Original article

Adiponectin is expressed in the pancreas of high-fat-diet-fed mice and protects pancreatic endothelial function during the development of type 2 diabetes

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Abstract

\textit{Aim}. – Adiponectin levels in skeletal muscle and adipose tissue have been reported to be involved in insulin resistance in rats fed with a high-fat diet (HFD). Our objective was to explore whether adiponectin is also expressed in the pancreas and what its potential role is during the development of type 2 diabetes (T2D) in outbred CD-1 mice.

\textit{Methods}. – Male 4-week-old outbred CD-1 mice were fed an HFD to induce a polygenic model of human T2D. Adiponectin expression was examined in mouse pancreas by quantitative real-time polymerase chain reaction (qPCR), western blots and immunofluorescence analyses. Human umbilical vein endothelial cells (HUVECs) were transfected with an adiponectin-expressing lentivirus to determine the effect of adiponectin on angiogenic function \textit{in vitro}.

\textit{Results}. – Feeding mice an HFD for 9 weeks resulted in constant hyperglycaemia, obesity, impaired glucose tolerance and insulin resistance. Additional hyperinsulinaemia emerged in mice fed an HFD for 18 weeks. Interestingly, aberrant expression of adiponectin was detectable in the pancreatic vascular endothelial cells (VECs) of mice fed with an HFD, but not in mice fed with regular chow (RC). Expression levels of pancreatic adiponectin varied during the development of T2D. This extraordinary expression of adiponectin in pancreatic VECs played a role in protecting endothelial function against potential damage by HFD. Our \textit{in vitro} study has demonstrated that adiponectin promotes angiogenic function.

\textit{Conclusion}. – These results reveal for the first time that adiponectin is expressed in pancreatic VECs of HFD-fed mice during the development of T2D as a protective adaptation in response to the HFD.
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\textbf{Keywords}: Adiponectin; Type 2 diabetes; Pancreas; Vascular endothelial cells; High-fat diet

\textit{Abbreviations}: AdipoR1, Adiponectin receptor 1; AdipoR2, Adiponectin receptor 2; AUC, Area under the curve; BW, Body weight; C/EBPα, CCAAT/enhancer-binding protein α; FBG, Fasting blood glucose; GFP, Green fluorescence protein; GTT, Glucose tolerance test; HFD, High-fat diet; HUVEC, Human umbilical vein endothelial cells; ITT, Insulin tolerance test; MANOVA, Multivariate analysis of variance; PPARγ, Peroxisome proliferator-activated receptor gamma; qPCR, Quantitative real-time PCR; RC, Regular chow; SEM, Standard error of mean; T2D, Type 2 diabetes; VEC, Vascular endothelial cells.

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1. Introduction

Type 2 diabetes (T2D) is a metabolic disease characterized by insulin resistance and pancreatic dysfunction. This metabolic disease results from the interaction of multiple genetic and environmental factors. T2D is a polygenic disease with multiple susceptibility loci, each of which contributes only a small increase in disease risk, but which together have a large physiological effect [1].

It is well accepted that a variety of adipokines, mainly produced by adipose tissue, are associated with insulin resistance and impaired insulin secretion, both of which contribute to T2D. Of these adipokines, adiponectin is often referred to as a “beneficial” adipokine possessing antidiabetic properties, including its “beneficial” effect on improving insulin sensitivity in skeletal muscle and the liver [2,3]. There is evidence to demonstrate that adiponectin is expressed not only in adipocytes, but also in mouse skeletal muscles, one of insulin’s target organs [4]. In addition, adiponectin levels in skeletal muscle tissue are correlated with high-fat-diet (HFD)-induced insulin resistance during weight gain in rats [9]. As for the pancreas, studies of mouse cell lines have reported that the insulin receptor is expressed on pancreatic beta cells and its signaling in beta cells influences insulin gene expression and insulin content [5]. Studies with genetically engineered mouse models have reported that downstream signaling activity of the insulin receptor in pancreatic islet cells is required for the positive feedback needed to stimulate insulin synthesis, and severe insulin resistance in pancreatic islets results in reduced insulin content [6]. Indeed, the effect of adiponectin on the pancreas has been substantially investigated [7,8]. For example, adiponectin was found to significantly augment insulin release from cultured mouse pancreatic endocrine cell lines and from purified rat islets when incubated in culture media containing high concentrations of glucose [8,9]. However, no study has yet reported whether adiponectin is expressed in the pancreas. In humans, the adiponectin gene is located on chromosome 3q27, which contains certain genetic determinants for T2D [10,11]. Thus, the aim of the present study was to determine whether adiponectin is expressed in the pancreas and, if so, whether local adiponectin plays a role in the pancreas during the development of T2D.

Outbred CD-1 mice are closely analogous to their human counterparts in terms of genetics, and possess a large number of genetic loci that predispose them to impaired glucose tolerance (IGT) and T2D [12]. In this study, outbred CD-1 mice were fed with an HFD to develop a polygenic T2D model that closely mimics the aetiology of human T2D – namely, a polygenic inheritance and the most commonly involved environmental factor (an HFD). Using this mouse model, our study investigated whether adiponectin is expressed in the pancreas and what its potential protective role is during the development of T2D.

2. Materials and methods

2.1. Experimental animals

All experimental procedures were approved by the Animal Ethics Committee of Peking University Health Science Center and performed in accordance with the institutional guidelines. Male outbred CD-1 mice 3 weeks of age were purchased from Charles River Laboratories via Vital River Laboratories (VRL, Beijing, China). They were kept in a temperature-controlled environment on a 12-h light/dark cycle with free access to food and water. Before initiating the experiment, the mice were acclimatized for 1 week. At the age of 4 weeks, the mice (weighing 15.3 ± 0.6 g) were randomly assigned to either a control group (n = 28) and fed with regular chow (RC) or an HFD group (n = 43) fed with the HFD; this time point became week 0 of the experiment, which lasted 18 weeks in total. The composition of each diet is shown in the Supplementary material associated with this article online. Fasting blood glucose (FBG) levels and body weight (BW) were monitored weekly. Weeks 1, 9 and 18 were set as time points for observing serum insulin levels and performing glucose tolerance tests (GTT) and insulin tolerance tests (ITT). Ages of the mice at these three time points were 5, 13 and 22 weeks, respectively. Ten RC-fed mice and seven HFD-fed mice at week 1 and 10 RC-fed mice and eight HFD-fed mice at week 9 were sacrificed for further analyses. At week 18, there were eight mice in the RC group and 28 mice in the HFD group. Mice from each group at the three time points were sacrificed after saline perfusion, and epididymal adipose tissue, hind leg skeletal muscles (biceps femoris, semimembranosus and semitendinosus muscles), liver and pancreas from these mice were collected and prepared for mRNA detection, western blots and immunofluorescence staining.

2.2. Criteria for identifying T2D

At week 18, three criteria were used to identify T2D in the mouse model. The criteria were generated from the FBG, GTT, and ITT test results. To set these criteria, the sum of the mean value and 1.645-fold of its standard deviation (STD) from the corresponding result were used to represent the upper limit of the 90% confidence interval (CI) of each corresponding result for the control RC-fed mice. Specifically, as FBG levels fluctuated throughout the 18 weeks, the criterion for FBG was generated as a mean value plus 1.645 STD of the FBG levels monitored at weeks 16, 17 and 18 in the RC group (criterion 1). The mean FBG levels of the HFD-fed mice at the same time periods were then compared with this criterion. Criteria for the GTT and ITT results were generated from the mean value plus 1.645 STD of the respective area under the curve (AUC) values of the corresponding test in the RC group at week 18, which were defined as criterion 2 and criterion 3, respectively. Thus, the three criteria were FBG = 123 mg/dL, AUC of GTT = 40723.2 mg/mL × min and AUC of ITT = 6691.3 mg/mL × min. If a test result from an HFD mouse was greater than or equal to the corresponding criterion, it could be concluded that the result was outside the upper 90% CI of test results for the RC-fed mice. T2D was identified when the corresponding test results from an HFD mouse at week 18 met two of the three criteria.
2.3. Insulin measurement

Blood samples were collected from mice fasted overnight to determine their fasting insulin levels, and collected again for postprandial insulin levels when the mice resumed free access to food (RC or HFD, as originally assigned) for 1 h. Blood samples were collected via one retro-orbital blood collection from one side of the periorbital sinus. Conventional radioimmunoassay (Beijing Kangyuan Ruide Biotechnology Co., Ltd, Beijing, China) was performed to determine serum insulin levels.

2.4. GTT and ITT

For the GTT, mice fasted overnight were intraperitoneally injected with n-glucose at a dose of 2 g/kg. Blood samples were obtained from snipped tail tips at 0, 30, 60, 90 and 120 min to check blood glucose levels using a glucometer (Johnson & Johnson, Johnson, China). ITT was performed to assess insulin sensitivity [13,14]. Porcine insulin (1 U/kg) was intraperitoneally injected into mice fasted for 5 h. Blood samples were collected and glucose levels were measured as was done for the GTT at 0, 5, 15, 30, 45 and 60 min.

2.5. RNA extraction, qPCR and standard PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The resulting cDNAs were subjected to quantitative real-time polymerase chain reaction (qPCR) or to standard PCR with primers (Table S1, Supplementary material associated with this article online) specific for adiponectin, adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), leptin, peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), CD31 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data were analyzed according to the qPCR protocol published in Nature Protocols [15].

2.6. Western blot analysis

Western blot analysis was performed as previously described [16]. Briefly, proteins extracted from the mouse pancreas and cells were resolved by 15% SDS–PAGE and then transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). Membranes were incubated with goat anti-adiponectin antibody (R&D Systems, Minneapolis, MN, USA), followed by Dylight™ 800-labeled rabbit anti-goat (KPL, Inc., Gaithersburg, MD, USA) and rabbit anti-β-actin (Sigma–Aldrich, Saint-Louis, MO, USA) antibodies and, finally, by Dylight 800-labeled goat anti-rabbit antibody (EarthOx, Millbrae, CA, USA).

2.7. Immunofluorescence analysis

All tissue samples were fixed in 4% paraformaldehyde and then cryosectioned. Ten-micron frozen sections were blocked with normal bovine serum albumin and incubated with primary antibodies overnight at 4 °C. Details of the antibodies are included in the Supplementary material associated with this article online. Slides were examined by confocal microscopy (TCS SP8; Leica, Solms, Germany).

2.8. Determination of serum adiponectin

Serum adiponectin levels in the experimental mice were determined using an adiponectin ELISA kit (Boster Biological Technology, Ltd., Wuhan, China) according to the manufacturer’s instructions.

2.9. Migration and tube formation assays

Migration activity was assessed using a modified Boyden chamber (Becton Dickinson, Franklin Lakes, NJ, USA). Briefly, a cell suspension (200 μL, 2.5 × 10^4 cells) in M199 with 1% FBS was added to the fibronectin-coated Transwell inserts. Then, 800 μL of M199 with 10% FBS was put into the lower chamber and the cells were then incubated for 8 h. Migrated cells on the lower side of the filter were fixed and stained with crystal violet, and eight random microscopy fields per well were quantified. For the in vitro tube formation assay, human umbilical vein endothelium cells (HUVECs) at 5 × 10^5 cells/well were seeded in a 24-well plate coated with Matrigel (Becton Dickinson). Tube formation was examined 4 h after seeding using an inverted phase-contrast microscope (Canon Inc., Tokyo, Japan). All tube-like structures (those exceeding six cells in length) were counted within five randomly selected fields of each well. All assays were performed in triplicate.

2.10. Statistical analysis

Statistical analyses were performed using SAS version 9.1 software. All data were presented as means ± SEM (standard error of mean). Data were analyzed with Student’s unpaired and paired t-tests, Wilcoxon signed-rank tests and multivariate analysis of variance (MANOVA) accordingly. Because FBG and BW were repeated measurement data, the difference between the two groups was evaluated using MANOVA. AUCs were calculated from the GTT and ITT results using the trapezoidal rule. The relationship between two variables was evaluated by calculating Spearman’s correlation coefficient.

3. Results

Study materials and methods and primer sequences for qPCR and standard PCR are summarized in Table S1, Supplementary material associated with this article online.

3.1. Polygenic mouse model of T2D induced by HFD

Outbred CD-1 mice were fed with an HFD for 18 weeks to establish a polygenic model of T2D. Three criteria based on FBG, GTT and ITT results were used to identify T2D in the HFD-fed mice. As a result, all 28 HFD-fed mice remaining at week 18 were identified as T2D mice. To investigate the progress or development of T2D, a total of three time points were used...
Fig. 1. Physiological parameters were compared between regular chow (RC)-fed and high-fat-diet (HFD)-fed mice. Fasting blood glucose (A) and body weight (B) were monitored weekly for 19 weeks (weeks 0–18). Arrows indicate the three time points (from left to right) of week 1, week 9 and week 18. Fasting (C) and postprandial (D) serum insulin levels, glucose tolerance tests (glucose AUC results shown in E) and insulin tolerance tests (glucose AUC results shown in F) were examined at weeks 1, 9 and 18. The number of experimental mice at week 18 (C–F) was n = 8 (RC-fed mice) and n = 27 (HFD-fed mice). The number of experimental mice at weeks 1 and 9 (C–F) was n = 8 (RC-fed mice) and n = 6 (HFD-fed mice). Data are means ± SEM. *P values are shown for each graph; **P < 0.01, ***P < 0.001.

for further analyses. At week 1, there was no difference in FBG, BW, serum insulin levels and blood glucose AUCs for GTT and ITT between the HFD-fed and RC-fed mice (Fig. 1A–F; Supplementary material associated with this article online, Fig. S1A). Up to week 9, HFD-fed mice had stable hyperglycaemia (P = 0.01) and increased BW (P = 0.014; Fig. 1A, B) as calculated by MANOVA. At week 9, HFD-fed mice also exhibited impaired glucose tolerance and insulin resistance (Fig. 1E, F; Supplementary material associated with this article online, Fig. S1B). At week 18, HFD-fed mice manifested more pronounced hyperglycaemia (P = 0.012; Fig. 1A), impaired glucose tolerance (Fig. 1E), insulin resistance as assessed by ITT (Fig. 1F; Supplementary material associated with this article online, Fig. S1C) and overt obesity (P = 0.0004; Fig. 1B). In addition, these HFD-fed mice displayed fasting and postprandial hyperinsulinaemia (Fig. 1C, D). It is noteworthy that postprandial serum insulin levels in RC-fed mice declined with age (from ages 5 to 22 weeks), but without statistical significance, and that the fasting serum insulin levels of these mice did not change with age. Postprandial serum insulin levels in HFD-fed mice also
did not decrease with age. At week 18, a relative but significant postprandial hyperinsulinaemia in HFD-fed mice compared with the respective insulin levels in RC-fed mice. It was also noteworthy that the ITT AUCs for RC-fed mice at weeks 9 and 18 tended to be lower than at week 1, showing increased insulin sensitivity with age in RC-fed mice. Thus, after 18 weeks of HFD, these mice all developed T2D with hyperglycaemia, impaired glucose tolerance, insulin resistance and hyperinsulinaemia.

### 3.2. Pancreatic adiponectin expression in HFD-fed vs RC-fed mice

In terms of the development of T2D, there is evidence that adiponectin levels in tissues are more relevant to the development of diet-induced insulin resistance than its levels in the circulation [17]. For this reason, adiponectin expression was sought in several insulin target organs, including adipose tissue, skeletal muscle and the liver, from RC and T2D mice at week 18. In addition, adiponectin expression in the pancreas, the insulin-producing organ was also looked for. Adiponectin was detectable in epididymal adipose tissue and skeletal muscle (Supplementary material associated with this article online, Fig. S2A–F), but not in the liver (Supplementary material associated with this article online, Fig. S2G–I). Eighteen weeks of HFD led to a significant decrease in skeletal muscle adiponectin (Supplementary material associated with this article online, Fig. S2D–F). Interestingly, adiponectin mRNA expression was found in the pancreas of HFD-fed mice, but not in that of RC-fed mice (Fig. 2C). Furthermore, adiponectin mRNA expression was measured by qPCR in these organs at weeks 1 and 9 in addition to week 18. Good-quality RNA from the pancreas was obtained from both RC- and HFD-fed mice (Supplementary material associated with this article online, Fig. S3A). As shown in Fig. 2A, there was no significant difference in adiponectin content of adipose tissue between RC and HFD-fed mice and their respective RC-fed mice at each time point. Compared with RC-fed mice, HFD-fed mice had markedly lower adiponectin content in skeletal muscle at week 18 (Fig. 2B). In fact, no adiponectin expression was detectable in the liver of any mice at all three time points (data not shown). In the pancreas of RC-fed mice, mRNA expression of adiponectin was almost undetectable at all three time points whereas, in contrast, adiponectin mRNA expression was high in the pancreas of HFD-fed mice (Fig. 2C). This extraordinary expression of pancreatic adiponectin in HFD-fed mice was detectable as early as 1 week after starting the HFD, reached its highest level at week 9 and then declined at week 18 (Fig. 2C). Protein levels of pancreatic adiponectin in HFD-fed mice at the three time points were confirmed using western blot analysis (Fig. 3A), whereas the weak bands of pancreatic adiponectin in RC-fed mice were evidently the result of “contamination” by circulating adiponectin, as no pancreatic expression of adiponectin in RC-fed mice was detectable at the mRNA level (Fig. 2C, Fig. 3A), which was similar to one report regarding “contamination” of adiponectin in the liver [18]. In the HFD-fed mice, although pancreatic adiponectin was extraordinarily expressed, neither pancreatic AdipoR1 nor AdipoR2 expression was lower than that in the RC-fed mice (Fig. 2D, E). To verify the production of adiponectin found in the pancreas induced by the HFD, the mRNA expression of leptin in the pancreas was examined. Leptin, another specific adipokine produced by adipose tissue, was not detectable in the pancreas of HFD-fed mice at all three time points (Fig. 2F). During the whole experimental period, serum adiponectin levels in both the RC and HFD groups were similar and did not alter with age (Supplementary material associated with this article online, Fig. S3B, C).

Adiponectin is transcriptionally regulated by various transcription factors, particularly PPARγ, in adipose tissue [19]. To investigate the mechanism underlying the newly discovered specific expression of adiponectin in the pancreas induced by HFD, PPARγ expression in the pancreas was examined by qPCR. Levels of pancreatic PPARγ mRNA in the HFD group were higher than in the RC group at every time point, while its levels in RC-fed mice did not fluctuate very much across the three time points (Fig. 3B). It is noteworthy that in HFD-fed mice, the expression pattern of PPARγ over all three time points was the same as that for adiponectin in the pancreas (Fig. 2C). Pancreatic PPARγ mRNA levels were positively correlated with pancreatic adiponectin mRNA levels \(P = 0.0002\), as shown by a linear correlation (Fig. 3C) only in HFD-fed mice. There was also a linear correlation between adiponectin and PPARγ mRNA levels in the adipose tissue of RC- and HFD-fed mice (Supplementary material associated with this article online, Fig. S3D), but not in skeletal muscle (data not shown). To further confirm that the upregulation of PPARγ was the reason for the extraordinary expression of pancreatic adiponectin in HFD-fed mice, mRNA expression of C/EBPα, which appears to depend on PPARγ activity was assessed [20]. In HFD-fed mice, C/EBPα expression showed the same pattern as PPARγ over the three time points (Fig. 3D). The difference in pancreatic C/EBPα expression between HFD-fed mice and RC-fed mice was statistically significant at week 9 \(P = 0.03;\) Fig. 3D).

### 3.3. Local adiponectin expression in the pancreas protects endothelial function despite HFD feeding

To confirm the aberrant protein expression of pancreatic adiponectin-induced by the HFD, the pancreas was examined by immunofluorescence analysis. No adiponectin could be detected in the pancreas of RC-fed mice (Supplementary material associated with this article online, Fig. S4A, F, K), which was in line with mRNA expression findings. For HFD-fed mice, the pancreas was co-stained with adiponectin/glucagon antibodies to label the pancreatic islets (Supplementary material associated with this article online, Fig. S4D, I, N), which helped to localize pancreatic adiponectin (Supplementary material associated with this article online, Fig. S4B–D, G–I, L–N). Leptin protein was undetectable in the pancreas of HFD-fed mice at all three time points (Supplementary material associated with this article online, Fig. S4E, J, O), which was consistent with mRNA results obtained by PCR. Specifically, immunofluorescence assay showed that adiponectin was expressed in the vascular endothelial cells (VECs) of the pancreas, which
was confirmed by the complete co-localization of adiponectin and CD31 (Supplementary material associated with this article online, Fig. S5B–D, F–H, J–L), a known VEC marker [21–23]. It has also been reported that HFD or free fatty acids can damage endothelial function in humans [24] and rodents [25]. CD31 expression is another indicator of endothelial function [22,23,26,27]. Thus, based on the special location of pancreatic adiponectin in our HFD-fed mice, mRNA levels of pancreatic CD31 were further measured at weeks 1, 9 and 18. In fact, mRNA levels of pancreatic CD31 in HFD-fed mice did not decline compared with RC-fed mice, and its expression pattern was similar to that of pancreatic adiponectin in HFD-fed mice (Supplementary material associated with this article online, Fig. S5Q; Fig. 2C).

3.4. Adiponectin enhances angiogenic function in HUVECs

Adiponectin was found to be expressed in pancreatic VECs in HFD-fed mice, and the expression pattern of CD31 mRNA was the same as for adiponectin mRNA, which suggests that adiponectin may play a role in the function of VECs. An in vitro endothelial cell system of HUVECs was used to examine the effect of adiponectin on endothelial function. It was found that adiponectin was strongly expressed in HUVECs transfected with adiponectin-expressing lentivirus (Supplementary material associated with this article online, Fig. S3D). First, a modified Boyden chamber assay showed that HUVECs transfected with an adiponectin-expressing lentivirus exhibited enhanced migration compared to controls transfected with an empty
lentivirus (Fig. 4A), and the significant difference ($P=0.014$) was confirmed by quantitative analysis (Fig. 4B). Furthermore, tube formation was significantly enhanced ($P=0.02$) in HUVECs transfected with green fluorescent protein (GFP)-tagged adiponectin compared with those transfected with empty GFP (Fig. 4C, D). These results suggest that adiponectin has a pro-angiogenic effect on endothelial cells, as indicated by the promotion of migration and tube formation of HUVECs.

4. Discussion

To investigate whether adiponectin is expressed in the pancreas and its role during the development of T2D, a polygenic T2D mouse model was generated by feeding outbred CD-1 mice an HFD in this study. This mouse model exhibited constant hyperglycaemia, insulin resistance and hyperinsulinaemia after 18 weeks of HFD, and closely mimicked the aetiology of human T2D, with polygenic inheritance and the main unhealthy environmental factor (the HFD). The development of T2D induced by the HFD was observed at three time points. Mice fed the HFD for 9 weeks exhibited constant hyperglycaemia, increased BW, impaired glucose tolerance and insulin resistance compared with RC-fed mice, but their hyperinsulinaemia was not significant at this time point. Up to week 18, HFD-fed mice manifested significant hyperinsulinaemia during both fasting and postprandial states compared with RC-fed mice. Regarding insulin resistance and hyperinsulinaemia, there have been controversial reports as to which comes first in the development of T2D [28]. According to the observed results in the present study, insulin resistance emerged first (larger glucose AUC during ITT and hyperglycaemia starting at or before week 9) and was followed by hyperinsulinaemia (at week 18) during the development of T2D induced by HFD in this outbred CD-1 mouse strain. It is interesting that in RC-fed mice at ages 5 to 22 weeks (time points from week 1 to week 18), fasting serum insulin levels did not change, whereas postprandial insulin levels had a tendency to decline. The ITT glucose AUCs for these control mice at ages 13 and 22 weeks (time points week 9 and 18) tended to be lower than at age 5 weeks. This result suggests that their insulin sensitivity tended to increase with age during this period, which was roughly equivalent to young childhood to early adulthood in humans; these mice were definitely not yet affected by senescence. This finding also implies that maturational growth continued with regard to insulin sensitivity with age during this period in our RC-fed mice. Nonetheless, this developmental process was disrupted when mice were treated with an HFD starting when they were just weaned, which resulted in insulin resistance in HFD-fed mice at later stages of maturity, as shown by their unreduced ITT AUCs, absolute fasting hyperinsulinaemia, relative but significant postprandial

Fig. 3. Adiponectin was detected by western blots (A). PPARγ mRNA expression in the pancreas (of HFD- and RC-fed mice) was detected at weeks 1, 9 and 18 by qPCR (B). A linear correlation was found between pancreatic PPARγ and pancreatic adiponectin at the mRNA level in HFD-fed mice (C). Pancreatic C/EBPα expression was tested by qPCR at the three time points (D). Five to nine mice were examined in each group. Data are means ± SEM. $P$ values are shown for each graph; $^*P<0.05$. 

Fig. 4. Adiponectin promotes migration and tube formation of HUVECs in vitro. A. Representative images of cell migration using a modified Boyden chamber assay. B. Quantitative analysis of migrated HUVECs transfected with an adiponectin-expressing lentivirus and empty lentivirus. C. Representative microscopy views of tube formation assay with HUVECs transfected with adiponectin and control HUVECs. D. Quantitative analysis of the tube formation assay. Data are means ± SEM; results are expressed relative to controls. P values were evaluated using Student’s t-test. Scale bars = 50 μm (A) and 100 μm (C). P values are given for each bar graph; *P < 0.05.

hyperinsulinaemia and pronounced hyperglycaemia compared with the respective test results of RC-fed mice. The finding that pancreatic adiponectin is expressed in mice fed an HFD in our study raises the following question of a new possibility: could hyperinsulinaemia at least partially result from the combined effects of overexpressed pancreatic adiponectin and pronounced hyperglycaemia during the development of T2D?

It was originally thought that adiponectin was specifically produced by adipose tissue; however, recent studies indicated that adiponectin was detectable within other cell types and tissues, such as mouse skeletal muscle tissues [4]. It has also been reported that adiponectin content in adipose tissue and skeletal muscle is associated with changes in BW and behaves like a “beneficial” adipokine with a protective response, demonstrating the close connection between skeletal muscle adiponectin and diet-induced insulin resistance [17]. Consistent with other reports [4,18], our present study has shown that adiponectin is not expressed in the liver, but is expressed in skeletal muscle as well as in adipose tissue. Similar to the report by Yang et al. [17], adiponectin expression levels in the skeletal muscle of HFD-fed mice at weeks 9 and 18 in our study were much lower. Moreover, our study has revealed for the first time that adiponectin was also expressed in the pancreas of HFD-fed mice, but not in that of RC-fed mice, as determined by qPCR. Also, compared with RC-fed mice, expression levels of pancreatic adiponectin receptors in HFD-fed mice were not downregulated. Thus, it can be reasonably assumed that adiponectin expression in the pancreas of HFD-fed mice will produce a net outcome of increased adiponectin signaling or local effects. During the development of T2D in our study, expression levels of pancreatic adiponectin changed with the highest levels appearing at week 9. This finding suggests that when mice are exposed to an HFD, the body is also triggered to locally produce a “beneficial” adipokine against HFD-induced damage to the pancreas.
Vascular endothelium is the “first-responder” to environmental stimuli, such as nutrients, cytokines, chemokines and physical activity [29,30]. In our study, adiponectin expression was induced in pancreatic VECs just 1 week after starting the HFD. In addition to its metabolic and anti-atherogenic effects, adiponectin possesses a newly reported property to stimulate new blood-vessel formation [31]. It is well known that diminished function of VECs is seen with diabetes [26,32]. CD31 is a significant indicator of VEC function and dysfunction [22,23,26,27]. Studies in humans [22] and of a human aortic endothelial cell line [23] have demonstrated that downregulation of CD31 is closely associated with VEC death and progression of atherosclerosis. Increased CD31 expression has been shown to limit endothelial cell dysfunction [21]. As it was predicted that pancreatic adiponectin might play a role in the function of pancreatic VECs, endothelial function was evaluated by checking CD31 expression. Despite the fact that a high-fat meal or free fatty acids can induce endothelial dysfunction [24,25], our study demonstrated no downregulation of pancreatic CD31 in HFD-fed mice compared with their respective RC-fed mice at each time point. Instead, at week 9 there was a trend towards increased CD31 expression in HFD-fed mice. It was also found that the expression pattern of pancreatic CD31 was similar to that of pancreatic adiponectin in HFD-fed mice. Our in vitro experiments using a HUVEC system demonstrated that transfection with adiponectin could promote migration and tube formation of these endothelial cells. This result implies that the overexpression of adiponectin in pancreatic VECs plays a protective role in maintaining endothelial function during the development of T2D induced by HFD.

It has been reported that endothelial signaling is required for the differentiation and function of pancreatic beta cells [33,34]. From this, it was inferred that the induced expression of adiponectin might exert an influence on the endocrine pancreas via the maintenance of vascular endothelial function. The beneficial effect of adiponectin itself on pancreatic islets and beta cells has been substantiated investigated [7,8]. There appears to be a general trend for adiponectin to increase glucose-stimulated insulin secretion in vitro, especially under stress conditions, such as lipotoxicity, glucotoxicity and insulin resistance [7,8]. In our present study, pancreatic adiponectin expression induced by HFD was followed by significant hyperinsulinemia at week 18 when mice were persistently exposed to the HFD and constant hyperglycaemia and insulin resistance. Postprandial insulin levels in HFD-fed mice lost the tendency shown in RC-fed mice to be reduced with age, exhibiting instead a relative postprandial hyperinsulinemia starting at week 9. Is it possible that, at a relatively early stage in the development of T2D, pancreatic adiponectin might augment insulin release by pancreatic islets, thereby resulting in hyperinsulinemia, including the relative postprandial hyperinsulinemia? The expression pattern of pancreatic adiponectin wherein levels increased at week 9 and then fell at week 18 implies that persistent exposure to an HFD will eventually lead to decreased adiponectin expression in the pancreas, and that the effect of adiponectin to protect endothelial function would finally diminish. However, to definitively answer the interesting question of whether overexpressed pancreatic adiponectin resulting from an HFD plays a role in hyperinsulinemia, more specific studies such as those using mouse models with related gene(s) knocked out are required to obtain more direct evidence.

Adiponectin levels faithfully mirror PPARγ activity and are considered a biomarker of PPARγ therapy efficacy and fibrogenic activity [35,36]. It is reported that adiponectin is transcriptionally regulated by various transcription factors in adipose tissue, particularly PPARγ [19]. PPARγ functions as a cis-regulatory element and may directly bind to the human proximal adiponectin promoter by forming heterodimers with retinoid X receptor (RXR) [19]. Our present study found that pancreatic PPARγ, C/EBPα and adiponectin were all expressed in the same pattern with higher mRNA levels in HFD-fed mice than in RC-fed mice. At the mRNA level, PPARγ expression was positively correlated with adiponectin in the pancreas of HFD-fed mice. These results suggest that HFD resulted in upregulation of PPARγ, as confirmed by the expression of C/EBPα, which then induced the expression of pancreatic adiponectin.

In summary, the present study has demonstrated for the first time the extraordinary expression of adiponectin in pancreatic VECs during the development of T2D induced by HFD in this outbred mouse model. This extraordinary expression may at least be triggered by upregulation of PPARγ at the transcriptional level. During the development of T2D by HFD, such local expression of adiponectin plays a protective role in the pancreas by protecting its endothelial function. Nevertheless, whether the aberrant expression of pancreatic adiponectin is at least partially responsible for hyperinsulinemia is worthy of further investigation.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data (French abstract; Figs. S1–S5 and Table S1) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.diabet.2014.05.004.
References


