

Supplementary Materials for

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

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Published 20 March 2012, *Sci. Signal.* **5**, ra23 (2012)
DOI: 10.1126/scisignal.2002519

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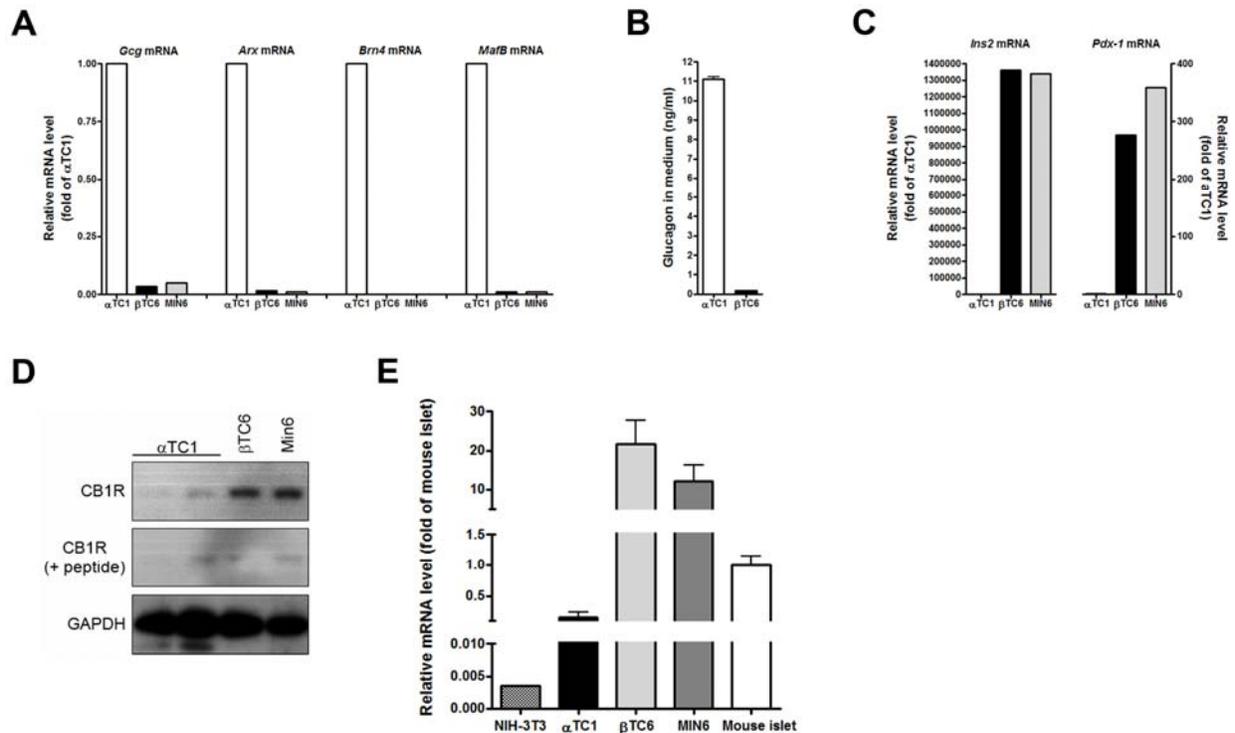


Fig. S1: CB1 receptor abundance in pancreatic β cell lines. (A) qRT-PCR analysis for *glucagon* (*Gcg*), *Arx*, *Brn4*, and *MafB* mRNA in mouse glucagonoma α TC1 cells and in mouse insulinoma β TC6 and MIN6 cells. All mRNA amounts were normalized to *18S* rRNA. Pancreatic α cells secrete glucagon and *Arx*, *Brn4* and *MafB* are the transcription factors implicated in pancreatic α cell fate specification. (B) Glucagon secretion from α TC1 and β TC6 cells. n=3 independent experiments. (C) qRT-PCR analysis for *insulin* (*Ins2*) and *Pdx-1* mRNA in α TC1, β TC6 and MIN6 cells. All mRNA amounts were normalized to *18S* rRNA. Pancreatic β cells secrete insulin and PDX-1 is a transcription factor that is involved in β -cell development and maturation, proliferation and augmentation of *insulin* gene transcription. (D) Western blot analysis for CB1 receptor in α TC1, MIN6, and β TC6 cells. The specificity of anti-CB1 receptor antibody was assessed by antigen pre-absorption with the corresponding blocking peptides. (E) qRT-PCR for *CB1R* mRNA in NIH-3T3, α TC1, β TC6, and MIN6 cells, and mouse islet. *CB1R* mRNA was normalized to *GAPDH* levels and NIH-3T3 cells were used as negative control. n=3 independent experiments. Data represent the mean \pm SEM.

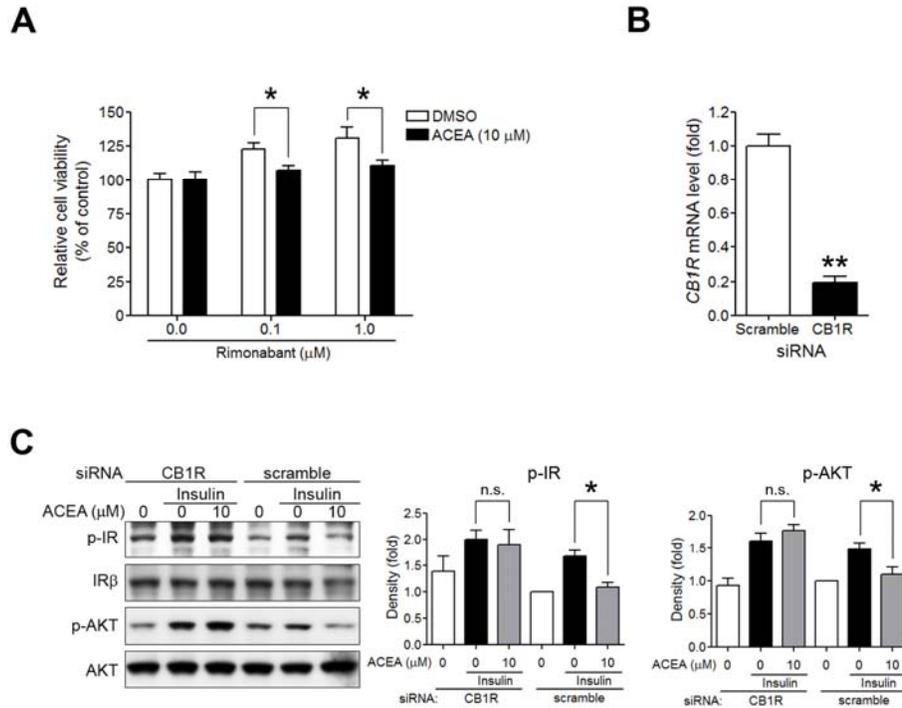


Fig. S2: Effects of ACEA depend on the CB1 receptor. (A) Cell viability of β IRWT cells exposed to rimonabant with or without ACEA. Data represent the mean \pm SEM from three independent experiments. $*P < 0.05$. (B) *CB1R* mRNA amounts in β IRWT cells transfected with scrambled or CB1R siRNAs. *CB1R* mRNA amounts were determined by qRT-PCR and normalized to *18S* mRNA. Data represent the mean \pm SEM from three independent experiments. $**P < 0.01$. (C) Phosphorylation of the insulin receptor and AKT in β IRWT cells exposed to insulin (10 nM) with ACEA or vehicle after transfection of the indicated siRNAs. Relative density for the indicated proteins is shown on the right. Data represent the mean \pm SEM from three independent experiments. $*P < 0.05$; n.s., not significant.

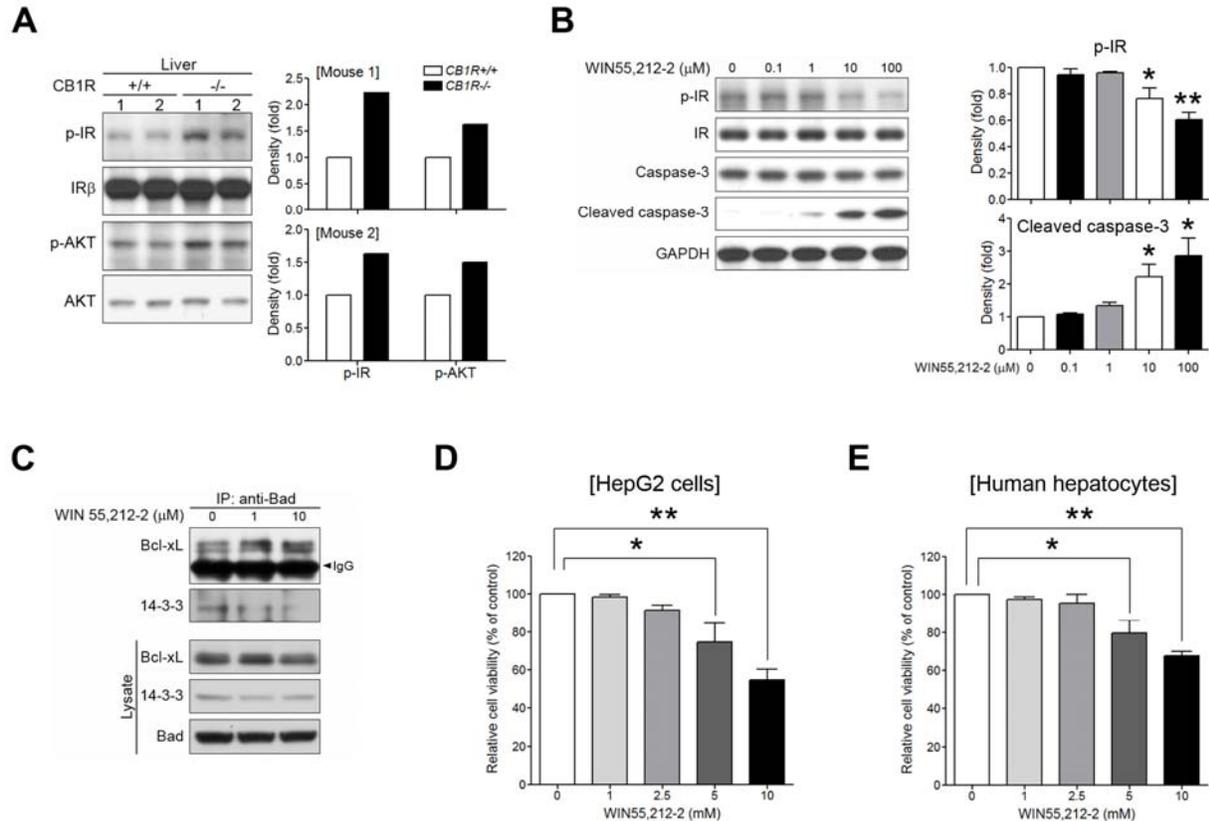
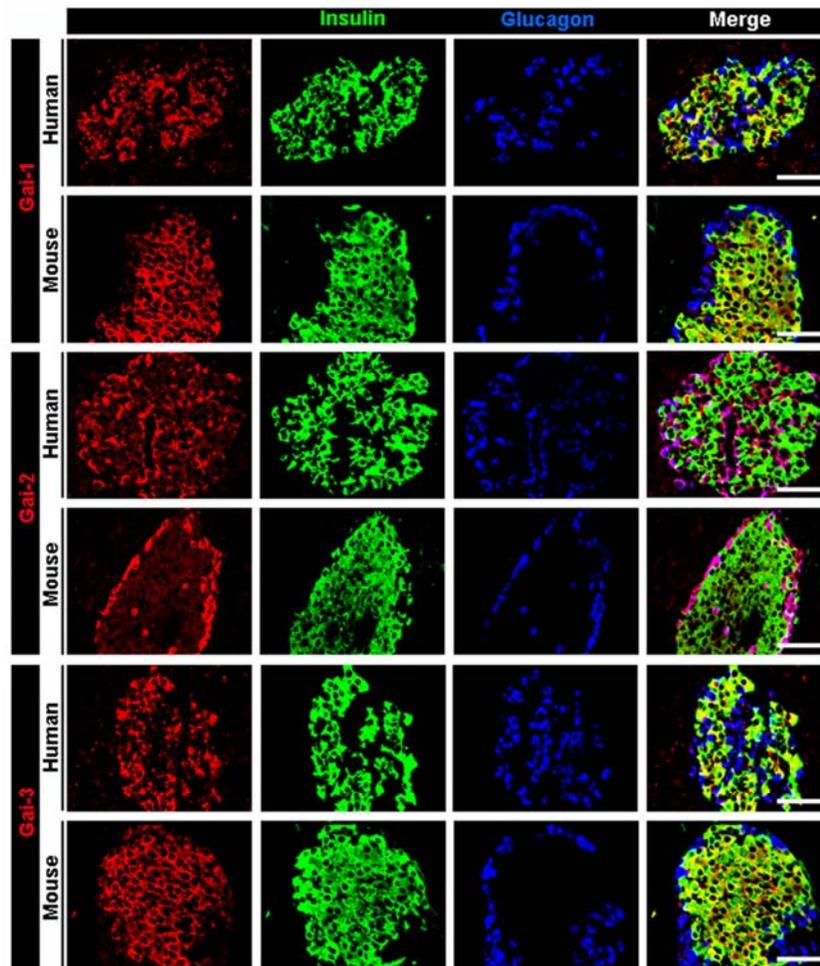
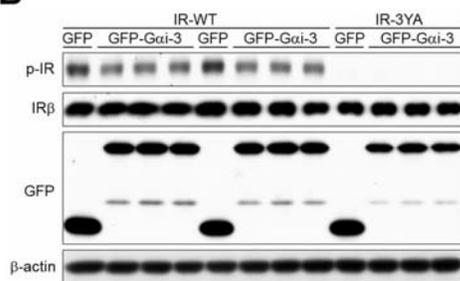


Fig. S3: Effects of WIN55,212-2 on human hepatocarcinoma HepG2 cells and primary human hepatocytes. (A) Western blot analysis of the indicated proteins in livers from *CB1R*^{+/+} and *CB1R*^{-/-} mice (n=2 animals per genotype). (B) Western blot analysis of the indicated proteins in HepG2 cells exposed to WIN55,212-2. Relative density for the indicated proteins is shown on the right. Data represent the mean \pm SEM from three independent experiments. **P* < 0.05; ***P* < 0.01. (C) Increase in the association of Bcl-xL with Bad by WIN55,212-2 in HepG2 cells. HepG2 cells exposed to WIN55,212-2 were subjected to immunoprecipitation with anti-Bad antibody and to Western blot analysis with anti-Bcl-xL or anti-14-3-3 antibody. Blots are representative of two independent experiments. (D and E) Relative cell viability of HepG2 cells (D) and primary human hepatocytes (E) exposed to WIN55,212-2 in serum-free media. Primary human hepatocytes were purchased from Invitrogen. Data represent the mean \pm SEM from three independent experiments. **P* < 0.05; ***P* < 0.01.

A



B



C

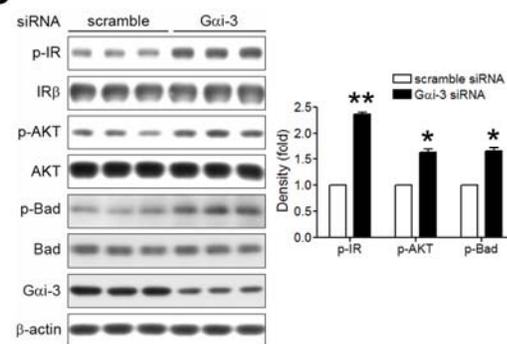


Fig. S4: Effects of $G\alpha_{i3}$ on insulin receptor signaling. (A) Immunostaining for $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ in human and mouse islets. Scale bar, 50 μm . Images are representative of least 20 islets. (B) Effects of $G\alpha_{i3}$ overexpression on phosphorylation of the insulin receptor in β IRKO cells reconstituted with IR-WT or IR-3YA. (C) Phosphorylation of the insulin receptor, AKT and Bad in β IRWT cells transfected with the indicated siRNAs. Relative densities for the indicated

proteins are shown on the right. Data represent the mean \pm SEM from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

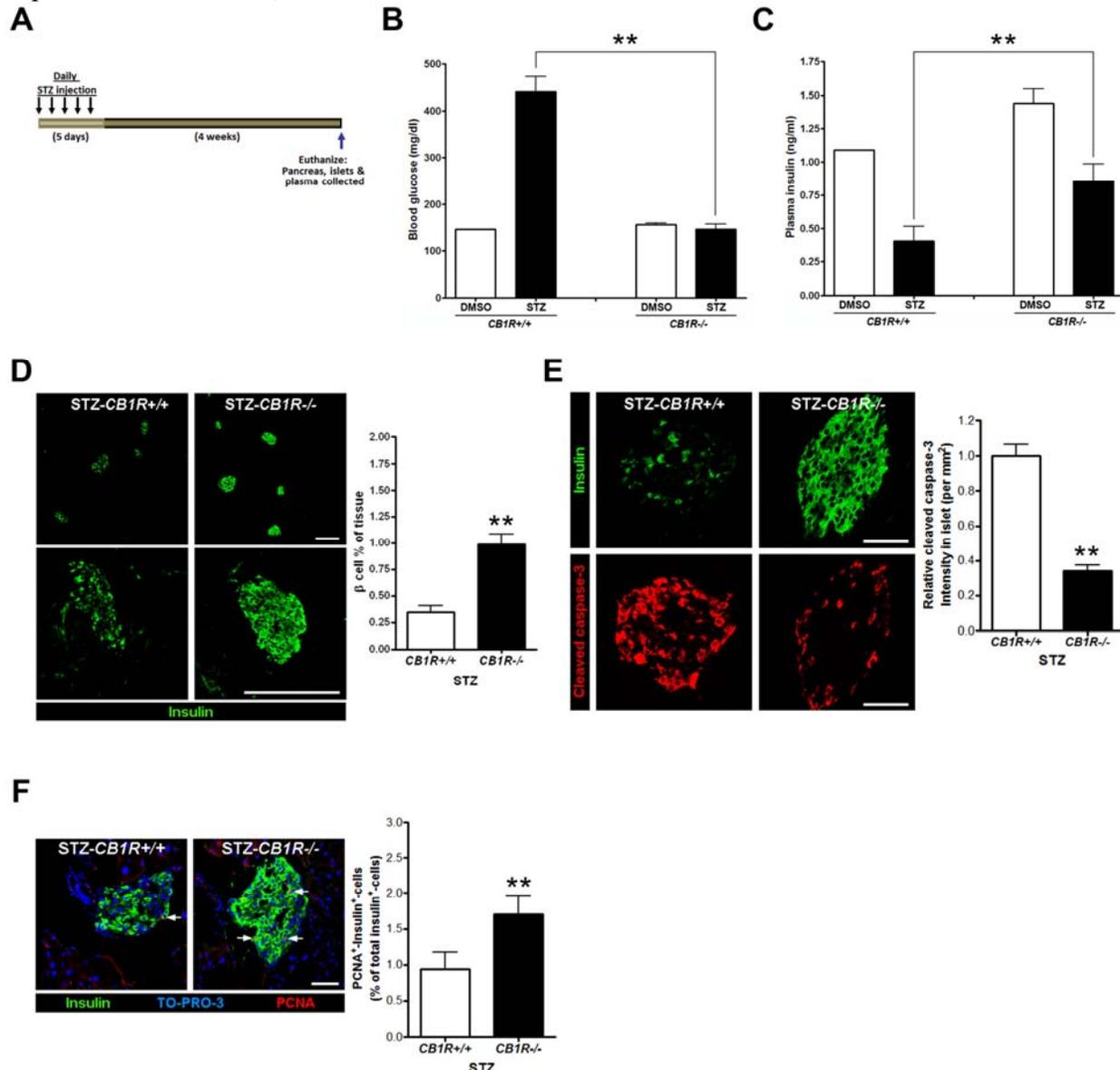


Fig. S5: Improved β cell mass due to enhanced β cell survival in STZ-treated $CB1R^{-/-}$ mice. (A) Experimental timeline of the study: STZ (50 mg/kg), streptozotocin. (B and C) Random blood glucose (B) and plasma insulin (C) concentrations of $CB1R^{+/+}$ (n=5) and $CB1R^{-/-}$ (n=5) mice 4 weeks after STZ treatment. (D) Representative images for insulin in pancreatic sections from $CB1R^{+/+}$ and $CB1R^{-/-}$ mice 4 weeks after STZ treatment. Scale bar, 200 μ m. The fraction of pancreas tissue area covered by β cells is shown on the right (n=3-5 animals per genotype). (E) Representative images for cleaved caspase-3 in islets of STZ-treated $CB1R^{+/+}$ and $CB1R^{-/-}$ mice of cohorts in (D). Scale bar, 50 μ m. Relative signal intensity for cleaved caspase-3 in islets is shown on the right (n=3 mice per genotype). (F) Representative images for PCNA-positive β cells of STZ-treated $CB1R^{+/+}$ and $CB1R^{-/-}$ 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=3-5 animals per genotype). Data represent the mean \pm SEM. ** $P < 0.01$.

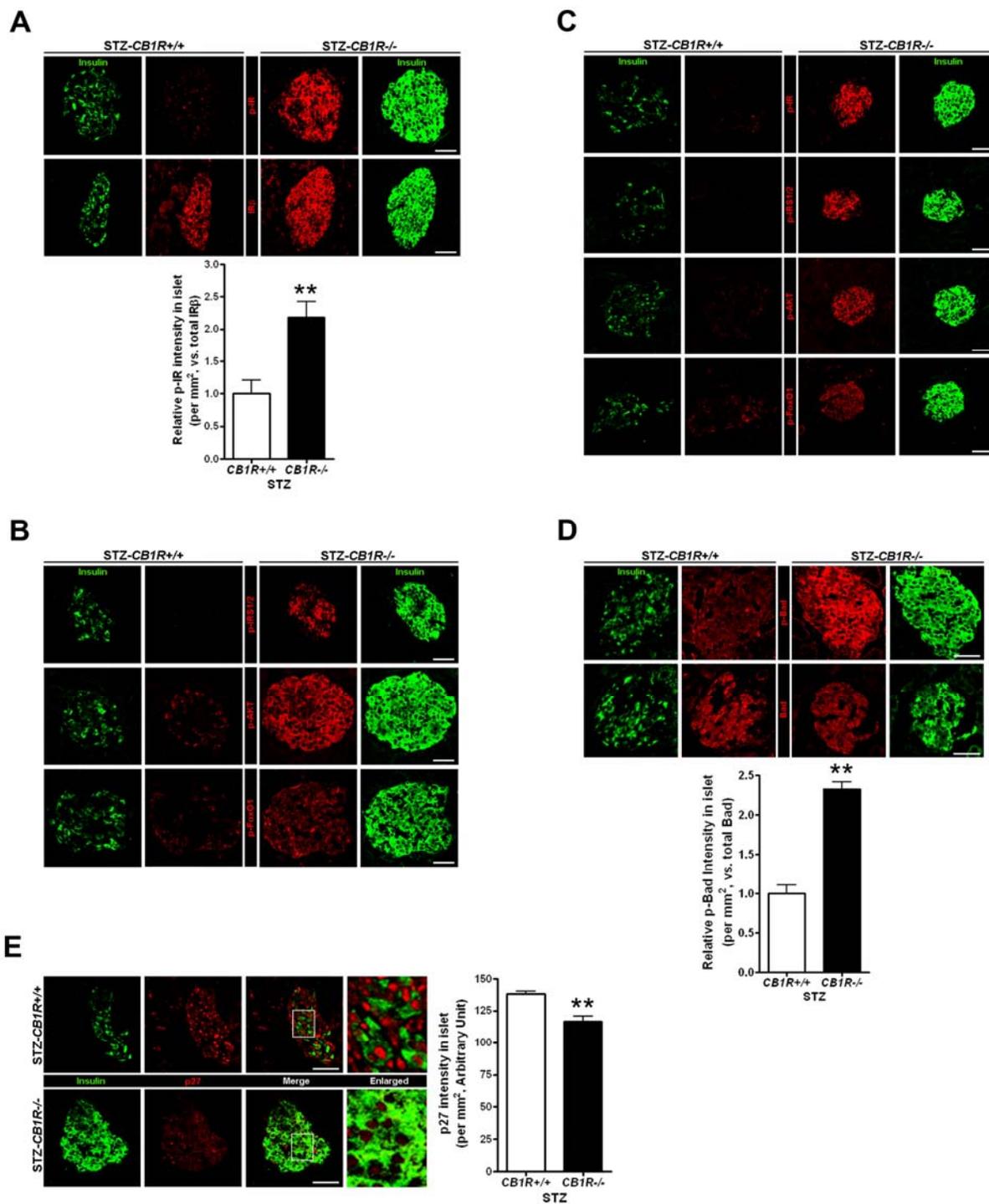


Fig. S6: Enhanced insulin signaling in β cells of STZ-treated $CB1R^{-/-}$ mice. (A) Representative images for insulin receptor and phosphorylated insulin receptor in islets of $CB1R^{+/+}$ and $CB1R^{-/-}$ mice 4 weeks after STZ treatment. Scale bar, 50 μ m. Relative signal intensity for phosphorylated insulin receptor in islets is shown on the bottom ($n=3$ animals per genotype). (B) Representative images for phosphorylated IRS1/2, AKT, and FoxO1 in islets of cohorts in (A). Scale bar, 50 μ m. Images are representative of least 20 islets from three animals

per genotype. (C) Representative images for phosphorylated IR, IRS1/2, AKT, and FoxO1 in smaller islets of STZ-treated *CB1R*^{-/-} mice compared with those of STZ-treated *CB1R*^{+/+} mice. Scale bar, 50 μ m. Images are representative of least 20 islets from three animals per genotype. (D) Representative images for Bad and phosphorylated Bad in islets of cohorts in (A). Scale bar, 50 μ m. Relative signal intensity for phosphorylated Bad in islets is shown on the bottom (n=3-5 animals per genotype). (E) Representative images for insulin and p27 in islets of cohorts in (A). Boxed areas were magnified, shown on the last panel, for better visualization. Relative p27 intensity in islets is shown on the right (n=4 animals per genotype). Scale bar, 50 μ m. Data represent the mean \pm SEM. ***P* < 0.01.

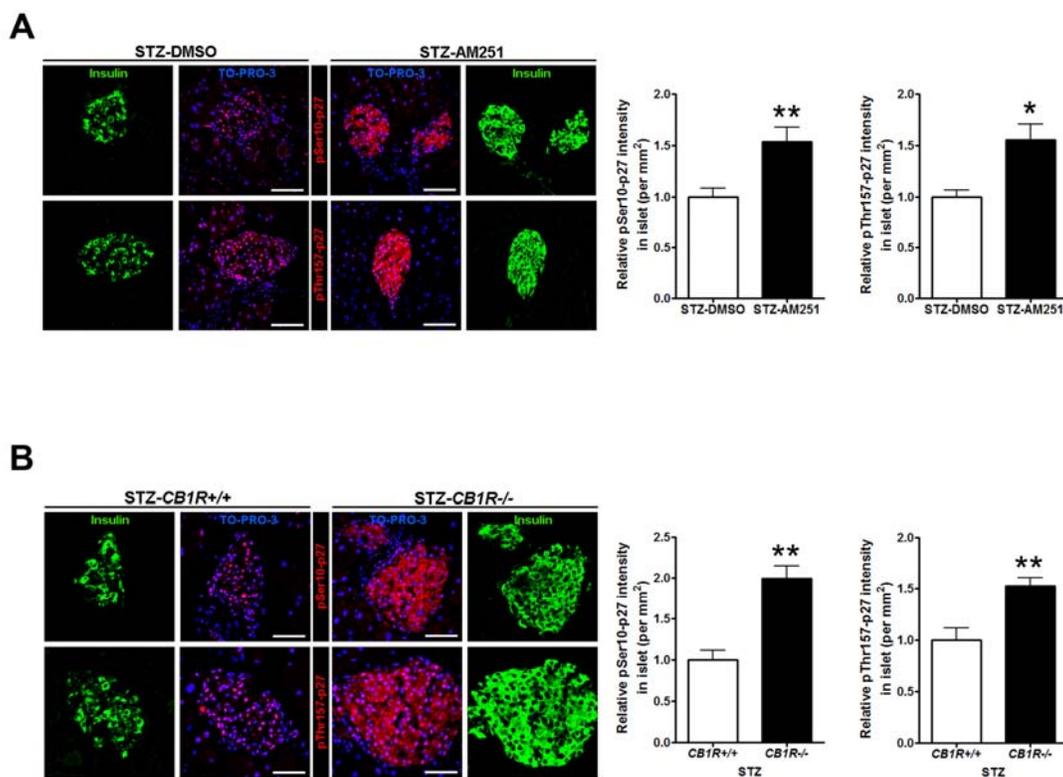


Fig. S7: Increased phosphorylation of p27 at Ser¹⁰ and Thr¹⁵⁷ in islets of STZ-treated mice by CB1 receptor blockade. (A) Representative images for p27 phosphorylated at Ser¹⁰ and Thr¹⁵⁷ in islets of DMSO- and AM251-injected mice after STZ treatment. Scale bar, 50 μ m. Relative signal intensities in islets are shown on the right (n=3-4 animals per genotype). (B) Representative images for p27 phosphorylated at Ser¹⁰ and Thr¹⁵⁷ in islets of STZ-treated *CB1R*^{+/+} and *CB1R*^{-/-} mice. Scale bar, 50 μ m. Relative signal intensities in islets are shown on the right (n=4-5 animals per genotype). Data represent the mean \pm SEM. **P* < 0.05; ***P* < 0.01.

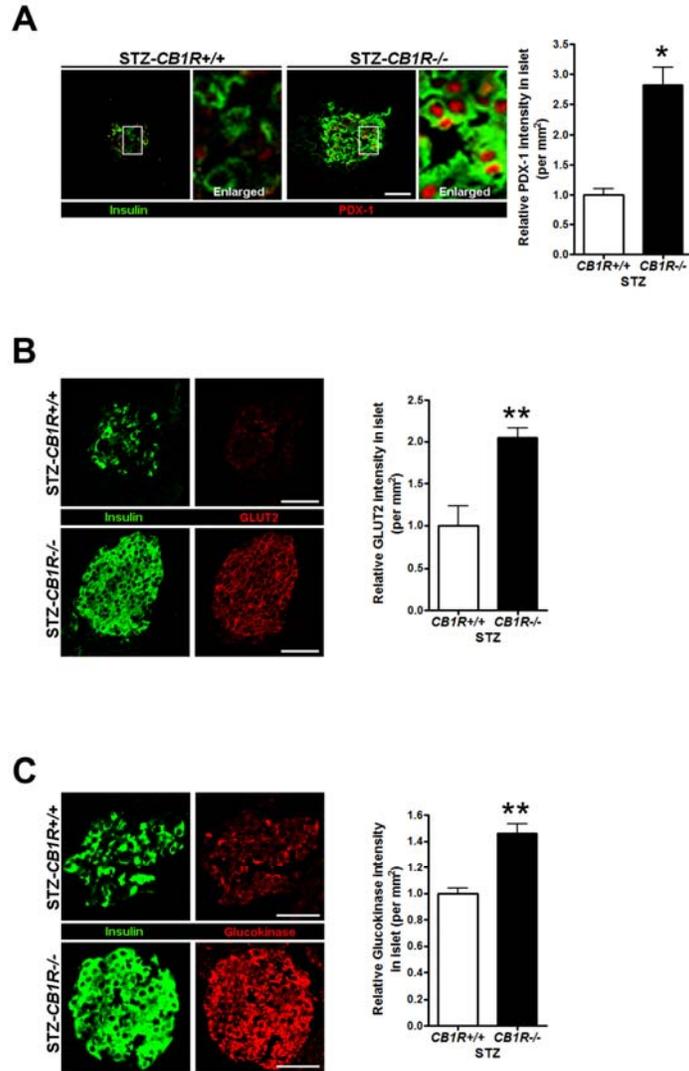


Fig. S8: Increased abundance of PDX-1, GLUT2, and glucokinase in β cells of STZ-treated $CB1R^{-/-}$ mice. (A to C) Representative images for PDX-1 (right-handed images are x4 magnifications) (A), GLUT2 (B) and glucokinase (C) in islets of STZ-treated $CB1R^{+/+}$ and $CB1R^{-/-}$ mice. Scale bar, 50 μ m. Relative signal intensities in islets are shown on the right of each image. Data represent the mean \pm SEM from n=3-5 animals per genotype. * $P < 0.05$; ** $P < 0.01$.

Marker	Host	Dilution		Source
		WB* (IP**)	IF [#]	
CB1R	rabbit	1:1000	1:100	Frontier Science Santa Cruz
	rabbit	(1:250 [$\mu\text{g}:\mu\text{g}$ protein])		
Insulin	guinea pig		1:500	Millipore/Linco Sigma
	mouse		1:300	
Glucagon	guinea pig		1:2000	Millipore/Linco Sigma Millipore/Linco
	mouse		1:500	
	rabbit		1:500	
IR β	rabbit	1:1000	1:50	Santa Cruz
p-IR (Tyr1162/1163)	rabbit	1:1000	1:50	
IRS1	rabbit	1:1000		Abcam
IRS2	rabbit	1:1000		Santa Cruz
p-IRS1/2 (Tyr612)	rabbit	1:1000	1:100	
AKT	rabbit	1:1000		Cell Signaling
p-AKT (Ser473)	rabbit	1:1000	1:100	
p-FoxO1 (Ser256)	rabbit		1:100	Abcam
PDX-1	rabbit		1:2000	Abcam
GLUT2	rabbit		1:200	Santa Cruz
Glucokinase	rabbit		1:100	
p27	mouse	1:5000	1:200	BD Bioscience
p-p27 (Ser10)	rabbit		1:100	Abcam
p-p27 (Thr157)	rabbit		1:100	
Cleaved caspase-3	rabbit	1:1000	1:100	Cell Signaling
Caspase-3	rabbit	1:1000		
Bad	rabbit	1:1000	1:100	Abcam Santa Cruz
	mouse	(1:250 [$\mu\text{g}:\mu\text{g}$ protein])		
p-Bad (Ser136)	rabbit	1:500		Cell Signaling Santa Cruz
	goat		1:200	
Bcl-xL	rabbit	1:1000		Cell Signaling
	mouse	1:1000		Santa Cruz
14-3-3	rabbit	1:1000		Cell Signaling
Gai-1	mouse	1:1000	1:100	Santa Cruz
Gai-2	rabbit		1:100	
Gai-3	rabbit	1:1000	1:100	
Flag M2	mouse	1:1000		Sigma
GFP	rabbit	1:1000		Abcam
GAPDH	mouse	1:10000		
β -actin	mouse	1:10000		
PCNA	mouse		1:1000	Sigma

*WB; western blot, **IP; immunoprecipitation, [#]IF; immunofluorescence

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