Histol Histopathol (2018) 33: 1167-1180

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Insulin degrading enzyme is up-regulated in pancreatic β cells by insulin treatment

Cristina M. Fernández-Díaz[1]*, Luis Escobar-Curbelo[2]*, J.F. López-Acosta[1], Carmen D. Lobatón[1], Alfredo Moreno[1], Julián Sanz-Ortega[2], Germán Perdomo[3] and Irene Cózar-Castellano[1]

[1]Institute of Molecular Biology and Genetics-IBGM (University of Valladolid-CSIC), Valladolid, [2]Clinical Hospital San Carlos, Madrid and [3]Department of Health Sciences, School of Health Sciences, University of Burgos, Burgos, Spain

*Both authors contributed equally

Summary. Insulin Degrading Enzyme (IDE) is an endopeptidase that degrades insulin and glucagon. *Ide* gene has been associated with type-2 diabetes mellitus (DM2). However, the physiological role(s) of IDE in glucose homeostasis and its potential therapeutic benefit remain not completely known.

To contribute in the understanding of IDE's role in glucose metabolism, we analyzed IDE protein level in pancreatic islets from two hyperinsulinemic mouse models, db/db and high-fat diet (HFD) mice, as well as in human islets from DM2 patients treated with oral hypoglycemic agents (OHAs) or insulin. IDE protein level was detected by staining and by western-blot. INS1E cells, rat and human islets were treated with insulin and IDE protein level was studied.

We have shown for the first time IDE staining in rodent and human tissue, using the proper negative control, IDE null mouse tissue. Our staining indicates that IDE is expressed in both beta- and alpha-cells, with higher expression in alpha-cells. Db/db and HFD mice islets showed increased IDE protein level. Interestingly, human islets from DM2 patients treated with OHAs showed decreased IDE protein level in beta-cells. Meanwhile, islets from insulin-treated DM2 patients showed augmented IDE protein level compared to OHAs patients, pointing to an upregulation of IDE protein level stimulated by insulin. These data correlate nicely with insulin-stimulated upregulation of IDE in cultured INS1E cells, as well as in rat and human islets.

In conclusion, our study shows that IDE is expressed in pancreatic beta- and alpha-cells of both rodents and humans, having higher expression in alpha-cells. Furthermore, insulin stimulates IDE protein level in pancreatic beta-cells. These results may have implications in how DM2 patient's treatment affects their beta-cell function.

Key words: Insulin-degrading enzyme, Type 2 diabetes, Insulin treatment, OHAs, Beta-cells, Alpha-cells, Rodent islets, Human islets

Offprint requests to: Irene Cózar-Castellano, PhD, Instituto de Biología y Genética Molecular (IBGM), Dpto. Fisiología y Bioquímica, Facultad de Medicina (5ª Planta), Universidad de Valladolid, C/ Ramón y Cajal, 7 47005 Valladolid, Spain. e-mail: irene.cozar@uva.es

DOI: 10.14670/HH-11-997

Introduction

Insulin-degrading enzyme (IDE) is a metalloprotease that degrades several peptides including insulin and glucagon. It is localized in cytoplasm, cell membranes, some organelles and it is also secreted into the extracellular space. IDE is ubiquitously expressed, supporting a multifunctional role. Although its most known function is to degrade circulating insulin in liver and kidneys (Duckworth et al., 1998; Pivovarova et al., 2016). The *Ide* gene is localized in one of the susceptibility loci linked to type-2 diabetes mellitus (DM2) (Gu et al., 2004; Schulze et al., 2007; Sladek et al., 2007), and some *Ide* polymorphisms have been associated with the DM2 population (Karamohamed et al., 2003; Kwak et al., 2008).

It has been proposed that IDE inhibition could be used therapeutically for DM2 treatment (Pivovarova et al., 2016; Tang, 2016). This line of reasoning is based on the assumption that *in vivo* inhibition of IDE could be used to reduce insulin clearance and thereby augment insulin levels in patients with DM2. Ideally, these

inhibitors should preferentially modulate IDE activity on insulin, without affecting degradation of other IDE substrates. Of note, IDE inhibitors that bind away from the catalytic cleft improve glucose tolerance in mice, suggesting that IDE might be a promising therapeutic target for the treatment of DM2 (Maianti et al., 2014). However, other IDE inhibitors have not exhibited similar results (Durham et al., 2015).

More controversial is the role of IDE in regulating glucose homeostasis in mice. Rather than improving glucose tolerance, deletion of the *Ide* gene in mice results in the development of pronounced glucose intolerance as well as insulin insensitivity (Farris et al., 2003; Abdul-Hay et al., 2011; Steneberg et al., 2013).

The function of IDE in pancreas remains largely unknown. Stenberg et al. demonstrated that IDE is required for insulin secretion in the IDE-KO mouse (Steneberg et al., 2013). However, there is paucity of data in the literature regarding IDE protein levels in pancreatic islet cells, and its potential relationship with pathophysiological conditions, such as DM2.

To better understand the pathophysiology of IDE in DM2, there is a pressing need to elucidate the basic biology of IDE in pancreatic endocrine cells. In the present study, we investigated IDE protein levels in alpha- and beta-cells in rodent models of obesity and DM2 and in cadaveric human pancreata from DM2 patients. Our findings suggest that IDE may play an important role in alpha cell function and point to insulin levels as a critical factor for the regulation of IDE protein levels.

Material and methods

Animal models

Animals were housed in ventilated cages on a 14-hours light, 10-hours dark schedule at the animal facility of the University of Valladolid (UVa, Spain). Cage enrichment included cotton bedding. Water and food were available ad libitum, consisting of normal chow. Animal experiments were approved by University of Valladolid Research Animal Ethical Committee and JCyL authorities (protocol #5003931).

Leptin receptor heterozygous mice BKS.Cg-+Leprdb/+ Lepr +/OlaHsd (db/+) were purchased (Harlan, UK) and then they were bred in our animal facility to obtain +/+ or db/db mice. 8 week old C57Bl6/J male mice were bought (Charles River, France) and they were fed standard (SD) or high fat diet (60% kcal% fat, Research Diets, USA) (HFD) for 18 weeks.

Three-month-old male Sprague Dawley rats were born at the University of Valladolid Animal facility; they were fed SD and water *ad libitum*.

Glucose and insulin determinations in mouse blood

Plasma samples were obtained from tail blood samples of mice under fasting (16h) or non-fasting conditions. Blood glucose levels were measured directly from tails using the Breeze2 Glucometer (Bayer, Germany). Plasma insulin levels were measured using Ultrasensitive Mouse Insulin ELISA (Mercodia AB, Sweden).

Human pancreas samples

Human pancreas samples were obtained from the biobank of the department of pathology/Biobank of the San Carlos Clinical Hospital in Madrid (protocol authorized by the Ethics Committee for Clinical Research # 12/202 and according to the Declaration of Helsinki). Human pancreata were classified into three groups: non-diabetic population (control), DM2 patients treated with oral hypoglycemic agents (DM2+OHAs) and DM2 patients undergoing insulin treatment (DM2+Insulin). Cases were categorized by sex and age. Controls without DM2 diagnosis were matched by age and sex. Control, n=9; DM2+OHAs, n=9; DM2+insulin, n=8.

The samples were selected using the following inclusion criteria: patients aged between 40 and 80 years; pancreas control in which morphologically normal islets were observed, suitable structure without tissue deteriorated in hematoxylin-eosin staining; samples have a minimum number of pancreatic islets by microscopic examination (n≥5 per slide).

A description of each subject included in the study is shown in Table 1.

Pancreatic islet isolation

Three-month-old male Sprague Dawley rats (fed SD), 3-month-old db/db and +/+ mice (fed SD); and 6-month old C57Bl6/J mice (fed SD or HFD) were euthanized by CO_2, and afterwards their pancreata were perfused with 1.7 mg/mL collagenase P (Roche Diagnostics, USA). Islets were isolated and purified as previously reported (Cozar-Castellano et al., 2004). Afterwards islets were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5.5 mM D-glucose, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The islets were maintained at 37°C with 5% CO_2 in a humidified atmosphere.

Human islets were obtained from Dr. Olle Körsgren lab at the University of Uppsala (Sweden) (European Consortium for Islet Transplantation).

Pancreas and islets staining and histomorphometry

Db/db and +/+ mice as well as HFD and SD mice were euthanized by CO_2, and afterwards their pancreata were dissected and fixed in 10% neutral buffer formalin overnight and embedded into paraffin blocks.

Human pancreas samples were obtained from cadaveric donors using ultrasound imaging to obtain tissue samples with minimum impact on the deceased body. Pancreas samples were obtained and fixed with

4% formaldehyde for 48-72 hours, and then embedded into paraffin blocks.

Five-micrometer pancreas serial sections were obtained and stained with: Guinea pig anti-insulin antibody (#7842, Abcam, UK), mouse anti-glucagon antibody (#10988, Abcam, UK) and rabbit anti-IDE antibody (#9210, Millipore, USA). The following secondary antibodies were used: Anti- Guinea pig-Alexa 488 (#A11073, Invitrogen Ltd, Europe), Anti-Mouse-Alexa 647 (#A21236, Invitrogen Ltd, Europe), Anti-Rabbit-Alexa 594 (#A11012, Invitrogen Ltd, Europe). Fluorescence images of the sections were acquired using a NIKON Eclipse 90i microscope associated with CCD NIKON camera (DSRi1), using a 20x objective.

Human and rat islets were treated with 500 nM insulin (Sigma-Aldrich, USA) at indicated time points. Afterwards, islets were fixed with 10% neutral buffer formalin for 1 h and embedded in paraffin blocks. Five-micrometer sections were obtained and stained as reported for pancreas section. Fluorescence images were acquired in non-saturated conditions using Confocal Leica TCS SP5 microscope, using a 63x oil immersion objective.

The number and area of pancreatic beta- or alpha-cells and/or IDE-positive cells; and the integrated density (for IDE fluorescence quantification) were quantified using ImageJ software (NIH, USA).

INS1E cells and pancreatic islets insulin treatment

INS1E cells were a gift of Dr. Pierre Maechler (University of Geneva, Switzerland). Cells were plated to a density 110,000 cells/cm^2 and they were grown in RPMI 1640 Glutamax (GIBCO, NY), 11 mM Glucose, 10 mM Hepes (Invitrogen Ltd, Europe), 0.05 mM β-Mercaptoethanol (Sigma-Aldrich, USA) 1 mM Sodium Pyruvate (Invitrogen Ltd, Europe), 5% FBS and 1% Penicillin- Streptomycin (Invitrogen Ltd, Europe). Cells were cultured at 37°C with 5% CO_2 in a humid atmosphere.

Cells were seeded in 24-well plates and treated in serum-free medium 24 hours before the experiment. 24 hours later, they were treated with serum free medium containing 500 nM insulin (Sigma-Aldrich, USA) at indicated time points.

Rat and human islets were plated in cell culture inserts into 24-well plates at a density of 400 IEq (islet equivalents) in serum-free medium overnight. 24 hours later, islets were treated with serum medium containing 500 nM insulin at indicated time points.

Western-blot

Islets from mice and humans were homogenized in lysis buffer and briefly sonicated. Proteins were quantified by the Micro BCA Kit (Thermo Scientific, USA), separated by SDS-PAGE, and then transferred to PDVF Immobilon-P membranes (Millipore, USA). Blots were incubated with the following antibodies: anti-actin (Sigma, USA) and anti-IDE (#9210, Millipore, USA). Bound peroxidase activity was visualized by the enhanced chemiluminescence kit

Table 1. Demographic data of pancreas donors included in the study.

	ID	Sex	Age	Treatment	Cause of death
Control	HC10	M	76		Respiratory insufficiency
	HC30	M	40		Septic shock
	HC23	M	54		Multiorgan failure
	HC 25	M	48		Multiorgan failure
	MC27	F	42		Unknown
	MC15	F	75		Status epilepticus
	MC22	F	52		Adult respiratory distress syndrome
	MC28	F	40		Cardiogenic shock
	MC21	F	63		Septic shock
DM2+OHAs	HA11	M	63	OHA (Metformin)	Cardiogenic shock
	HA15	M	65	OHA (unknown)	Septic shock
	HA16	M	65	OHA (unknown)	Septic shock
	HA21	M	59	OHA (unknown)	Unknown
	HA7	M	78	OHA (Metformin)	Unknown
	MA9	F	76	OHA (Metformin)	Cerebral haemorrhage
	MA13	F	65	OHA (Metformin)	Massive pulmonary embolism
	MA19	F	58	OHA (Metformin)	Post surgery shock
	MA16	F	69	OHA (unknown)	Unknown
DM2+Insulin	HI2	M	77	Insulin (unknown)	Septic shock
	HI16	M	60	Insulin (NPH)	Cardiogenic shock
	HI15	M	67	Insulin (Mixtard 30)	Acute Pulmonary Edema
	HI18	M	44	Insulin (unknown)	Septic shock
	MI12	F	63	Insulin (unknown)	Urological sepsis
	MI9	F	71	Insulin (Lantus)	Pulmonary aspergillosis
	MI6	F	75	Insulin (NPH)	Cardiogenic shock
	MI5	F	72	Insulin (unknown)	Respiratory insufficiency

Immun-Star WesternC (Bio-Rad, EEUU). ImageJ software (NIH, USA) was used for processing and analysis of data. Results were normalized to control values on each membrane.

Glucose stimulated insulin secretion (GSIS) and survival after IDE inhibition

INS1E cells were seeded on cell culture 24-well plates at a density of 200,000 cells per well for 72 hours. Afterwards, they were treated for 4 hours with 500 nM human insulin (#I9278, Sigma-Aldrich, USA), or for 30 min with 2mM 1-10 Phenantroline (#131377, Sigma-Aldrich, USA), or both. After insulin/Phenantroline treatment MTT and GSIS were performed.

MTT assay

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay method. MTT was added to each well at a concentration of 0.5 mg/mL and plates were incubated at 37°C for 1 hour in the absence of light. The medium was replaced with 150 µL dimethyl sulfoxide (DMSO) and stirred for 20 min to dissolve the formazan crystals. The absorbance for each well was measured at 562 nm using a Microplate reader MB-580 HEALES microplate (Shenzhen Heales Technology Development Co., China).

GSIS

INS1E cells were maintained the last 2 h in glucose-free culture medium. The cells were then washed twice and preincubated for 30 min at 37°C in glucose-free HEPES balanced salt solution (HBSS) (114 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH2P O_4, 1.16 mmol/l $MgSO_4$, 20 mmol/l HEPES, 2.5 mmol/l $CaCl_2$, 25.5 mmol/l $NaHCO_3$, and 0.2% bovine serum albumin [essentially fatty acid free], pH 7.2). Next, cells were washed once with glucose-free HEPES balanced salt solution (HBSS) and then insulin secretion was stimulated by using static incubation for 30 min period in 1 ml of the same buffer containing 2 or 16 mmol/l glucose respectively. Secretion samples were measured by Rat Insulin ELISA (Mercodia AB, Sweden).

Statistical analysis

Statistical analysis of data was performed using the Graph Pad Prism4 (GraphPad Software, Inc., La Jolla, CA, USA). Distribution of variables was checked using the Kolmogorov-Smirnov test. Comparisons between two groups were performed using Student's t-test; comparisons between more than two groups were performed by ANOVA followed by Tukey's Multiple Comparison Test. Differences were considered significant at $p<0.05$.

Results

IDE is expressed in pancreatic alpha- and beta-cells of rodents and humans

To determine whether IDE is expressed in insulin- and glucagon-producing cells, we validated our antibody for IDE staining using pancreata of IDE null mice (Abdul-Hay et al., 2011); no staining was observed in the null tissue (Fig. 1). Staining of multiple tissues revealed that IDE is expressed in both beta- and alpha-cells of wild type mouse and human pancreata (Fig. 1). Of note, IDE staining was more intense in glucagon positive-cells of both species relative to beta-cells or other pancreatic cells (such as exocrine cells), indicating a higher expression of IDE in alpha-cells.

IDE protein level is augmented in islet-cells of preclinical models of hyperinsulinemia

To investigate whether IDE protein level varies in the context of preclinical hyperinsulinemia we investigated islet IDE protein level in db/db and HFD mouse models. Twelve-week-old db/db mice were hyperinsulinemic (5.0 ng/mL vs. 0.5 ng/mL, $p<0.05$) and diabetic (450 mg/dL vs. 150 mg/dL, $p<0.05$). Twenty-six-week-old male C57Bl6J mice fed with a HFD were hyperinsulinemic (16.9 ng/mL vs. 1.0 ng/mL, $p<0.05$) and mildly hyperglycemic (153 mg/dL vs. 119 mg/dL, $p<0.05$). Insulin and glucose levels are non-fasting.

We stained pancreata of WT and db/db mice for IDE, insulin and glucagon. IDE-integrated intensity was measured for alpha- and beta-cells and those intensities were then divided by glucagon and insulin areas respectively (Fig. 2A). We did not detect IDE protein level differences in pancreata freshly obtained from db/db versus wild-type mice. Surprisingly, however, when islets were isolated and western-blots were performed, a significant 50% increase in IDE protein level was detected (Fig. 2B,C). Similar results were observed for HFD-treated pancreata: the integrated intensity of IDE was similar in HFD-treated mice as compared to control animals fed a standard diet (SD) (Fig. 2D); but there is a significant 30% increase in IDE protein level in HFD isolated islets when detected by western-blotting (Fig. 2E,F). Of note, WT (+/+) pancreata (Fig. 2A), SD and HFD pancreata (Fig. 2D) showed augmented IDE levels in alpha- compared to beta-cell population.

Human type-2 diabetic islets show impaired beta-cell to alpha-cell ratio

Before studying expression of IDE in human pancreata of control and diabetic patients, we characterized their alpha- and beta-cell populations. A histomorphometric study was performed in parallel using two methods of quantification (a) alpha- or beta-

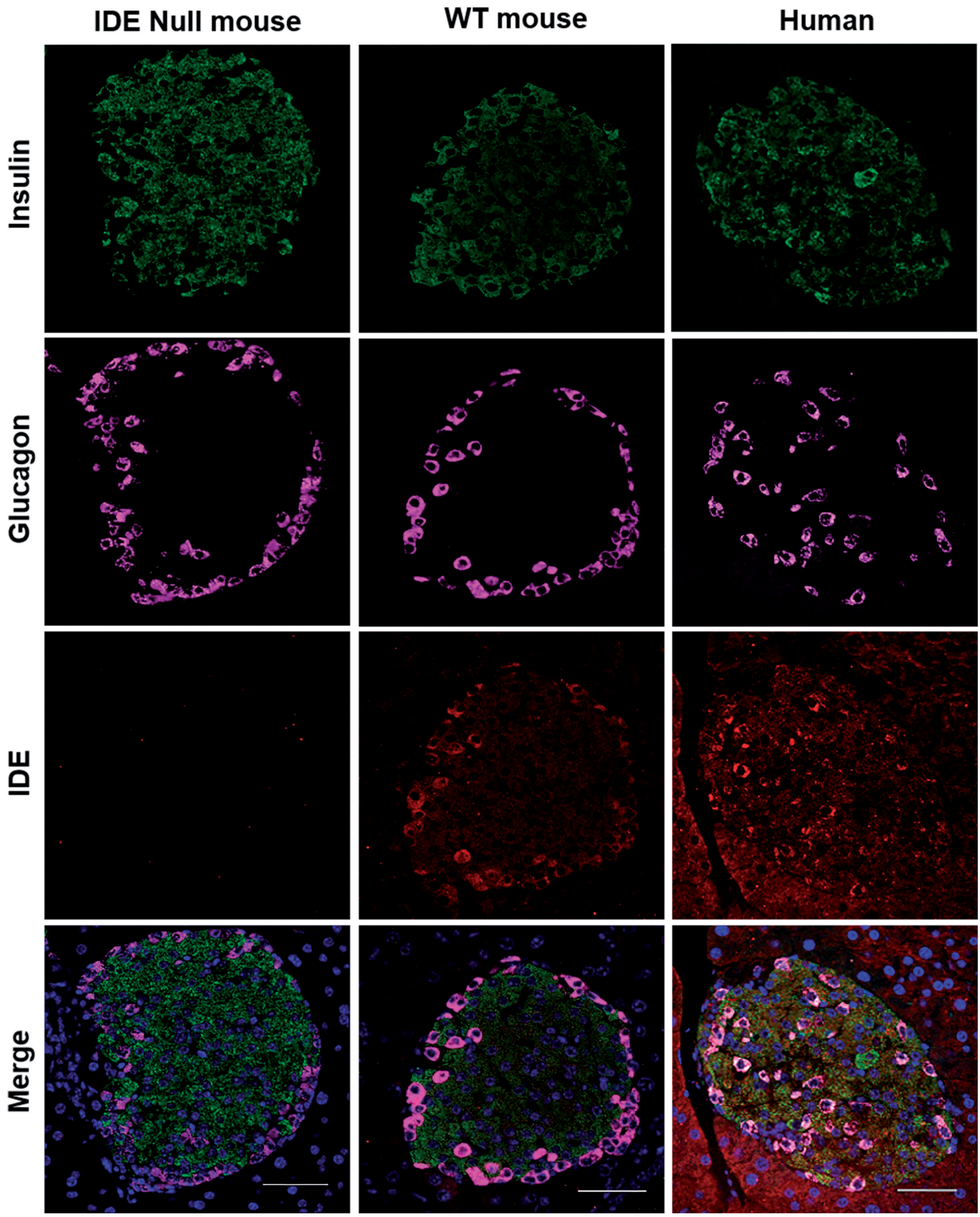

Fig. 1. Validation of IDE antibody for immunostaining. Insulin (green), glucagon (pink), IDE (red) and DAPI (blue) were stained in IDE null and wild-type (WT) mice, as well as in human pancreata. Scale bars: 50 µm.

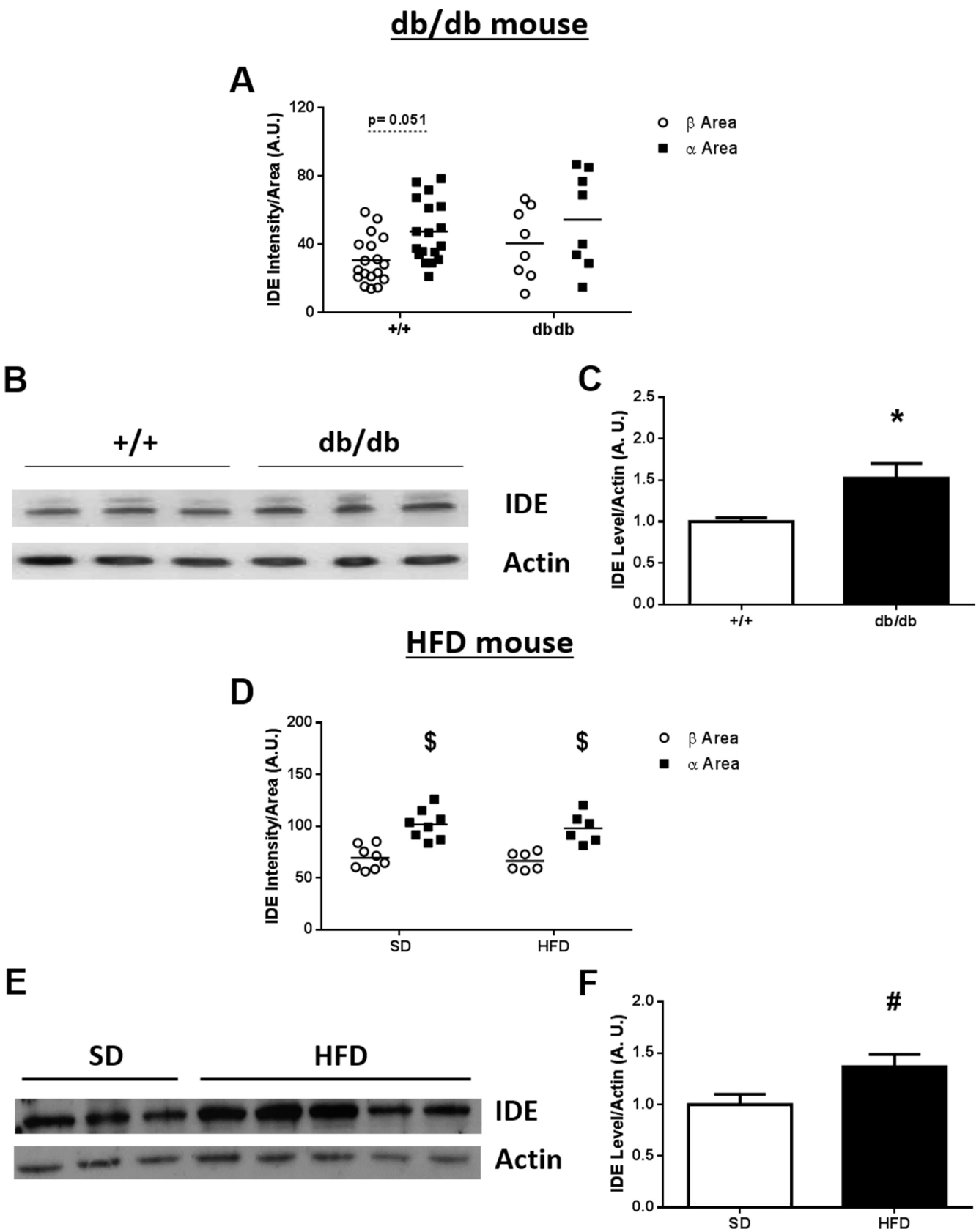

Fig. 2. IDE expression in preclinical models of diabetes and obesity. **A.** IDE integrated density per insulin and glucagon area was measured in pancreas of wild-type (+/+) or db/db mice. N=18 +/+; N=9 db/db **B.** Representative IDE western blot of +/+ and db/db islets. **C.** Quantification of IDE in islets of +/+ and db/db mice. N=4 +/+; N=5 db/db. **D.** IDE integrated density per insulin and glucagon area was measured in pancreas of mice fed with standard diet (SD) or high-fat diet (HFD). N=8 SD; N=6 HFD. **E.** Representative IDE western blot of SD and HFD islets. **F.** Quantification of IDE in islets of SD and HFD mice. N=3 SD; N=5 HFD. One pancreas section was used per animal for each kind of staining. *p<0.05 versus control (+/+); $ p<0.05 versus β Area; # p<0.05 versus SD.

cell number per total islet cell number and (b) alpha- or beta-cell area per pancreas area.

Representative pictures of insulin and glucagon staining in control, DM2+OHAs and DM2+insulin pancreata are shown in Fig. 3A,B. DM2 pancreata (detailed information in Table 1) showed no difference in beta-cell numbers when compared to control pancreata, but a significant 40% decrease in beta-cell area in insulin-treated DM2 patients (Fig. 4A). Presenting an opposite profile, DM2 pancreata displayed a significant 30% increase in alpha-cell numbers (Fig. 4B), but no differences in alpha-cell area. These results point to an increase in alpha-cell numbers per islet without increasing alpha-cell area per pancreas.

Interestingly, pancreas from DM2 patients showed a 50% reduction in beta-/alpha-cell ratio independently of the method used for its quantification (Fig. 4C). These data are indicative of impaired DM2 pancreatic endocrine cell distribution, which parallels dysfunction in the secretion of insulin and glucagon (Kahn et al., 2014; Moon and Won, 2015).

IDE protein level is down-regulated in islets of DM2 patients treated with OHAs and up-regulated in DM2 patients treated with insulin

Understanding that different anti-diabetic treatments might differentially affect IDE protein level, we elected to separately investigate IDE protein levels in pancreatea from DM2 patients treated with OHAs versus those treated with insulin. Representative pictures of insulin/IDE and glucagon/IDE staining in control, DM2+OHAs and DM2+insulin pancreata are shown in Fig. 5. Quantification of IDE intensity per beta- or alpha-cell area showed a 100-300% increase in IDE protein levels in alpha-cells versus beta-cells (Fig. 6A). This result quantitatively demonstrates that IDE is expressed in higher amounts in alpha than in beta-cells. These results nicely correlate to those for rodents in Fig. 2A,D.

Interestingly, whereas DM2+OHAs pancreata showed a significant 40% decrease in IDE protein level in beta-cells compared to control subjects, DM2+insulin

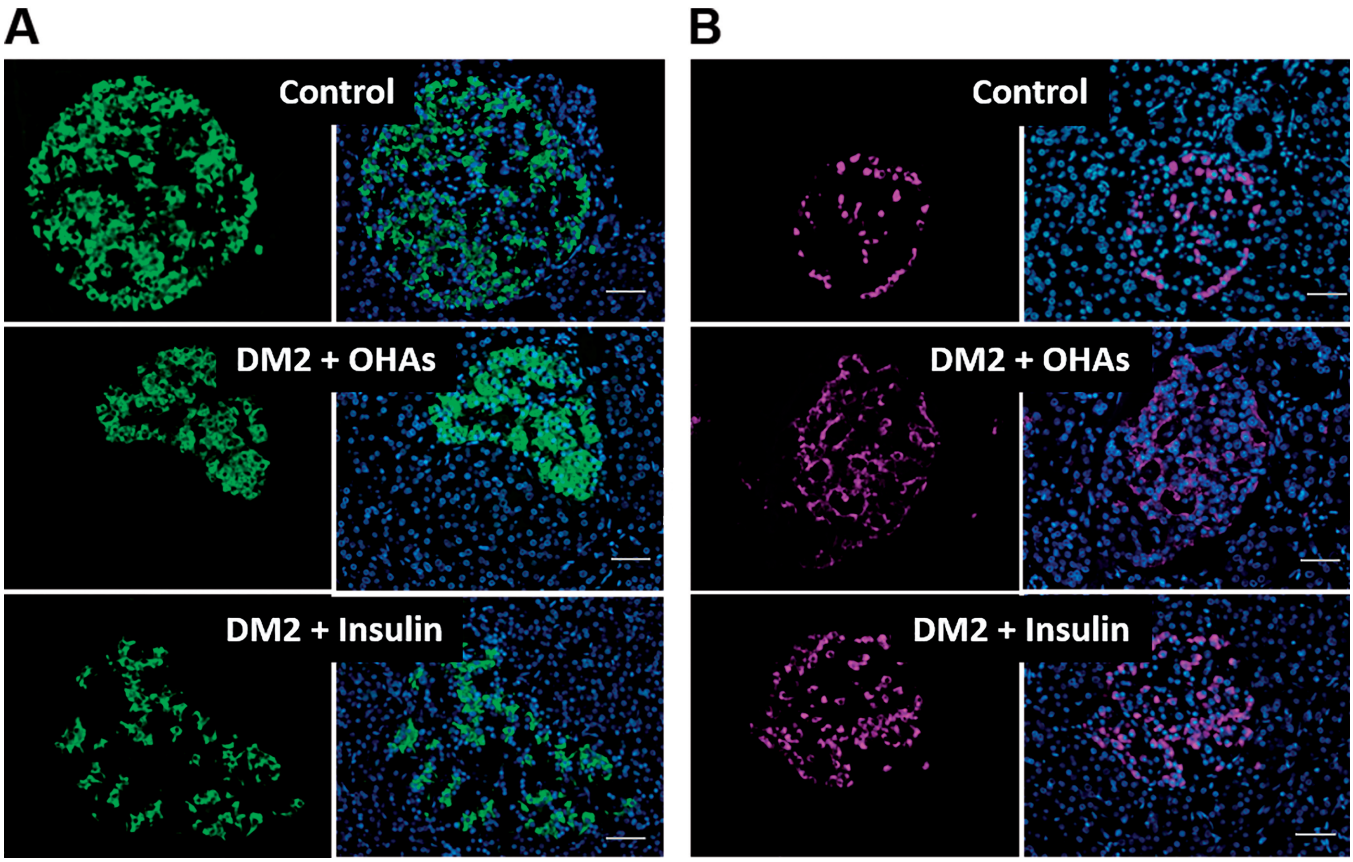

Fig. 3. β- and α-cells distribution in human pancreata. Control, non-diabetic donor; DM2, type-2 diabetic donor; DM2+OHAs, type-2 diabetic donor treated with oral anti-diabetic agents; DM2+insulin, type-2 diabetic donor treated with insulin. **A, B.** Representative pictures of insulin staining (green), glucagon (pink) and DAPI (blue). Scale bars: 50 μm.

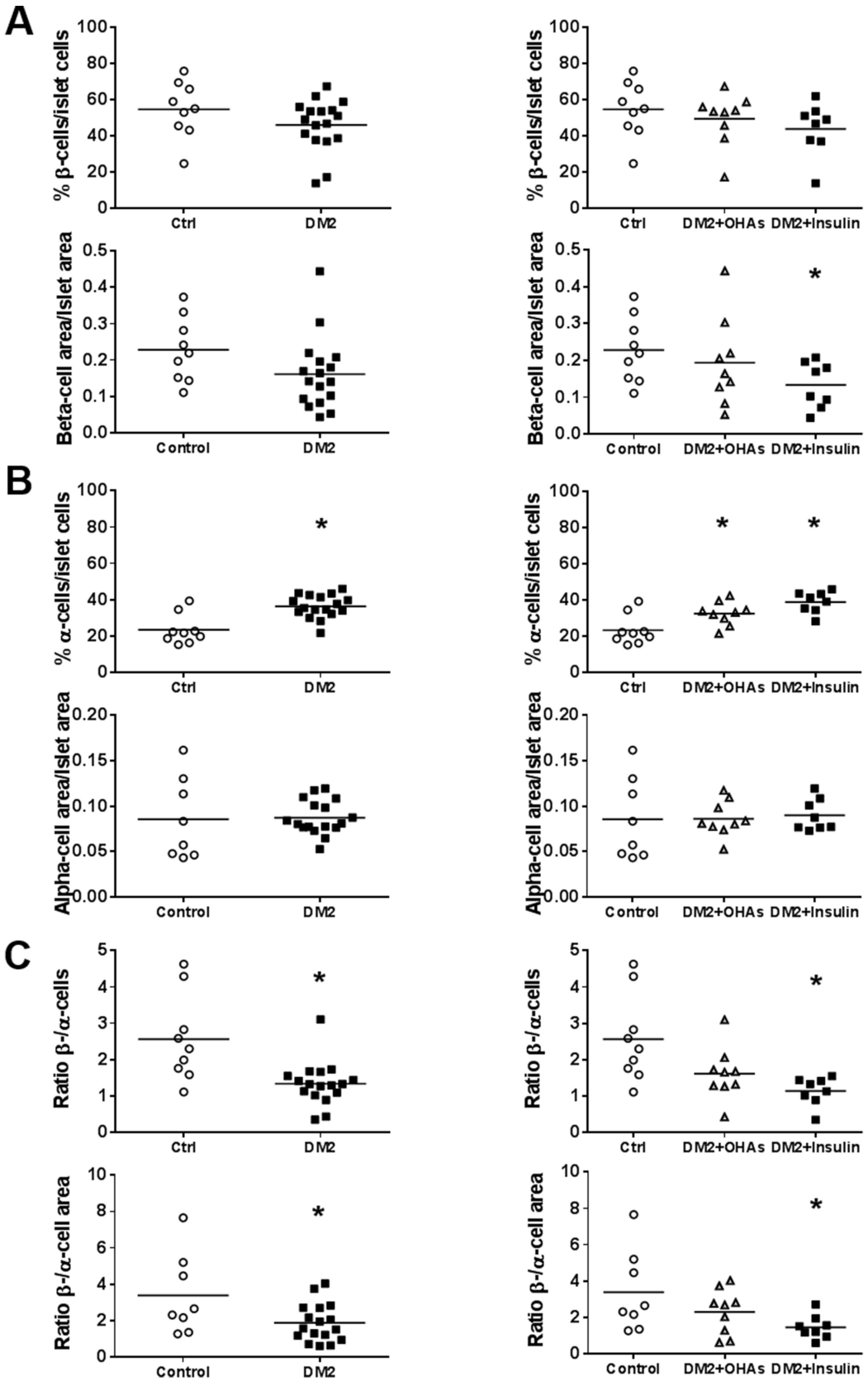

Fig. 4. β- and α-cells quantification in human pancreata. **A.** Percentage of insulin-positive cells per total islet cell number; and beta-cell area/islet area. **B.** Percentage of glucagon-positive cells per total islet cell number; and alpha-cell area/islet area. **C.** Ratio of beta- per alpha-cell number; and ratio beta- per alpha-cell area.

pancreata displayed increased IDE protein level as compared to DM2+OHAs (Fig. 6B,C). This is an interesting outcome that distinguishes two different kinds of type-2 diabetics. In alpha-cells, on the other hand, we did not detect significant changes in IDE protein levels (Fig. 6D,E).

Of note, diabetes reduces IDE protein levels in beta-cells, but under insulin treatment IDE is upregulated returning to normal (Fig. 6C). These results point to a potential effect of insulin on stimulation of IDE levels.

Insulin treatment induces IDE protein level in INS1E and islet cells in vitro

In view of our results in DM2 patients, showing decreased IDE protein level in OHAs-treated diabetics but increased IDE protein level in insulin-treated-DM2 subjects, we hypothesized that insulin produces IDE up-regulation. In an attempt to elucidate the potential influence of insulin treatment on IDE levels, we cultured INS1E cells and isolated rat and human islets and treated them *in vitro* with insulin for a period of 1 to 4 h. Afterwards, IDE protein level was measured by western blotting and/or immunostaining. IDE level in INS1E cells was found to be significantly increased by 40-70% after 4 h of insulin treatment (Fig. 7A-D); similarly, IDE was increased by 60% in rat and human islets (Fig. 8A-E respectively). Interestingly, IDE is 50% increased in pancreatic- beta-cells. These results suggest that insulin is responsible for the relative upregulation of IDE observed in insulin-treated diabetic patients versus OHAs-treated patients.

Implications of IDE activity on beta-cell biology

To shed light on the possible physiological role of the insulin-mediated IDE up-regulation on beta-cell survival and function, we have performed experiments treating INS1E cells with the IDE inhibitor Phenantroline +/- insulin. We have measured cell viability by MTT observing no changes in beta-cell survival after 30 min Phenantroline treatment (Fig. 9A). Furthermore, IDE inhibition showed impaired beta-cell function as measured by GSIS. Interestingly, 4 h insulin treatment, although increasing IDE protein levels (Fig. 7), impairs GSIS as well (Fig. 9B). Thus, both, increment and decrease of IDE function, result in inhibition of insulin secretion.

Discussion

Diabetes is a costly and increasingly common disease which requires a clearer understanding of its molecular mechanisms, improved treatments with fewer side effects, as well as novel biomarkers to determine in an easy and non-invasive way the evolution of the

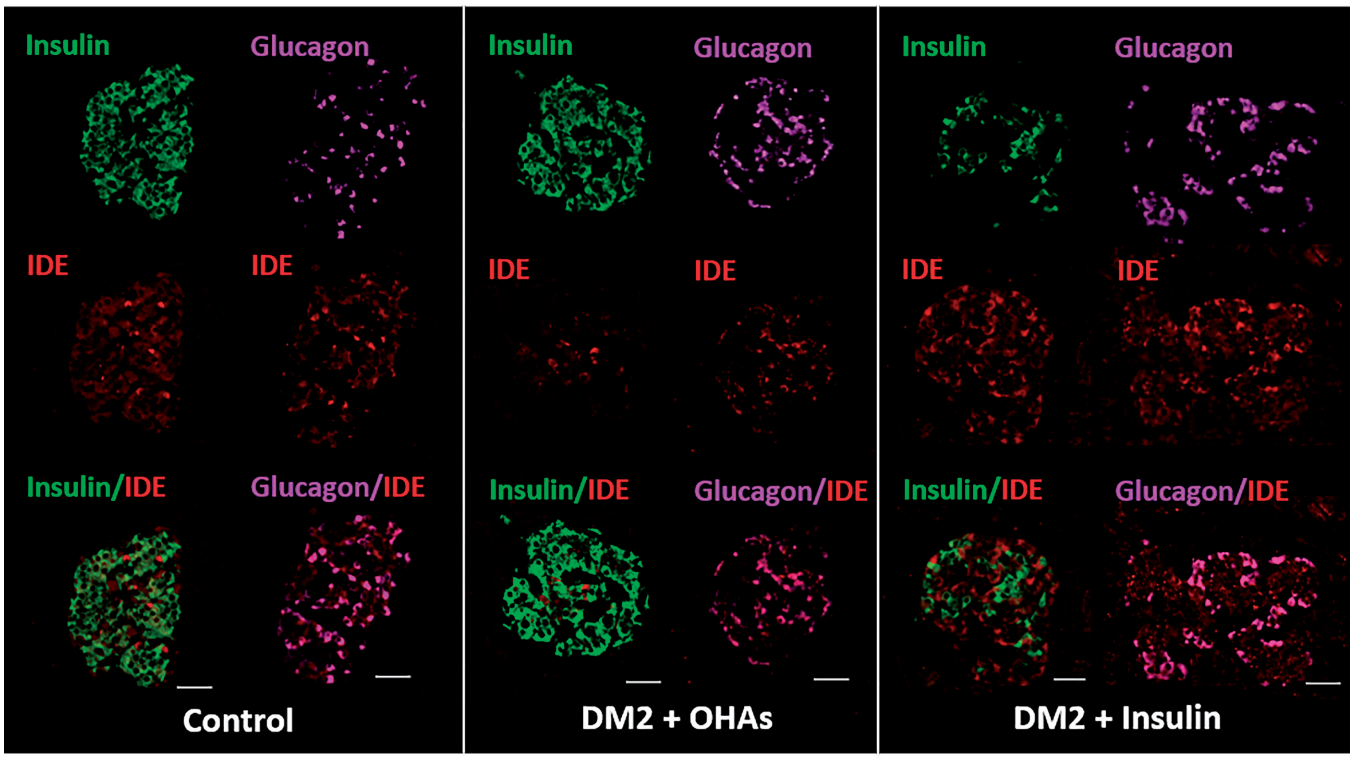

Fig. 5. IDE staining in endocrine human pancreata. Representative pictures of IDE/insulin (green/pink) and IDE/glucagon staining (red/pink). Scale bars: 50 μm.

disease and the response to patients' treatments. The *Ide* locus has been genetically linked to the risk of suffering both diabetes and hyperinsulinemia (Karamohamed et al., 2003; Gu et al., 2004; Schulze et al., 2007; Sladek et al., 2007; Kwak et al., 2008), yet, surprisingly, IDE protein levels have not previously been quantified by staining in any animal or human tissue, neither under physiological nor pathophysiological conditions. Here, we have conducted for the first time a cell-specific study in beta- and alpha-cells comparing IDE protein levels in human DM2 patients versus control subjects, as well as in multiple murine models of hyperinsulinemia. Furthermore, we conducted the first comparative study of IDE protein levels on OHAs versus insulin-treated DM2 patients.

Our results highlight the relevance of IDE in DM2 and include a number of findings. First, in agreement with previous publications (Yoon et al., 2003), DM2 patients show an impaired beta-/alpha-cell ratio. It is relevant to emphasize that using different histomorphometric analysis in the same samples sometimes ends in controversial results as seen in the literature (Sakuraba et al., 2002; Butler et al., 2003; Yoon et al., 2003; Rahier et al., 2008). Beta-/alpha-cell ratio results are greatly consistent in our hands, showing by both methodologies to be decreased in diabetic population. Second, IDE was found to be highly expressed in pancreatic alpha-cells, with lower levels present in beta-cells. Third, IDE levels were increased in islet cells from db/db and HFD mice as compared to their respective controls. Interestingly, western-blotting showed to have higher sensitivity than fluorescence quantification to this end. Fourth, IDE protein level was decreased in beta-cells of DM2+OHAs patients, but

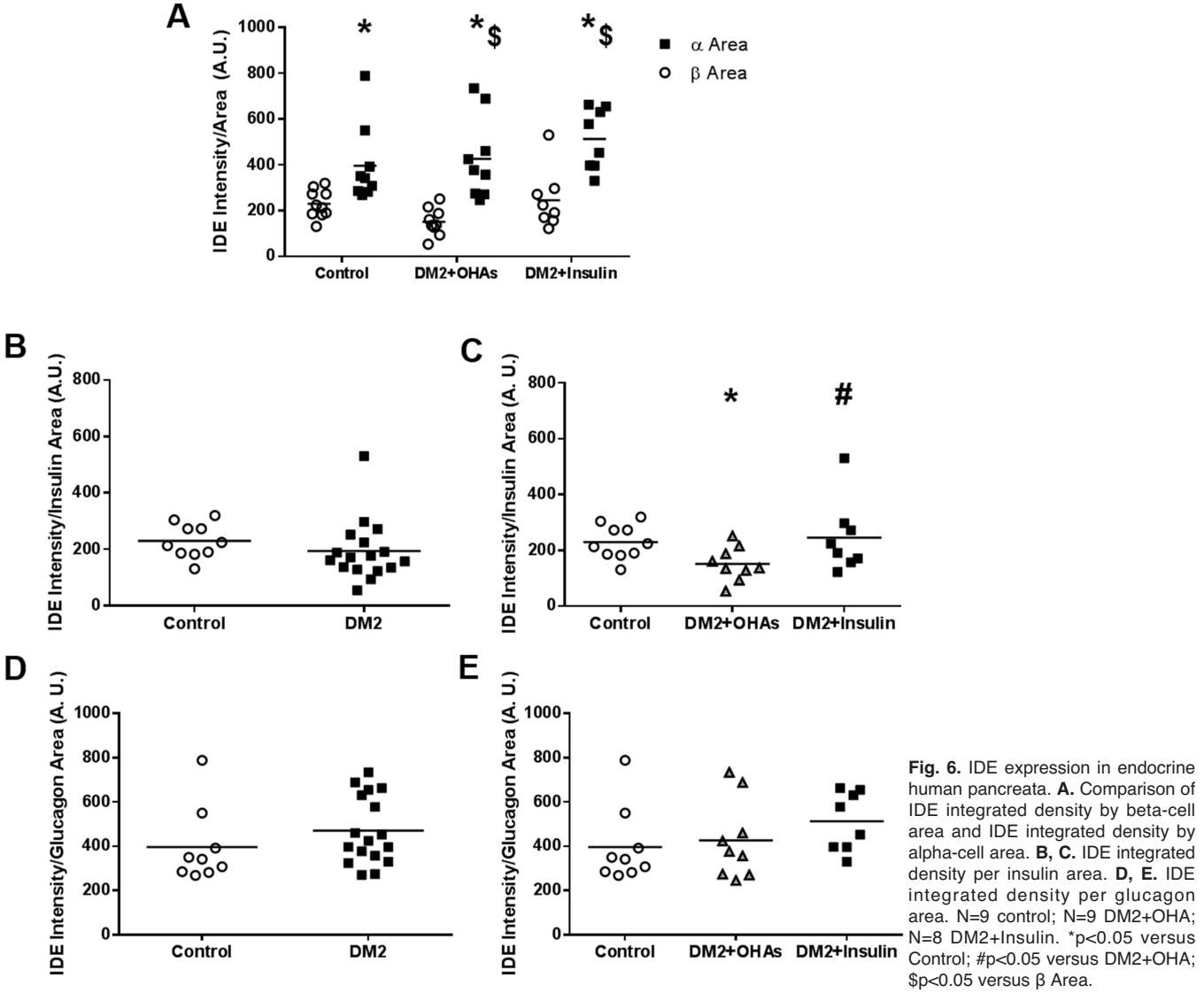

Fig. 6. IDE expression in endocrine human pancreata. **A.** Comparison of IDE integrated density by beta-cell area and IDE integrated density by alpha-cell area. **B, C.** IDE integrated density per insulin area. **D, E.** IDE integrated density per glucagon area. N=9 control; N=9 DM2+OHA; N=8 DM2+Insulin. *p<0.05 versus Control; #p<0.05 versus DM2+OHA; $p<0.05 versus β Area.

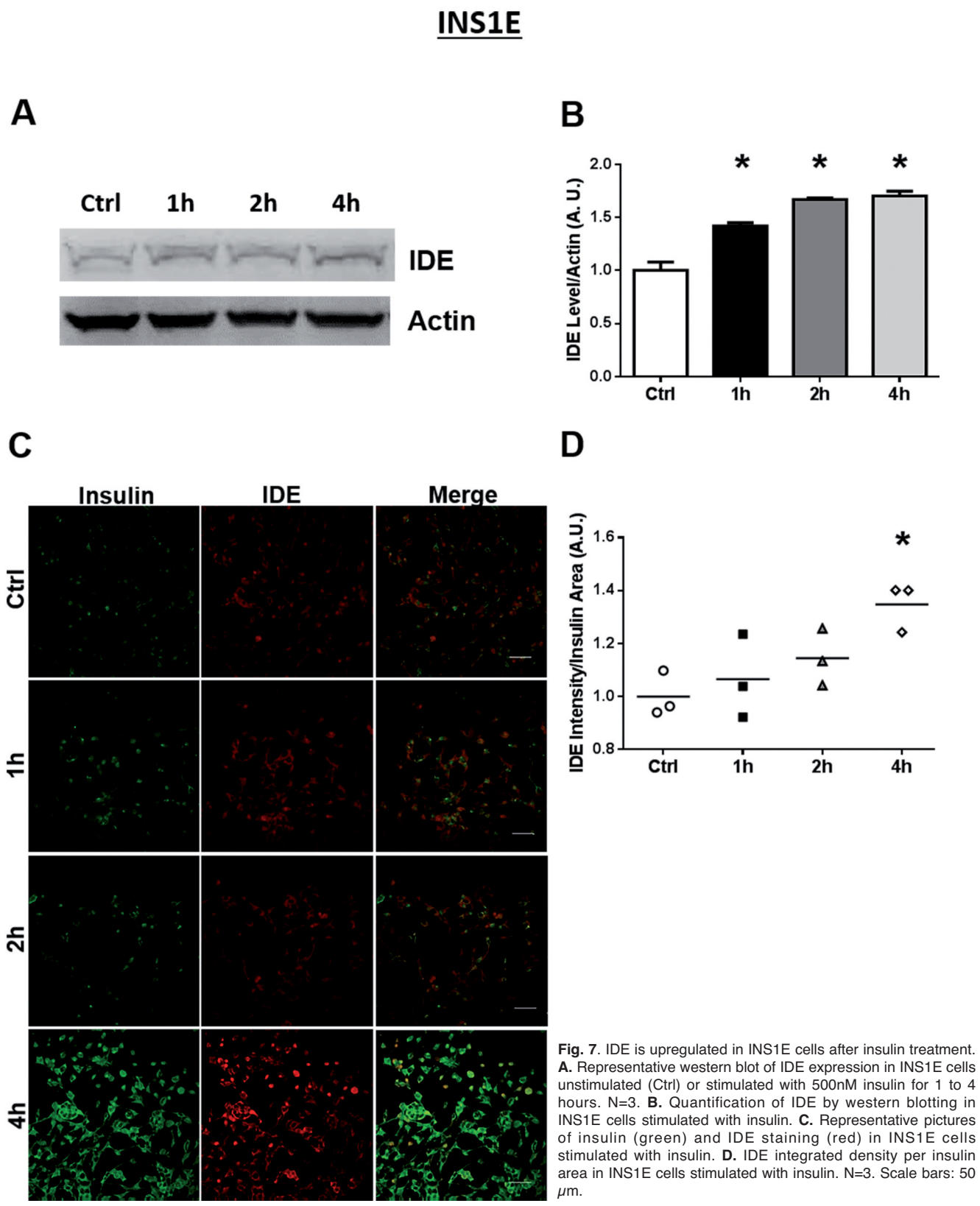

Fig. 7. IDE is upregulated in INS1E cells after insulin treatment. **A.** Representative western blot of IDE expression in INS1E cells unstimulated (Ctrl) or stimulated with 500nM insulin for 1 to 4 hours. N=3. **B.** Quantification of IDE by western blotting in INS1E cells stimulated with insulin. **C.** Representative pictures of insulin (green) and IDE staining (red) in INS1E cells stimulated with insulin. **D.** IDE integrated density per insulin area in INS1E cells stimulated with insulin. N=3. Scale bars: 50 μm.

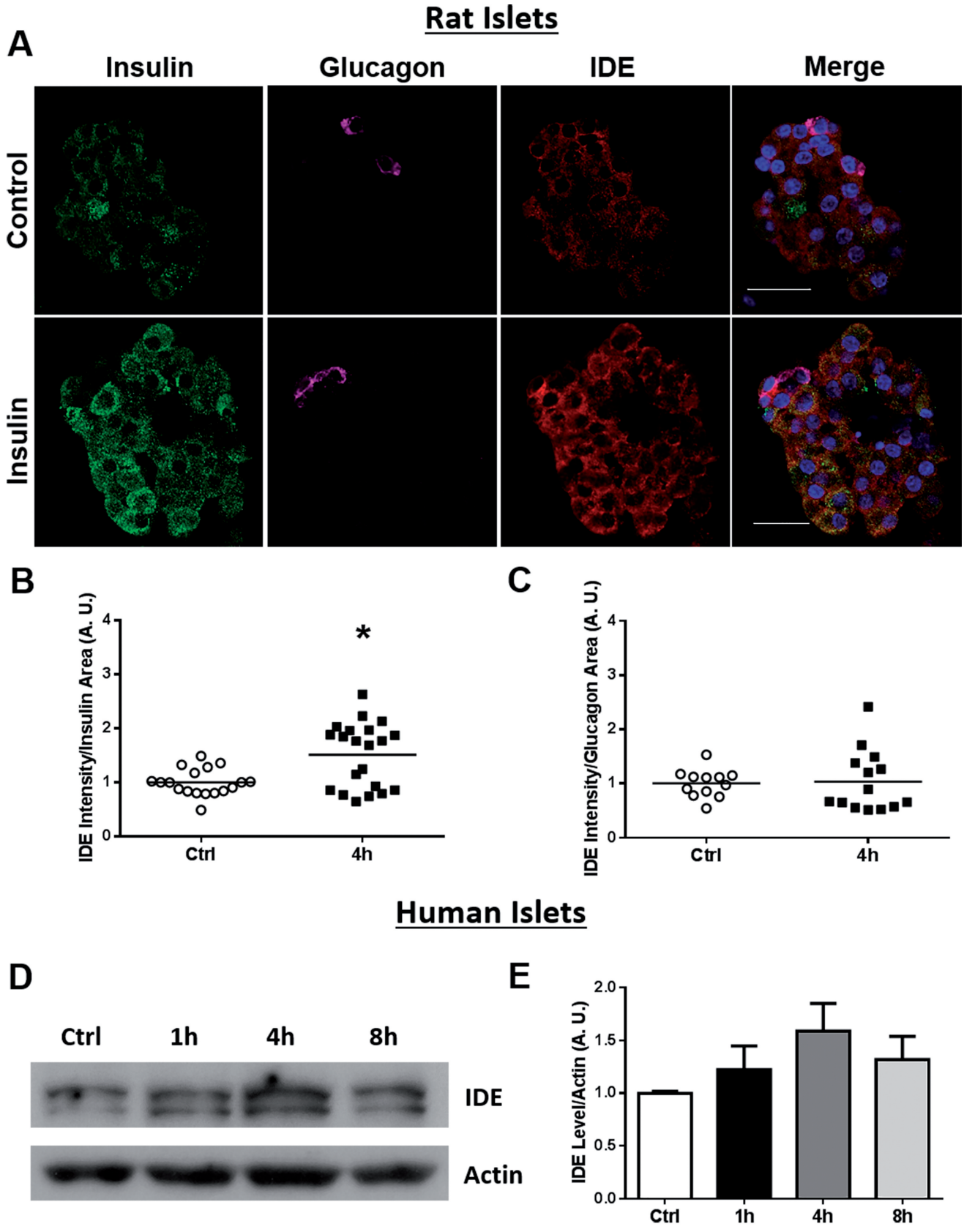

Fig. 8. IDE is upregulated in human and rodent pancreatic islet-cells after insulin treatment. **A.** Representative pictures of insulin (green) and IDE staining (red) in rat islets stimulated with insulin. **B.** IDE integrated density per insulin area in rat islets stimulated with insulin. N=18-21. **C.** IDE integrated density per glucagon area in rat islets stimulated with insulin. N=12-14. **D**. Representative western-blot of IDE expression in human islets no stimulated (Ctrl) or stimulated with 500 nM insulin for 1 to 8 hours. **E.** Quantification of IDE by western blotting in human islets stimulated with insulin. N=2 different human islet preparations. *p<0.05 versus Control. Scale bars: 25 µm.

comparatively upregulated in beta-cells of DM2+insulin patients. Finally, we established that insulin treatment of isolated rodent and human islets induces IDE overexpression.

Steneberg et al. (2013) have previously shown by western-blot that IDE protein level is decreased by 40% in whole islets of DM2 patients compared to controls. This study did not report the age, sex or treatment of these patients, but their results nicely correlate with our finding in beta-cells of DM2+OHAs patients. Steneberg and colleagues study showed that genetic deletion of IDE impairs glucose stimulated insulin secretion in beta-cells which is a hallmark of DM2. Our results go one step further: here we have characterized two different populations of DM2 patients, treated with OHAs versus insulin, and we have performed specific detection of IDE in beta- and alpha-cells showing that loss of IDE levels occurs specifically in beta-cells but not alpha-cells.

Furthermore, although beta-cells from OHAs-treated DM2 subjects show reductions in IDE protein levels, as reported by Steneberg and colleagues, insulin-treated subjects showed no changes relative to controls. Insulin-treated subjects, however, showed significant increases in IDE protein level in beta-cells relative to OHAs-treated patients. These results nicely correlate with those in hyperinsulinemic islets of db/db and HFD mice. At the same time, this finding suggested that insulin treatment stimulates elevated IDE protein levels, which we confirmed through multiple experiments in INS1E cells and rodent and human islets. IDE activity inhibition using Phenantroline suggests a physiological role of IDE on insulin secretion, in accordance with Steneberg et al (Steneberg et al., 2013). Interestingly, the result of GSIS after insulin stimulus may suggest an IDE-independent mechanism for GSIS in hyperinsulinemia.

IDE upregulation under high insulin conditions may be part of a counterregulatory islet adaptation to produce insulin clearance in this hyperinsulinemic microenvironment. This is possible because insulin not cleared by liver and kidney is ultimately removed by other insulin-sensitive cells (Duckworth et al., 1998).

Other studies in liver have already shown that insulin levels can regulate IDE protein expression. An obesity-induced hyperinsulinemic mouse model showed lower IDE levels that were upregulated after taurine conjugated bile acid (TUCDA) (Vettorazzi et al., 2017). Interestingly, a different study by the same group has shown that hyperinsulinemia caused by glucocorticoid treatment may be due to decreased IDE activity (Protzek et al., 2016).

Our findings reveal new insights into the involvement of IDE in endocrine pancreatic function as well as its response to DM2 subjects treated with different anti-diabetic therapies. At the same time, they raise a significant number of new questions. Why does insulin simulate the expression of IDE? Does this represent a homeostatic mechanism whereby high insulin levels promote the degradation of insulin? Is it helpful or harmful that IDE levels return to normal in DM2+insulin beta-cells?

Insulin is secreted through the classical secretory pathway; meanwhile IDE is not (Zhao et al., 2009). While they might be expressed in the same cells, IDE and insulin would not be expected to reside within the same cell compartments. If not, where does IDE interact with insulin? Our findings open new lines of thinking around this intriguing protein. Further research is warranted to investigate the subcellular localization of

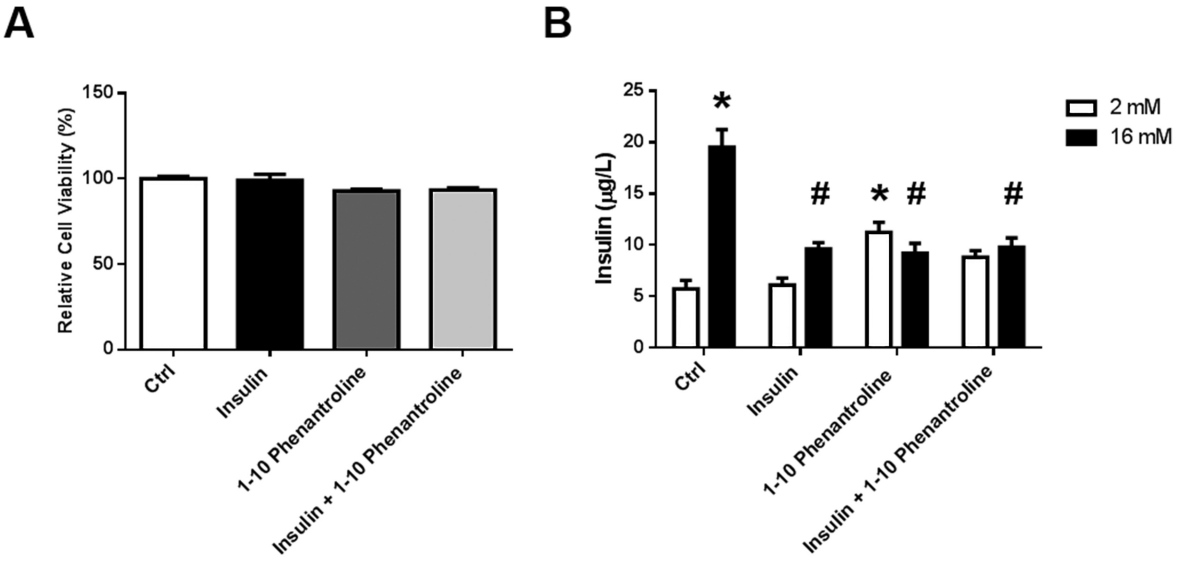

Fig. 9. Beta-cell viability and function after insulin treatment. **A.** Cell viability was measured in INS1E cells by MTT after 4h insulin, 30 min Phenantroline or Insulin+Phenatroline treatment (500 nM insulin; 2 mM 1-10 Phenantroline). **B.** Glucose stimulated insulin secretion was measured after 4h insulin, 30 min Phenantroline or Insulin+Phenatroline treatment. N=6 for both experiments. *p<0.05 versus Ctrl-2mM; #p<0.05 versus Ctrl-16mM.

IDE and to identify possible sites of interaction with insulin inside and outside the beta-cell.

Acknowledgements. This research has been funded by Ministerio de Economia y Competitividad-Government of Spain and FEDER (SAF2014-58702-C2-1-R and SAF2014-58702-C2-2-R) to IC and to GP respectively.

Authors acknowledge Dr. Malcolm Leissring (University of California-Irvine, USA), Dr. Rupangi Vasavada (Mount Sinai School of Medicine, NY, USA) and Dr. David A. Cano (Intituto de Biomedicina de Sevilla-IBIS, Spain) for their thoughtful discussions of the ideas in this report.

Conflict of interest. All authors declare no conflict of interest.

Author contributions. MCF, LE and JFL researched data and contributed to discussion. AM, CDL and JS reviewed/edited the manuscript and contributed to discussion. IC and GP conceptualized the research, supervised experiments and wrote the manuscript. All authors approved the final version of this manuscript.

References

Abdul-Hay S.O., Kang D., McBride M., Li L., Zhao J. and Leissring M.A. (2011). Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance. PLoS One 6, e20818.

Butler A.E., Janson J., Bonner-Weir S., Ritzel R., Rizza R.A. and Butler P.C. (2003). Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52, 102-110.

Cozar-Castellano I., Takane K.K., Bottino R., Balamurugan A.N. and Stewart A.F. (2004). Induction of beta-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. Diabetes 53, 149-159.

Duckworth W.C., Bennett R.G. and Hamel F.G. (1998). Insulin degradation: progress and potential. Endocr. Rev. 19, 608-624.

Durham T.B., Toth J.L., Klimkowski V.J., Cao J.X., Siesky A.M., Alexander-Chacko J., Wu G.Y., Dixon J.T., McGee J.E., Wang Y., Guo S.Y., Cavitt R.N., Schindler J., Thibodeaux S.J., Calvert N.A., Coghlan M.J., Sindelar D.K., Christe M., Kiselyov V.V., Michael M.D. and Sloop K.W. (2015). Dual exosite-binding inhibitors of insulin-degrading enzyme challenge its role as the primary mediator of insulin clearance *in vivo*. J. Biol. Chem. 290, 20044-20059.

Farris W., Mansourian S., Chang Y., Lindsley L., Eckman E. A., Frosch M. P., Eckman C.B., Tanzi R.E., Selkoe D.J. and Guenette S. (2003). Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain *in vivo*. Proc. Natl. Acad. Sci. USA 100, 4162-4167.

Gu H.F., Efendic S., Nordman S., Ostenson C. G., Brismar K., Brookes A.J. and Prince J.A. (2004). Quantitative trait loci near the insulin-degrading enzyme (IDE) gene contribute to variation in plasma insulin levels. Diabetes 53, 2137-2142.

Kahn S.E., Cooper M.E. and Del Prato S. (2014). Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. Lancet 383, 1068-1083.

Karamohamed S., Demissie S., Volcjak J., Liu C., Heard-Costa N., Liu J., Shoemaker C.M., Panhuysen C.I., Meigs J.B., Wilson P., Atwood L.D., Cupples L.A., Herbert A. and NHLBI Framingham Heart Study. (2003). Polymorphisms in the insulin-degrading enzyme gene are associated with type 2 diabetes in men from the NHLBI Framingham Heart Study. Diabetes 52, 1562-1567.

Kwak S.H., Cho Y.M., Moon M.K., Kim J.H., Park B.L., Cheong, H.S., Shin H.D., Jang H.C., Kim S.Y., Lee H.K. and Park K.S. (2008). Association of polymorphisms in the insulin-degrading enzyme gene with type 2 diabetes in the Korean population. Diabetes Res. Clin. Pract. 79, 284-290.

Maianti J.P., McFedries A., Foda Z.H., Kleiner R.E., Du X.Q., Leissring M.A., Tang W.J., Charron M.J., Seeliger M.A., Saghatelian A. and Liu D.R. (2014). Anti-diabetic activity of insulin-degrading enzyme inhibitors mediated by multiple hormones. Nature 511, 94-98.

Moon J.S. and Won K.C. (2015). Pancreatic alpha-Cell Dysfunction in Type 2 Diabetes: Old Kids on the Block. Diabetes Metab. J. 39, 1-9.

Pivovarova O., Hohn A., Grune T., Pfeiffer A.F. and Rudovich N. (2016). Insulin-degrading enzyme: new therapeutic target for diabetes and Alzheimer's disease? Ann. Med. 48, 614-624.

Protzek A.O., Rezende L.F., Costa-Junior J.M., Ferreira S.M., Cappelli A.P., de Paula F.M., de Souza J.C., Kurauti M.A., Carneiro E.M., Rafacho A. and Boschero A.C. (2016). Hyperinsulinemia caused by dexamethasone treatment is associated with reduced insulin clearance and lower hepatic activity of insulin-degrading enzyme. J. Steroid Biochem. Mol. Biol. 155 (Pt A), 1-8.

Rahier J., Guiot Y., Goebbels R.M., Sempoux C. and Henquin J.C. (2008). Pancreatic beta-cell mass in European subjects with type 2 diabetes. Diabetes Obes. Metab. 10 (Suppl). 4, 32-42.

Sakuraba H., Mizukami H., Yagihashi N., Wada R., Hanyu C. and Yagihashi S. (2002). Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. Diabetologia 45, 85-96.

Schulze M.B., Al-Hasani H., Boeing H., Fisher E., Doring F. and Joost H.G. (2007). Variation in the HHEX-IDE gene region predisposes to type 2 diabetes in the prospective, population-based EPIC-Potsdam cohort. Diabetologia 50, 2405-2407.

Sladek R., Rocheleau G., Rung J., Dina C., Shen L., Serre D., Boutin P., Vincent D., Belisle A., Hadjadj S., Balkau B., Heude B., Charpentier G., Hudson T.J., Montpetit A., Pshezhetsky A.V., Prentki M., Posner B.I., Balding D.J., Meyre D., Polychronakos C. and Froguel P. (2007). A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 445, 881-885.

Steneberg P., Bernardo L., Edfalk S., Lundberg L., Backlund F., Ostenson C.G. and Edlund H. (2013). The type 2 diabetes-associated gene ide is required for insulin secretion and suppression of alpha-synuclein levels in beta-cells. Diabetes 62, 2004-2014.

Tang W.J. (2016). Targeting Insulin-Degrading Enzyme to Treat Type 2 Diabetes Mellitus. Trends Endocrinol. Metab. 27, 24-34.

Vettorazzi J.F., Kurauti M.A., Soares G.M., Borck P.C., Ferreira S.M., Branco R.C.S., Michelone L.S.L., Boschero A.C., Costa J.M. and Carneiro E.M. (2017). Bile acid TUDCA improves insulin clearance by increasing the expression of insulin-degrading enzyme in the liver of obese mice. Sci. Rep. 7, 14876.

Yoon K.H., Ko S.H., Cho J.H., Lee J.M., Ahn Y.B., Song K.H., Kang S.K., Kim H.S., Lee I.K. and Bonner-Weir S. (2003). Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. J. Clin. Endocrinol. Metab. 88, 2300-2308.

Zhao J., Li L. and Leissring M.A. (2009). Insulin-degrading enzyme is exported via an unconventional protein secretion pathway. Mol. Neurodegener. 4, 4.

Accepted May 4, 2018