Insulin downregulates the expression of the Ca\textsuperscript{2+}-activated nonselective cation channel TRPM5 in pancreatic islets from leptin-deficient mouse models

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Abstract We recently proposed that the transient receptor potential melastatin 5 (TRPM5) cation channel contributes to glucose-induced electrical activity of the β cell and positively influences glucose-induced insulin release and glucose homeostasis. In this study, we investigated Trepm5 expression and function in pancreatic islets from mouse models of type II diabetes. Gene expression analysis revealed a strong reduction of TREPM5 mRNA levels in pancreatic islets of db/db and ob/ob mice. The glucose-induced Ca\textsuperscript{2+} oscillation pattern in db/db and ob/ob islets mimicked those of TRPM5
−/− islets. Leptin treatment of ob/ob mice not only reversed the diabetic phenotype seen in these mice but also upregulated Trepm5 expression. Leptin treatment had no additional effect on Trepm5 expression levels when plasma insulin levels were comparable to those of the vehicle-injected control group. In murine β cell line, MIN6, insulin downregulated TRPM5 expression in a dose-dependent manner, unlike glucose or leptin. In conclusion, our data show that increased plasma insulin levels downregulate TRPM5 expression in pancreatic islets from leptin-deficient mouse models of type 2 diabetes.

Keywords Diabetes type 2 · TRPM5 · ob/ob mice · db/db mice · MIN6 cells

Introduction

Diabetes type 2 mainly results from a combination of lifestyle factors (such as obesity, sedentary lifestyle, unhealthy eating habits…) and genetics. Moreover, environmental factors probably influence gene expression in almost all cells, including β cells. This can have an effect on the susceptibility to disruptions in glucose homeostasis and metabolic regulation and on the risk profile for diabetes type 2 [9, 19, 35]. It has been proposed that individuals at risk of diabetes type 2 carry one or more polymorphisms in genes encoding ion channels or in genes that regulate ion channel function, membrane targeting, or expression [2]. As a result, such genetic backgrounds will be reflected in small changes in β cell electrical activity that will immediately be mirrored by changes in insulin secretion. Thus, knowledge of the biophysical basis of insulin secretion and glucose-induced electrical activity and identification of ion channels with altered activity in type 2 diabetes is of great clinical significance in the diagnosis and treatment of this disease. Previous studies from our laboratory indicated the transient receptor potential melastatin 5 (TRPM5) as an important player in the electrical activity from the β cell and glucose-induced insulin release [6]. TRPM5 is a member of the large family of TRP channels [10, 27], a group of cation channels with diverse expression patterns that are shown to be important in human diseases [10, 22, 23, 27] and suggested to have significant roles in the pancreatic β cell [7]. TRPM5 is a nonselective monovalent cation channel that is impermeable to Ca\textsuperscript{2+} but directly activated by intracellular Ca\textsuperscript{2+} [11, 30]. The channel contributes to membrane depolarization during electrically silent intervals, thereby facilitating the initiation of
a new burst of activity. As a result, islets of Trpm5 knockout (Trpm5\(^{-/-}\)) mice show mainly slow glucose-induced changes in membrane potential (\(V_m\)) and oscillations of the intracellular \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}\)]), whereas islets of wild type (WT) mice are characterized by fast, slow, and mixed oscillations [6]. Accordingly, Trpm5\(^{-/-}\) islets release less insulin and Trpm5\(^{-/-}\) mice display lower plasma insulin levels and moderate glucose intolerance [3, 6]. This pre-diabetic phenotype in Trpm5\(^{-/-}\) mice suggests that TRPM5 might be a candidate for predisposition of type 2 diabetes. Interestingly, genetic variation within the Trpm5 locus is shown to associate with pre-diabetic phenotypes in subjects at increased risk for type 2 diabetes [13].

Two widely studied genetic models of type 2 diabetes are the leptin receptor-deficient db/db and the leptin-deficient ob/ob mouse, which develop morbid obesity due to impaired leptin signaling and the characteristic hyperinsulinemia and hyperglycemia of type 2 diabetes [4, 5, 18]. We detected loss of Trpm5 expression levels in the pancreatic islets of these mouse models, which could be rescued by recovery of the leptin pathway and the diabetic phenotype. Our data indicate that high insulin levels are important in downregulation of Trpm5 in mouse models with impaired leptin signaling.

Research design and methods

Mice

All animal work was conducted following the ethical approval of the KU Leuven Ethical Committee and according to national and international guidelines. Db/db, ob/ob mice, and control mice in C57BL/6J background were obtained from The Jackson Laboratory. Only male mice were used for experiments. Chow was purchased from Ssniff Spezialdiäten GmbH (Germany). The high glucose chow contained ≥50 % glucose and ≥12+ oligosaccharides/dextrines, whereas the high fat chow contained 30.2 % crude fat. All other animals received normal chow (containing 4.7 % sugar and 3.3 % crude fat).

Preparation of islets

Islets were isolated from male mice via collagenase digestion as described previously [6]. The isolated islets were either immediately used (RNA extraction) or cultured overnight (\(\text{Ca}^{2+}\) imaging and incubation experiments) in islet culture medium (advanced RPMI medium 1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 4 mM Glutamax) at 5 % \(\text{CO}_2/95 \% \text{O}_2\).

Glucose tolerance tests, plasma glucose, and insulin levels

All blood samples were obtained via tail bleeding. Plasma glucose levels were measured directly in the blood using an Accu-Check Aviva glucose meter (Roche, USA). Plasma insulin was measured using a commercially available ELISA kit (Crystal Chem. Inc., IL, USA) after addition of 2 \(\mu\)l EDTA (0.5 M) to 30-\(\mu\)l blood and centrifugation of the blood sample (4 °C, 12 min, 3,400 rpm). For the glucose tolerance tests, glucose (2.5 g/kg body weight) was injected intraperitoneal (IPGTT) in overnight fasted mice. Glucose and insulin levels were measured at 0, 15, 30, 60, and 120 min and at 0 and 30 min after glucose administration, respectively.

Quantitative PCR

Total RNA from freshly isolated or cultured pancreatic islets was extracted with the RNasy mini kit (Qiagen). The quantity and quality of the RNA samples were assessed by use of the Experion RNA StdSens analysis kit (Bio-Rad, USA) and only RNA samples with a RNA quality indicator value above 5 were used for further experiments. Complementary DNA was synthesized by using Ready-To-Go You-Prime first-strand beads (GE Healthcare, UK). Triplicate cDNA samples from each independent preparation were analyzed by quantitative real-time polymerase chain reactions (qPCR) in the 7500 real-time PCR system (Applied Biosystems) using specific TaqMan gene expression assays for Trpm5 located in the boundary of exons 8–9 and 16–17 (Applied Biosystems; TaqMan assay Mm00498442_m1 and Mm01129032_m1, respectively). β-actin and TATA box binding protein were identified as most stably expressed reference genes by the geNorm analysis [36] and further used as endogenous controls for accurate normalization of qPCR results (Applied Biosystems, TaqMan assay Mm_00446973_m1).

Chemicals

Leptin (Sigma-Aldrich) was dissolved at a concentration of 1 mg/ml in 20 mM Tris/HCl (pH 8.0) and further diluted to a concentration of 0.1 mg/ml in PBS (Invitrogen). Vehicle solution consisted of a 10× dilution of 20 mM Tris/HCl (pH 8.0) in PBS. Insulin (Sigma-Aldrich) was dissolved in diluted hydrochloric acid (pH=2–3) and further diluted in Milli-Q water. Diazoxide (Sigma-Aldrich) was dissolved in 0.1 M NaOH and further diluted in Milli-Q water. Tyrophan AG490 (Sigma-Aldrich) was diluted in 100 % EtOH and further diluted in Milli-Q water.

Cell culture and incubation experiments

Islets from ob/ob mice were isolated and cultured overnight in culture medium prior to starting the incubation. The next day, islets from each mouse were divided into two groups and incubated either in islet culture medium or with 200 ng/ml leptin added. After a 48-h incubation time, islets were collected and RNA was extracted immediately.
MIN6 cells, a murine pancreatic β cell line, was cultured in MIN6 culture medium (Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM Glutamax, β-mercaptoethanol, and pyruvate) at 5% CO2/95% O2. Cells with a confluence of about 80% were used for incubation experiments. After incubation, MIN6 cells were collected and frozen in liquid nitrogen prior to RNA extraction. MIN6 cells were kindly provided by Dr. E. Yamato (Osaka University, Osaka, Japan).

[Bath temperature was controlled by a SC-20 dual in-line heater/cooler (Warner Instruments) and monitored by a TA-29 thermometer (Thermometrics) positioned in close vicinity of the islet. Islets were loaded with 1 μM Fura-2 acetoxyethyl ester (TelLabs) for 1 h at 37 °C in culture medium. [Ca2+]i, from Fura-2 loaded islets was measured monitoring fluorescence ratio (F350/F380) every second (after correction for background fluorescence) at 37 °C. [Ca2+]i was measured using a monochromator based system consisting of a Polychrome IV monochromator (TILL Photonics) with an additional TILL photonics photomultiplier, both controlled by Pulse software (HEKA Elektronik). Standard extracellular solution for Ca2+ imaging measurements contained (in millimolar) 120 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgCl2, 10 Hepes, at pH 7.4 with NaOH, with different concentrations of glucose added as indicated.

Data analysis

Origin 7.0 software (OriginLab Corporation, Northhampton, MA, USA) and Microsoft Excel were used for data analysis. Data are represented as mean ± SEM, unless mentioned differently. Normality assumptions were tested with the Shapiro–Wilk test. Statistical analysis was performed with the student’s t test, either one-sample (alternative hypothesis: mean≠1) or two-sample, unless mentioned differently. P value <0.05 was considered to represent a significant difference.

Results

Decreased expression of Trpm5 in pancreatic islets of db/db and ob/ob mice

Both db/db and ob/ob mice suffer from obesity and dramatically elevated plasma glucose and insulin levels as compared to age-matched WT mice (see Table 1). Moreover, they show a severely impaired glucose tolerance (see Fig. 1a, b). Interestingly, Trpm5 expression was dramatically decreased in islets from db/db mice at all ages (TaqMan assay located in a boundary of Trpm5 exons 19–20, WT 1.00±0.25 vs. db/db 0.08±0.02, p=0.006 at 5 weeks; WT 1.00±0.26 vs. db/db 0.02±0.00, p=0.005 at 10 weeks; WT 1.00±0.28 vs. db/db 0.06±0.03, p=0.00008 at 15 weeks; n=5–10 mice per group; see Fig. 1c). Comparable results were also obtained with a specific Trpm5 TaqMan assay targeting a boundary of exons 8–9: WT 1.00±0.08 vs. db/db 0.04±0.01, n=3 per group, p=0.0003 for 10-week-old mice. Similar to db/db animals, islets of ob/ob mice also demonstrated downregulation of Trpm5 expression (WT 1.0±0.49 vs. ob/ob 0.06±0.05, p=0.007, n=7–8 mice per group, see Fig. 1d). Furthermore, islets of heterozygous db/+ mice, which do not differ from WT mice in their metabolic parameters (see Table 1), did not show alteration of Trpm5 expression when compared with WT (WT 1.0±0.24 vs. db/+ 1.54±0.05, p=0.21, n=2–3 per group).

During Fura-2-based Ca2+ imaging, islets from both db/db and ob/ob mice respond to a stimulus of 10-mM glucose with an oscillatory increase in the intracellular Ca2+ concentration ([Ca2+]i) (see Fig. 2a), although some islets (4 out of 21 db/db islets and 5 out of 23 ob/ob islets) responded with a sustained increase in [Ca2+]i (plateau), a feature that was never detected in WT islets. Whereas the average [Ca2+]i increase during the glucose stimulus was comparable in all tested islet groups (see Fig. 2b), the average frequency of oscillations in db/db and ob/ob islets was significantly lower than in WT islets and reached levels observed in Trpm5−/− islets (0.40±0.06 and 0.43±0.04 peaks per min, respectively; see Fig. 2c). Moreover, they specifically lack fast glucose-induced oscillations, reminiscent of what is shown for Trpm5−/− islets (see Fig. 2c). These data suggest that the downregulation of Trpm5 is reflected at the functional level during glucose-induced Ca2+-signaling in the pancreatic β cell.

| Table 1 | Metabolic phenotype of WT, db/db, and ob/ob mice at several ages |
|---------------------------------|------------------|------------------|------------------|
| Bodyweight (g)                  | Plasma glucose (mg/dl) | Plasma insulin (ng/ml) |
|---------------------------------|------------------|------------------|
| 5 weeks                         |                   |                  |
| WT (n=10)                       | 18.6±0.5          | 173±7            | 1.36±0.18        |
| Db/+ (n=5)                      | 17.8±0.1          | 151±6            | 1.4±0.22         |
| Db/db (n=5)                     | 25.2±0.8***      | 312±44***        | 13.04±0.95***    |
| 10 weeks                        |                   |                  |
| WT (n=10)                       | 23.8±0.4          | 155±12           | 1.51±0.16        |
| Db/db (n=5)                     | 42.6±0.8***      | 336±38***        | 15.9±0.6***      |
| 15 weeks                        |                   |                  |
| WT (n=9)                        | 27.7±0.4          | 134.1±4.4        | 0.92±0.17        |
| Db/db (n=5)                     | 48.6±0.6***      | 259.8±31.1***    | 19.05±1.95***    |
| Ob/ob (n=7)                     | 54.3±1.2***      | 237.1±32.2*      | 22.7±0.8***      |

Comparison of bodyweight, plasma glucose, and plasma insulin levels in WT, db/db, and ob/ob mice at 5, 10, or 15 weeks of age as indicated. *p<0.05, **p<0.01, ***p<0.001 versus age-matched WT mice.
Fig. 1 Downregulation of Trpm5 expression in islets from db/db and ob/ob mice. a, b Plasma glucose (a) and insulin (b) levels in WT, db/db, and ob/ob mice measured during an intraperitoneal glucose tolerance test at several time points as indicated. N=5–10 mice per group. Asterisk and number sign indicate significant difference of WT mice versus db/db and ob/ob mice, respectively (**p<0.01, ***p<0.001). c, d QPCR experiments showing mRNA expression of Trpm5 in freshly isolated pancreatic islets from WT and db/db mice at several ages as indicated (c) or from WT and ob/ob mice at 15 weeks of age (d). Data are normalized to the average Trpm5 expression in islets from age-matched WT mice. N=5–12 per group, **p<0.01, ***p<0.001.

Fig. 2 Glucose-induced Ca2+-signaling in islets from db/db and ob/ob mice correlates with a loss of TRPM5. a Representative examples of Ca2+-oscillations in isolated pancreatic islets of db/db, ob/ob, WT, and Trpm5−/− mice during stimulation with 10 mM glucose. b Average increase in ratio (F350/F380) in islets from db/db, ob/ob, WT, and Trpm5−/− islets during glucose stimulation. One-way ANOVA revealed no difference between the four groups (overall p=0.1769; n=21–33 per group from 3–7 mice). c Frequency of oscillations in db/db, ob/ob, WT, and Trpm5−/− islets, counted as the number of peaks per minute. An increase of 15% was considered to be an oscillation, when 100% is the amplitude between the baseline and the highest level reached in 10 mM glucose. One-way ANOVA revealed a difference between the four groups (overall p=0.00013; n=21–33 per group from 3–7 mice) with the subsequent mean comparison Bonferroni test showing a difference of WT islets compared to db/db, ob/ob, and Trpm5−/− islets (*p<0.05, ***p<0.001 vs. WT islets). Data from WT and Trpm5−/− islets are adapted from [6].
No effect of dietary compounds on Trpm5 expression

One of the most striking features of db/db and ob/ob mice is the pronounced obesity, caused by hyperphagia. This increased food intake leads to higher intake of specific dietary compounds, such as glucose and fat. To investigate whether this could influence Trpm5 expression levels in pancreatic islets, we fed normal WT mice during 14 weeks with a diet containing either ≥50% glucose and ≥12% oligosaccharides/dextrines (= high glucose diet) or 30.2% fat (= high fat diet). After 14 weeks, the high fat (but not the high glucose) fed mice had a significantly increased bodyweight (normal diet 29.71±0.53 g; high glucose diet 30.34±1.04 g, p=0.56 vs. normal diet; high fat diet 46.23±0.67 g, p=5.13×10^{-11} vs. normal diet; n=4–6 per group; see Fig. 3a). There was a tendency in both groups towards higher plasma glucose levels (normal diet 166.6±2.8 mg/dl; high glucose diet 177.7±4.4 mg/dl, p=0.042 vs. normal diet; high fat diet 175.5±4.0 mg/dl, p=0.078 vs. normal diet, n=4–6 per group; see Fig. 3b) and plasma insulin levels were slightly elevated by both diets (normal diet 0.8±0.07 ng/ml; high glucose diet 1.36±0.22 ng/ml, p=0.025 vs. normal diet; high fat diet 2.9±0.37 ng/ml, p=0.00046 vs. normal diet, n=4–6 per group; see Fig. 3c). However, neither the high glucose diet (normal diet 1.0±0.07 vs. high glucose diet 0.93±0.1, p=0.7096; n=4–6 per group) nor the high fat diet (normal diet 0.99±0.14 vs. high fat diet 0.98±0.18, p=0.92, n=4–6 per group; see Fig. 3d) altered islet Trpm5 expression, suggesting that dietary compounds are not responsible for the altered Trpm5 expression in db/db and ob/ob islets. As the high fat mice also developed obesity, comparable to that observed in db/db mice, these data exclude obesity as such being causative of impaired Trpm5 expression.

Leptin treatment converts diabetic phenotype and rescues Trpm5 expression in ob/ob mice

Five-week-old ob/ob mice were daily injected with a dose of 1 mg/kg bodyweight leptin i.p. during 5 weeks and compared to ob/ob mice that received daily vehicle injections. Leptin injections decreased the daily food intake per mouse (vehicle-injected 5.83±0.05 vs. leptin-injected 3.31±0.19 g/day/mouse). During the course of the experiment, the bodyweight of the vehicle-treated group increased much faster as compared to the leptin-injected group, with a statistically significant difference starting from day 8, reaching bodyweights of 44.5±1.7 g for the vehicle-injected group and 35.6±1.0 g for the leptin-injected group after 36 days (n=10 per group, p=0.000663, see also Fig. 4a). After 5 weeks, both plasma glucose levels (vehicle-injected 188.2±9.6 mg/dl vs. leptin-injected 175.5±4.0 mg/dl, p=0.042 vs. normal diet; high fat diet 175.5±4.0 mg/dl, p=0.078 vs. normal diet, n=4–6 per group; see Fig. 3b) and plasma insulin levels (normal diet 0.8±0.07 ng/ml; high glucose diet 1.36±0.22 ng/ml, p=0.025 vs. normal diet; high fat diet 2.9±0.37 ng/ml, p=0.00046 vs. normal diet, n=4–6 per group; see Fig. 3c). However, neither the high glucose diet (normal diet 1.0±0.07 vs. high glucose diet 0.93±0.1, p=0.7096; n=4–6 per group) nor the high fat diet (normal diet 0.99±0.14 vs. high fat diet 0.98±0.18, p=0.92, n=4–6 per group; see Fig. 3d) altered islet Trpm5 expression, suggesting that dietary compounds are not responsible for the altered Trpm5 expression in db/db and ob/ob islets. As the high fat mice also developed obesity, comparable to that observed in db/db mice, these data exclude obesity as such being causative of impaired Trpm5 expression.

Leptin treatment converts diabetic phenotype and rescues Trpm5 expression in ob/ob mice

Five-week-old ob/ob mice were daily injected with a dose of 1 mg/kg bodyweight leptin i.p. during 5 weeks and compared to ob/ob mice that received daily vehicle injections. Leptin injections decreased the daily food intake per mouse (vehicle-injected 5.83±0.05 vs. leptin-injected 3.31±0.19 g/day/mouse). During the course of the experiment, the bodyweight of the vehicle-treated group increased much faster as compared to the leptin-injected group, with a statistically significant difference starting from day 8, reaching bodyweights of 44.5±1.7 g for the vehicle-injected group and 35.6±1.0 g for the leptin-injected group after 36 days (n=10 per group, p=0.000663, see also Fig. 4a). After 5 weeks, both plasma glucose levels (vehicle-injected 188.2±9.6 mg/dl vs. leptin-
injected 152.2±7.7 mg/dl; p=0.0098, n=10 per group) and plasma insulin levels (vehicle-injected 16.2±2.0 ng/ml vs. leptin-injected 5.7±1.2 ng/ml; p=0.00025, n=10 per group) were dramatically decreased due to leptin treatment (see Fig. 4b, c). Moreover, the leptin treatment restored glucose tolerance in ob/ob mice (Fig. 5). Interestingly, islet Trpm5 expression in islets of leptin-treated mice was upregulated as compared to islets of vehicle-injected mice (vehicle 0.96±0.25 vs. leptin 6.52±0.61; p=2.1×10^{-6}, n=7 per group). These data imply that downregulation of Trpm5 expression in islets from ob/ob and db/db mice results from the disruption of the leptin pathway and/or the consequent diabetic phenotype.

No additional effect of leptin on Trpm5 expression when plasma insulin levels are equal between leptin- and vehicle-injected ob/ob mice

In order to investigate whether the recovery of the leptin pathway (without altering the metabolic phenotype) would be sufficient to upregulate Trpm5 expression, we injected 9-week-old ob/ob mice for 2 days with either 1 mg leptin per kilogram bodyweight or vehicle. Since leptin will have a dramatic and immediate effect on food intake and consequently on the metabolic phenotype, animals were divided in two groups as follows: one group of mice had free access to food for the whole duration of the experiment (Fig. 6a), whereas a second group was put on food restriction, meaning that they received 3 g of food per day and were fasted overnight during the last night before islet isolation (Fig. 6b). Bodyweight was not altered by this short period of leptin treatment, neither in the fed ad libitum group (vehicle-injected 44.1±1.7 g vs. leptin-injected 42.7±1.1 g; p=0.52, n=4 mice per group) nor in the food restriction group (vehicle-injected 41.0±0.7 g vs. leptin-injected 39.2±1.5 g; p=0.33, n=4 mice per group). Also, plasma glucose levels did not change after 2 days of leptin treatment for both groups: vehicle-injected 245.5±68.2 mg/dl versus leptin-injected 167.5±4.9 mg/dl; p=0.30, n=4 mice per group for the fed ad libitum mice and vehicle-injected 179±18 mg/dl versus leptin-injected 155±17 mg/dl; p=0.38, n=4 mice per group for the mice put on food restriction. However, plasma insulin was dramatically decreased by leptin treatment in the fed ad libitum mice (vehicle-injected 16.9±1.4 ng/ml vs. leptin-injected 6.2±1.0 ng/ml; p=0.0008, n=4 mice per group). In contrast, the mice that were on food restriction displayed normal insulin levels (4.4±1.6 ng/ml vs. 2.7±1.2 ng/ml, respectively, p=0.41, n=4 mice per group). Interestingly, Trpm5 was upregulated by leptin treatment in mice that had free access to food (vehicle-injected 0.96±0.43 vs. leptin-injected 3.65±0.53; p=0.0092, n=4 mice per group) but not in mice that were on food restriction (vehicle-injected 0.95±0.36 vs. leptin-injected 1.08±0.31, p=0.86, n=4 mice per group). These data strongly suggest that plasma insulin levels are a critical factor in the regulation of islet Trpm5 expression.
Insulin levels are important for downregulation of Trpm5

We incubated the insulinoma cell line MIN6 cells with several factors altered in these mouse models for type 2 diabetes. First of all, both mouse strains suffer from a defect in the leptin signaling pathway. Activation of the leptin pathway in MIN6 cells by incubation with 200 ng/ml leptin for 48 h had no influence on Trpm5 expression (control 1.00±0.05 vs. leptin 1.17±0.13, n=3 per group, p=0.22; Fig. 7a). Furthermore, disrupting leptin signaling by adding the compound Tyrophostin AG490 to the incubation medium for 1 week had no effect on Trpm5 expression in MIN6 cells (leptin 1.00±0.08 vs. leptin+AG490 0.85±0.09, n=3 per group, p=0.279). Similarly, recovery of the leptin pathway in ob/ob islets by incubation with 200 ng/ml leptin for 48 h had no influence on Trpm5 expression (islet Trpm5 expression 1.27±0.22 vs. control, n=4, one-paired t test p=0.30; Fig. 7b, c). These data strongly suggest that the disrupted leptin pathway is not the cause of the downregulation of Trpm5.

Second, db/db and ob/ob mice suffer from high plasma glucose levels. However, incubation of MIN6 cells with 25 mM glucose did not alter Trpm5 expression as compared to expression in cells incubated in 5.5 mM glucose (5.5 mM glucose+250 μM diazoxide 1.0±0.0 vs. 25 mM glucose+250 μM diazoxide 1.13±0.12, p=0.39, n=3–6 per group; Fig. 7d), implying no influence of high glucose levels on Trpm5 expression.

Finally, diabetic db/db and ob/ob mice display elevated plasma insulin levels. Incubation of MIN6 cells for 1 week with increasing concentrations of insulin dose-dependently decreased Trpm5 expression (control 1.0±0.0; 10 nM insulin 0.74±0.05, p=0.0082; 100 nM insulin 0.61±0.05, p=0.00027; n=4–7 per group; Fig. 7e), indicating that high plasma insulin levels are indispensable for the downregulation of Trpm5 in pancreatic islets from diabetic mice.

**Discussion**

Trpm5 was previously identified as a critical component in the electrical activity of the pancreatic β cell and in glucose-induced insulin release [6]. Since Trpm5−/− mice show a pre-diabetic phenotype, it is conceivable that mutations or altered expression patterns of Trpm5 are involved in the pathogenesis of type 2 diabetes mellitus. Expression of Trpm5 in the small intestine has been shown to be negatively correlated with blood glucose concentrations in type 2 diabetic patients [38]. Moreover, genetic variation of Trpm5 is shown to be associated with pre-diabetic phenotypes in a population of European ancestry [13]. Although the functional relevance of these mutations remains to be shown, these data strongly suggest a link between TRPM5 and type 2 diabetes. In this study, we provide evidence that elevated plasma insulin levels are important for the downregulation of Trpm5 expression in pancreatic islets from animal models with impaired leptin signaling.

Trpm5 mRNA expression was almost 20-fold downregulated in islets from db/db and ob/ob mice, two mouse models of type 2 diabetes. Both mouse models suffer from a defect in leptin-signaling, resulting in hyperphagia, obesity, hyperinsulinism, hyperglycemia, and diabetes [5, 18]. We systematically tested whether any of these factors underlies the downregulation of Trpm5. Interestingly, the glucose-induced Ca2+-signaling in islets from db/db and ob/ob mice correlated well with a loss of TRPM5. Previous studies from our laboratory show that Trpm5−/− islets display a reduced frequency of oscillations in Ca2+ and Vm, specifically due to a lack of fast oscillations [6]. During glucose stimulation, db/db and ob/ob islets showed a frequency of Ca2+ oscillations comparable to Trpm5−/− islets and significantly lower than oscillations in WT islets. They never showed simple fast glucose-induced oscillations as detected in a population of WT islets. Irregular Ca2+ oscillations and, more specifically, a lack of fast
oscillations in db/db and ob/ob islets had been shown before [31, 32]. These results suggest that islets from db/db and ob/ob mice display reduced TRPM5 channel activity, consistent with the downregulation of Trpm5 mRNA expression.

As mentioned above, genetic variation of Trpm5 has been shown to associate with pre-diabetic phenotypes [13]. Notably, these mutations were all found in intron sequences, suggesting alternative splicing. Using Trpm5-specific TaqMan assays located in distinct exon boundaries (8–9 and 19–20), we did not find any evidence for the presence of alternative splice variants of Trpm5 in the islets of db/db mice. Another possible explanation is that some of these mutations might be a part of cis-regulatory elements involved in transcriptional regulation of Trpm5. It has been recently shown that transcriptional regulation of the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial chloride channel, depends on the cis-regulatory elements located in intron 11 of CFTR (100 kb distal to the promoter) [12, 26]. These critical cis-acting elements recruit multiple transcription factors that tune the tissue-specific gene expression.

Both db/db and ob/ob mice suffer from hyperphagia that leads to obesity and consequently to diabetes. This increased food provisions results in higher intake of certain compounds such as sugars and fat, suggesting that these food constituents might be responsible for the downregulation of Trpm5. However, neither a high glucose nor a high fat diet for 14 weeks had any influence on Trpm5 expression in pancreatic islets after 14 weeks. As the mice fed with a high fat diet showed a marked obesity, comparable to that detected in db/db mice, we can also exclude obesity as being responsible for Trpm5 downregulation. Although both diets slightly elevated plasma glucose and insulin levels, the metabolic phenotype
detected in these mice was much less severe as compared to db/db and ob/ob mice.

Leptin has several effects on glucose homeostasis in the body. It reduces hepatic glucose production, increases glucose uptake from skeletal muscle cells, and reduces insulin synthesis and release from β cells [1, 33, 37]. Insulin released from the β cell increases secretion of leptin from adipose tissue that in turn will reduce insulin secretion. This so called adipo-insular axis is proposed to be disturbed in type 2 diabetes due to leptin resistance of the β cell, leading to increased insulin release [20, 37]. Interestingly, leptin has also been shown to alter the expression of several genes in pancreatic β cells [34]. The proinsulin gene and protein phosphatase 1 gene are repressed by leptin-dependent pathways, whereas the gene encoding the suppressor of cytokine signaling 3 protein is a leptin-induced gene in pancreatic β cells. Thus, it is conceivable that leptin signaling might be directly involved in the regulation of Trpm5 expression. Reconstitution of leptin signaling by i.p. injections in ob/ob mice recovered Trpm5 expression both after long-term (5 weeks) and short-term (2 days) treatment. After long-term treatment with leptin, ob/ob mice display normal (i.e., comparable to WT) bodyweight, plasma glucose, and insulin levels and a normal IPGTT profile, as expected [28]. On the other hand, 2 days of leptin treatment not only normalized plasma insulin levels but also increased the Trpm5 expression level, indicating that either leptin signaling as well as plasma insulin levels regulate Trpm5 expression. In contrast, incubation of either MIN6 or isolated ob/ob islets with leptin had no influence on Trpm5 expression. Similarly, disruption of leptin signaling in MIN6 cells had no effect on Trpm5 expression. Strikingly, when leptin-treated and -untreated ob/ob mice were placed on a food-restricted diet for 2 days, resulting in significantly decreased insulin levels in both groups, there was no additional effect of leptin treatment on Trpm5 expression. Thus, all these data contradict with a direct role of leptin signaling in Trpm5 gene regulation.

Hyperglycemia and hyperinsulinism lead to altered expression patterns of several genes in β cells [15–17, 21, 24]. Whereas incubation of MIN6 cells with glucose did not change the expression pattern of Trpm5, insulin dose-dependently decreased Trpm5 expression in MIN6 cells. These data are consistent with results obtained from leptin-
injected ob/ob mice. Taken together, our data indicate that the elevated plasma insulin level in db/db and ob/ob mice is major responsible for the downregulation of Trpm5. It is clear that plasma insulin levels have to be substantially elevated to cause downregulation of Trpm5, as mice fed with a high-fat diet (where plasma insulin levels were only slightly elevated to 0.5nM) showed no alteration in Trpm5 levels. Moreover, it can be difficult to explain the discrepancy between in vitro insulin concentrations (10–100nM) needed to obtain downregulation of Trpm5 and concentrations of plasma insulin levels measured in vivo in db/db and ob/ob mice (2–4 nM). However, it is clear that plasma insulin levels do not indicate actual insulin levels sensed by the β cell in its micro-environment. Moreover, the time scales of the performed experiments differed (mice of 5, 10, and 15 weeks old vs. 1 week incubation of MIN6 cells with insulin), and it is well accepted that insulin might trigger events on different time scales [16]. An immediate effect of insulin is proposed to be the opening of KATP channels by activation of PI3kinase and production of PI(3,4,5)P3 [14]. Whereas this is triggered in seconds or only a few minutes, events such as gene transcription and translation require several minutes to hours and even longer when protein synthesis of transcription factors is involved [16].

What could be the physiological relevance of insulin altering expression level of the Trpm5 gene? The dynamics of insulin secretion could partly explain this phenomenon [16]. The autocrine effect of secreted insulin on its own secretion is still a matter of debate and both negative and positive feedback mechanisms are reported [16, 17]. Indeed, insulin is released in a pulsatile manner, a feature believed to be important to maintain insulin sensitivity not only in peripheral cells but also in β cells. Interestingly, impairment of pulsatile insulin release is an early marker for β cell dysfunction in type 2 diabetes and is even detected in relatives of patients with type 2 diabetes [25, 29]. Steadily elevated plasma insulin levels are believed to cause a negative feedback on insulin secretion [29]. In this regard, the elevated plasma insulin levels detected in db/db and ob/ob mice might hamper its own secretion partly by downregulation of Trpm5, a positive regulator of insulin release. Thus, insulin-dependent downregulation of Trpm5 expression may represent a compensatory mechanism in order to prevent excessive insulin secretion and protect against hyperinsulinism. How insulin would downregulate TRPM5 expression is still unclear. Possible mechanisms include a direct influence of insulin-induced intracellular signaling on Trpm5 promoter activity, through cis- and trans-acting elements [24]. Alternatively, insulin regulates the expression of several microRNA’s [8]. However, the putative Trpm5 promoter region does not contain insulin-responsive elements, nor have any specific mRNA’s been identified which interact with the Trpm5 gene.

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