Apolipoprotein A-I Increases Insulin Secretion and Production From Pancreatic β-Cells via a G-Protein-cAMP-PKA-FoxO1-Dependent Mechanism

Arterioscler Thromb Vasc Biol. 2014;34:2261-2267; originally published online August 21, 2014;
doi: 10.1161/ATVBAHA.114.304131

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/34/10/2261

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/08/21/ATVBAHA.114.304131.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Apolipoprotein A-I Increases Insulin Secretion and Production From Pancreatic β-Cells via a G-Protein-cAMP-PKA-FoxO1–Dependent Mechanism


Objective—Therapeutic interventions that increase plasma levels of high-density lipoproteins and apolipoprotein A-I (apoA-I) A-I, the major high-density lipoprotein apolipoprotein, improve glycemic control in people with type 2 diabetes mellitus. High-density lipoproteins and apoA-I also enhance insulin synthesis and secretion in isolated pancreatic islets and clonal β-cell lines. This study identifies the signaling pathways that mediate these effects.

Approach and Results—Incubation with apoA-I increased cAMP accumulation in Ins-1E cells in a concentration-dependent manner. The increase in cAMP levels was inhibited by preincubating the cells with the cell-permeable, transmembrane adenylyl cyclase inhibitor, 2’5’ dideoxyadenosine, but not with KH7, which inhibits soluble adenylyl cyclases. Incubation of Ins-1E cells with apoA-I resulted in colocalization of ATP-binding cassette transporter A1 with the Gα subunit of a heterotrimeric G-protein and a Gα subunit-dependent increase in insulin secretion. Incubation of Ins-1E cells with apoA-I also increased protein kinase A phosphorylation and reduced the nuclear localization of forkhead box protein O1 (FoxO1). Preincubation of Ins-1E cells with the protein kinase A–specific inhibitors, H89 and PKI amide, prevented apoA-I from increasing insulin secretion and mediating the nuclear exclusion of FoxO1. Transfection of Ins-1E cells with a mutated FoxO1 that is restricted to the nucleus confirmed the requirement for FoxO1 nuclear exclusion by blocking insulin secretion in apoA-I–treated Ins-1E cells. ApoA-I also increased Irs1, Irs2, Ins1, Ins2, and Pdx1 mRNA levels.


Key Words: apolipoprotein A-I ■ diabetes mellitus, type 2

Recent evidence suggests that high-density lipoproteins (HDL) play an important role in glucose metabolism and that they may protect against the development of type 2 diabetes mellitus.1 People with type 2 diabetes mellitus typically have lower HDL cholesterol levels than healthy subjects.2,3 Low plasma HDL cholesterol levels are also predictive of progression to type 2 diabetes mellitus in people with pre–diabetes mellitus.4 Increasing HDL levels with reconstituted HDL infusions consisting of apolipoprotein A-I (apoA-I), the main HDL apolipoprotein, complexed with phosphatidylcholine in people with type 2 diabetes mellitus reduces blood glucose levels, increases plasma insulin levels, and improves pancreatic β-cell function.5 Treatment with the cholesteryl ester transfer protein inhibitor, torcetrapib, which increases plasma HDL cholesterol levels by ≈70%, also improves glycemic control in people with type 2 diabetes mellitus.6 ApoA-I seems to be responsible for conferring antidiabetic properties to HDL. Thus, apoA-I–deficient mice have impaired glucose tolerance, decreased skeletal muscle and hepatic AMP-activated protein kinase (AMPK) phosphorylation, as well as increased hepatic glucose production.7 Mice that over-express human apoA-I, by contrast, have improved insulin sensitivity and do not become obese when fed a high-fat diet.8 A single infusion of apoA-I also improves glucose tolerance and increases insulin secretion in insulin-resistant mice.9 We have previously reported that treatment of isolated islets and the MIN6 clonal β-cell line with either apoA-I or reconstituted HDL increases insulin secretion and insulin gene transcription under both basal and glucose-stimulated conditions.10 These effects were dependent, at least in part, on the ATP-binding cassette transporters, ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), and scavenger receptor class B type 1...
We have also reported that apoA-I increases insulin secretion from MIN6 cells in an ABCA1- and SR-BI–dependent manner.10 To confirm this in Ins-1E cells, transfections were performed with scrambled small interfering RNA (siRNA), ABCA1 siRNA, SR-BI siRNA, and ABCG1 siRNA, which decreased ABCA1, SR-BI, and ABCG1 protein expression by 56.8±4.6%, 74.0±7.9%, and 70.1±5.2%, respectively (Figure IIA and IIB in the online-only Data Supplement). Transfection of Ins-1E cells with scrambled siRNA or ABCG1 siRNA had no effect on apoA-I–mediated insulin secretion. When, by contrast, ABCA1 and SR-BI were knocked down, insulin secretion decreased from 165.0±25.4 to 59.5±13.7 and 52.0±15.4 ng insulin/mg protein, respectively (P<0.005 for both versus scrambled siRNA–transfected cells; Figure IIC in the online-only Data Supplement).

As reported previously,10 incubation of Ins-1E cells with apoA-I under basal and high glucose conditions did not alter cellular cholesterol levels (Table II in the online-only Data Supplement). Although this suggests that apoA-I increases insulin secretion from Ins-1E cells by a mechanism that is independent of cellular cholesterol depletion, the possibility that the insulin secretion was increased because of alterations in the subcellular localization of cholesterol cannot be excluded.

**ApoA-I Increases Insulin Secretion From Ins-1E Cells Independent of the Akt/Protein Kinase B, Janus Kinase 2, Protein Kinase C, and AMPK Signaling Pathways**

Because the Akt/protein kinase B (PKB) signaling pathway plays a key role in β-cell function and insulin secretion,13 this pathway is activated by HDL in endothelial cells.18 Next, we asked whether this could explain the increased insulin secretion in apoA-I–treated Ins-1E cells. Figure III in the online-only Data Supplement shows that apoA-I does not increase Akt phosphorylation in Ins-1E cells under basal (2.8 mmol/L) glucose conditions. Similarly, apoA-I did not activate the protein kinase C, janus kinase 2, or AMPK pathways,7,11,12 in Ins-1E cells (Figure III in the online-only Data Supplement).

**ApoA-I Increases cAMP Accumulation in Ins-1E Cells in a Transmembrane Adenylyl Cyclase–Dependent Manner**

Because apoA-I increases cAMP levels in fibroblasts and Chinese hamster ovary cells,10 cAMP levels were also quantified in Ins-1E cells. These experiments were conducted under low-glucose conditions to minimize glucose-mediated cAMP induction.19 Incubation with apoA-I increased Ins-1E cAMP levels in a concentration-dependent manner (Figure 1A). At final apoA-I concentrations of 0.5 and 1.0 mg/mL, Ins-1E cAMP levels increased from 20.1±4.8 to 57.6±5.9 pmol/mg cell protein (P<0.01) and 95.5±12.1 pmol/mg cell protein (P<0.001), respectively.

To determine whether the increase in cAMP levels involved transmembrane or soluble adenylyl cyclases, Ins-1E cells were preincubated with 2′5′ dideoxyadenosine or KH7, which respectively inhibit transmembrane and soluble adenylyl cyclases, before addition of apoA-I (Figure 1B). Incubation with apoA-I increased Ins-1E cAMP levels from 45.3±4.7 to 119.2±42.6 pmol/mg cell protein in the control cells, (P<0.05). When the cells were treated with KH7 before incubation with
ApoA-I Increases Colocalization of ABCA1 With the Heterotrimeric G-Protein \( \alpha \) Subunit

Because the \( \alpha \) subunit of heterotrimeric G-proteins can activate adenyl cyclases, we next asked whether this is the link between the interaction of apoA-I with ABCA1 and transmembrane adenyl cyclase activation. Transfection of Ins-1E cells with heterotrimeric G-protein \( \alpha \) subunit siRNA reduced \( \alpha \) protein levels by 62.2±9.7% relative to control (Figure 2A and 2B). Incubation with apoA-I increased insulin secretion in the scrambled siRNA-transfected and -nontransfected cells to the same extent (Figure 2C). When cells in which the \( \alpha \) subunit was knocked down were incubated with apoA-I, insulin secretion decreased from 189.6±25.2 ng/mg protein (control) to 98.35±25.96 ng/mg protein \((P<0.001\) versus scrambled siRNA-transfected cells; Figure 2C). Incubation with apoA-I also increased the colocalization of ABCA1 and the \( \alpha \) subunit (Figure 2D), with the Pearson coefficient increasing from 0.04±0.03 to 0.17±0.05 in Ins-1E cells incubated with PBS \((P<0.001\) versus negative control) and to 0.3±0.04 in cells incubated with apoA-I \((P<0.001\) versus PBS; Figure 2E). The specificity of the anti-ABCA1 antibody was confirmed by establishing that colocalization of ABCA1 and the \( \alpha \) subunit was minimal when ABCA1 was knocked down in Ins-1E cells (Figure IV in the online-only Data Supplement).

ApoA-I Increases Insulin Secretion From Ins-1E Cells and Primary Islets in a PKA-Dependent Manner

To determine if PKA was activated by the increase in cAMP levels, Ins-1E cells were incubated with apoA-I under basal conditions. This increased PKA phosphorylation by 103.9±19.6% at 15 minutes (Figure 3A and 3B; \(P<0.001\) versus control) and by 54.2±19.6% \((P<0.001\) versus control) at 30 minutes. PKA phosphorylation levels had returned to baseline by 60 minutes. Incubation of primary mouse islets with apoA-I for 15 minutes increased PKA phosphorylation by 76.4±15.1% \((P<0.01;\) Figure 3C and 3D).

Incubations were also performed with the PKA-specific inhibitors, H89 and PKI amide. Incubation of Ins-1E cells with H89 abolished the apoA-I–mediated increase in cAMP (Figure 3A). Hence, apoA-I increases cAMP levels in Ins-1E cells in a transmembrane adenyl cyclase–dependent manner. A, Ins-1E cells were incubated for 15 minutes with apoA-I (final concentration, 0.125–1 mg/mL) in the presence of 0.5 mmol/L IBMX. B, Ins-1E cells were preincubated for 30 minutes with 30 \(\mu\)mol/L KH7 and 50 \(\mu\)mol/L 2’-dideoxyadenosine (ddAdo) or PBS, then incubated for 15 minutes in the presence of 0.5 mmol/L IBMX with (black bars) or without (white bars) apoA-I (final concentration, 1 mg/mL). cAMP was extracted from cells with 0.1 mol/L HCl and quantified by ELISA. Values represent the mean±SD (n=4) of 3 separate experiments. **\(P<0.01\), ****\(P<0.001\).

apoA-I, cAMP levels increased from 32.8±0.9 to 125.0±26.7 pmol/mg cell protein \((P<0.01)\). Pretreatment with the transmembrane adenyl cyclase inhibitor 2’-dideoxyadenosine, by contrast, abolished the apoA-I–mediated increase in cAMP.

Figure 1. Apolipoprotein A-I (ApoA-I) increases cAMP levels in Ins-1E cells in a transmembrane adenyl cyclase–dependent manner. A, Ins-1E cells were incubated for 15 minutes with apoA-I (final concentration, 0.125–1 mg/mL) in the presence of 0.5 mmol/L IBMX. B, Ins-1E cells were preincubated for 30 minutes with 30 \(\mu\)mol/L KH7 and 50 \(\mu\)mol/L 2’-dideoxyadenosine (ddAdo) or PBS, then incubated for 15 minutes in the presence of 0.5 mmol/L IBMX with (black bars) or without (white bars) apoA-I (final concentration, 1 mg/mL). cAMP was extracted from cells with 0.1 mol/L HCl and quantified by ELISA. Values represent the mean±SD (n=4) of 3 separate experiments. **\(P<0.01\), ****\(P<0.001\).

Figure 2. Apolipoprotein A-I (ApoA-I) induces colocalization of ATP-binding cassette transporter A1 (ABCA1) and a heterotrimeric G\(_\alpha\) subunit. A, Ins-1E cells were transfected with \(\alpha\) siRNA or scrambled siRNA, then lysed and subjected to Western blotting for \(\alpha\) subunit and \(\beta\)-actin. B, Quantification of \(\alpha\) expression in siRNA-transfected cells. C, Transfected Ins-1E cells were incubated for 1 hour without (white bars) or with (black bars) apoA-I (final concentration, 1 mg/mL) under basal (2.8 mmol/L) glucose conditions. Insulin levels in the medium were quantified by radioimmunoassay. D, Ins-1E cells were seeded on coverslips, transfected with a vector encoding for hemagglutinin (HA)-\(\alpha\) subunit, serum starved for 6 hours, then incubated for 5 minutes with or without apoA-I (final concentration, 1 mg/mL). The cells were fixed, immunostained with a DyLight-488–labeled ABCA1 antibody and an Alexa-657–labeled HA-tag antibody, and imaged using a total internal reflection fluorescence microscope. Representative images from 3 separate experiments are shown. Arrows indicate points of colocalization. Scale bar, 20 \(\mu\)m. E, Quantification of the Pearson coefficient. All values represent the mean±SD (n=4 for B and C, n=20 fields of view for E). ****\(P<0.001\) compared with controls.
apoA-I for 1 hour under basal conditions increased the concentration of insulin in the medium from 61.1±8.5 to 171.6±12.7 ng insulin/mg cell protein (Figure 3E, white bars, P<0.001). Preincubation of the cells with H89 and PKI amide (Figure 3E, black and gray bars, respectively) abolished the apoA-I–mediated increase in insulin secretion. Preincubation of Ins-1E cells (Figure 3F) and primary islets (Figure 3G) with H89 and PKI amide under high glucose conditions also abolished the apoA-I–mediated increase in insulin secretion (Figure 3F).

ApoA-I Mediates Nuclear Translocation of FoxO1 in a PKA-Dependent Manner

As reported previously, 94.9±8.4% of the total cellular FoxO1 was localized in the nucleus of Ins-1E cells after 6 hours of serum starvation (Figure 4A, top).20 The intracellular distribution of FoxO1 was not affected by incubation with H89 for 30 minutes. When, by contrast, the cells were incubated with apoA-I, only 14.3±18.8% of the FoxO1 remained in the nucleus (P<0.001 versus control). Preincubation of the cells with H89 before incubation with apoA-I increased the nuclear localization of FoxO1 to 86.8±12.7% (P<0.001 versus incubation with apoA-I alone).

Ability of ApoA-I to Increase Long-Term Insulin Secretion From Ins-1E Cells Is Dependent on Nuclear Exclusion of FoxO1

To determine whether the ability of apoA-I to increase long-term insulin production depends on continued nuclear exclusion of FoxO1, Ins-1E cells were transiently transfected with pCMV5 vectors encoding for wild-type FoxO1, FoxO1-ADA (a transcriptionally active FoxO1 mutant that resides exclusively in the nucleus), or an empty pCMV5 vector. FoxO1 protein expression was comparable in the FoxO1 and FoxO1-ADA transfected cells (Figure 5A and 5B). Incubation of the transfected cells with PBS had no effect on insulin secretion (Figure 5C, white bars). Incubation with apoA-I increased the insulin concentration in the medium from 287.8±62.4 and 259.6±59.0 to 836.2±34.5 and 789.0±98.5 ng insulin/mg cell protein in the cells transfected with empty vector and the vector encoding for FoxO1, respectively (P<0.001 versus PBS for both; Figure 5C, black bars). Insulin secretion was reduced to 401.0±55.69 ng insulin/mg cell protein when the FoxO1-ADA–transfected cells were incubated with apoA-I (P<0.001).

ApoA-I Increases Expression of FoxO1 Target Genes and β-Cell Survival Genes

As reported previously,10 Ins1 and Ins2 mRNA levels increased 2.1-fold (P<0.001), and 2.8-fold (P<0.005), respectively, when Ins-1E cells were incubated for 16 hours with apoA-I. Insulin receptor substrate 1 (IRS1) and IRS2 mRNA levels increased 4.7-fold (P<0.01) and 2.9-fold, respectively (P<0.05) (Figure 6). Nuclear exclusion of FoxO1 by apoA-I also increased transcription of the β-cell survival factor Pdx1 5.3-fold (P<0.001).21

Discussion

Emerging evidence suggests that increasing plasma HDL cholesterol and apoA-I levels improves glycaemic control in people with type 2 diabetes mellitus.5,6 The present study indicates that this may relate to the ability of HDL and apoA-I to increase insulin secretion and production in pancreatic β-cells6,10,22 and the activation of a G-protein–cAMP–PKA–FoxO1 signaling pathway. The results also establish that apoA-I increases transcription of insulin production genes and expression of the β-cell survival gene, Pdx1.

Insulin secretion is impaired in pancreatic β-cells with elevated cholesterol levels,23 and this detrimental effect can be rectified by

---

**Figure 3.** Apolipoprotein A-I (ApoA-I) increases insulin secretion via a protein kinase A (PKA)–dependent mechanism. A, Ins-1E cells were cultured in serum-free medium for 12 hours, then incubated with apoA-I (final concentration, 1 mg/mL) for 5 to 60 minutes. Cell lysates were Western blotted for phosphorylated PKA (pPKA), total PKA, and β-actin. B, Quantification of PKA phosphorylation in Ins-1E cells incubated for 5 to 60 minutes in the absence or presence of apoA-I. C, Isolated primary mouse islets (n=50) were cultured in serum-free medium for 24 hours, then incubated with apoA-I (final concentration, 1 mg/mL) for 15 minutes. Cell lysates were Western blotted for pPKA, total PKA, and β-actin. D, Quantification of PKA phosphorylation in isolated primary islets. E and F, Ins-1E cells were preincubated for 30 minutes with H89 (final concentration, 20 μmol/L, black bars), PKI amide (final concentration, 5 μmol/L, gray bars), or an equivalent volume of dimethyl sulfoxide (DMSO; white bars), then incubated for 1 hour with apoA-I (final concentration, 1 mg/mL) or PBS in Krebs-Ringer bicarbonate HEPES (KRBH) buffer containing 1.67 mmol/L glucose, or PBS in KRBH buffer containing 16.7 mmol/L glucose. Insulin levels in the medium were quantified by radioimmunoassay (RIA). G, Primary mouse islets were preincubated for 30 minutes with H89 (final concentration, 20 μmol/L, black bars), PKI amide (final concentration, 5 μmol/L, gray bars), or an equivalent volume of dimethyl sulfoxide (DMSO; white bars) in Krebs-Ringer bicarbonate HEPES (KRBH) buffer containing 1.67 mmol/L glucose, then incubated for 1 hour with apoA-I (final concentration, 1 mg/mL) or PBS in KRBH buffer containing 16.7 mmol/L glucose. Insulin levels in the medium were quantified by radioimmunoassay (RIA). Values represent the mean±SD (n=4) of 3 separate experiments. **P<0.01, ***P<0.005, ****P<0.001 compared with control.
removing cholesterol from the cells with agents such as cyclodextrin that act as cholesterol scavengers. Because apoA-I is the main acceptor of cholesterol that is exported from cells via ABCA1 and SR-B1, depletion of excess cholesterol is a potential mechanism by which apoA-I may have enhanced insulin secretion in Ins-1E cells. However, we have reported previously, and confirm that this is not the case in the present study, as incubation with apoA-I increased insulin secretion in an ABCA1- and SR-B1–dependent manner without altering cellular cholesterol levels.10 This led us to ask whether apoA-I increases β-cell insulin secretion by activating cell signaling pathways.

Because activation of adenylyl cyclases, the secondary messenger cAMP, and PKA can also release insulin from β-cells28,29 and improve β-cell survival by increasing expression of IRS-230 and reducing apoptosis,31 this pathway was also explored. Support for involvement of this pathway in the apoA-I–mediated increase in insulin secretion in Ins-1E cells comes from studies showing that HDL increase intracellular cAMP levels in cholesterol-loaded fibroblasts.32 The results showing that apoA-I activates ≥1 transmembrane adenylyl cyclases, increases intracellular cAMP levels, and activates PKA in Ins-1E cells are consistent with insulin secretion being increased by activation of this pathway. It is noteworthy that this is the same mechanism by which the incretin, glucagon-like peptide-1, increases β-cell insulin synthesis and secretion.33

One of the key findings to emerge from the present study is that the interaction of apoA-I with ABCA1 increases insulin secretion by activating a transmembrane adenylyl cyclase via the Gαs subunit of a heterotrimeric G-protein. Sequence analysis shows that ABCA1 shares a striking homology

Figure 4. Apolipoprotein A-I (ApoA-I) mediates nuclear exclusion of forkhead box protein O1 (FoxO1) in a protein kinase A–dependent manner. A, Ins-1E cells were seeded on coverslips, serum starved for 6 hours, preincubated with H89 (final concentration, 20 μmol/L) or an equivalent volume of dimethyl sulfoxide (DMSO) for 30 minutes, then incubated for 30 minutes with or without apoA-I (final concentration, 1 mg/mL). The cells were fixed and immunostained with an anti-FoxO1 antibody, an Alexa-488–labeled secondary antibody, and DAPI (4',6-diamidino-2-phenylindole). Representative images of FoxO1 distribution from 3 separate experiments are shown. Scale bar, 20 μm. B, Quantification of nuclear FoxO1 localization. Values represent the mean±SD (n=30 cells). ***P<0.001 compared with controls.

Figure 5. Nuclear exclusion of forkhead box protein O1 (FoxO1) is required for increased long-term insulin secretion by apolipoprotein A-I (apoA-I). A, Ins-1E cells were transfected with vectors encoding wild-type FoxO1, constitutively expressed nuclear FoxO1-ADA or an empty vector. B, Western blotting and quantification of whole cell lysates for FoxO1 24 and 36 hours after transfection. C, Ins-1E cells were transfected with empty vector or vectors encoding wild-type FoxO1 or FoxO1-ADA, then incubated for 24 hours. The transfected cells were incubated for an additional 16 hours at 37°C in low-glucose (2.8 mmol/L) RPMI (Roswell Park Memorial Institute)-1640 medium in the absence (white bars) or presence (black bars) of apoA-I (1 mg/mL). Insulin levels in the medium were determined by RIA. Values represent the mean±SD (n=3) for 3 separate experiments. ***P<0.001 vs control.
with regions of the glucagon-like peptide-1 receptor and the glucose-dependent insulinotropic polypeptide receptor, which also bind to the Go, heterotrimeric G-protein subunit.34,35 Figure V in the online-only Data Supplement. This binding site consists of a KLK/R motif in the first intracellular domain of ABCA1 that is N-terminally flanked by several hydrophobic residues. When this is considered in light of the observation that apoA-I also activates the cAMP-PKA signaling pathway, it follows that the immediate (1 hour) glucose-mediated increase in insulin secretion in Ins-1E cells is unlikely to involve K+ ATP channel closure.10,28 FoxO1 localization, or increased insulin synthesis.

Because PKA can also directly phosphorylate FoxO1 in endothelial cells,36 we asked whether this might explain the previously reported apoA-I–mediated increase in insulin synthesis in MIN6 cells10 and in Ins-1E cells in the present study. The result in Figure 4 showing that apoA-I excludes FoxO1 from the nucleus of Ins-1E cells is consistent with this notion. It also agrees with the observed activation of transcription factors that upregulate the Ins1 and Ins2 genes37 and the increase in Pdx1 mRNA levels. The combined effects of these events increase insulin gene transcription.38 As described previously,39 this suggests that nuclear FoxO1 functions as an insulin repressor in Ins-1E cells. The ability of apoA-I to increase Pdx1 gene transcription also has the capacity to improve β-cell survival.40

In conclusion, this study sheds new light on the molecular basis by which apoA-I increases insulin synthesis and secretion in pancreatic β-cells and provides an insight as to why therapies that increase apoA-I and HDL levels improve glycemic control in people with type 2 diabetes mellitus and the metabolic syndrome. The results also identify apoA-I and possibly apoA-I mimetic peptides, as incretin-like molecules that are potentially attractive agents for treating people with type 2 diabetes mellitus and poor glycemic control.

Acknowledgments

B.J. Cochran and K.-A. Rye designed the studies, analyzed and interpreted the results, and prepared the article. B.J. Cochran, R.J. Bioendial, L. Hou, C.N. Glaros, and J. Rossy acquired the results. K.-A. Rye is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. P.J. Barter and S.R. Thomas interpreted the results and assisted with article preparation.

Sources of Funding

This work was supported by the National Health and Medical Research Council of Australia (grant 482800).

Disclosures

None.

References


Downloaded from http://atvb.ahajournals.org/ by guest on October 1, 2014
Supplementary Figure I: ApoA-I increases insulin release from Ins-1E cells in a concentration-dependent manner. Ins-1E cells were incubated for 1 h with apoA-I (final concentration 0.063-1.0 mg/mL). The insulin content in the medium was quantified by RIA. Values represent the mean±SD (n=4) of two separate experiments. **** P < 0.001 vs control.
Supplementary Figure II: ApoA-I increases insulin release from Ins-1E cells in an ABCA1- and SR-BI-dependent manner. (A and B) Ins-1E cells were transfected with ABCA1 siRNA, SR-BI siRNA and ABCG1 siRNA. The transfected cells were lysed and protein expression was quantified by western blotting. (C) Transfected cells were incubated in the absence or presence of apoA-I (final concentration 1 mg/mL) under basal (2.8 mM) glucose conditions and the insulin content in the media was quantified by RIA. Values represent the mean±SD (n=3) of three separate experiments. ***P<0.005, **** P < 0.001 vs control.
Supplementary Figure III: Incubation of Ins-1E cells with apoA-I does not activate the PI3K/Akt, PKC, JAK2 or AMPK signaling pathways. Ins-1E cells were cultured in serum-free medium for 12 h, then incubated with apoA-I (final concentration 1 mg/mL) for 5-60 min. Cell lysates were western blotted for phosphorylated Akt (pAkt), total Akt, phosphorylated PKC (pPKC), total PKC, phosphorylated JAK2 (pJAK2), total JAK2, phosphorylated AMPK (pAMPK), total AMPK and β-actin. The ratio of phosphorylated to total protein was determined and expressed relative to control. Values represent mean±SD (n=3).
Supplementary Figure IV: Confirmation of ABCA1 antibody specificity. (A and B) Ins-1E cells were transfected with scrambled siRNA or ABCA1 siRNA. Transfected cells were lysed and protein expression was quantified by western blotting. (C) Ins-1E cells seeded on glass coverslips were transfected with siRNA. The cells were fixed, immunostained with a DyLight-488 labeled ABCA1 antibody and imaged using a total internal reflection fluorescence microscope. Representative images from three separate experiments are shown. Scale bar=20 µm. All values represent the mean±SD (n=3). ****P<0.001 compared to controls.
ABCA1: DVGLPPSKLK
GLP-1R: VICIVVSKLK
GIPR: ILGILLSKLK
GCGR: IVQLLLVAKLR

Supplementary Figure V: ABCA1 displays sequence homology to known Gαs subunit binding regions of the glucagon-like peptide-1 receptor (GLP-1R), gastric inhibitor polypeptide receptor (GIPR) and glucagon receptor (GCGR). Conserved residues are highlighted. Hydrophobic residues are underlined.
Supplementary Table I: Sequence of oligonucleotide primers used for RT-PCR.

<table>
<thead>
<tr>
<th></th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS1</td>
<td>5'-ACTGGACGTACAGGCAGAATGA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGAGGAAGACGTAGGTCCTGCTGCT-3'</td>
</tr>
<tr>
<td>IRS2</td>
<td>5'-TGCGGAACAGCGTCGCTGAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GACGGGTAGCGGCTGAAAGG-3'</td>
</tr>
<tr>
<td>Ins1</td>
<td>5'-ACCTGGTGAGGCTCTGTACCT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGCGGGTCCTCCACTTCACGA-3'</td>
</tr>
<tr>
<td>Ins2</td>
<td>5'-CGGAAACCTGACAGCAGGCTGCA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCCACGCTCACCACACACA-3'</td>
</tr>
<tr>
<td>Pdx1</td>
<td>5'-GAAATCCACAAAGCTCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGTAGGCTGACGCTGCTCT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CACCGCGAGTACACCTTC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCCATACCCACCACATCACC-3'</td>
</tr>
<tr>
<td>HSPcb</td>
<td>5'-GATCCTCACATCCTCTTAGG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTGTCATCCAGTTGTGCT-3'</td>
</tr>
</tbody>
</table>
Supplementary Table II: Incubation of Ins-1E cells with apoA-I does not alter cellular cholesterol levels.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Treatment</th>
<th>Unesterified Cholesterol (nM/mg protein)</th>
<th>Esterified Cholesterol (nM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
<td>PBS</td>
<td>230.4±56.9</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.8</td>
<td>apoA-I</td>
<td>247.2±41.6</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>PBS</td>
<td>184.1±22.3</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>apoA-I</td>
<td>190.5±15.4</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Ins-1E cells were incubated at 37 °C for 1 h without or with apoA-I (final concentration 1 mg/mL) under basal (2.8 mM) or high (25 mM) glucose conditions. The cells were lysed and the cholesterol was extracted and quantified by HPLC. Values represent the mean±SD (n=3) of two separate experiments.

nd<sup>a</sup>: Not detected
Materials and Methods

Cell culture. Ins-1E rat insulinoma cells (provided by Prof. Claes Wollheim and Prof. Pierre Maechler, University of Geneva) were cultured at 37 °C in a humidified, 5% CO₂ atmosphere with RPMI-1640 medium supplemented with 10% (v/v) FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µM β-mercaptoethanol. Cells were used between passages 70-90. All incubations were performed in Krebs-Ringer bicarbonate HEPES (KRBH) medium containing 1 mM CaCl₂, 5 mM NaHCO₃, 25 mM HEPES (pH 7.4) with 0.1% (w/v) BSA under basal (2.8 mM) or high (25 mM) glucose conditions.

ApoA-I. The apoA-I used in the studies was donated by CSL, Ltd, Parkville, Australia. The samples were chromatographed on a Q-Sepharose Fast-Flow column attached to an AKTA FPLC system prior to use. The preparations were confirmed to be >95% pure by electrophoresis on 20% SDS-polyacrylamide PhastGels (GE Healthcare, Little Chalfont, United Kingdom) and Coomassie Blue staining.

Islet Isolation. Pancreatic islets were isolated from 12 week old C57Bl6 male mice, essentially as previously described. Liberase TL (05401020001, Roche Applied Science, Indianapolis, IN) was injected into the pancreas, prior to excision and digestion at 37 °C for 12 min. Islets were purified using a custom made Histopaque 1100 solution (Sigma-Aldrich, St Louis, MO) and hand picked under a dissection microscope. This procedure was approved by the Sydney Local Health District Animal Ethics Committee.

cAMP Assay. Ins-1E cells (0.5 × 10⁶ cells/well) were incubated at 37 °C in the presence or absence of apoA-I. Cells were pre-incubated for 15 min with 2',5'-dideoxyadenosine (2’5’ ddAdo, final concentration 50 µM) (D7408, Sigma-Aldrich) or KH7 (13243, final concentration 30 µM) (Cayman Chemical, Ann Arbor, MI) to block transmembrane and soluble adenylyl cyclases, respectively. Control cells were pre-incubated with an equivalent volume of DMSO. All experiments were conducted in the presence of IBMX (final concentration 0.5 mM) (I5879, Sigma-Aldrich) to inhibit phosphodiesterase activity. At the completion of the incubations cAMP was extracted from the cells with 0.1 M HCl and quantified by ELISA (581001, Cayman Chemical).

TIRF microscopy. Ins-1E cells (1 × 10⁶ cells/well) were grown on glass coverslips and transfected with pcDNA3-HA-Gαs (a gift from Dr. Denis Dupré, Dalhousie University, Canada) using Lipofectamine 2000 (11668019, Life Technologies, Carlsbad, CA). After 24 h, the cells were serum starved for 6 h and incubated with or without apoA-I for 5 min. The cells were fixed in 10% (v/v) neutral buffered formalin (HT501128, Sigma-Aldrich) for 10 min, permeabilized with PBS containing 0.3% (v/v) Triton X-100 and incubated overnight at 4 °C with a DyLight 488-conjugated rabbit anti-mouse ABCA1 antibody (1:250 dilution, NB400-105G, Novus Biologicals, Littleton, CO) and Alexa647-conjugated mouse anti-HA Tag antibody (1:100 dilution, 3444, Cell Signaling, Technology, Beverly, MA). The cells were washed and imaged on a total internal reflection fluorescence microscope (ELYRA; Zeiss) with a 100× oil-immersion objective with a numerical aperture of 1.46. Pearson’s
coefficient was calculated using the JACP plugin\textsuperscript{5} for Image J (NIH, MD). Images with one channel rotated 90 degrees were used as the negative control.

**Cell signalling assays.** Ins-1E cells were grown in 12-well plates (1 × 10\textsuperscript{6} cells/well) to ~70% confluency, serum-starved for 12 h, then incubated with apoA-I (final concentration 1 mg/mL) for the time periods indicated. When the incubations were complete the cells were lysed with ice-cold radioimmune precipitation assay buffer, electrophoresed on 4-12\% gradient SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with a rabbit anti-mouse PKA phosphorylated at Thr197 (pPKA) (1:1000, 5661, Cell Signaling Technology), rabbit anti-mouse PKA (1:1000, 5842, Cell Signaling Technology), rabbit anti-mouse Akt phosphorylated at Ser473 (pAkt) (1:1000, 4058, Cell Signaling Technology), rabbit anti-mouse Akt (1:1000, 4685, Cell Signaling Technology), rabbit anti-mouse PKC phosphorylated at Thr638 (pPKC) (1:1000, 9375, Cell Signaling Technology), rabbit anti-mouse PKC (1:1000, 2056, Cell Signaling Technology), rabbit anti-mouse JAK2 phosphorylated at Tyr1008 (pJAK2) (1:1000, 8082, Cell Signaling Technology), rabbit anti-mouse JAK2 (1:1000, 3230, Cell Signaling Technology), rabbit anti-mouse AMPK phosphorylated at Thr172 (pAMPK) (1:1000, 2535, Cell Signaling Technology), rabbit anti-mouse AMPK (1:1000, 5831, Cell Signaling Technology), and rabbit anti-mouse β-actin (1:5000, ab1801, Abcam, Cambridge, UK) antibodies. A polyclonal sheep anti-rabbit-HRP was used as the secondary antibody (1:5000, ab6795, Abcam). The blots were developed with ECL Prime (RPN2236, GE Healthcare Life Sciences, Little Chalfont, UK) and imaged using a ChemiDoc MP system (BioRad, Hercules, CA). Band intensities were quantified using ImageJ (NIH, MD).

PKA activity was inhibited by pre-incubating Ins-1E cells or islets for 30 min with H89 (final concentration 20 µM) (9844, Cell Signaling Technology) or the PKA inhibitor, 14-22 amide (final concentration 5 µM) (476485 Merck Millipore, Billerica, MA). Control cells were incubated with an equivalent volume of DMSO. Insulin concentrations were measured by radioimmunoassay (RIA) (RI-13K, Merck). Cells were lysed as described above and cellular protein levels determined by BCA assay (23225, Thermo Scientific, Rockford, IL).

**Confocal microscopy.** Ins-1E cells (1 × 10\textsuperscript{6} cells/well) were grown on glass coverslips, serum starved for 6 h and incubated with or without apoA-I and H89, or an equivalent volume of DMSO, for 30 min. The cells were fixed in 10\% (v/v) neutral buffered formalin (HT501128, Sigma-Aldrich) for 10 min, permeabilized with PBS containing 0.3\% (v/v) Triton X-100 and incubated overnight at 4 °C with a rabbit anti-mouse FoxO1 monoclonal primary antibody (1:500 dilution, 2880, Cell Signaling Technology). The cells were washed, incubated with an Alexa488-conjugated mouse anti-rabbit secondary antibody (1:500 dilution, A-11034, Life Technologies) for 1 h at room temperature and mounted in DAPI-containing Vectashield mounting medium.
Confocal images were acquired using a confocal laser scanning microscope (Leica, SPEII, objective 40x/1.15 NA Wetzlar, Germany) and processed using Volocity 3-D Image Analysis software (Perkin Elmer, Waltham, MA).

**Transfections.** Ins-1E cells were grown to 70% confluency in 12-well plates (~1×10⁶ cells/well) and transfected with pCMV5-FoxO1, pCMV5-FoxO1-ADA or an empty vector (Addgene, Cambridge, MA) using Lipofectamine 2000 (11668019, Life Technologies). Expression of FoxO1 in the transfected cells was quantified after 24 and 36 h. Cells were lysed as above and western blotted using a rabbit anti-mouse FoxO1 monoclonal antibody (1:1000). To determine whether apoA-I increases long-term insulin secretion in a FoxO1-dependent manner, Ins-1E cells that had been transfected with pCMV5-FoxO1, pCMV5-FoxO1-ADA or empty vector were incubated for a further 24 h. The cells were then incubated with apoA-I (final concentration 1 mg/mL) for an additional 16 h. The insulin concentration in the medium was determined by RIA and total cell protein as previously described.

Ins-1E cells at 70% confluency were also transfected with ABCA1 siRNA (L-092341, Thermo Scientific), ABCG1 siRNA (L-093864, Thermo Scientific), and SR-BI siRNA (L-098018, Thermo Scientific) using DharmaFECT (T-200, Thermo Scientific) or the Gαs subunit siRNA (E-092648, Thermo Scientific) using Accell Delivery Media (B-005000, Thermo Scientific). Protein expression relative to β-actin was quantified 72 h after transfection by western blotting whole cell lysates using rabbit anti-mouse ABCA1 (NB400-105, Novus Biologicals), rabbit anti-human ABCG1 (sc-20795, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-mouse SR-BI (ab52629, Abcam), rabbit anti-human Gαs subunit (bs3939R, Bioss Antibodies, Woburn, MA) and rabbit anti-mouse β-actin.

**Quantitative Real-Time PCR.** Total RNA was extracted from Ins-1E cells using TRI reagent (T9424, Sigma-Aldrich). cDNA was synthesised with iScript (170-8891, BioRad) and gene expression levels were quantified with iQ SYBR Green Super mix (170-8887, BioRad) as previously described. Primer sequences are shown in Supplementary Table I.

**Quantification of Ins-1E cholesterol levels.** Ins-1E cells (5×10⁶ cells) were incubated in the presence of absence of apoA-I under basal (2.8 mM) and high (25 mM) glucose conditions for 1 h at 37 °C. Cells were washed with PBS and lysed with water. Cholesterol content of lysates was quantified by HPLC as described previously.

**Statistics.** Results are presented as mean±SD of at least three independent experiments. Multiple comparisons were analysed as appropriate by one-way ANOVA or two-way ANOVA with Tukey’s post-test. A value of P < 0.05 was considered significant.
References


