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The Myokine Irisin Is Released in Response to Saturated Fatty Acids and Promotes Pancreatic Beta-Cell Survival and Insulin Secretion.

Running title: Irisin effects on pancreatic beta-cells

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Abstract

This study explored the role of irisin as a new pancreatic beta-cell secretagogue and survival factor and its potential role in the communication between skeletal muscle and pancreatic beta-cells under lipotoxic conditions. Recombinant irisin stimulated insulin biosynthesis and glucose-stimulated insulin secretion (GSIS) in a PKA-dependent manner, and prevented saturated fatty acid-induced apoptosis in human and rat pancreatic beta-cells, as well as in human and murine pancreatic islets, via AKT/BCL2 signaling. Treatment of myotubes with 0.5 mM palmitate for 4 h, but not with oleate, promoted an increase in irisin release in the culture medium. Moreover, increased serum levels of irisin were observed in mice fed with a high-fat diet. The conditioned medium from myotubes exposed to 0.5 mM palmitate for 4 h significantly reduced apoptosis of insulin-secreting INS-1E cells, and this was abrogated in the presence of an irisin neutralizing antibody. Thus, the myokine irisin has the ability to promote beta-cell survival and to enhance GSIS, and may thus participate in the communication between skeletal muscle and beta-cells under conditions of excess saturated fatty acids.

Abbreviations

AKT: v-akt murine thymoma viral oncogene homolog · BrdU: Bromodeoxyuridine · ELISA: Enzymatic Linked Immunosorbent Assay · FFAs, free fatty acids · HUVECs: human umbilical vein endothelial cells.

47 Irisin is a newly discovered muscle-derived hormone, produced by cleavage of the membrane
48 protein fibronectin type III domain-containing protein 5 (FNDC5) and proposed to bridge
49 exercise with metabolic homeostasis (1). Irisin levels correlate with markers of insulin
50 resistance in non-diabetic subjects, while they are reduced in overt type 2 diabetes (2,3). The
51 biological functions of irisin include effects on multiple tissues (1,4–7). Irisin also improves
52 glucose tolerance and insulin sensitivity and increases energy expenditure in both obese and
53 diabetic mice (8). However, the effects of irisin on pancreatic beta-cells are not known.

54 If chronically in excess, saturated free fatty acids (FFAs) can reduce insulin biosynthesis (9)
55 and secretion (10) and promote beta-cell apoptosis (11,12), a phenomenon termed
56 «lipotoxicity». Excessively high plasma levels of FFAs, particularly long-chain saturated
57 FFAs, also foster insulin resistance (13). Palmitate can directly impair insulin signaling in
58 skeletal muscle cells (14) and alter the expression of some myokines (15).

59 Skeletal muscle, the major site of insulin-stimulated glucose disposal (16), has been recently
60 identified as a secretory organ able to release myokines. It is thus plausible that this organ
61 might interact with the endocrine pancreas by releasing myokines that adjust insulin secretion
62 to the actual insulin need for appropriate peripheral glucose utilization (17,18). In this study,
63 we have investigated the effects of irisin on pancreatic beta-cells and release of this myokine
64 by skeletal muscle cells.

65

Research Design and Methods

Animals. Animal experimentations were conducted following approval by the Ethics Committee (CESA) of IRSG, Biogem, Italy (internal ID 0907), in accordance with the NIH Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and regulations of Italy and EU. CD-1 mice were purchased from Charles River Laboratories (Calco, LC, Italy). The high fat diet (HFD, 60% of energy from fat) was from Mucedola, Italy.

Pancreatic islets and cell culture. Mouse and human islets were isolated and cultured as previously described (19,20). Cell lines were cultured in monolayer at 37 °C in a humidified incubator gassed with 5% CO_2. Rat L6 skeletal myoblasts (passage 5-38) were grown in modified Eagle's medium (MEM) supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin and 1% nonessential amino acids. FBS was replaced with 2% horse serum (HS) to induce myotube differentiation. Human skeletal muscle satellite cells were isolated from *rectus abdominis* muscle biopsies by trypsin digestion, grown in F-12 nutrient mixture (Ham) supplemented as above, and differentiated in 2% HS. Rat insulin-secreting INS-1E cells (passage 15-30; a kind gift from C. B. Wollheim, University of Geneva, Geneva, Switzerland) were grown in 11 mM glucose unless otherwise indicated (21). Human pancreatic insulin-releasing 1.1B4 cells (passage 15-40; purchased from ECACC, European Collection of Cell Cultures, Sigma-Aldrich, St Louis, MO, USA) were grown as described (22). All reagents for cell culture were from Life Technologies, Carlsbad, CA, USA. Palmitate and oleate were obtained from Sigma-Aldrich, St Louis MO, and prepared as previously reported (12,21).

Cells were preincubated with recombinant irisin (AdipoGen SA, Liestal, Switzerland) for the indicated doses and times. The chemical inhibitors AKT Inhibitor VIII (2.5 μM) or H-89 (5

90 μM) (from Calbiochem, Merck Millipore, Darmstadt, Germany) were added 30 min or 60 min
91 before irisin.

92 **Cell transfection.** L6 cells were transfected with 30 nM *Fndc5* small interfering RNA
93 (s141378; Life Technologies, Arlsbad, CA, USA) using Lipofectamine RNAiMAX
94 Transfection Reagent (6 μl/well; Gibco Invitrogen) for 48 h.

95 **Immunoblotting.** Cells lysates were obtained and analyzed by immunoblotting, as described
96 (12). A list of the antibodies used is shown in Supplementary Table 1.

97 **Gene expression analysis by quantitative RT-PCR.** RNA isolation, cDNA synthesis, and
98 mRNA quantitation were carried out as described (12). A list of the primer sequences used is
99 shown in Supplementary Table 2.

100 **Apoptosis and cell proliferation assays.** Apoptosis was measured by using the Cell Death
101 Detection ELISA^{PLUS} kit (Roche Biochemicals Indianapolis Indiana, USA) and by assessing
102 caspase-3 cleavage. Proliferation was assessed with the BrdU ELISA Kit (colorimetric)
103 (Abcam, Cambridge, UK).

104 **Irisin and insulin assays.** Irisin concentrations were measured using an irisin competitive
105 ELISA Kit (AdipoGen SA, Liestal, Switzerland). Insulin levels were measured by a mouse-
106 specific ELISA (Mercodia AB, Sylveniusgatan, Uppsala, Sweden). GSIS was performed as
107 described (12).

108 **Statistical analyses.** Data were analyzed by the Student's t test or ANOVA, as appropriate,
109 and are presented as mean ± SE. Statistical significance was set at p value <0.05.

110

111 **Results**

112 **Irisin prevents palmitate-induced beta-cell apoptosis and induces insulin secretion.**

113 INS-1E cells treated with 0.5 mM palmitate for 24 h showed increased caspase-3 cleavage, as
114 expected (*p<0.05 vs control without palmitate; Fig. 1A). However, pretreatment with
115 recombinant irisin for 24 h reduced this response in a dose-dependent manner (†p<0.05 vs
116 palmitate alone; Fig. 1A). A similar anti-apoptotic effect of irisin was observed in human
117 pancreatic 1.1B4 cells and in murine and human pancreatic islets (†p<0.05 vs palmitate; Fig.
118 1B). Irisin prevented palmitate-induced apoptosis through a mechanism involving B-cell
119 lymphoma 2 (BCL2) and BCL2-associated X protein (BAX), key apoptosis regulatory
120 proteins. While palmitate increased BAX expression and, consequently, reduced the
121 BCL2/BAX ratio, pretreatment with irisin increased BCL2 expression and restored the
122 BCL2/BAX ratio (*p<0.05 vs control without palmitate; †p<0.05 vs palmitate; Fig. 1C).
123 Treatment of INS-1E cells with irisin increased AKT phosphorylation (#p<0.05 vs basal; Fig.
124 1D), which is known to upregulate BCL2 expression (23). When INS-1E cells were pretreated
125 with the AKT Inhibitor VIII, irisin-mediated AKT phosphorylation was abrogated, and
126 augmentation of BCL2 protein expression (Fig. 1E) and inhibition of palmitate-induced
127 apoptosis (Fig. 1F) were prevented (*p<0.05 vs control without palmitate; †p<0.05 vs
128 palmitate; @p<0.05 vs control without AKT Inhibitor VIII). Therefore, irisin exerts anti-
129 apoptotic effects by activating AKT and increasing BCL2.

130 Exposure of murine islets and rat INS-1E cells to irisin also increased *Insulin* mRNA levels
131 and insulin content, and augmented GSIS (*p<0.05 vs basal, §p<0.05 vs 3 mM glucose; Figs.
132 2A-G). These responses were dependent on PKA activation, since they were abrogated by the
133 PKA inhibitor H-89 (*p<0.05 vs basal; †p<0.05 vs irisin alone; Figs. 2D-G), as was the irisin-
134 induced CREB phosphorylation at 25 mM glucose (*p<0.05 vs basal; †p<0.05 vs irisin; Fig.

135 2H). Moreover, irisin slightly increased proliferation of INS-1E cells (*$p<0.05$ vs basal;
136 Supplementary Fig. 1).

137 **Saturated FFAs regulate FNDC5 expression and irisin release by skeletal muscle cells.**

138 Treatment of rat myotubes with palmitate for 4 h resulted in increased FNDC5 mRNA levels
139 and protein content (*$p<0.05$ vs basal; Fig. 3A, B) and caused a 3-fold higher release of irisin
140 in the culture medium compared with untreated myotubes (*$p<0.05$ vs basal; Fig. 3C). By
141 contrast, in myotubes treated with palmitate for 24 h FNDC5 mRNA levels and protein
142 content were significantly reduced (*$p<0.05$ vs basal; Fig. 3A, B), and irisin release in the
143 culture medium was unchanged (Fig. 3C). Moreover, oleate, a monounsaturated FFA, did not
144 stimulate irisin release (Supplementary Fig. 2). Human myotubes showed a similar regulation
145 of irisin release by palmitate (Fig. 3E), even though FNDC5 mRNA levels were unchanged
146 (Fig. 3D). Moreover, using a siRNA to FNDC5 prevented the palmitate-induced increase of
147 FNDC5 protein expression while irisin release was unaffected (Supplementary Fig. 3).
148 Validation of the ELISA kit and irisin antibody is shown in Supplementary Fig. 4.

149 Serum irisin levels were also measured *in vivo* in CD-1 mice fed with a standard diet (SD) or
150 high fat diet (HFD) for 63 days. The HFD resulted in markedly elevated triglyceride levels
151 (data not shown) and caused a rapid increase of serum irisin concentrations, which persisted at
152 later times (#$p<0.05$ vs baseline; †$p<0.05$ vs mice fed with a SD; Fig. 3F).

153 **Irisin secreted by L6 myotubes promotes INS-1E cell survival.**

154 The role of myotube-derived irisin on regulation of beta-cell survival was investigated next.
155 Culture medium was collected from L6 myotubes exposed to 0.5 mM palmitate for 4 h
156 (PM4h) or 24 h (PM24h), or from untreated L6 myotubes (PM0h), and the effects on beta-cell
157 apoptosis were evaluated. Samples of L6-free culture medium with (CTR2) or without

158 (CTR1) 0.5 mM palmitate were used as additional controls. CTR1 and PM0h had no effects
159 on apoptosis of INS-1E cells, while CTR2 increased INS-1E apoptosis, as expected (*p<0.05
160 vs CTR1; †p<0.05 vs CM; Fig. 4A, B). However, apoptosis was increased further when INS-
161 1E cells were exposed to PM24h. By contrast, apoptosis markers were significantly reduced
162 when INS-1E cells were exposed to PM4h (*p<0.05 vs CTR1; †p<0.05 vs PM0h; #p<0.05 vs
163 CTR2; Fig. 4A, B), and this was abrogated by a neutralizing antibody directed against irisin
164 ($^{@}p$<0.05 vs PM4h; Fig. 4C, D), which also inhibited the irisin-induced increase of UCP-1 in
165 3T3-L1 adipocytes (Supplementary Fig. 5). Thus, irisin released by cultured myotubes in
166 response to palmitate exerts anti-apoptotic effects on beta-cells.
167

168 **Discussion**

169 Here we demonstrate for the first time that recombinant irisin protects beta-cells from
170 palmitate-induced apoptosis, through a mechanism involving AKT/BCL2 signaling (Fig. 1).
171 Similarly, in the HUVECs irisin partly suppressed high glucose-induced apoptosis by
172 affecting BCL2, BAX and caspase expression (7). Furthermore, irisin promotes insulin
173 biosynthesis and secretion in a PKA-dependent manner (Fig. 2). The beneficial effects of
174 irisin on beta-cells resemble those of GLP-1 and its analogs, enhancing the secretory function
175 and survival of beta-cells in a comparable way (24). Like GLP-1, irisin induces cyclic AMP
176 generation (data not shown) and activates PKA and AKT, suggesting the existence of a
177 specific receptor that has not been yet identified.

178 Short-term treatment of rat myotubes with palmitate results in increased FNDC5 mRNA
179 levels and protein content (Fig. 3A, B) and raises irisin levels in the culture medium 3-fold
180 compared with untreated myotubes (Fig. 3C). The mechanism by which saturated FFAs
181 induce irisin release from skeletal muscle cells is still unclear, and may involve the specific
182 activation of FNDC5 cleavage by protease(s). In human myotubes, higher irisin release in the
183 culture medium in response to palmitate occurs in the absence of changes in FNDC5 mRNA
184 levels (Fig. 3D, E). Furthermore, in rat myotubes, FNDC5 knock-down prevents the
185 palmitate-induced increase of FNDC5 protein expression, without affecting irisin release
186 (Supplementary Fig. 3). Altogether, these results suggest independent regulation of FNDC5
187 expression and cleavage, respectively, by FFAs. Irisin release appears to be also influenced by
188 the type of FFA, since oleate, a monounsaturated FFA, does not stimulate irisin release from
189 myotubes (Supplementary Fig. 2).

190 Mice receiving a HFD and gaining weight display an early increase in serum irisin levels
191 which persists thereafter (Fig. 3F). This is in line with the observed positive correlation

192 between serum irisin levels and the BMI, markers of adiposity and HOMA-IR index in
193 humans (3). Circulating irisin is not released exclusively by skeletal myotubes, since also
194 adipose tissue secretes irisin (25). However, approximately 72% of circulating irisin is
195 estimated to be released by skeletal muscle (1), even if tissue-specific FNDC5 knockout mice
196 are not available to address this issue.

197 We could not target the actions of irisin on pancreatic beta-cells *in vivo* due to the lack of
198 identification of the irisin receptor and difficulty in neutralizing circulating irisin in mice.
199 However, the results with the CM from palmitate-treated myotubes indicate that also
200 myotube-derived irisin, like recombinant irisin, can prevent apoptosis in beta-cells (Fig. 4).
201 The anti-apoptotic effect of PM4h in INS-1E cells was abrogated by an irisin neutralizing
202 antibody (Fig. 4C, D); furthermore, PM24h, in which irisin levels were not increased (Fig.
203 3C), did not counteract beta-cell apoptosis (Fig. 4A, B). However, in contrast to recombinant
204 irisin, PM4h did not enhance GSIS (Supplementary Fig. 6). Since distinct biological effects of
205 irisin are known to be elicited at different doses (1,4–7), it is possible that higher irisin
206 concentrations than those in PM4h are required to enhance GSIS. Alternatively, other
207 myokines in PM4h could counteract the action of irisin on insulin secretion or differences
208 between recombinant and cell derived irisin may exist.

209 In conclusion, the myokine irisin has anti-apoptotic actions on pancreatic beta-cells and
210 stimulates insulin biosynthesis and secretion. Under conditions of excess saturated FFAs,
211 irisin release from skeletal muscle may be promoted as an adaptive response, enhancing beta-
212 cell survival to compensate for the increased insulin resistance and challenge of glucose
213 tolerance.

214

215

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221 **Duality of Interest.** The authors declare that AN, NM and FG are named inventors of a
222 pending patent application related to the work described. FG has received grant support from
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225 **Author Contributions.** AN, NM and FG designed the study. GB and RS acquired data. NM,
226 GB and RL performed experiments and data analysis. AN, NM and GB performed data
227 analysis and interpretation. AC, GB, RS and RL participated in interpretation and discussion
228 of the data. AN, NM and FG wrote the article. PM, LL, and SP critically reviewed the
229 manuscript for intellectual content. All the authors approved the final version of the
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231

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322

323

324 **Figure Legends**

325

326 **Figure 1.** Effects of irisin on palmitate-induced apoptosis in pancreatic beta-cells. Rat (INS-
327 1E) and human (1.1B4) beta-cells and murine and human islets were treated with or without
328 recombinant irisin (10-100 nM) for 24 h and then incubated with or without 0.5 mM palmitate
329 for 24 h. A: Apoptosis was measured by assessing caspase-3 cleavage in INS-1E insulin-
330 secreting cells (n = 11 independent experiments). Densitometric analysis of the related bands
331 was expressed as relative optical density of the bands, corrected using β-actin as a loading
332 control and normalized against untreated control. B: Apoptosis was evaluated by measuring
333 cytoplasmic oligonucleosomes with an ELISA assay (data expressed as percentage of
334 untreated control) in INS-1E cells (n = 7 independent experiments), murine islets (n = 3
335 independent experiments), 1.1B4 HPC (n = 9 independent experiments) and human islets (n =
336 3 independent experiments). C: BCL2 and BAX protein content was measured by
337 immunoblotting and quantified by densitometry in INS-1E cells. The BCL2/BAX ratio was
338 also calculated (n = 8 independent experiments). D: AKT phosphorylation was measured by
339 immunoblotting and quantified by densitometry (n = 6 independent experiments) in INS-1E
340 cells treated with 100 nM recombinant irisin for different times. E and F: INS-1E cells were
341 treated with or without 100 nM recombinant irisin for 24 h in presence or absence of the AKT
342 inhibitor VIII (2.5 μM) for 60 min and then incubated with or without 0.5 mM palmitate for
343 24 h. (E) AKT phosphorylation and BCL2 protein content was measured by immunoblotting
344 and quantified by densitometry (n = 8 independent experiments). Densitometric analysis of
345 the related bands was expressed as relative optical density of the bands, corrected using β-
346 actin as loading controls and normalized against untreated control. (F) Cell death was
347 evaluated by measuring cytoplasmic oligonucleosomes with an ELISA assay and expressed as
348 percentage of untreated control. Data are expressed as mean ± SE. *p <0.05 vs control

349 without palmitate; †p <0.05 vs palmitate; #p<0.05 vs basal; @p < 0.05 vs control without
350 AKT Inhibitor VIII. Palm, palmitate.
351
352 **Figure 2.** Effects of irisin on insulin biosynthesis and glucose-induced insulin secretion
353 (GSIS). A-C: Murine islets were incubated with 100 nM recombinant irisin for 60 min. A:
354 *Insulin* gene expression was evaluated by q-RT PCR analysis normalized to *18S* (n = 4
355 independent experiments). B: GSIS was measured after 60 min at 3 mM glucose (white bars =
356 basal secretion) followed by 60 min at 25 mM glucose (dark bars = stimulated secretion) (n =
357 3 independent experiments). Secretion is normalized to protein concentration and is expressed
358 as a percentage of untreated control. C: Fold increase of 25 mM vs 3 mM GSIS. D-G: INS-1E
359 cells were treated with or without 5 μM H-89 for 30 min before incubation with 100 nM
360 recombinant irisin for 60 min. D: *Insulin* gene expression was evaluated by q-RT PCR
361 analysis normalized to *Gusb* gene expression (n = 5 independent experiments). E: Insulin
362 content was evaluated by an ELISA assay (n = 9 independent experiments). F: GSIS was
363 measured after 60 min at 3 mM glucose (white bars = basal secretion) followed by 60 min at
364 25 mM glucose (dark bars = stimulated secretion) (n = 18 independent experiments).
365 Secretion is normalized to protein concentration and is expressed as a percentage of untreated
366 control. G: Fold increase of 25 mM vs 3 mM GSIS. H: INS-1E cells were cultured in medium
367 at 25 mM glucose and were treated with or without 5 μM H-89 for 30 min before incubation
368 with 100 nM recombinant irisin for 10 min. CREB phosphorylation was measured by
369 immunoblotting and quantified by densitometry. Densitometric analysis of the related bands
370 was expressed as relative optical density of the bands, corrected using β-actin as loading
371 control and normalized against untreated control (n = 7 independent experiments). Data are
372 expressed as mean ± SE. §p < 0.05 vs 3 mM glucose; *p < 0.05 vs basal; †p < 0.05 vs irisin.
373 Palm, palmitate.

374

375 **Figure 3.** Effect of saturated FFAs on FNDC5 expression and irisin release by skeletal
376 muscle cells. L6 rat skeletal muscle cells were exposed to 0.5 mM palmitate for 4 h or 24 h.
377 A: *Fndc5* gene expression was evaluated by q-RT PCR analysis and normalized to *Gusb* gene
378 expression (n = 6 independent experiments). B: FNDC5 protein expression was evaluated by
379 immunoblotting and quantified by densitometry (n = 8 independent experiments).
380 Densitometric analysis of the related bands was expressed as relative optical density of the
381 bands, corrected using β-actin as a loading control and normalized against the untreated
382 control. C: Irisin secretion in the culture medium was measured by ELISA assay and was
383 expressed as percentage of untreated control (n = 3 independent experiments). Irisin
384 concentration in the conditioned medium (CM) of L6 cells treated with 0.5 mM palmitate for
385 4 h was 2.1 nM. D and E: Human skeletal muscle cells were exposed to 0.5 mM palmitate for
386 4 h. D: *FNDC5* gene expression was evaluated by q-RT PCR analysis and normalized to *18S*
387 gene expression (n = 6 independent experiments). E: Irisin secretion in culture medium was
388 measured by ELISA assay and was expressed as percentage of untreated control (n = 6
389 independent experiments). F: From weaning at the age of 5 weeks onwards CD-1 mice
390 received a standard diet (SD); then, they were randomized to either a high-fat diet (HFD) or
391 the SD for an additional 63 days. Blood samples were collected from the tail vein. Serum
392 irisin levels in mice fed with HFD (black circles; n = 15) or SD (white circles; n = 15) during
393 the observation were measured by ELISA assay and were expressed as fold over baseline
394 (left) or as nM (right; data from days 0, 2 and 21 in the HFD group). Data are expressed as
395 mean ± SE. **p* <0.05 vs basal; #*p* <0.05 vs baseline; †*p* <0.05 vs SD. Palm, palmitate.

396

397 **Figure 4.** Effects of irisin secreted by L6 myotubes on INS-1E cell survival. Samples of
398 conditioned medium (CM) were obtained by culturing L6 rat myotubes for 0 h (PM0h), 4 h

399 (PM4h) or 24 h (PM24h) with 0.5 mM palmitate. Culture medium not exposed to L6 cells
400 with (CTR2) or without (CTR1) 0.5 mM palmitate was used as additional control. CM was
401 collected, transferred into 6-well plates containing INS-1E cells, and left for 24 h. A: INS-1E
402 cells apoptosis. Cell death was evaluated by measuring cytoplasmic oligonucleosomes with an
403 ELISA assay (data expressed as percentage of CTR1) (left) or by assessing caspase-3
404 cleavage (right) (n = 6 independent experiments). B: INS-1E cells were cultured with PM4h
405 with or without a neutralizing antibody directed against irisin. Cell death was evaluated by
406 measuring cytoplasmic oligonucleosomes with an ELISA assay (data expressed as percentage
407 of CTR1) (left) or by assessing caspase-3 cleavage (right) (n = 4 independent experiments).
408 Densitometric analysis of the related bands was expressed as relative optical density of the
409 bands, corrected using β-actin as a loading control and normalized against CTR1. Data are
410 expressed as mean ± SE. §$p < 0.05$ vs basal; *$p < 0.05$ vs CTR1; †$p < 0.05$ vs PM0h; #$p <$
411 0.05 vs CTR2; @$p < 0.05$ vs PM4h.