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SIGNALING AND CELL PHYSIOLOGY

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

Barbara Colsoul · Griet Jacobs · Koenraad Philippaert · Grzegorz Owsianik · Andrei Segal · Bernd Nilius · Thomas Voets · Frans Schuit · Rudi Vennekens

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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 *Trpm5* expression and function in pancreatic islets from mouse models of type II diabetes. Gene expression analysis revealed a strong reduction of *Trpm5* mRNA levels in pancreatic islets of *db*/*db* and *ob*/*ob* mice. The glucose-induced Ca^{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 *Trpm5* expression. Leptin treatment had no additional effect on *Trpm5* 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.

B. Colsoul · G. Jacobs · K. Philippaert · G. Owsianik · A. Segal · B. Nilius · T. Voets · R. Vennekens (✉)
Laboratory of Ion Channel Research, KU Leuven,
Herestraat 49, bus 802, Leuven 3000, Belgium
e-mail: rudi.vennekens@med.kuleuven.be

F. Schuit
Gene Expression Unit, Department of Cellular and Molecular Medicine, KU Leuven, Leuven 3000, Belgium

B. Colsoul
Biomedical Engineering—Health Engineering,
Group T International Engineering School, KU Leuven,
Leuven 3000, Belgium

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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^{2+} but directly activated by intracellular Ca^{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 Ca^{2+} concentration ($[Ca^{2+}]_i$), 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 (Ca^{2+} imaging and incubation experiments) in islet culture medium (advanced RPMI medium 1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM Glutamax) at 5 % CO_2/95 % 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 μl EDTA (0.5 M) to 30-μl 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 RNeasy 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. Tyrphostin 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 in 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 % CO_2/95 % O_2. 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).

$[Ca^{2+}]_i$ measurements

Bath temperature was controlled by a SC-20 dual in-line heater/cooler (Warner Instruments) and monitored by a TA-29 thermistor (Thermometrics) positioned in close vicinity of the islet. Islets were loaded with 1 μM Fura-2 acetoxymethyl ester (TefLabs) for 1 h at 37 °C in culture medium. $[Ca^{2+}]_{cyt}$ from Fura-2 loaded islets was measured monitoring fluorescence ratio (F_{350}/F_{380}) every second (after correction for background fluorescence) at 37 °C. $[Ca^{2+}]_{cyt}$ 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 Ca^{2+} imaging measurements contained (in millimolar) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 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. Similarly 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±0.058, $p=0.007$, $n=7$–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).

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/+	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

During Fura-2-based Ca^{2+} 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 Ca^{2+} concentration ($[Ca^{2+}]_i$) (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 $[Ca^{2+}]_i$ (plateau), a feature that was never detected in WT islets. Whereas the average $[Ca^{2+}]_i$ 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 Ca^{2+}-signaling in the pancreatic β cell.

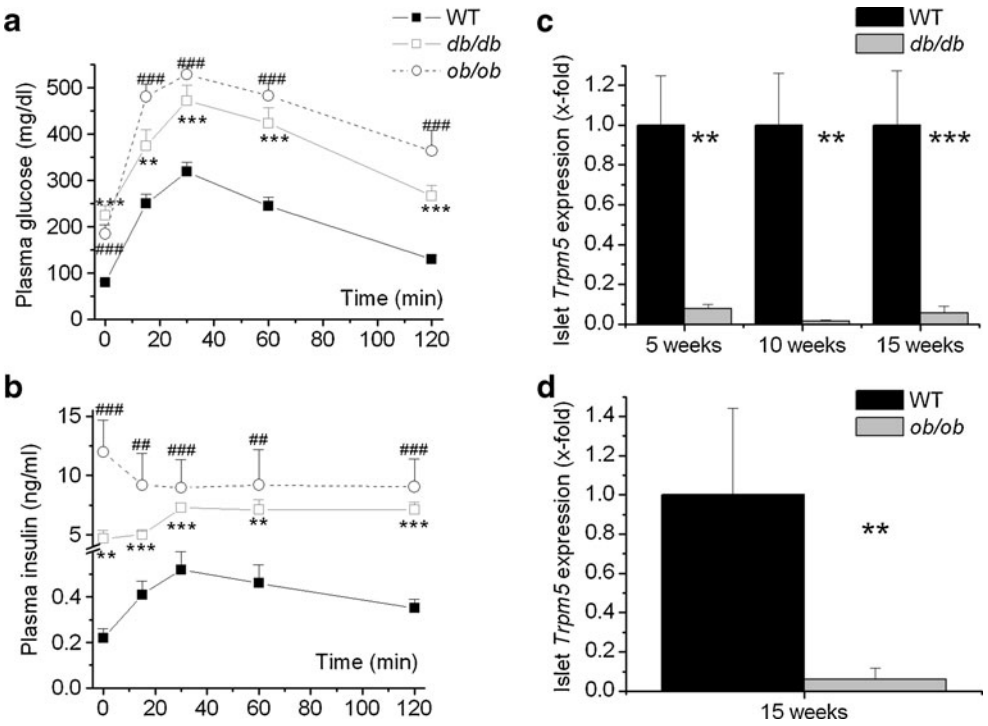

Fig. 1 Downregulation of *Trpm5* expression in islets from *db*/*db* and *ob*/*ob* mice. **a**, **b** Plasma glucose (**a**) and insulin (**b**) levels in WT, *db*/*db*, and *ob*/*ob* mice measured during an intraperitoneal glucose tolerance test at several time points as indicated. $N = 5–10$ mice per group. *Asterisk* and *number sign* indicate significant difference of WT mice versus *db*/*db* and *ob*/*ob* mice, respectively (**$p < 0.01$, *** <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, **$p < 0.01$, ***$p < 0.001$

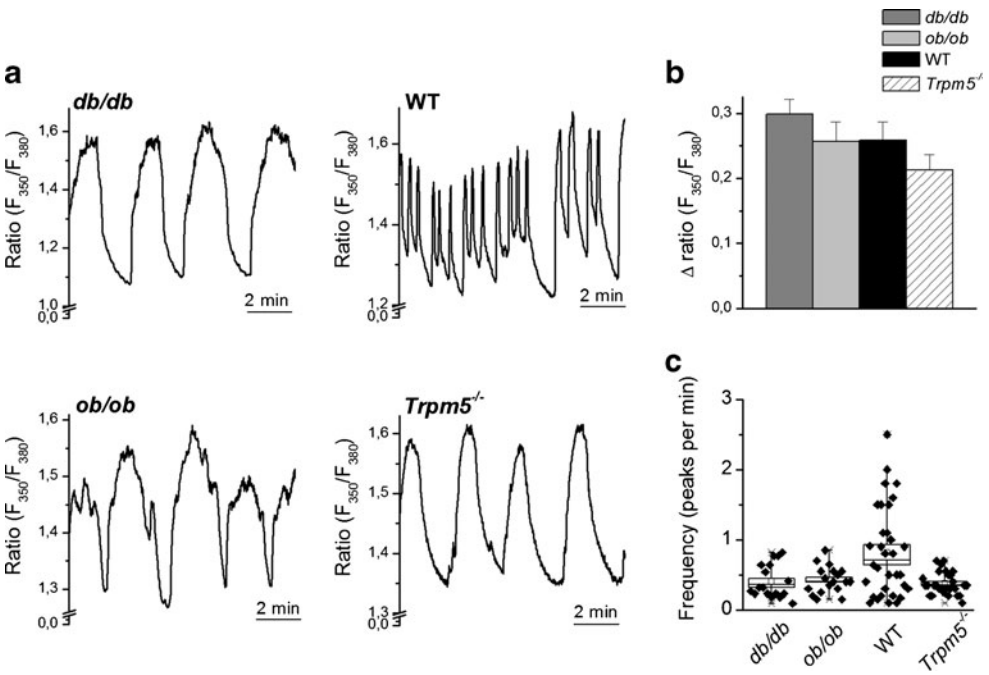

Fig. 2 Glucose-induced Ca^{2+}-signaling in islets from *db*/*db* and *ob*/*ob* mice correlates with a loss of TRPM5. **a** Representative examples of Ca^{2+}-oscillations in isolated pancreatic islets of *db*/*db*, *ob*/*ob*, WT, and *Trpm5*$^{-/-}$ mice during stimulation with 10 mM glucose. **b** Average increase in ratio (F_{350}/F_{380}) 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, $p=5.13^{-11}$ 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-

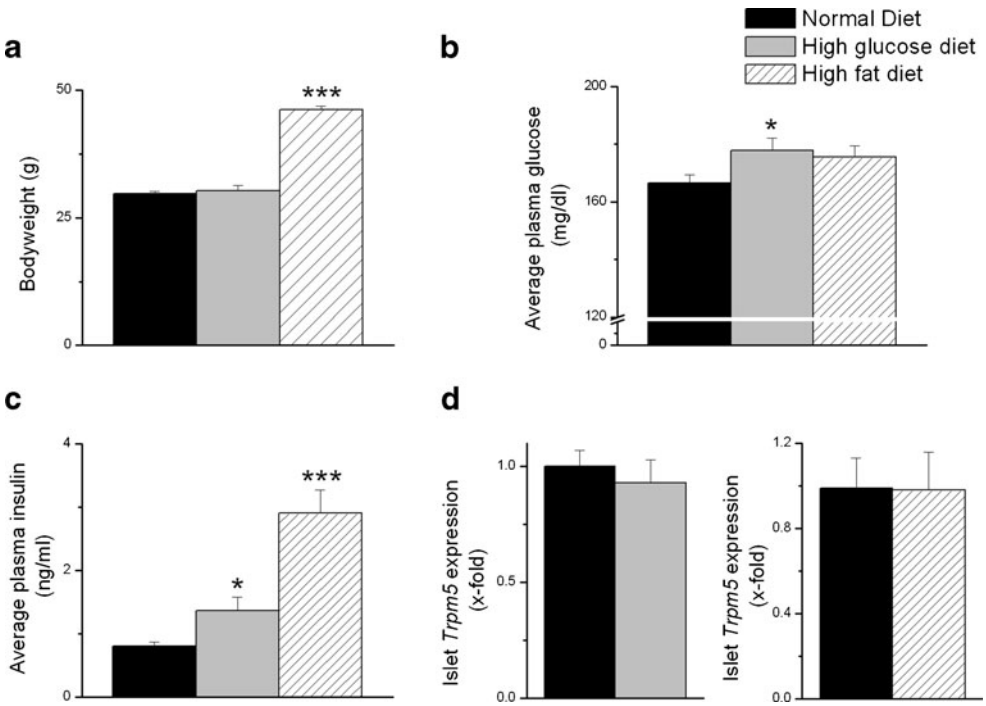

Fig. 3 No effect of dietary compounds on *Trpm5* expression. **a**–**c** Metabolic phenotype of WT mice that received either a high glucose diet (containing ≥50 % glucose and ≥12+ oligosaccharides/dextrines) or a high fat diet (containing 30.2 % fat) during 14 weeks. **a** Bodyweight in WT mice that had received a normal, a high glucose, or a high fat diet during 14 weeks. **b**, **c** Average plasma glucose (**b**) and insulin levels (**c**) over the course of the experiment in mice receiving a normal, a high glucose, or a high fat diet during 14 weeks. **d** Islet *Trpm5* expression levels after 14 weeks of diet. *Trpm5* expression is normalized to the average *Trpm5* expression in islets from age-matched mice that received a normal diet. $N=4$–6 mice per group, $^{*}p<0.05$, $^{***}p<0.001$ vs. WT mice on a normal diet

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; $p=2.1^{-6}$, $n=7$ 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.

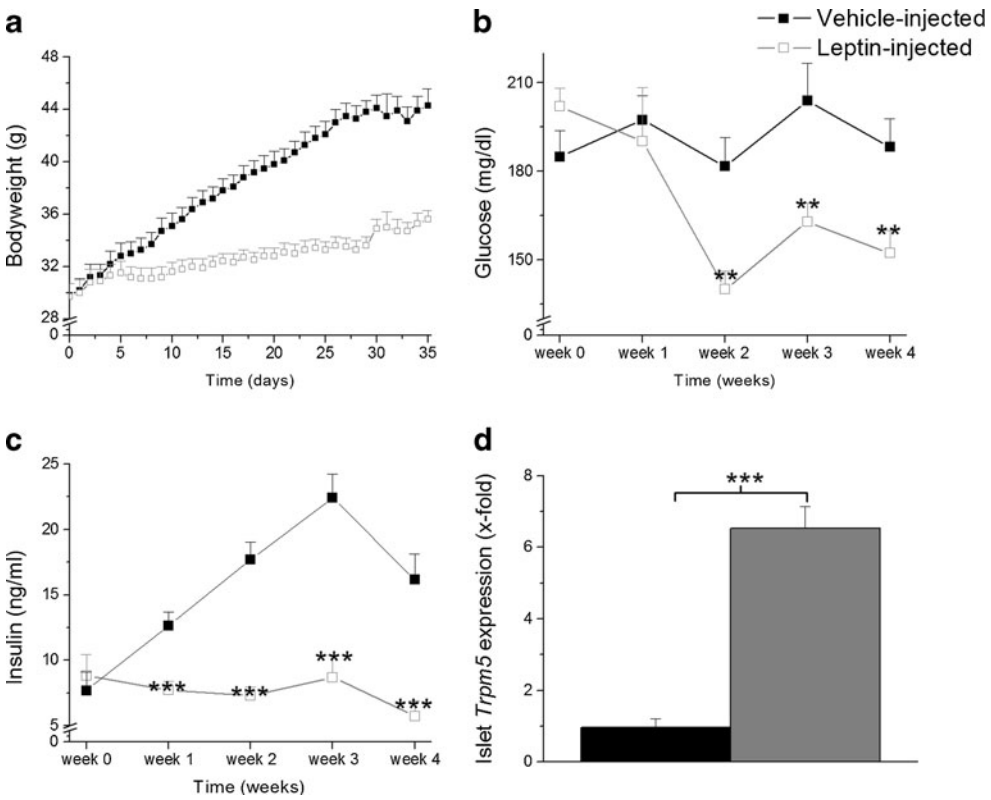

Fig. 4 Leptin treatment of leptin-deficient *ob*/*ob* mice causes a reversal of the diabetic phenotype and an upregulation of islet *Trpm5* expression. Metabolic phenotype of o*b*/*ob* mice that were injected either with 1 mg leptin/kg bodyweight or with vehicle during 5 weeks. **a**–**c** Bodyweight (**a**), plasma glucose (**b**), and plasma insulin levels (**c**) were measured on a daily (bodyweight) or a weekly (plasma glucose and insulin) basis. There was a statistically significant difference of bodyweight between the two groups starting from day 8. **d** *Trpm5* mRNA expression in freshly isolated pancreatic islets from vehicle- and leptin-injected *ob*/*ob* mice after 5 weeks of treatment. Data were normalized to the average *Trpm5* expression in islets from vehicle-injected mice. $N=10$ mice per group, $^{**}p<0.01$, $^{***}p<0.001$

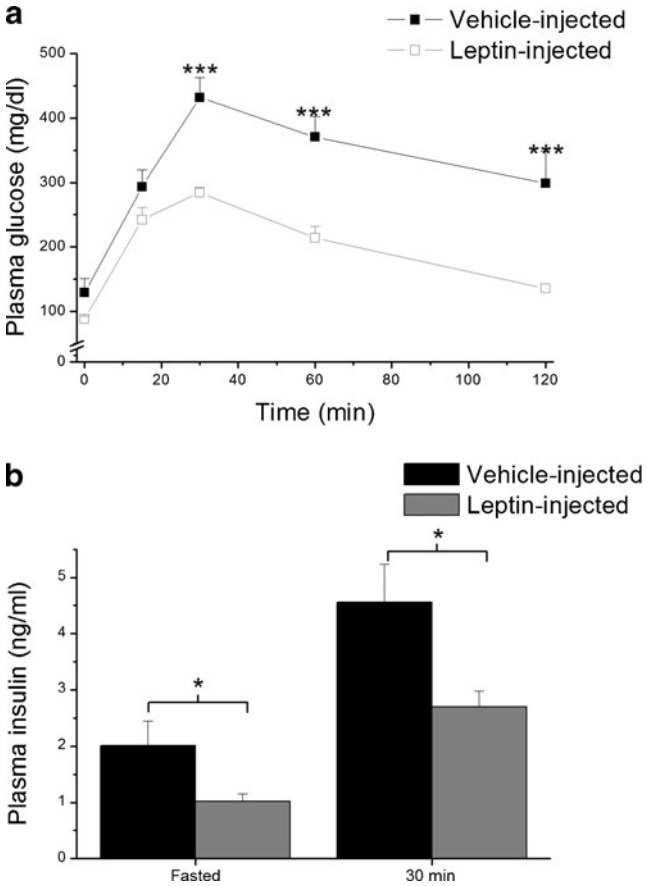

Fig. 5 IPGTT in *ob*/*ob* mice injected during 5 weeks with leptin or vehicle. **a** Plasma glucose levels in *ob*/*ob* mice that were injected with leptin or vehicle during 5 weeks. Mice received an i.p. injection of 2.5 g glucose/kg bodyweight and plasma glucose was measured at several time points as indicated. **b** Plasma insulin levels after overnight fasting and 30 min after i.p. glucose injection of 2.5 g glucose/kg bodyweight. $N=10$ mice per group, $*p<0.05$, $***p<0.001$

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 Tyrphostin AG490 to the incubation medium for 1 week had no effect on *Trpm5* expression in MIN6 cells (leptin 1.0±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 Ca^{2+}-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 Ca^{2+} and V_m, specifically due to a lack of fast oscillations [6]. During glucose stimulation, *db*/*db* and *ob*/*ob* islets showed a frequency of Ca^{2+} 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 Ca^{2+} oscillations and, more specifically, a lack of fast

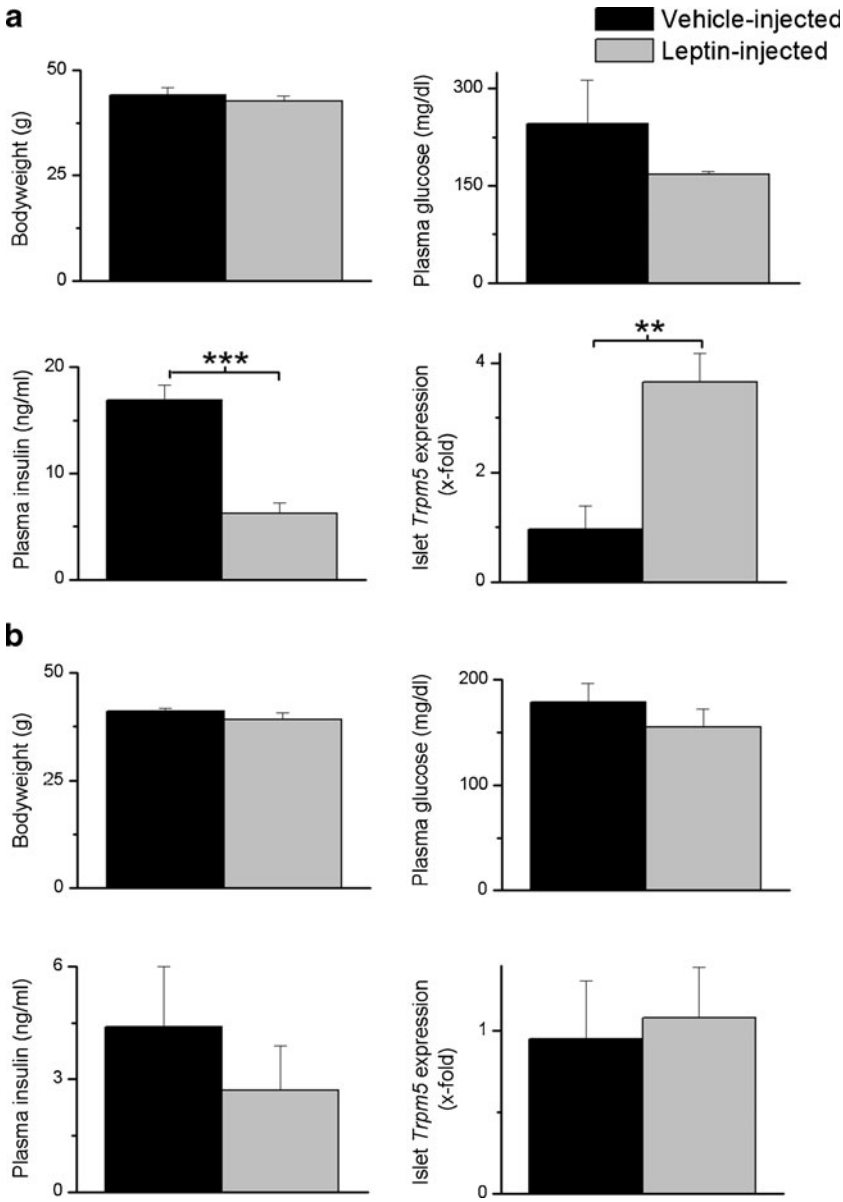

Fig. 6 Effect of leptin injection on islet *Trpm5* expression and circulating insulin levels in control-fed and food-restricted mice. Bodyweight, plasma glucose, plasma insulin, and islet *Trpm5* expression in *ob*/*ob* mice that were injected for 2 days with either 1 mg leptin/kg bodyweight or vehicle. Mice were divided in two groups: they had either free access to food during the course of the experiment (**a**) or were put on food restriction (**b**), meaning that they received 3 g of food per mouse per day and were fasted overnight during the last night before islet isolation. *Trpm5* expression in islets from leptin-injected mice was normalized to the average *Trpm5* expression in islets from vehicle-injected mice that had followed the same food protocol. $N=4$ mice per group, $^{**}p<0.01$, $^{***}p<0.001$

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

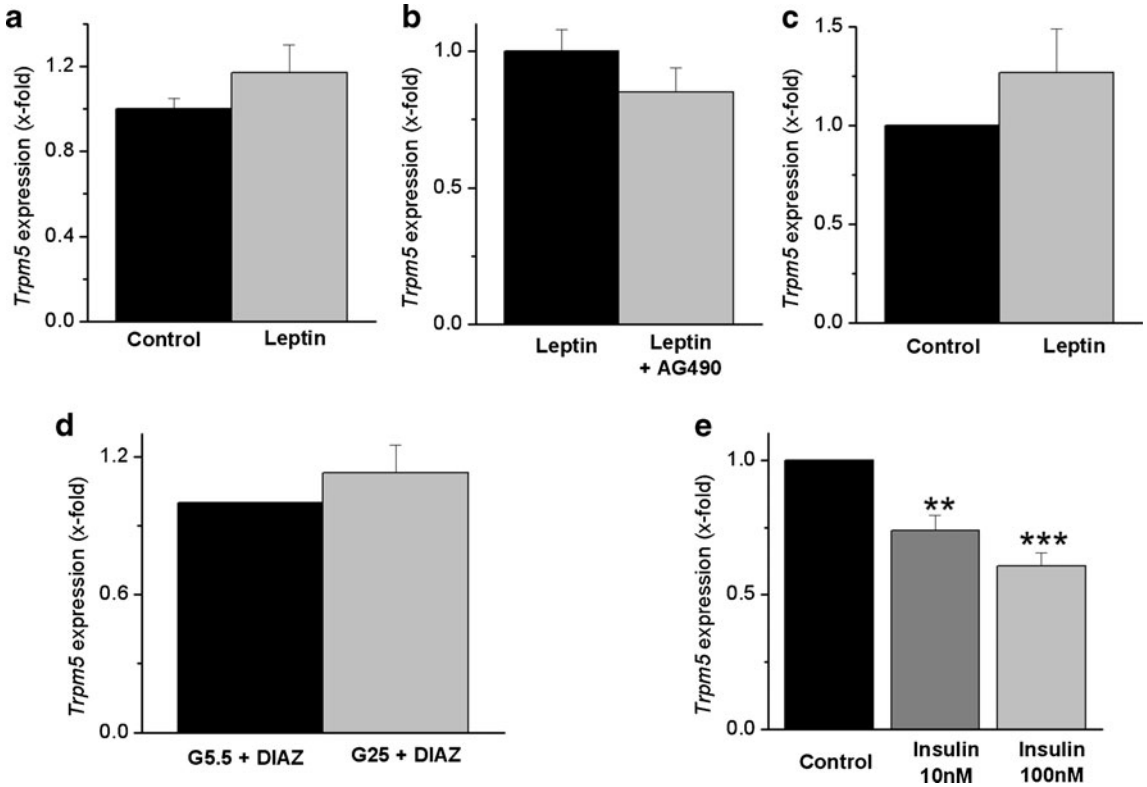

Fig. 7 Insulin downregulates *Trpm5* expression in Min6 cells. **a**, **b** *Trpm5* expression in the insulinoma cell line MIN6 incubated for 1 week with 200 ng/ml leptin (**a**) or with 200 ng/ml leptin+30 μM Tyrphostin AG490 (**b**). Control medium contained either no leptin (**a**) or 200 ng/ml leptin (**b**). Expression was normalized to *Trpm5* expression in MIN6 cells incubated in control medium. $N=3$ per group. **c** *Trpm5* expression in *ob*/*ob* islets incubated with 200 ng/ml leptin for 48 h. Expression was normalized to expression in islets from the same animal incubated in control medium. $N=4$ per group, $p=0.149$ (one-paired t test). **d** *Trpm5* expression in MIN6 cells incubated for 1 week with 25 mM glucose+ 250 μM diazoxide. Expression was normalized to *Trpm5* expression in MIN6 cells incubated in control medium containing 5.5 mM glucose+ 250 μM diazoxide. $N=3$–7 per group. **e** *Trpm5* expression in MIN6 cells after incubation with either 10 or 100 nM insulin during 1 week. The incubation medium contained 5.5 mM glucose and expression was normalized to *Trpm5* expression in MIN6 cells incubated in control medium without insulin. $N=7$ per group, $^{**}p<0.01$, $^{***}p<0.001$

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 might 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 K_{ATP} channels by activation of PI_3kinase and production of $PI(3,4,5)P_3$ [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 miRNA's been identified which interact with the *Trpm5* gene.

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Conflict of interest The authors have no competing financial interests to declare.

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