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Pharmacological potential of novel agonists for FFAR4 on islet and enteroendocrine cell function and glucose homeostasis

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Short Title: Selective FFAR4 agonists and beta cell function

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23 **Abstract**

24 **Background:** To investigate the metabolic effects of FFAR4-selective agonists on islet and
25 enteroendocrine cell hormone release and the combined therapeutic effectiveness with DPP-
26 IV inhibitors.

27 **Methods:** Insulinotropic activity and specificity of FFAR4 agonists were determined in clonal
28 pancreatic BRIN-BD11 cells. Expression of FFAR4 was assessed by qPCR and western
29 blotting following agonist treatment in BRIN-BD11 cells and by immunohistochemistry in
30 mouse islets. Acute *in-vivo* effects of agonists was investigated after intraperitoneal (i.p.) or
31 oral administration in lean and HFF-obese diabetic mice.

32 **Results:** GSK137647 (10^{-11}-10^{-4} M) and Compound-A (10^{-10}-10^{-4} M) stimulated insulin
33 secretion at 5.6mM ($p<0.05$-$p<0.001$) and 16.7mM ($p<0.05$-$p<0.001$) glucose in BRIN-BD11
34 cells, with no cytotoxicity effects as assessed by MTT. FFAR4 antagonist (AH-7614) abolished
35 the insulintropic effect of GSK137647 ($p<0.05$-$p<0.001$), whilst FFAR1 antagonist (GW1100)
36 had no effect. Incubation of BRIN-BD11 cells with GSK137647 and Compound-A increased
37 FFAR4 ($p<0.01$) gene expression at 16.7 mM glucose, with a corresponding increase in FFAR4
38 ($p<0.01$) protein concentrations. FFAR4 upregulation was attenuated under normoglycaemic
39 conditions. Immunohistochemistry demonstrated co-localisation of FFAR4 and insulin in
40 mouse islets. Orally administered GSK137647 or Compound-A (0.1 µmol/kgBW)
41 monotherapy and combinational therapy with Sitagliptin improved glucose tolerance
42 ($p<0.001$), increased plasma insulin ($p<0.001$), GLP-1 ($p<0.05$), GIP ($p<0.05$), decreased DPP-
43 IV activity ($p<0.01$-$p<0.001$) and induced satiety ($p<0.001$) in HFF mice.

44 **Conclusions:** Specific FFAR4 agonism improves glucose tolerance through insulin and
45 incretin secretion, with enhanced DPP-IV inhibition in combination with Sitagliptin.

General significance: These findings have for the first time demonstrated that selective FFAR4 activation regulates both islet and enteroendocrine cell function with agonist combinational therapy, presenting a promising strategy for the treatment of type-2-diabetes.

Keywords: FFAR4, specificity, insulin, incretin, DPP-IV inhibition, combinational therapy

1 Introduction:

FFAR4 (GPR120) is a rhodopsin-like G-protein coupled receptor that is activated by unsaturated fatty acids (C16-22) and long chain saturated fatty acids (C14-18) [1-2]. The human FFAR4 gene is encoded on chromosome 10.q23.3 [3]. With respect to tissue distribution, FFAR4 is extensively expressed in peripheral tissues, intestines, lungs, spleen and pro-inflammatory macrophages [4]. Furthermore, recent studies have demonstrated that FFAR4 is abundantly expressed in the pancreatic islet, with further analysis demonstrating its expression in clonal pancreatic β-cell lines, including MIN6, RINm5f and INS-1E [5-7].

Previously considered as orphan receptors, recent studies have shown FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), FFAR4 (GPR120) and GPR84 to be activated by free fatty acid (FFA) molecules [3, 4, 8]. FFAR3 and FFAR2 exhibit specificity towards short chain fatty acids, GPR84 is activated by medium chain fatty acids, whereas FFAR1 and FFAR4 are activated by long chain fatty acids [3, 4]. Furthermore, FFAR4 and FFAR1 share 10% sequence homology and can be activated by similar endogenous ligands (Omega-3-fatty acids), which warrants the utilisation of receptor specific agonists to evaluate the therapeutic potential of FFAR4 [2, 8].

68 FFAR4 has been hypothesised to act as a lipid sensor in the body, and has been proven to have
69 involvement in the regulation of inflammation, adipogenesis, and glucose homeostasis [1, 7,
70 9]. Interestingly, it has been reported that a mutation in the FFAR4 gene (R270H) is linked
71 with the development of obesity. The p.R270H variant impairs the signalling response of
72 FFAR4 upon FFA binding, with subsequent defects observed to intracellular calcium
73 mobilisation and GLP-1 secretion in intestinal cells [10, 11]. Further studies have
74 demonstrated that FFAR4 knockdown with siRNA impaired the anti-apoptotic effects of
75 omega-3 fatty acids in serum-starved STC-1 cells. Thus, indicating the potential proliferative
76 and anti-apoptotic effects of FFAR4 in pancreatic beta cells [12].

77 Numerous studies have identified the involvement of FFAR4 in the gastrointestinal (GI) tract,
78 including the mediation of glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide
79 (GIP) and cholecystokinin (CCK) secretion from intestinal L-cells, K-cells and I-cells, with
80 high FFAR4 expression observed in the intestinal STC-1 and GLUTag cell lines [1, 12, 13].
81 FFAR4 activation has been shown to mediate GLP-1 secretion when tested with its endogenous
82 agonist α-linolenic acid [1, 14, 15]. However, other studies suggest that FFAR4 has no role in
83 GLP-1 release [16]. Previous findings have shown FFAR4 to mediate insulin-sensitising and
84 anti-inflammatory properties in peripheral tissues [9].

85 The expression and biological function of FFAR4 in the intestinal tract has been heavily
86 documented, however the role of FFAR4 in pancreatic beta cell function was not investigated
87 until recently [7]. A number of FFAR4 agonists were demonstrated to have regulatory role in
88 glucose dependent insulin secretion in mouse islets, including endogenous docosahexaenoic
89 acid (DHA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA) and synthetic GW-9508
90 [7, 8]. In addition, these agonists demonstrated insulinotropic and glucose lowering properties
91 in *in-vivo* [7]. However, the selectivity of endogenous FFAR4 agonists (ALA, DHA, EPA)

92 remains uncertain as activation of FFAR1 may contribute to the effects observed, whilst
93 synthetic GW9808 has been shown to exhibit 100-fold greater potency towards FFAR1 over
94 FFAR4 [17, 18].

95 Upon activation, FFAR4 primarily couples to Gαq, which stimulates an array of secondary
96 messenger signalling pathways through phospholipase C (PLC), including intracellular
97 calcium and mitogen-activated protein kinases [4, 7]. The mechanism of FFAR4 mediated
98 insulin secretion from the pancreatic beta cell is not conclusive; however, studies have shown
99 a range of FFAR4 agonists to induce intercellular calcium release, indicating the potential
100 involvement of inositol trisphosphate on intracellular calcium stores through PLCβ signalling
101 [7]. FFAR4 activation with ALA and DHA leads to the rapid and transient phosphorylation of
102 the receptor of HEK293 cells [19]. Although FFAR4 has been shown to act predominately
103 through PKC signalling, DHA has also shown to activate G-protein coupled receptor kinase
104 (GPK6) upon FFAR4 phosphorylation, with Thr(347), Ser (350), and Ser(357) shown to be
105 major phosphorylation sites in the C-terminal tail of FFAR4 [19].

106 Recently, Oh *et al.,* have reported an orally available, selective, high affinity, small FFAR4
107 agonist (Compound A) that exhibits a range of anti-diabetic effects [20]. Oral administration
108 of Compound A improved glucose tolerance, insulin sensitivity and exerted anti-inflammatory
109 effects on macrophages in high fat fed obese mice [20]. Sparks *et al.,* recently identified a
110 potent FFAR4 agonist GSK137647 [21] and preliminary *in-vitro* analysis has demonstrated
111 that GSK137647 augmented insulin secretion in MIN6 cells, with a modest increase in GLP-1
112 secretion from the NCl-H716 intestinal cell line [21, 22]. *In-vivo* findings showed that
113 GSK137647 induced GLP-1 release by mouse circumvallate papillae [22]. The highly selective
114 properties of this agonist suggested that it was suitable to evaluate FFAR4 activation in
115 pancreatic beta cells.

116 Due to the regulatory role of FFAR4 activation on insulin and incretin secretion [1, 7, 14], a
117 promising approach using selective FFAR4 agonists combined with a dipeptidyl peptidase-4
118 (DPP-IV) inhibitor may offer therapeutic potential [23, 24]. The present study has assessed the
119 effect of potent DPP-IV inhibitor (Sitagliptin) in combination with Compound A and
120 GSK137647 on glucose tolerance and insulin secretion in high fat fed (HFF)-induced diabetic
121 mice. This research aims to investigate the acute metabolic effects and of FFAR4 agonist
122 monotherapy and combinational therapy on islet and enteroendocrine cell function, using
123 pancreatic cells and diabetic mice.

124

125 **2 Materials and methods:**

126 **2.1 Materials:**

127 FFAR4 agonists Compound A and GSK137647 were purchased from Cayman Chemicals
128 (Michigan, USA) and Tocris (Bristol, UK) respectively. Sitagliptin phosphate monohydrate
129 was obtained from Apexbio Technology LLC (Texas, USA). Thiazolyl blue tetrazolium
130 bromide (MTT) was received from Sigma (Poole, UK). Rabbit anti-GPR120 polyclonal IgG
131 antibody (H-155) was purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA) and
132 guinea pig anti-insulin from Abcam (Cambridge, UK).

133 **2.2 Insulin secretion:**

134 Generation and characterization of the insulin-secreting BRIN-BD11 cells were outlined
135 previously [25]. BRIN-BD11 cells were cultured with RPMI-1640 media (11.1 mM glucose)
136 containing antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 10% foetal calf
137 serum at 37°C in 95% air and 5% carbon dioxide. For acute insulin secretion studies, cells were
138 detached using trypsin/EDTA and incubated overnight in 24-well plates with 150,000 cells per

139 well. Cells were then pre-incubated for 40 min at 1.1 mmol/l glucose in Krebs buffer
140 (comprising 4.7 mmol/l KCL, 115 mmol/l NaCl, 1.28 mmol/$CaCl_2$, 10 mmol/l $NaHCO_3$, 5 g/l
141 bovine serum albumin, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$.7H2O, pH 7.4). Test
142 incubations were then performed at 37ºC for 20 min. Supernatants were removed, then frozen
143 at −20ºC until determination of insulin by radioimmunoassay [26]. All FFAR4 ligands (1
144 mg/ml) were dissolved in 50% DMSO prior to preparation in Krebs buffer. Compound A and
145 GSK137647 at 10^{-12}-10^{-4} mol/l were tested at 5.6 and 16.7 mmol/l glucose. To determine the
146 selectivity of the agonists towards FFAR4, the FFAR1 antagonist GW1100 (10^{-5} mol/l), and
147 FFAR4 antagonist AH-7614 (10^{-5} mol/l) were utilised to antagonise the respective receptors.
148 Receptor antagonists were co-incubated with Compound A and GSK137647 on BRIN-BD11
149 cells, with insulin secretory responses determined.

150 **2.3 Cytotoxicity assessment by tetrazolium (MTT):**

151 BRIN-BD11 cells were cultured overnight in 96-well plates. Incubations were performed as
152 described for insulin secretory analysis. Test solutions were decanted, then 1mg/ml of working
153 MTT solution was added and incubated for 2 h at 37ºC. MTT solution was removed and
154 replaced with 200 µl of DMSO. The plate was placed on an orbital shaker for 5 min to mix the
155 formazan into the solvent. The optical density of each well was recorded at 560 nm with the
156 background absorbance at 670 nm removed. The resultant optical density was proportional to
157 the viable cell quantity.

158 **2.4 Acute effects of FFAR4 agonists *in-vivo*:**

159 All animal experiments were carried out in accordance with the UK Animal (Scientific
160 Procedures) Act 1986. Male lean and HFF Swiss TO mice (Harlan UK, 30-34 weeks old, 55–
161 67 g) were individually housed in an air-conditioned room at 22 ± 2ºC with a 12-h light: 12-h
162 dark cycle. Drinking water was supplied ad libitum. Animals were maintained on a high fat

163 diet (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Dietex
164 International Ltd., Witham, UK) from 8 weeks of age for a total of 150 days to evoke dietary-
165 induced obesity-diabetes (DIO). Another group of mice was maintained on standard rodent diet
166 (10% fat, 30% protein, 60% carbohydrate; percent of total energy 12.99 kJ/g, Trouw Nutrition,
167 Cheshire, UK) and used as a model of normal controls. Similar high-fat diets, containing a
168 large percentage of energy from fat, are used routinely in obesity-diabetes research [27-29].
169 Mice (n=6, fasted 18 h) received an oral or IP administration of glucose alone (18 mmol/kg
170 body weight) or in combination with FFAR4 agonists (0.1 μmol/kg body weight). All FFAR4
171 ligands (1 mg/ml) were dissolved in 50% DMSO prior to preparation in saline. Blood samples
172 were obtained by a cut from the tip of the tail vein of conscious mice at the times indicated in
173 Figures, and centrifuged at 16,060 × *g* for 3 min at 4ºC. Plasma glucose was measured using
174 an automated glucose oxidase procedure with a Beckman glucose analyser (Beckman-Coulter,
175 High Wycome, UK) and insulin determined by radioimmunoassay [26]. Intestinal hormone
176 secretion was assessed using ELISA; total GLP-1 (Millipore) and total GIP (Millipore). DPP-
177 IV activity was evaluated by Gly-Pro-AMC cleavage [30]. In a second series of experiments,
178 18 h fasted normal mice were used to assess the effects of agonist treatment on food intake.
179 Mice received oral administration of saline alone (0.9% (w/v) NaCl) or in combination with
180 agonist (0.1 μmol/kg body weight) and food intake measured at 30 min intervals.

181 **2.5 Gene expression analysis by qPCR:**

182 mRNA was extracted from clonal pancreatic BRIN-BD11 cells following exposure to agonist
183 treatment, using an RNeasy Mini kit adhering to manufacturer's protocol (Qiagen, UK).
184 Isolated mRNA (3 μg) was converted to cDNA using SuperScript II Reserve Transcriptase.
185 Amplification parameters were set at 95ºC for denaturation, 58ºC for primer annealing and
186 72ºC for elongation for a total of 40 cycles, followed with melting curve analysis, with

187 temperature range set at 60ºC to 90ºC. Values were analysed using the Livak method and
188 normalised to GAPDH expression.

189 **2.6 FFAR4 protein concentrations using western blotting:**

190 BRIN-BD11 cells were seeded at a density of 1,000,000 cells per well in 6-well plates and
191 allowed to attach overnight. After 4 h exposure to 10^{-4}M GSK137647 and Compound A, total
192 protein was extracted at 4ºC for 10 min using RIPA buffer containing 150 mM NaCl, 1.0%
193 Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 7.6 and protease
194 inhibitor cocktail (Sigma, UK). Total protein concentration was determined using Bradford
195 reagent (Sigma, UK). Equal amounts of protein were prepared in aliquots with Laemmli buffer
196 (1 µg/µl), then boiled at 95ºC for 10 min. Samples (25 µg per well) were loaded onto pre-cast
197 gels (NUPAGE 4–12% Bis–Tris gels, Invitrogen, UK) and subjected to SDS-PAGE (70 V, 90
198 min). After transfer to nitrocellulose membrane for 16 h at 90 mA, membranes were blocked
199 with 5% skimmed milk and probed with rabbit anti-FFAR4 (1:150) (Santa Cruz, US)/mouse
200 anti-β-actin (1:2500) (Cell signalling, US). Membranes were probed with ECL horseradish
201 peroxidase donkey anti-rabbit IgG/ECL horseradish peroxidase sheep anti-mouse IgG
202 (1:10000) (GE Healthcare, UK) and detected using Luminata Forte HRP substrate (Millipore,
203 UK), with images captured using the G:BOX Chemi XX9 imager (Syngene, UK). Data were
204 normalised to β-actin and presented relative to untreated control.

205 **2.7 Tissue distribution of FFAR4 by immunohistochemistry:**

206 Pancreatic tissue from lean and HFF NIH Swiss mice was excised and cut at 8µm using a
207 microtome. Sections were placed on slides and dried for 2 h on a hot plate at 37ºC. After
208 incubation, wax was removed and tissue re-hydrated in ethanol (100%), ethanol (95%), ethanol
209 (80%) and distilled water for 5 min each. Slides were incubated in 50 mM sodium citrate for
210 20 min at 90ºC for antigen retrieval. BSA (2.5%) was added to each slide (200 µl) for 45 min.

211 Primary antibodies (200 µl) at optimal dilutions (FFAR4 1:100, Insulin 1:300) were added and
212 incubated at 4ºC overnight. Slides were washed, followed by secondary antibody (1:400)
213 incubation at 37ºC for 45 min. The slides were washed, then DAPI (0.1 µg/ml) added and
214 incubated at 37ºC for 15 min. Slides were washed, then mounted.

215 **2.8 Statistics**

216 All data was analysed with Prism (v.5.0, GraphPad Software Inc. CA, USA) and expressed
217 as mean ± S.E.M. All *in-vivo* glucose tolerance test data (glucose, insulin, GLP-1, GIP and
218 DPP-IV activity) were analysed using two-way analysis of variance (ANOVA) followed by
219 the Bonferroni *post-hoc* test. Area under the curve (AUC) was calculated using trapezoidal
220 rule with baseline correction. All other data including AUC were analysed using Student's *t*-
221 test (non-parametric, with two-tailed P values and 95% confidence interval). $p<0.05$ was
222 considered to be statistically significant.

223

224 **3 Results:**

225 **3.1 Determination of FFAR4 agonist selectivity on insulin secretion.**

226 The insulinotropic response and specificity of the novel synthetic FFAR4 agonists (Compound
227 A, GSK137647) at 10^{-12}-10^{-4} mol/l were assessed using clonal pancreatic BRIN-BD11 cells.
228 At 5.6 mM glucose, Compound A at 10^{-10}-10^{-4} mol/l augmented insulin secretion by 1.2- to
229 1.9-fold ($p<0.05$-$p<0.001$), with a half maximal effective concentration (EC_{50}) of 2.9×10^{-7}
230 mol/l, while GSK137647 was more potent (EC_{50} of 2.2×10^{-7} mol/l) with a 1.5- to 2.1-fold
231 increase at 10^{-8}-10^{-4} mol/l ($p<0.05$-$p<0.001$) (Figure 1 A, B). At 16.7 mM glucose, both
232 agonists exhibited enhanced insulinotropic activity. Compound A at 10^{-7}-10^{-4} mol/l augmented
233 insulin secretion from 1.8- to 2.4 fold ($p<0.05$-$p<0.001$) and GSK137647 at 10^{-11}-10^{-4} mol/l

234 enhanced insulin secretion by 1.4- to 2.8-fold ($p<0.05$-$p<0.001$), with EC_{50} values of 3.0 x 10^{-8} mol/l (Compound A) and 1.2 x 10^{-10} mol/l (GSK137647) (Figure 1 C, D). Neither agonist
236 affected cell viability when assessed by MTT (Figure 1).

237 To investigate the selectivity of the agonists, both Compound A and GSK137647 were co-
238 incubated with selective FFAR1 and FFAR4 antagonists. The insulin secretory response of
239 Compound A and GSK137647 was not influenced by incubation with the FFAR1 antagonist
240 GW1100 (10^{-5} mol/l), with similar insulinotropic responses being observed in the presence and
241 in the absence of GW1100 (Figure 1). In contrast, the FFAR4 antagonist AH-7614 (10^{-5} mol/l)
242 significantly impaired the insulinotropic responses of Compound A and GSK137647. At 5.6
243 mM glucose in the presence of the FFAR4 antagonist, Compound A (10^{-6}-10^{-4} mol/l)
244 augmented insulin secretion by 1.25- to 1.3-fold ($p<0.05$), corresponding to a 65% decrease in
245 insulin output compared with control. Furthermore, the insulinotropic effect of GSK137647
246 was abolished in the presence of the FFAR4 antagonist (Figure 1 A, B). At 16.7 mM glucose
247 in the presence of the FFAR4 antagonist, Compound A at 10^{-5}-10^{-4} mol/l increased insulin
248 secretion by 1.7- to 1.8-fold ($p<0.05$), corresponding to a 65% reduction compared to agonist
249 alone. GSK137647 at 10^{-7}-10^{-4} mol/l only augmented insulin secretion by 1.7- to 2.1 fold in
250 the presence of the antagonist ($p<0.01$) (Figure 1 C, D), reflecting a 40% decrease in
251 insulinotropic action.

252 **3.2 Expression of FFAR4 in high fat fed pancreatic tissue and BRIN-BD11 cells:**

253 Immunohistochemistry revealed high expression and areas of co-localisation of FFAR4 and
254 insulin in pancreatic islets from lean and HFF mice (Figure 2 A-H). The pancreatic BRIN-
255 BD11 cell line was used to confirm FFAR4 gene expression in pancreatic beta cells. At 5.6
256 mM glucose, FFAR4 agonists Compound A ($p<0.05$) and GSK137647 ($p<0.05$)
257 downregulated FFAR4 receptor mRNA expression in BRIN-BD11 cells (Figure 2 I). When

exposed to 16.7 mM glucose, FFAR4 agonists (Compound A ($p<0.01$) and GSK137647 ($p<0.01$) increased FFAR4 mRNA expression (Figure 2 J). Western blotting was conducted to determine complimentary FFAR4 protein concentrations after agonist treatment. GSK137647 upregulated FFAR4 protein by 1.9 fold ($p<0.05$) in hyperglycaemic conditions, whilst Compound A had no significant effect (Figure 2 L). FFAR4 protein concentrations were not altered by either agonist under normoglycaemic (5.6 mM glucose) conditions (Figure 2 K).

3.3 Acute effects of FFAR4 agonists on glucose tolerance and insulin secretion *in-vivo*

An oral glucose tolerance test (OGTT) was performed to assess the anti-diabetic activity of Compound A and GSK137647 in fasted HFF mice. Compound A and GSK137647 were assessed alone or in combination with the DPP-IV inhibitor (Sitagliptin). FFAR4 antagonist AH-7614 was utilised to determine agonist specificity *in-vivo*. Oral administration of Compound A and GSK13647 improved glucose tolerance ($p<0.05$-0.001) (Figure 3 A, C), with AUC data showing decreases with Compound A and GSK137647 by 26% ($p<0.05$) and 18% ($p<0.05$) respectively (Figure 3 E, G). In combination with Sitagliptin, these compounds exhibited a further improvement on glucose excursion by 5-11%. The FFAR4 antagonist impaired the glucose lowering properties of Compound A and GSK137647 by 77% ($p<0.05$) and 89% ($p<0.05$) respectively (Figure 3 A, C).

These effects on blood glucose control were accompanied by relative changes in insulin secretion. Agonising FFAR4 with Compound A ($p<0.05$) and GSK137647 ($p<0.001$) increased plasma insulin by 20% and 54% when assessed with AUC data (Figure 3 F, H). Compound A in combination with Sitagliptin demonstrated an additive 12% insulinotropic effect compared to Compound A alone, whilst GSK137647 combinational therapy resulted in a 9% reduction compared to agonist alone (Figure 3 B, D). The FFAR4 antagonist AH-7614 inhibited the insulinotropic response of Compound A by 30% and GSK137647 by 76% ($p<0.001$).

GSK137647 increased plasma GLP-1 ($p<0.05$) and GIP ($p<0.05$), whilst Compound A increased plasma GLP-1 ($p<0.05$) but had no effect GIP secretion (Figure 4 A, B, D, E). GSK137647 ($p<0.01$) and Compound A ($p<0.01$) reduced DPP-IV activity, with activity further diminished when administered in combination with Sitagliptin ($p<0.001$) (Figure 4 C, F).

In addition to oral treatment, Compound A and GSK137647 were administered also by intraperitoneal injection to lean mice (Figure 5 A). Compound A significantly improved glucose excursion ($p<0.01$), with GSK137647 ($p<0.05$) eliciting a similar response.

3.4 Acute effects of FFAR4 agonists on appetite suppression in lean Swiss TO mice

Compound A induced satiety after 30 min with further effects observed over the 3 h time period ($p<0.05$-$p<0.001$) (Figure 5 B). Sitagliptin impaired the satiation effect of Compound A at 60 min. GSK137647 inhibited food intake after 30 min, with lasting effects throughout the experimental timeframe ($p<0.01$-$p<0.001$) (Figure 5 C). Combination of GSK137647 with Sitagliptin impaired the appetite suppressive effects of the agonist from 60 min to 180 min ($p<0.05$-$p<0.001$).

4 Discussion:

Recent interest in long chain fatty acid receptors has intensified due to identification of their involvement in the maintenance of glucose homeostasis through GPCR signalling. FFAR1 (GPR40) [31], GPR55 [32, 33] and GPR119 [33, 34] have been previously reported to regulate islet function and hormone secretion. In particular, orally administered FFAR1 agonist TAK-875 (Fasiglifam) entered stage III clinical trials with promising anti-diabetic effects of equal

304 potency to the sulphonylurea glimepiride, whilst omitting the risk of hypoglycaemia [35, 36].
305 However, signs of liver toxicity were observed towards the latter stages of the trial [37].

306 FFAR4 (GPR120) is a rhodopsin (class A) type receptor which has recently been shown to
307 have anti-diabetic properties through the regulation of insulin and incretin secretion [1, 7, 8].
308 Recent studies have identified the expression and involvement of FFAR4 in the pancreatic islet
309 and intestinal tract, with expression also identified in adipose tissue, lungs and pro-
310 inflammatory macrophages. FFAR4 shares 10% homology with FFAR1 and has high affinity
311 for long chain fatty acids. However, many FFAR4 agonists can act as dual agonists with
312 FFAR1, such as synthetic GW9508, therefore the identification of selective FFAR4 agonists is
313 required to fully evaluate the role of the receptor in the maintenance of glucose homeostasis
314 [7, 8].

315 In the present study, the therapeutic potential of two novel synthetic agonists (Compound A,
316 GSK137647), which have been shown to exhibit selective properties towards FFAR4, were
317 assessed [20-21]. Insulin secretion studies demonstrated that both agonists enhanced glucose
318 stimulated insulin secretion from pancreatic BRIN-BD11 cells. Compound A and GSK13647
319 displayed similar potencies at basal glucose levels (EC_{50} of ~10^{-7} mol/l). Stimulatory glucose
320 concentrations, GSK137647 exhibited an EC_{50} of 10^{-10} mol/l, compared with an EC_{50} of 10^{-8}
321 mol/l for Compound A. Neither agonist imparted adverse effects on cell viability as assessed
322 using MTT.

323 To demonstrate the selectivity of the novel agonists towards FFAR4, potent antagonists for
324 FFAR4 and FFAR1 were employed. In the presence of FFAR1 antagonist (GW1100), the
325 insulin secretory responses to both Compound A and GSK137647 was relatively unaffected.
326 In contrast, the FFAR4 antagonist (AH-7614) impaired the insulinotropic properties of both
327 agents, suggesting that the two agonists stimulate glucose dependent insulin secretion through

328 FFAR4 and not FFAR1 in the pancreatic beta cell. Numerous endogenous FFAR4 ligands have
329 been shown to stimulate other fatty acids receptors, including FFAR1 [3, 8].

330 Immunohistochemistry demonstrated the highly abundant expression and co-localisation of
331 FFAR4 and insulin in both the pancreas of lean and diabetic mice. Consistent with this, gene
332 expression analysis demonstrated an upregulation of FFAR4 in BRIN-BD11 cells exposed to
333 hyperglycaemia, suggesting that FFAR4 may have a regulatory role in islets exposed to
334 diabetic stress factors. Interestingly, agonist treatment under normoglycaemic conditions
335 significantly attenuated FFAR4 gene expression, with no effect demonstrated on FFAR4
336 protein concentrations. The glucose responsive properties of the receptor expression promotes
337 FFAR4 as a novel therapeutic target.

338 In HFF mice, both Compound A and GSK137647 demonstrated acute glucose lowering and
339 insulinotropic properties. Previously, FFAR4 activation was shown to augment glucagon
340 release from pancreatic alpha cells [38]. Although glucagon opposes the biological actions of
341 insulin, improved glucose tolerance is exhibited upon FFAR4 agonist treatment. This indicates
342 superior secretory actions of FFAR4 agonism on insulin releasing beta cells. Interestingly,
343 FFAR4 activation has also been shown to inhibit somatostatin release from pancreatic delta
344 cells, which may have indirectly enhanced the insulinotropic actions of FFAR4 agonists
345 observed in this study [39]. Although numerous glucoregulatory hormones are released upon
346 FFAR4 activation, the anti-inflammatory effects and potentiation of glucose uptake in
347 peripheral tissues may have also attributed to the glucose lowering effects exhibited by FFAR4
348 [9].

349 Conflicting results have been reported of the effect of FFAR4 activation of GLP-1 secretion
350 [1, 16]. In the present study, GSK137647 was shown to induce GLP-1 and GIP secretion, whilst
351 Compound A only stimulated GLP-1 secretion. To prolong the bioactivity of endogenously

352 released incretins, the DPP-IV inhibitor Sitagliptin was examined in combination with the
353 FFAR4 agonists. Under these conditions, Compound A and GSK137647 exhibited enhanced
354 glucose lowering capabilities, by stimulating incretin (GLP-1, GIP) and insulin secretion
355 through FFAR4 activation, supplemented with prolonged incretin action through DPP-IV
356 inhibition. DPP-IV inhibition however countered the inhibitory effects of both FFAR4 agonists
357 on feeding activity. Further studies are required to fully understand the mechanism but
358 inhibition of DPP-IV-mediated degradation of PYY(1-36) to the active form PYY(3-36) seems
359 likely [40].

360 To establish that the glucose lowering properties of the agonists was not based solely on
361 incretin secretion, each agonist was administered i.p. in combination with glucose. Both
362 GSK137647 and Compound A improved glucose excursion confirming that FFAR4 activation
363 also directly stimulates beta cell function. The selectivity of the novel agonists was tested using
364 the FFAR4 antagonist AH-7614, which significantly impaired the insulinotropic and glucose
365 lowering capabilities of GSK137647 and Compound A.

366 In conclusion, selective FFAR4 agonists enhance glucose stimulated insulin secretion in a
367 concentration-dependent manner, whilst exhibiting no affinity for FFAR1. Expression analysis
368 demonstrated glucose responsive properties of FFAR4 expression under hyperglycaemic
369 stress, a novel finding which may aid the development of future anti-diabetic therapeutics.
370 Activation of FFAR4 was associated with acute stimulatory effects on GLP-1 and GIP
371 secretion. GSK137647 was the most potent agonist in terms of insulin and incretin secretion,
372 suggesting that this agonist should be considered for further investigation. In addition, it has
373 been shown for the first time that FFAR4 agonist combinational therapy with sitagliptin further
374 improves glucose tolerance and may provide a novel approach for the treatment of type 2
375 diabetes.

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378 methodology, validation, formal analysis and writing – original draft. MGM conducted the
379 investigation. PRF involved in the writing- review and editing and supervision. AMMK
380 involved in the conceptualization, formal analysis, supervision writing – review and editing.

381

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Figure Legends

Figure 1. Acute insulinotropic effects of FFAR4 agonists (A, C) Compound A and (B, D) GSK137647 alone and in combination with FFAR4 antagonist AH-7614 (10^{-5} mol/l) or FFAR1 antagonist GW1100 (10^{-5} mol/l) at 5.6 mM and 16.7 mM glucose in clonal pancreatic BRIN-BD11 cells. Alanine (10 mmol/l) was used as positive control. MTT cell viability analysis demonstrating cytotoxicity of (A, C) Compound A and (B, D) GSK137647 on BRIN-BD11 cells. Hydrogen peroxide (1 mmol/l) was used as a positive control. Values are mean ± SEM (n=8) for insulin secretion and (n=4) for cell viability. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to saline control. †$p<0.05$, ††$p<0.01$, †††$p<0.001$, compared to agonist alone.

Figure 2. Localisation of (A, B) 4', 6 diamidino-2-phenylindole (DAPI) nuclear stain, (C, D) FFAR4, (E, F) insulin and (G) double immunofluorescence of FFAR4 and insulin in high fat fed (A, C, E, G) and lean (B, D, F, H) pancreatic islets at X40 magnification. Examples of double immunofluorescence indicated by white arrows. qPCR and western blot analysis demonstrating the effect of FFAR4 agonist treatment on FFAR4 mRNA and protein concentrations at (I, K) 5.6 mM and (J, L) 16.7 mM in clonal pancreatic BRIN-BD11 cells after 4 h treatment. Expression was normalised to GAPDH for qPCR (n=3) and β-actin for western blotting (n=2; two independent experiments with two technical replicates). Values are presented as mean ± SEM.*$p<0.05$, **$p<0.01$, compared to glucose control.

Figure 3. Acute effects of FFAR4 agonists Compound A and GSK137647 on plasma glucose (A, C), insulin (B, D) and respective AUC (E-H). Glucose (18 mmol/kg bw) was administered orally alone or in combination with FFAR4 agonist Compound A or GSK137647 (0.1 µmol/kg bw) and either the FFAR4 antagonist AH-7614 (0.1 µmol/kg bw) or Sitagliptin (50 mg/kg bw) to HFF mice (n = 6). Values are presented as mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$,

compared to HFF glucose control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

Figure 4. Acute effects of FFAR4 agonists Compound A and GSK137647 (0.1 µmol/kg bw) on circulating (A, D) total GLP-1, (B, E) total GIP and (C, F) DPP-IV activity. Glucose (18 mmol/kg bw) was administered orally alone or in combination with FFAR4 agonist to HFF mice (n = 6). Values are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, compared to HFF glucose control.

Figure 5. Acute effects of i.p. administration of FFAR4 agonists Compound A and GSK137647 (0.1 µmol/kg bw) on (A) glucose tolerance and (B, C) cumulative food intake. Glucose (18 mmol/kg bw) was administered i.p. in combination with FFAR4 agonist to lean Swiss TO mice for glucose tolerance (n = 6). For satiety analysis, FFAR4 agonists were orally administered alone or in combination with DPP-IV inhibitor (Sitagliptin) to 18 h fasted lean Swiss TO mice. *p<0.05, **p<0.01, ***p<0.001, compared to saline control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

Supplementary data

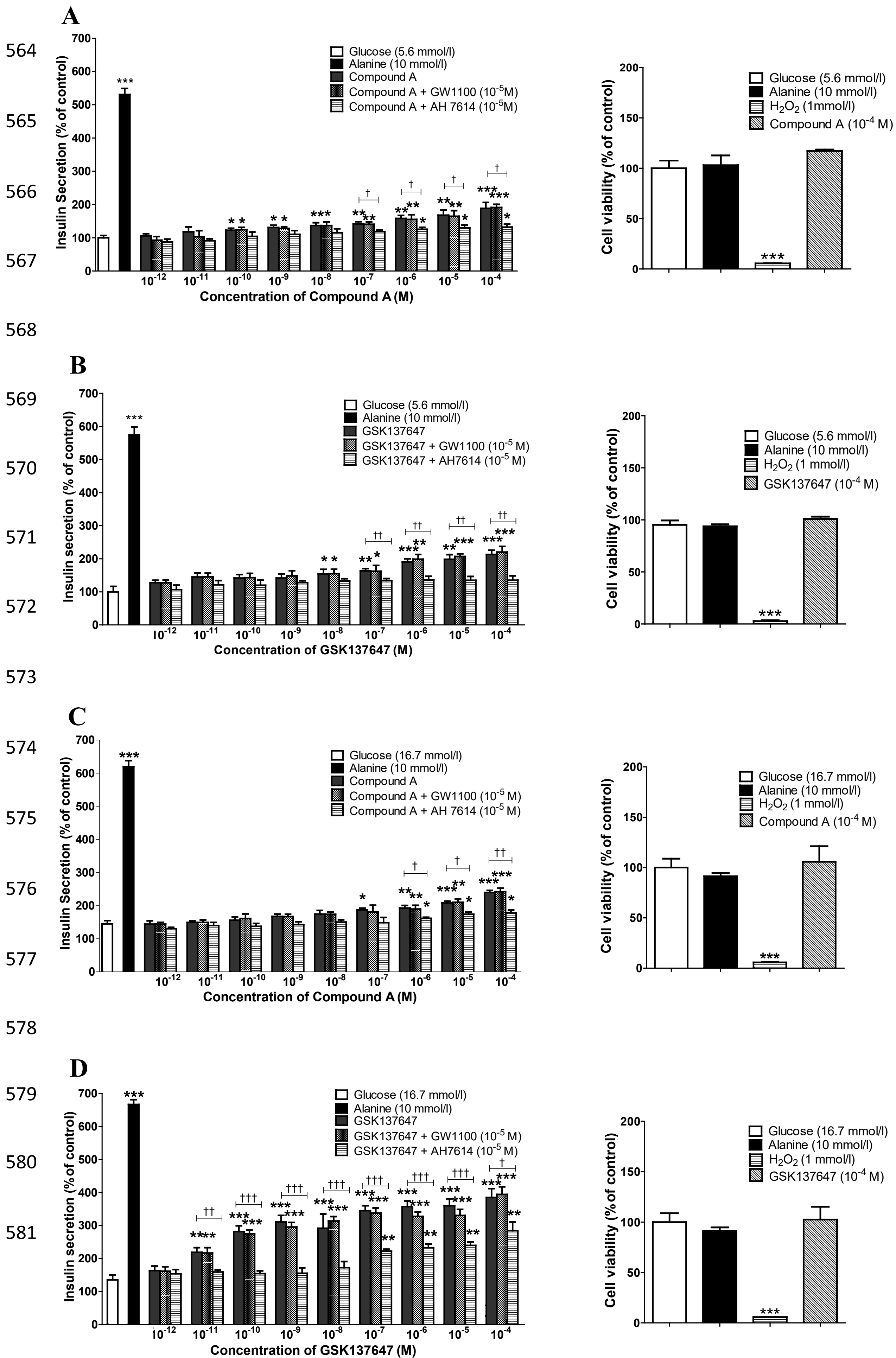
Figure 1. Effects of Compound A and GSK137647, with half maximum effective concentration (EC_{50}) values, on insulin release from clonal pancreatic BRIN-BD11 cells at (A) 5.6 mM and (B) 16.7 mM glucose concentrations. Results are the mean ± SEM (n=8).

Figure 2. Effect 150-day high fat fed diet on glucose tolerance and insulin secretion in 18 h fasted Swiss TO mice. Animals were subjected to an oral glucose tolerance test (18 mmol/kg bw) with (A, B) glucose tolerance, (C, D) insulin secretory response, (E) fasting plasma glucose

and (F) bodyweight determined. Results are the mean ± SEM (n=6). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to lean control.

Figure 3. Effect 150-day high fat fed diet on glucose tolerance and insulin secretion in 18 h fasted Swiss TO mice. Animals were subjected to an insulin sensitivity test (40U/kg bodyweight; dissolved in 0.9% saline, i.p. injection). (A) Plasma glucose and (B) respective AOC are shown. Results are mean ± SEM (n=8). **$p<0.01$, compared to lean control.

563 **Figure 1**



582 Figure 2.

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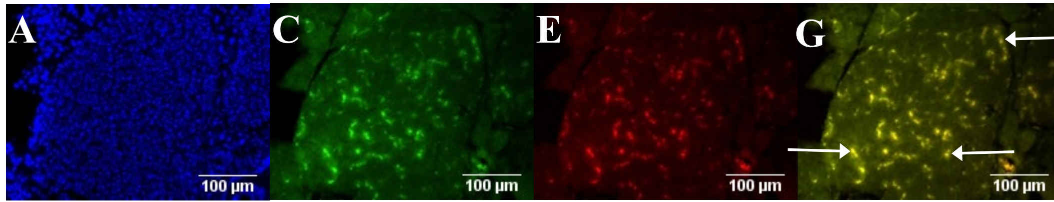

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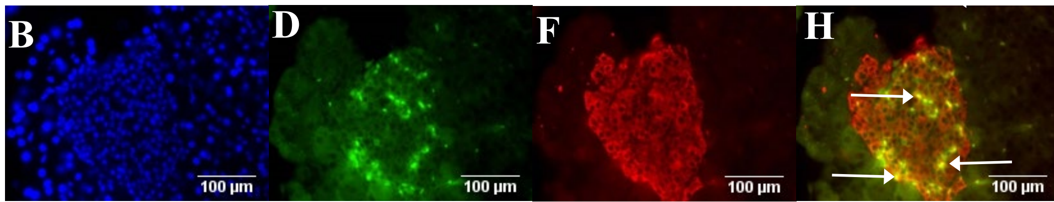

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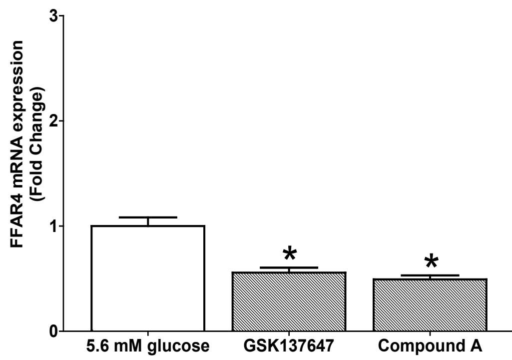

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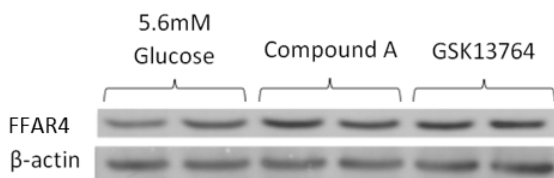

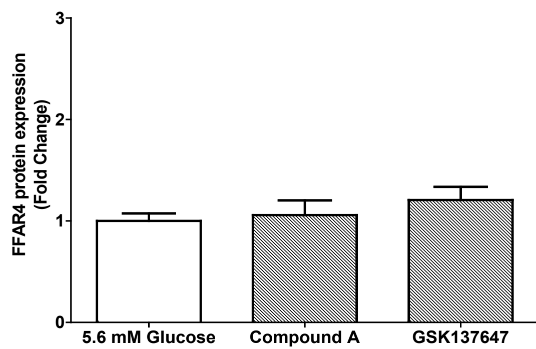

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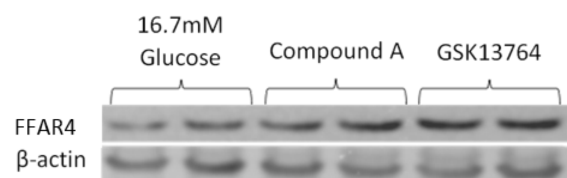

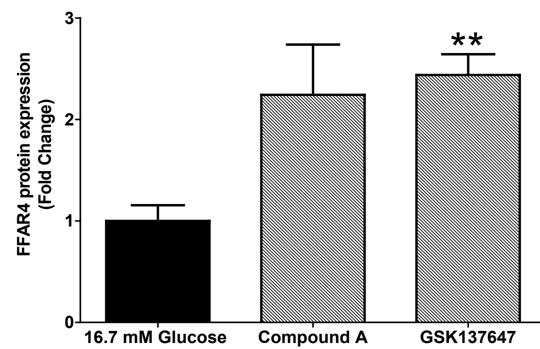

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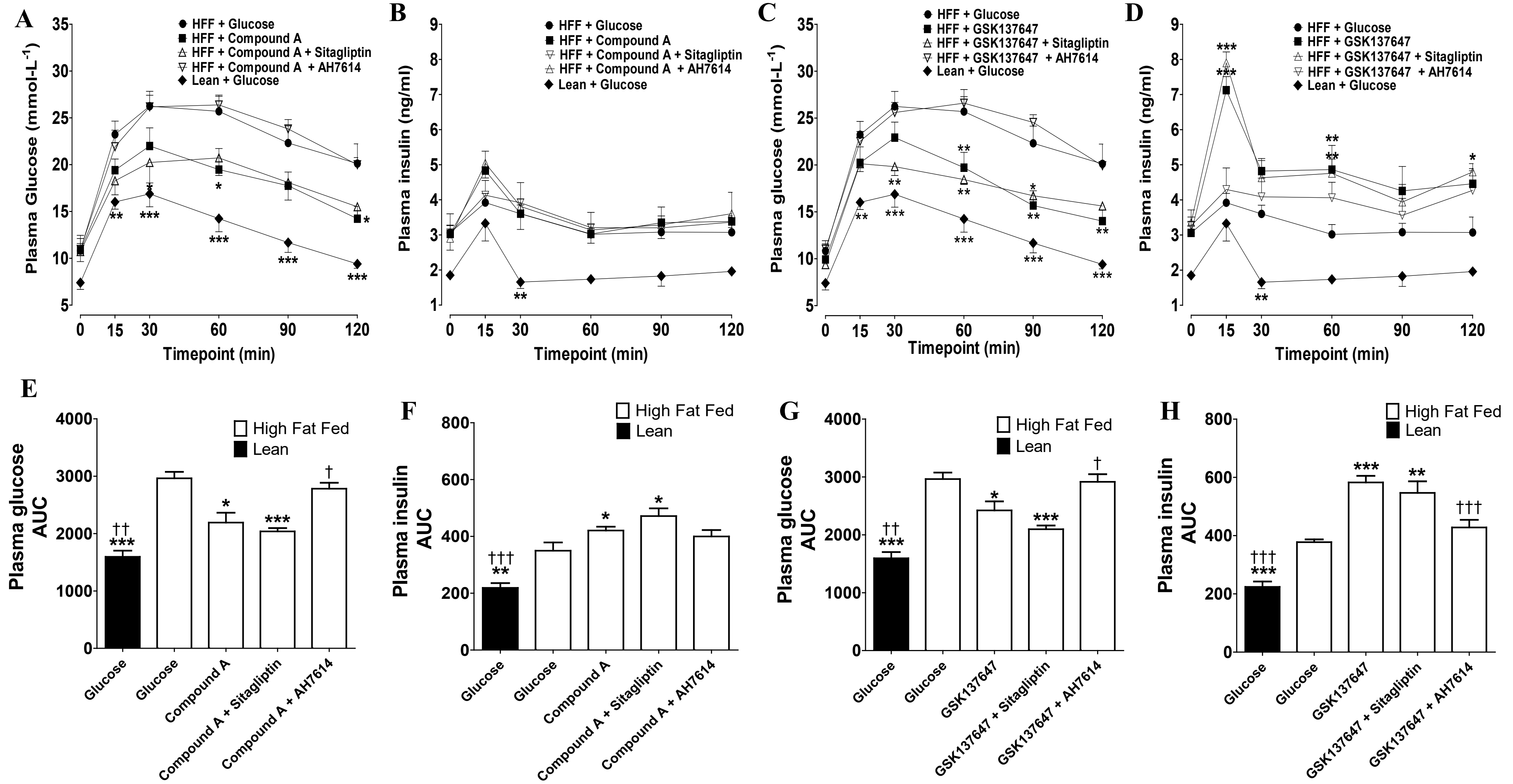

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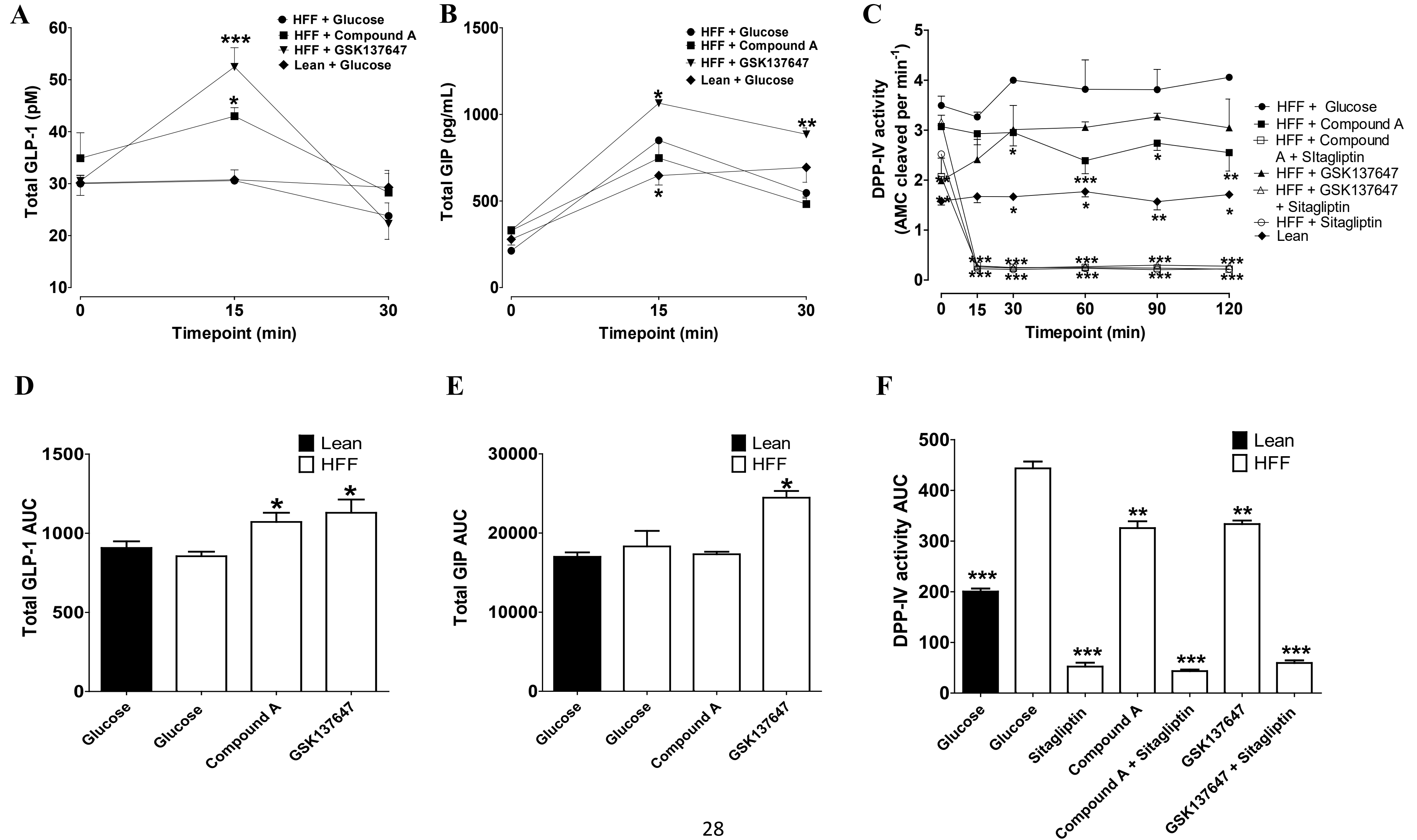

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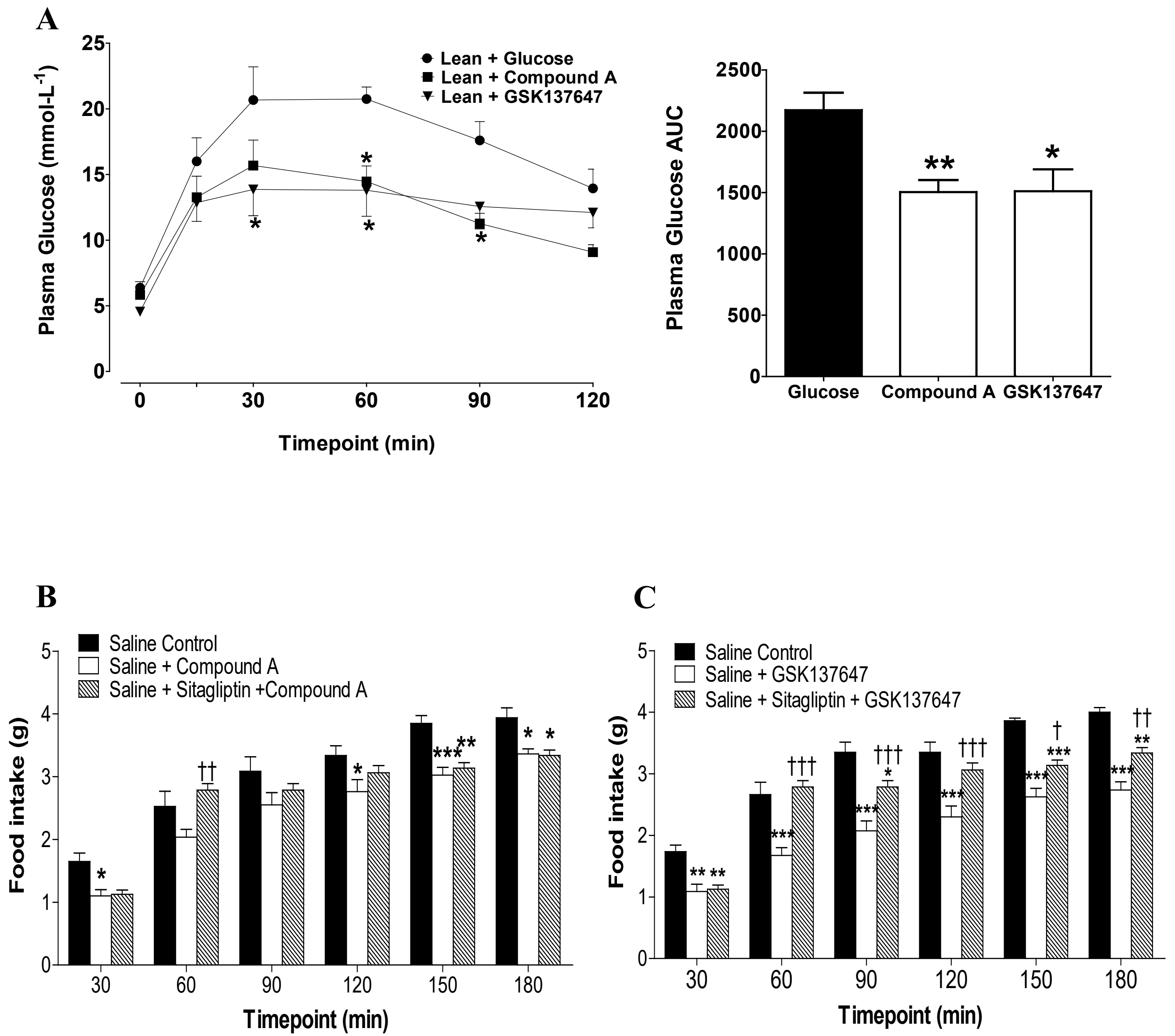
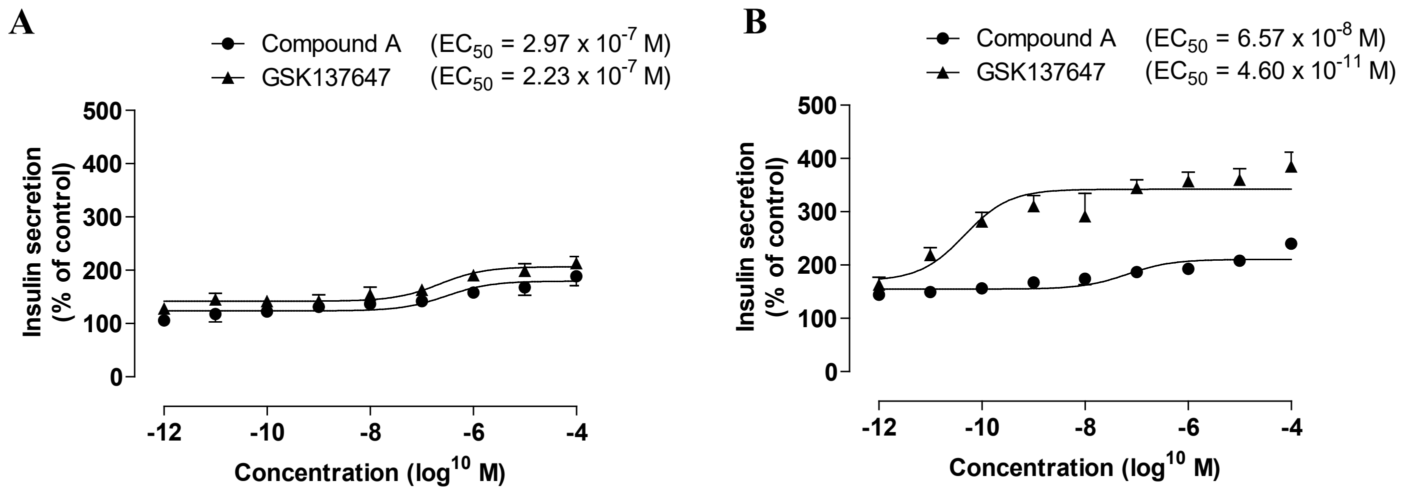


Supplementary material

Figure 1



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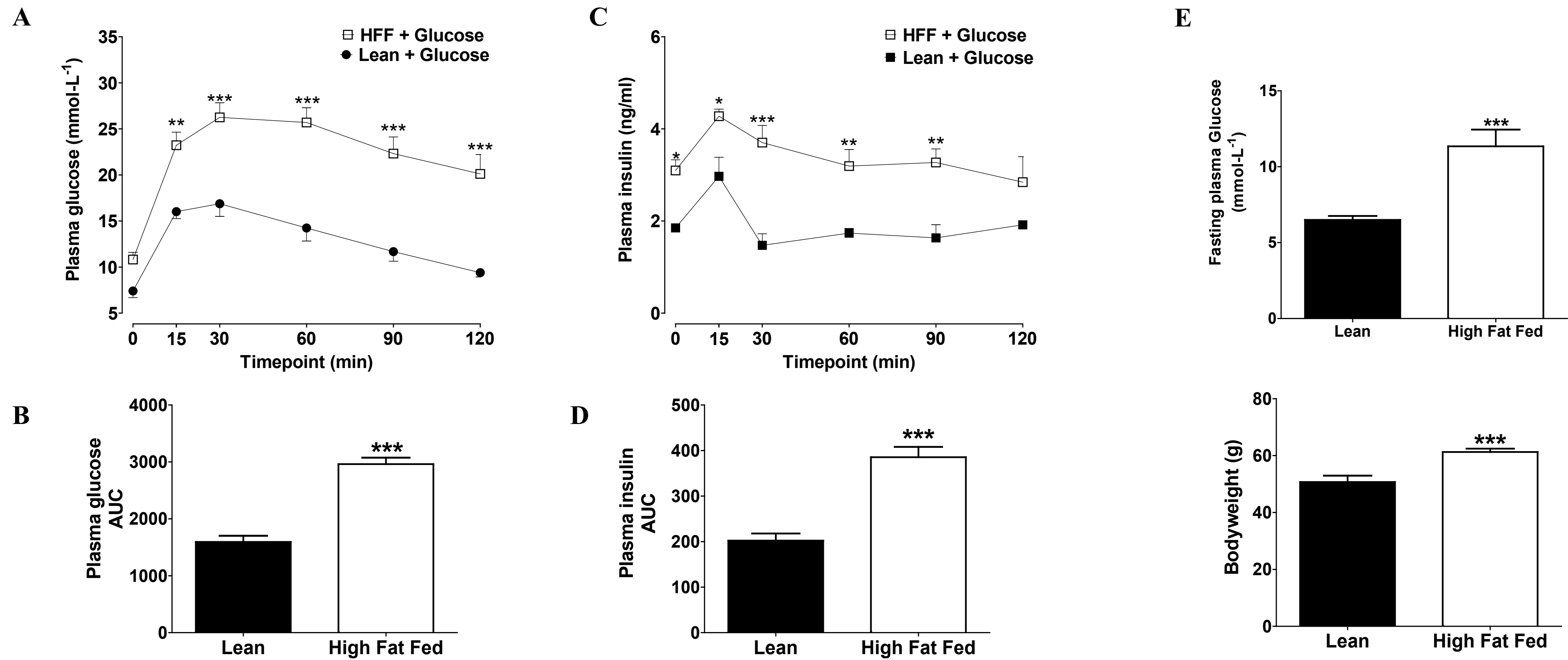

Figure 3

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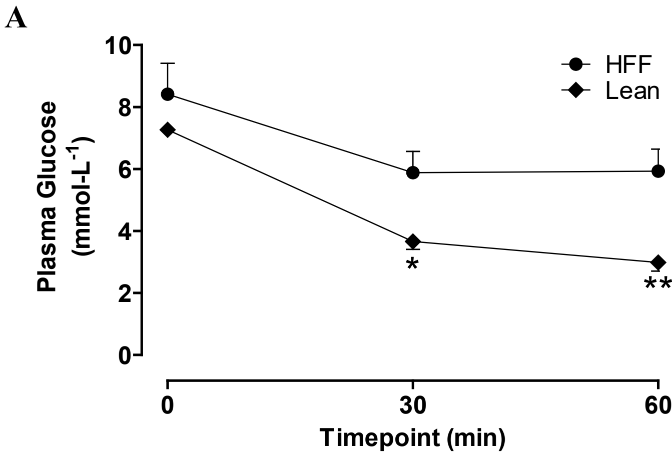

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