treated counterparts (Fig. 5E). We also observed on-going β -cell proliferation in the STZ-AM251 mice, as evidenced by the increase in PCNA-positive nuclei (Fig. 5F).

We further confirmed the effects of CB1 receptor modulation on β -cell survival and proliferation by injecting low dose of STZ into *CB1R*^{-/-} mice (fig. S5A). Consistent with the results in the STZ-AM251 mice, deletion of the gene encoding the CB1 receptor also resulted in lower blood glucose and increased plasma insulin concentrations (fig. S5, B and C); it also improved islet architecture and increased insulin content and β -cell mass, resulting from enhanced β -cell survival and proliferation (fig. S5, D–F) compared to STZ-treated *CB1R*^{+/+} mice. The combination of these morphological and metabolic data suggest that normalization of blood glucose and insulin concentrations, islet architecture and β -cell mass by CB1 receptor antagonism after a diabetes-inducing injury occurs as a result of increased β -cell survival and proliferation.

CB1 receptor blockade enhances insulin receptor signaling in β cells of STZ-treated mice

We next evaluated whether the increased β -cell survival and growth seen by CB1 receptor antagonism in STZ-injected mice was associated with changes in insulin receptor signaling. Phosphorylation of the insulin receptor, IRS1/2, AKT, and FoxO1 were significantly increased in AM251-treated mice compared with DMSO-treated mice (Fig. 6, A and B), and STZ-treated *CB1R*^{-/-} mice also showed increased phosphorylation of the insulin receptor, IRS1/2, AKT, and FoxO1, compared with STZ-treated *CB1R*^{+/+} mice (fig. S6, A and B). Moreover, phosphorylation of the insulin receptor, IRS1/2, AKT, and FoxO1, as well as intra-islet insulin content, were higher in STZ-treated *CB1R*^{-/-} mice (although islet size was reduced) compared with STZ-treated *CB1R*^{+/+} mice (fig. S6C). Consistent with increased phosphorylation of AKT caused by CB1 receptor antagonism, AM251-treated mice had increased phosphorylation of Bad compared with DMSO-treated mice despite similar total abundances of Bad (Fig. 6C). The same effects were observed in STZ-treated *CB1R*^{-/-} mice (fig. S6D).

We examined the abundance and subcellular localization of p27 because AKT also regulates p27 activity by affecting both its abundance and subcellular localization through the FoxO family (12, 44) and by direct phosphorylation (14, 15). Furthermore, accumulation of p27 in the nuclei of β cells contributes to β -cell failure during the development of diabetes (19), and *p27*^{-/-} mice show reduced susceptibility to STZ-induced diabetes (45). Immunostaining

of pancreatic sections from AM251-treated mice showed a significant decrease in both the total amount and nuclear localization of p27 in β cells, and most p27 was localized in the cytoplasm (Fig. 6D). The same effects were also observed in STZ-treated *CB1R*^{-/-} mice (fig. S6E). The decrease in nuclear p27 most likely resulted from a decrease in the total abundance of p27 as well as from increased phosphorylation of p27 at Ser¹⁰ or Thr¹⁵⁷ (fig. S7, A and B). These modifications modulate p27 cytoplasmic localization and inhibit its function (14, 15), and the non-phosphorylated form of p27 accumulates predominantly in the nucleus of β cells of IRS2-null and leptin receptor-null mice, causing diabetes due to deficient β -cell mass and proliferation (19). Taken together, these results suggest that inhibition of CB1 receptor signaling promotes β -cell survival as well as proliferation following a diabetes-inducing injury by facilitating increased insulin receptor signaling.

AKT-mediated phosphorylation of FoxO1 in β cells results in the increased abundance of PDX-1 (16), a transcription factor that promotes *insulin* gene transcription and the increased abundance of glucose transporter2 (GLUT2) and glucokinase, part of the glucose-sensing machinery of β cells. The abundance of PDX-1, GLUT2 and glucokinase was increased in β cells of AM251-treated mice compared with DMSO-treated mice (Fig. 7, A–C). A similar pattern was evident in pancreatic sections from STZ-injected *CB1R*^{-/-} mice (fig. S8, A–C), suggesting that CB1 receptors could contribute to β -cell function and survival by regulating the abundance of PDX-1, GLUT2 and glucokinase (Fig. 7D).

DISCUSSION

There is growing interest in the role of endogenous cannabinoids in the regulation of cell death and survival. Their pro-apoptotic and anti-proliferative effects have been reported in various cancer cells and, at least in part, result from inhibition of the PI3K-AKT cascade (30–32). Our data suggest a model for the direct regulation of insulin receptor activity by CB1 receptors in which physical and functional crosstalk between the CB1 and insulin receptors directly influences cell survival and growth (Fig. 7D). CB1 receptors form a heteromeric complex with insulin receptor and G α_i when activated, which in turn impairs autophosphorylation and kinase activity of the insulin receptor. G α_i mediates the formation of the complex as well as the inhibitory effects of CB1 receptors on insulin receptor kinase activity by directly binding to the activation loop of the insulin receptor. This leads to reduced AKT-mediated phosphorylation of the pro-apoptotic Bad protein that in turn paves the way to cell death. We also found that WIN55,212-2 treatment not only decreased insulin receptor phosphorylation, increased cleaved caspase-3 and Bad activity, and decreased cell viability in human hepatocarcinoma HepG2 cells, but also decreased cell viability in primary human hepatocytes, suggesting that the effects of cannabinoids are not unique to pancreatic β cells.

CB1 receptor agonists have been reported to exert CB1 receptor-independent effects under certain conditions, but we believe this is unlikely in β cells because the receptor antagonist AM251 prevented the inhibitory effect of the agonist ACEA on β -cell proliferation and because siRNA directed against the CB1 receptor abolished the ability of its agonists to inhibit exogenous insulin-stimulated phosphorylation of the insulin receptor and β -cell proliferation (25). Additionally, the CB1 receptor antagonists that we tested increased the viability of β cells from wild-type mice in the nano-molar range (Fig. 1H), an effect that was prevented by ACEA (fig. S2A).

We and others have reported that endogenous cannabinoids influence insulin action by influencing insulin receptor signaling in insulin-sensitive tissues (25, 33, 34). In skeletal muscle cells, insulin-stimulated phosphorylation of AKT was impaired by endogenous cannabinoids and enhanced by CB1 receptor antagonists (33, 34), and CB1 receptors

inhibited pancreatic β -cell proliferation in an insulin receptor-dependent manner (25). We had hypothesized that the close proximity of the CB1 receptors to insulin receptors would allow their involvement in influencing insulin receptor-mediated signaling (25), because these receptors as well as $G\alpha_i$ proteins are present within lipid rafts (46–48), membrane microdomains with a distinct structural composition that appear to act as platforms for facilitating protein-protein interactions involved in intracellular signaling pathways (49). The association of the insulin receptor with the CB1 receptor was strengthened by a CB1 receptor agonist and by substitution of Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³ residues of the insulin receptor with Ala, but was attenuated by insulin, and the CB1 receptor primarily bound to the activation loop of IR β , which contains the autophosphorylation sites (Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³). The association of the insulin and CB1 receptors is likely mediated by $G\alpha_i$, because knockdown of $G\alpha_i$ by siRNA diminished the association between these two receptors. Indeed, $G\alpha_i$ co-localized with the insulin receptor at the cell membrane and directly bound to the activation loop of IR β and more IR β associated with active GTP-bound $G\alpha_i$ than with inactive GDP-bound $G\alpha_i$. This observation is consistent with our previous finding that CB1 receptor agonism increased the amount of GTP-bound $G\alpha_i$ and its association with the insulin receptor (25). Taken together, our findings are indicative of physical and functional crosstalk between CB1 receptor and IR β , with $G\alpha_i$ protein acting as an intermediary. Indeed, $G\alpha_i$ mediated the inhibitory effects of the CB1 receptor on autophosphorylation and kinase activity of the insulin receptor, as well as downstream signaling through direct binding to the activation loop of IR β . The activation loop within the tyrosine kinase domain of the insulin receptor undergoes a major conformational change upon autophosphorylation of Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³, resulting in unrestricted access of ATP and protein substrates to the kinase active site and stabilization of the conformation of the triple-phosphorylated activation loop (3). Therefore, these results further support our hypothesis that $G\alpha_i$ activated by the CB1 receptor directly associates with unphosphorylated insulin receptor at Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³, preventing a conformational change that secures the activation loop in a catalytically competent configuration upon ligand binding (25).

In mammals the absolute number of β cells reflects a dynamic balance between β -cell growth and death. An inadequate expansion of β -cell mass to compensate for increased insulin demand, followed by the eventual loss of β cells due to apoptosis, is a hallmark of diabetes mellitus. This is most apparent in type 1 diabetes mellitus when ongoing autoimmunity causes destruction and consequent loss of β cells. Although β -cell mass is highly variable in human populations, declines in β -cell mass due to increased apoptosis have also been observed in patients with type 2 diabetes mellitus (50). A β -cell threshold seems to exist below which hyperglycemia will occur (51) and obese people with diabetes mellitus have reduced β -cell mass because of increased apoptotic rates (50, 52). Insulin acting through insulin receptors is a key growth and survival factor in most mammalian cells, including β cells (16–20, 53, 54). From the data in this report, we propose that the endogenous cannabinoid system that is intrinsic to islets (25) is a pathway by which β cells could influence their own survival and growth. The amounts of endogenous cannabinoids are reported to be increased in both the circulation and in the pancreas in diabetic and obese states (21, 22, 55, 56), and, additionally, increased amounts of endogenous cannabinoid are associated with increased DAGL α (which is an endogenous cannabinoid synthetic enzyme) and decreased FAAH (which is an endogenous cannabinoid degrading enzyme) amounts in β cells (22). Thus, by impeding insulin receptor autophosphorylation in insulin-sensitive tissues in type 2 diabetes, increased endogenous cannabinoid 'tone' within islets (due to increased endogenous cannabinoid synthesis, reduced degradation, or receptor abundance or activity) likely contributes to the lack of glucose responsiveness of β cells and the development of insulin resistance. Indeed, pharmacologic blockade of the CB1 receptor in obese *fa/fa* Zucker rats (57) and *db/db* mice (25) lead to decreased blood glucose

concentrations and preserved β -cell mass. We showed in our previous report (25) that pharmacological blockade and genetic deficiency of the CB1 receptor in normal mice leads to decreased blood glucose concentrations and increased β -cell mass due to enhanced insulin receptor signaling. This is also true in STZ-treated mice. Thus, blockade of the CB1 receptor may have effects also in type 1 diabetes mellitus. These data might suggest that in animal models of both type 1 and type 2 diabetes mellitus, endogenous cannabinoid tone is increased in β cells and contributes to decrease β -cell mass. However, it was previously observed that, unlike the pancreas of obese *fa/fa* Zucker rats and DIO mice (22, 55), no alteration of endogenous cannabinoid amounts and CB1 receptors occurs in the pancreas of STZ-treated mice (22).

Direct follow-up of this work could include generation of a specific β -cell CB1 receptor-null mouse. The predicted phenotype would be a mouse whose β cells would be more resistant to apoptosis. Mice that globally lack the CB1 receptor are more resistant to β -cell apoptosis and have larger islets (25). Another potential follow-up would be development of a peripherally-acting CB1 receptor antagonist with poor brain penetrance to lessen psychiatric side effects that are potentially life-threatening. It might be useful therapies in type 1 and 2 diabetes mellitus to influence insulin receptor activity and β -cell function in the remaining β cells.

MATERIALS AND METHODS

Materials and Reagents

Sources and dilutions of primary antibodies used in western blotting, immunoprecipitation and immunostaining are listed in Table S1. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), WIN55,212-2, arachidonyl-2-chloroethylamide (ACEA), and AM251 were obtained from Cayman chemical. Rimobabant and SLV-319 were obtained from Dr. John F. McElroy (Jenrin Discovery). The caspase-3 and -7 inhibitor Ac-DNLD-CHO was from Calbiochem. Recombinant IR β (amino acids 941–1343) fused to GST, recombinant IRS1 consisting of the p30 fragment (IRS1-p30, amino acids 516–777), and recombinant G α_{i1} were obtained from Calbiochem. Insulin and streptozotocin (STZ) were from Sigma. The human insulin receptor and G α_{i3} cDNA were amplified by RT-PCR from a human pancreas total RNA (stratagene), with oligo-dT (18bp) for the reverse transcription. The insulin receptor cDNA was incorporated into a mCerulean-N1 vector between 5'-HindIII and 3'-AgeI sites for IR-cerulean with the cerulean epitope at its C-terminus. The cerulean epitope of IR-cerulean was then replaced with a 3 $\times$ Flag epitope between the 5'-AgeI and 3'-BsrGI sites to make Flag-tagged IR. The Flag-tagged insulin receptor mutant (IR-3YA) was generated from wild-type insulin receptor (IR-WT) using a Quikchange II XL site-directed mutagenesis kit (stratagene). Three tyrosine residues at positions 1158, 1162 and 1163 of the wild-type insulin receptor were replaced with alanines. IR β deletion mutants were amplified from Cerulean-insulin receptor and cloned into mVenus-C1 vector. The G α_{i3} cDNA was incorporated into an mVenus-C1 vector between 5'-XhoI and 3'-EcoRI sites for Venus-G α_{i3} with the Venus epitope at its N-terminus.

Cell culture, transfection, and cell viability assays

Wild-type and insulin receptor-deficient β cells were established from control and β -cell-specific insulin receptor knockout mice, respectively (17, 25, 36, 37). All β -cell line and α TC1 cells were maintained in DMEM with 10% FBS (Invitrogen). Transfections of siRNAs (Santa Cruz) for G α_{i3} and Bad and the expression vectors for G α_{i3} and insulin receptor were carried out using Lipofectamine RNAiMAX or 2000 (Invitrogen). Scramble siRNA (Silencer Negative Control #1; Ambion) or empty vector was transfected as negative control. For exogenous insulin treatment, cells starved overnight in DMEM containing 2

mM glucose and 0.1% FBS were pre-treated with CB1 receptor agonists for 15 min before insulin stimulation for 10 min with or without CB1 receptor agonists. For cell viability and cytotoxicity studies, cells were plated into 96-well plates and incubated for 2 days with complete media. The viability and cytotoxicity of the cells were determined 48 hours after treatment with CB1 receptor agonists or antagonists in DMEM containing 0.1% FBS using the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega), according to the manufacturer's instructions. To test the knockdown effects of Bad or Gα_{i1} on the cell viability, β cells from wild-type mice transfected with the indicated siRNAs for 24 hours were treated with WIN55,212-2 in the media containing 0.1% FBS.

RNA isolation and quantitative real-time PCR (qRT-PCR)

We prepared total RNA from isolated islets or cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. After reverse transcription, the resulting materials were used for qRT-PCR amplification using gene-specific primer pairs and SYBR Green PCR master mix (Applied Biosystems).

In vitro binding Assay

Recombinant Gα_{i1} (2 μg) was incubated with GTPγS or GDP for 90 min before incubation with 2 μg of GST or GST-IRβ and glutathione-Sepharose 4B beads (Amersham Pharmacia) in binding buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA) containing protease and phosphatase inhibitor cocktails, after which the beads were extensively washed in the same buffer, and the adsorbed proteins were subjected to Western blot analysis with the primary antibodies and with an HRP-conjugated secondary antibody. Blots were visualized by ECL (GE Health).

Immunoprecipitation

Cell lysates extracted using RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA) containing protease and phosphatase inhibitor cocktails were incubated with the appropriate antibody overnight at 4 °C and subsequently incubated with protein A/G beads for 3 hours. Beads were washed 3 times with RIPA buffer and subjected to western blot analysis. Normal IgG was used as negative control. For domain-mapping studies, lysates of β cells from knockout mice transfected with IRβ deletion mutants were subjected to immunoprecipitation with normal rabbit IgG and anti-CB1 receptor or Gα_{i3} antibodies and Western blot analysis with anti-GFP antibody.

In vitro kinase assay

For Fig. 2B, IRβ immune complexes from β cells from wild-type mice exposed to insulin with ACEA or vehicle were washed two times with RIPA buffer and once with tyrosine kinase buffer (50 mM HEPES at pH 7.4, 20 mM MgCl₂, 0.1 mM MnCl₂, and protease and phosphatase inhibitors) and then resuspended in tyrosine kinase buffer containing 25 μM ATP and IRS1-p30 (2 μg). The reaction mixtures were incubated for 30 min at 30°C, after which the reaction was terminated by the addition of SDS sample buffer. Samples were subjected to Western blot analysis. For Fig. 4D, IRβ immune complexes from β cells from knockout mice reconstituted with wild-type or the tyrosine phosphorylation deficient mutant were washed and incubated in tyrosine kinase buffer containing 25 μM ATP, IRS1-p30 (2 μg), and GTP-bound Gα_{i1} (2 μg) for 30 min at 30°C. Recombinant Gα_{i1} was incubated with GTPγS for 90 min before addition.

Immunofluorescence

To detect caspase-3 activation in αTC1 and β-cell lines, the cells were treated with WIN55,212-2, fixed, and incubated with anti-cleaved caspase-3 antibody, followed by

secondary antibodies (Invitrogen) along with TO-PRO-3 (Invitrogen) for nuclear staining. To detect endogenous IR β and G α_{i3} in wild-type β cells, cells were incubated with anti-IR β and anti-G α_{i3} antibodies, followed by secondary antibodies. Images were viewed using a LSM-710 confocal microscope (Carl Zeiss MicroImaging).

Animal experiments

All animal care and experimental procedures followed US National Institutes of Health guidelines and were approved by the US National Institute on Aging Animal Care and Use Committee. *CB1R*^{-/-} mice and their wild-type littermates were developed and backcrossed to a C57Bl/6J background, as previously described (39). For regeneration experiments, low-dose (50 mg/kg) of STZ were administered by daily intraperitoneal (i.p.) injection into 2-month-old CD1 or *CB1R*^{-/-} and *CB1R*^{+/+} mice (n=5 per group) for 5 days. DMSO or AM251 (10 mg/kg) were then administered into CD1 mice by daily i.p. injection without STZ. Three weeks after STZ withdrawal, pancreata and plasma were collected for the metabolic and morphological analyses. Blood glucose concentration was measured from tail-vein blood using a glucometer (Elite, Bayer Inc.) and plasma insulin was measured using rat/mouse insulin ELISA kit (Crystal Chem). Pancreata were rapidly dissected, fixed in 4% paraformaldehyde (Sigma), immersed in 20% sucrose before freezing, and then sectioned at a thickness of 7 μ m. After antigen unmasking, the slides were blocked with 5% BSA/PBS and incubated at 4°C with a specific primary antibody, followed by secondary antibodies along with TO-PRO-3, in some cases, for nuclear staining. Slides were viewed using a LSM-710 confocal microscope. Signal intensity and the number of nuclear p27- or PCNA-positive β cells were assessed using LSM Image Browser software (Carl Zeiss) or ImageJ software (<http://rsb.info.nih.gov/ij/>). For the analysis of β -cell and total pancreas area, digital images of multiple sections from 3–5 mice per group, separated by at least 200 μ m from each section, at a magnification of $\times 10$ were obtained and the cross-sectional areas of pancreata and β cells (insulin-positive cells) were determined using LSM Image Browser software (Carl Zeiss). The relative cross-sectional area of β cells was determined by quantification of the cross-sectional area covered by insulin-positive cells divided by the cross-sectional area of total pancreas tissue. The number of cells that are positive for both insulin and nuclear p27 or PCNA were quantified as a percentage of the total number of insulin-positive cells in the sections.

Statistical analysis

Quantitative data are presented as the mean $\pm$ SEM. Differences between mean values were compared statistically by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc comparison. Comparisons were performed by using Graphpad Prism or SAS ver 9.1. A *p* value of < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are deeply grateful Dr. J. Pickel, NIMH Transgenic Core/NIH, provided the *CB1R*^{-/-} mice and the animal facilities of NIA/NIH carried out the genotyping and husbandry. We also thank Dr. John F. McElroy for providing reagents.

Funding: This work was supported by the Intramural Research Program of the National Institute on Aging (NIA)/NIH. E.K.L. is supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (20110013116) and the Catholic Medical Center Research Foundation. R.N.K. is supported by NIH RO1 DK 67536 and 68721.

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FIG. 1. Activation of CB1 receptors induces caspase-3-mediated β -cell death in an insulin receptor-dependent manner

(A and B) Relative cell viability (A) and cytotoxicity (B) of αTC1 and MIN6 cells exposed to CB1 receptor agonists (WIN55,212-2, AEA or 2-AG) in serum-free media ($n=3$ independent experiments). (C) Activation of caspase-3 by WIN55,212-2 in MIN6 and αTC1 cells. αTC1 and MIN6 cells exposed to WIN55,212-2 in serum-free media were subjected to immunostaining with anti-cleaved caspase-3 antibody. Scale bar, 50 μm . (D) Effect of the caspase-3 inhibitor Ac-DNLD-CHO on WIN55,212-2-induced decrease in MIN6 cell viability ($n=3$ independent experiments). (E) Activation of caspase-3 by WIN55,212-2 in β cells from wild-type mice (βIRWT) and or from mice with a β -cell-specific knockout of the

insulin receptor (β IRKO) was assessed by immunostaining with anti-cleaved caspase-3 antibody. Scale bar, 50 μ m. (F) Relative cell viability of β IRWT and β IRKO cells exposed to WIN55,212-2 in serum-free media (n=3 independent experiments). (G) Abundance of the indicated proteins in β IRWT and β IRKO cells exposed to WIN55,212-2. Relative densities for the indicated proteins are shown on the bottom (n=3 independent experiments). p, phosphorylated. (H) Relative cell viability of β IRWT and β IRKO cells exposed to the CB1 receptor (CB1R) antagonists AM251, rimonabant, or SLV-319 (n=3 independent experiments). Data represent the mean $\pm$ SEM. * P < 0.05; ** P < 0.01; n.s., not significant.

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FIG. 2. Regulation of insulin-stimulated insulin receptor kinase activity and Bad phosphorylation by CB1 receptors in β cells

(A) Abundance of the indicated proteins in MIN6 cells exposed to insulin with ACEA or vehicle. Relative densities for the indicated proteins are shown on the right ($n=3$ independent experiments). (B) In vitro kinase assay using $IR\beta$ immunoprecipitated from $\beta IRWT$ cells exposed to insulin with ACEA or vehicle. Blots are representative of two independent experiments. (C) Regulation of Bad function by AKT. (D) Regulation of insulin-induced Bad interaction with Bcl-xL or 14-3-3 by ACEA in $\beta IRWT$ cells. Relative densities for the indicated proteins are shown on the right ($n=3$ independent experiments). (E) Abundance of the indicated proteins in $\beta IRKO$ cells exposed to ACEA after

reconstitution with wild-type insulin receptor (IR-WT) or a mutant with Ala substitutions for Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³ (IR-3YA) (n=3 independent experiments). (F) Bad abundance after siRNA transfection in β IRWT cells. (G) Effects of WIN55,212-2 on caspase-3 activation in β IRWT cells transfected with scrambled or Bad siRNA (n=3 independent experiments). (H) Comparison of the viability of β IRWT cells transfected with scrambled or Bad siRNA (n=3 independent experiments). (I) Effects of WIN55,212-2 on the viability of β IRWT cells transfected with the indicated siRNAs (n=3 independent experiments). Data represent the mean $\pm$ SEM. * P < 0.05; ** P < 0.01; n.s., not significant.

FIG. 3. CB1 receptors interact with IRβ and Gα_i

(A) Interaction of endogenous CB1 receptor with IRβ in βIRWT cells exposed to ACEA or vehicle. Relative density for IRβ is shown on the right (n=3 independent experiments). (B) Interaction of endogenous CB1 receptor with IRβ in βIRWT cells exposed to insulin with or without ACEA. Relative density for IRβ is shown on the right (n=3 independent experiments). (C) Increased interaction of the CB1 receptor with IRβ by substitution of Tyr¹¹⁵⁸, Tyr¹¹⁶², and Tyr¹¹⁶³ with Ala. βIRKO cells transfected with empty vector, IR-WT or IR-3YA were exposed to ACEA and subjected to co-immunoprecipitation assay. Relative density for IRβ is shown on the bottom (n=2 independent experiments). (D) Schematic representation of Venus-tagged IRβ deletion mutants. (E) Domain-mapping studies in βIRKO cells transfected with Venus-IRβ deletion mutants. (F) Role of Gα_i in the CB1 receptor-IRβ interaction. βIRKO cells transfected with scrambled or Gα_{i3} siRNA were reconstituted with IR-WT and subjected to co-immunoprecipitation with anti-CB1 receptor antibody. Blots are representative of two independent experiments. Data represent the mean ± SEM. *P < 0.05.

FIG. 4. $G\alpha_i$ inhibits insulin receptor kinase activity by direct insulin receptor binding (A) Co-localization of $IR\beta$ and $G\alpha_{i3}$ in β IRWT cells. Scale bar, 20 μ m. (B) Interaction of $G\alpha_{i3}$ with Venus- $IR\beta$ -1 or -3 in β IRKO cells. (C) In vitro binding assay carried out with GST- $IR\beta$ and GDP- or GTP-bound $G\alpha_{i1}$. Blots are representative of two independent experiments. (D) Effects of recombinant $G\alpha_{i1}$ on the insulin receptor kinase activity. Relative densities for the indicated proteins are shown on the bottom ($n=2$ independent experiments). (E) Phosphorylation of IRS1/2 and AKT in β IRWT cells exposed to insulin with ACEA or vehicle after transfection of the indicated siRNAs. Relative densities for the indicated proteins are shown on the bottom ($n=3$ independent experiments). (F) Comparison of the viability between β IRWT cells transfected with scrambled or $G\alpha_{i3}$ siRNA ($n=3$ independent experiments). (G) Effects of WIN55,212-2 on the viability of β IRWT cells transfected with the indicated siRNAs ($n=3$ independent experiments). Data represent the mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; n.s., not significant.

FIG. 5. AM251, a CB1 receptor antagonist, improves β -cell mass in STZ-treated mice because of enhanced β -cell survival

(A) Experimental timeline for streptozotocin (STZ, 50 mg/kg) and AM251 (10 mg/kg) treatment in two-month-old wild-type CD1 mice. (B and C) Random blood glucose (B) and plasma insulin (C) concentrations of DMSO- and AM251-injected mice ($n=5$ animals per group) after STZ treatment. (D) Representative images for insulin in islets of DMSO- and AM251-injected mice after STZ treatment. Scale bar, 200 μ m. The fraction of pancreatic tissue area covered by β cells is shown on the bottom ($n=3-5$ animals per group). (E) Representative images for cleaved caspase-3 in islets of control and STZ-treated mice of cohorts in (D). Scale bar, 50 μ m. Relative signal intensity for cleaved caspase-3 in islets is

shown on the bottom (n=3 animals per group). (F) Representative images for PCNA-positive β cells of STZ-treated mice of cohorts in (D). Arrows denote PCNA-positive cells. Scale bar, 50 μ m. Quantification of PCNA-positive β cells is shown on the right (n=3 animals per group). Data represent the mean $\pm$ SEM. * P < 0.05; ** P < 0.01.

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FIG. 6. AM251 enhances insulin receptor signaling in β cells of STZ-treated mice

(A) Representative images for insulin receptor and phosphorylated insulin receptor in islets of DMSO- and AM251-injected mice after STZ treatment. Scale bar, 50 μ m. Relative signal intensity for phosphorylated insulin receptor in islets is shown on the right (n=3 animals per group). (B) Representative images for p-IRS1/2, p-AKT and p-FoxO1 in islets of cohorts in (A). Scale bar, 50 μ m. Relative signal intensity for the indicated proteins in islets is shown on the right (n=3 animals per group). (C) Representative images for Bad and p-Bad in islets of cohorts in (A). Scale bar, 50 μ m. Relative signal intensity for p-Bad in islets is shown on the right (n=3–5 animals per group). (D) Representative images for insulin and p27 in islets of cohorts in (A). Boxed areas were magnified, shown on the last panel, for better

visualization. Scale bar, 50 μm . Relative p27 intensity and percentage of nuclear p27-positive β cells in islets are shown on the bottom (n=3–5 animals per group). Data represent the mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

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FIG. 7. Increased abundance of PDX-1, GLUT2, and glucokinase in β cells of AM251-injected mice after STZ treatment

(A–C) Representative images for PDX-1 (right-handed images are $\times 4$ magnifications) (A), GLUT2 (B) and glucokinase (C) in islets of cohorts in (A). Scale bar, 50 μm . Relative signal intensities in islets are shown on the bottom of each image. Data represent the mean $\pm$ SEM from $n=3-5$ animals per group. * $P < 0.05$; ** $P < 0.01$. (D) Schematic unifying the regulation of insulin receptor signaling by endogenous cannabinoids upon cell growth and survival. Binding of endogenous cannabinoids (ECs) to CB1 receptors activates the $G\alpha_i$ class of heterotrimeric proteins and increases the association between $G\alpha_i$ and the insulin receptor that counteracts the effects of insulin on autophosphorylation and kinase activity of

the insulin receptor, resulting in decreased cell growth and survival. In pancreatic β cells, CB1 receptor signaling through $G\alpha_i$ leads to decreased PDX-1, GLUT2 and glucokinase abundance.