Loss of insulin-induced activation of TRPM6 magnesium channels results in impaired glucose tolerance during pregnancy

Anil V. Nair1,2, Berthold Hoche1,2,3,4, Sjoerd Verkaart3, Femke van Zeeland3, Thiemo Pfab4,5, Torsten Slowinski4,5, You-Peng Chen6,7, Karl Peter Schlingmann8, André Schaller1, Sabina Gallati1, René J. Bindels9, Martin Konrad10, and Joost G. Hoenderop1,2

1Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, 6500 HB, Nijmegen, The Netherlands; 2Institute of Nutritional Science, University of Potsdam, D-14469 Potsdam, Germany; 3Center for Cardiovascular Research/Institute of Pharmacology, Campus Mitte, Charité, 10115 Berlin, Germany; 4Research and Early Development, F. Hoffmann-La Roche, BS-4070 Basel, Switzerland; 5Department of Nephrology, Campus Benjamin Franklin, Charité, 12203, Berlin, Germany; 6Department of Nephropathy, Campus Mitte, Charité, 10117 Berlin, Germany; 7Department of Infectious Diseases, The First Affiliated Hospital of Jinan University, Guangzhou, China; 8Department of General Pediatrics, University Children’s Hospital Münster, 48149 Münster, Germany; and 9Division of Human Genetics, University Hospital, CH-3010 Bern, Switzerland

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Hypomagnesemia affects insulin resistance and is a risk factor for diabetes mellitus type 2 (DM2) and gestational diabetes mellitus (GDM). Two single nucleotide polymorphisms (SNPs) in the epithelial magnesium channel TRPM6 (V1393I and K1584E) were predicted to confer susceptibility for DM2. Here, we show using patch clamp analysis and total internal reflection fluorescence microscopy, that insulin stimulates TRPM6 activity via a phosphoinositide 3-kinase and Rac1-mediated elevation of cell surface expression of TRPM6. Interestingly, insulin failed to activate the genetic variants TRPM6 (V1393I) and TRPM6(K1584E), which is likely due to the inability of the insulin signaling pathway to phosphorylate TRPM6(T1399) and TRPM6(K1584E). Moreover, by measuring total glycosylated hemoglobin (TGH) in 997 pregnant women as a measure of glucose control, we demonstrate that TRPM6(V1393I) and TRPM6(K1584E) are associated with higher TGH and confer a higher likelihood of developing GDM. The impaired response of TRPM6(V1393I) and TRPM6(K1584E) to insulin represents a unique molecular pathway leading to GDM where the defect is located in TRPM6.

Results

Insulin Stimulates TRPM6 Channel Activity Specifically by Engaging IR.

TRPM6 is expressed along the distal convoluted tubule (DCT) in the apical membrane where it reabsorbs Mg2+ (15) and thus plays a direct connection between Mg2+ deficiency and the occurrence of metabolic diseases came from the identification of a monogenic disease primarily characterized by significant hypomagnesemia that was caused by a mutation in a mitochondrial tRNA (9). Moreover, in a recent genome-wide association (GWA) study, it was demonstrated that certain SNPs nominally associated with hypomagnesemia also correlate with fasting glucose levels, again supporting the hypothesis of a direct link between Mg2+ and metabolic defects (10). Recently, Song et al. (11) suggested by conducting a nested case control study that two common nonsynonymous SNPs of the epithelial Mg2+ channel TRPM6, V1393I and K1584E, might confer susceptibility to DM2 in postmenopausal women with low Mg2+ intake.

Mg2+ regulates ion channels, cellular processes, and serves as a cofactor for ample essential metabolic reactions. It is involved in multiple steps of the insulin signal transduction pathways such as insulin secretion, binding, and receptor activity (5, 6, 12), suggesting a direct connection between Mg2+ deficiency and the occurrence of metabolic diseases.
Insulin activates TRPM6 channels by specifically engaging IR. (A) Immunohistochemical staining of the insulin receptor (IR, in green) and the Na\textsuperscript{+},Cl\textsuperscript{−} cotransporter (NCC, in red) on renal mouse sections. (B) mRNA transcripts of IR were identified in the COPAS sorted DCT fragments using PCR. (C) Similarly, the IR expression in HEK293T cells was identified. Milli-Q water was taken as a control. (D) Dose–response curve of insulin-induced current in TRPM6-transfected HEK293T cells indicating half maximal effective concentration of 0.16 nM (n = 17–43). (E) The I/V relation of cells transfected with plasmids of mock (a, n = 9), mock pretreated with 10 nM of insulin (b, n = 10), TRPM6 (b, n = 43), and TRPM6 pretreated with 10 nM of insulin (b; n = 24). (F) Averaged current density (nA/pF) at +80 mV of TRPM6-transfected HEK293T cells, pretreated with 10 nM S961, the IR blocker, with (●) or without (□) insulin pretreatment.

Insulin-Mediated Activation of TRPM6 Is Kinase Activity Independent. TRPM6 consists of an ion channel unit fused to an α-kinase. To determine whether the α-kinase domain plays a role in insulin-mediated stimulation of TRPM6 activity, HEK293T cells expressing the phosphotransferase-deficient mutant (17) TRPM6(K1583R) were treated for 1 h with 10 nM insulin. Whole-cell patch clamp analysis showed that insulin stimulated the activity of TRPM6 (K1583R) channels similar to that of wild-type TRPM6 (Fig. 2 C and D). The I/V relations (Fig. 2 D) and total protein expression (Fig. S2B) of the TRPM6 variants were comparable.

Cyclin-Dependent Kinase 5 Is Involved in TRPM6 Activation by Insulin. To deduce why the TRPM6 SNPs were insensitive to insulin, the molecular mechanism responsible for the insulin-induced TRPM6 stimulation was investigated. Cross-species protein sequence comparison showed potential phosphorylation sites in the vicinity of the SNPs (Fig. 2 E and F). The NetPhosK server (http://www.cbs.dtu.dk/services/NetPhosK/) predicted T1583 as a potential phosphorylation target of cyclin-dependent kinase 5 (CDK5) and S1587 as a protein kinase A (PKA) or C (PKC) site. Roscovitine, an inhibitor of CDK5, abolished insulin-mediated...
stimulation of TRPM6 (Fig. 2G). Insulin (10 nM, 1 h) was added to the bath solution 20 min after starting the treatment with roscovitine (20 μM). The I/V relations of TRPM6 were not affected by roscovitine treatment (Fig. 2H). To substantiate the involvement of CDK5, the mRNA encoding the kinase was down-regulated by small-interference RNA. This treatment prevented the stimulatory action of insulin on TRPM6 channel activity and down-regulated CDK5 protein expression significantly (Fig. S2 C and D).

Phosphomimetic Substitution of T1391 and S1583 Recovers Insulin-Mediated Activation of TRPM6(V1393) and TRPM6(K1584E). To confirm the role of T1391 in insulin-mediated TRPM6 activation, a phosphorylation-deficient mutant T1391A was constructed. Insulin (10 nM) treatment for 1 h did not affect the whole-cell current of this mutant (Fig. 3A). The I/V curves (Fig. 3B), basal activity, and protein expression (Fig. S3A) of TRPM6(T1391A) were identical to that of wild-type TRPM6. To pinpoint the exact role of T1391 in insulin-mediated TRPM6 activation, a channel mutant mimicking the phosphorylated state (TRPM6(T1391D)) was inserted in TRPM6(V1393I). Contrary to TRPM6(T1391A) and TRPM6(V1393I), 10 nM insulin treatment for 1 h increased both the TRPM6(T1391D) and the TRPM6(T1391D + V1393I)-mediated whole-cell current (Fig. 3 C and D). Protein expression of TRPM6(T1391D) and the TRPM6(T1391D + V1393I) was comparable to that of wild-type TRPM6 (Fig. S3B). To address the insensitivity of TRPM6(K1584E) activity to insulin, the phosphorylation-deficient mutant TRPM6(S1583A), the second site predicted by NetPhosK, the constitutive phosphorylation mimicking mutant TRPM6(S1583D) and the double mutant TRPM6(S1583D + K1584E) were constructed. Whole-cell patch clamp analysis after insulin (10 nM, 1 h) treatment of these mutants showed that S1583 is indeed necessary for insulin-mediated wild-type TRPM6 stimulation. Moreover, the insulin-dependent activation of TRPM6(K1584E) was recovered by the constitutive activity of S1583 (Fig. 3 E–H). The I/V relationship and protein expression of the mutants were comparable to that of wild-type TRPM6 (Fig. 3 F and H and Fig. S3 C and D).

Insulin Increases Cell Surface Expression of TRPM6. Increased TRPM6 activity by the Rac1(G12V) implies the notion that IR signaling promotes the mobility of TRPM6 containing endomembranes toward the plasma membrane. To this end, we used the potential of total internal reflection fluorescent (TIRF) microscopy to visualize the trafficking of eGFP–TRPM6-containing vesicles at the plasma membrane (22). Detailed observation of eGFP–TRPM6 in transiently transfected HEK293T cells using TIRF microscopy revealed a highly motile punctate distribution pattern. The fluorescence intensity of the cell in the TIRF plane was monitored and measured for 14 min before insulin application to obtain a basal intensity level (Materials and Methods) and subsequently for 22 min after application. The quantification of the fluorescence signals showed a clear augmentation of fluorescence intensity after insulin treatment (Fig. 4D). The first row of Fig. 4E shows the TIRF images of eGFP–TRPM6-expressing cells measured as a control at 0, 26, and 36 min. The second row of Fig. 4E depicts TIRF images before and 12 and 22 min after insulin application. The observed increase in fluorescence could be due to plasma membrane movement or expansion rather than trafficking of the vesicles. To address this
Rescue of Insulin-Mediated Cell Surface Recruitment of TRPM6(V1391I) and TRPM6(K1584E). Electrophysiological studies showed that T, V1393I, S1583, and K1584 residues are essential for insulin-dependent activation of TRPM6. To substantiate that the rescue of insulin-mediated activity of SNP baring channels was due to an increase in cell surface recruitment, appropriate phosphomimetic substitutions and SNPs were introduced into the eGFP–TRPM6 vector and subjected to TIRF analysis. Introducing the V1393I or K1584E mutation in TRPM6 or ablating of the proposed phosphorylation site T1393A or S1583A in close vicinity prevented insulin-mediated recruitment of TRPM6 to the cell surface (Fig. 4 F, G, J, and K). The cell surface recruitment of TRPM6(V1391I) was restored by introducing the phosphomimetic residue, T1391D, into the channel. Similarly, introducing S1583D into TRPM6(K1584E) increased the cell surface accumulation upon insulin treatment (Fig. 4 H, I, L, and M). Coexpression of Rac1(G12V) did not restore cell surface recruitment in the absence of the appropriate phosphomimetic substitutions (Fig. S4 C–I).

Role of TRPM6 SNPs in Gestational Diabetes Mellitus. To assess the clinical relevance of our unique finding that, unlike TRPM6, TRPM6(V1393I) and TRPM6(K1584E) were not stimulated by insulin, 997 women from the Berlin Birth Cohort (BBC) (13, 14) were analyzed. Detailed data analysis of the entire study population including the maternal TRPM6(V1393I) and TRPM6(K1584E) genotypes is shown in Table 1 and Tables S1 and S2. Total glycosylated hemoglobin (TGH) level at delivery, as a measure of insulin resistance/glycemic control in this cohort of pregnant women who were nondiabetic and otherwise healthy before pregnancy, was measured. The more frequent SNP TRPM6(V1393I) was also associated with a higher likelihood of developing a significant impairment in glycemic control (TGH ≥ 7.0%). All carriers of the rare AA V1393I genotype were also carriers of the GG K1584E genotype. Therefore, the odds ratio for the more frequent GG K1584E genotype was calculated. The resulting odds ratio for TGH ≥ 7.0% for the maternal GG K1584E genotype was 4.59 (95% confidence interval: 1.59–13.23. (Table 1 and Tables S1 and S2). Here, we show that the two SNPs in TRPM6 are associated with altered TGH levels.

Discussion

The molecular etiology of hypomagnesemia and its clinical complications associated with insulin resistance and/or GDM/DM2 is poorly understood, despite a growing body of evidence but is rescued by constitutive phosphorylation. (F) eGFP–TRPM6(V1393I), ○, n = 15; ●, n = 7. (G) eGFP–TRPM6(T1393A) ○, n = 5; ●, n = 3. (H) eGFP–TRPM6(T1393D) ○, n = 4; ●, n = 7. (I) eGFP–TRPM6(V1393D + V1393I) ○, n = 5; ●, n = 4. (J) eGFP–TRPM6(K1584E), ○, n = 5; ●, n = 6. (K) eGFP–TRPM6(S1583A) ○, n = 15; ●, n = 19. (L) eGFP–TRPM6(S1583D) ○, n = 11; ●, n = 7. (M) eGFP–TRPM6(S1583D + K1584E) ○, n = 10; ●, n = 16. Images are the average of 20 frames taken at 0.2-s intervals at the indicated time point. Data shown are mean ± SEM. *P < 0.05 compared with wild-type TRPM6 and **P < 0.05 compared with wild-type TRPM6 pretreated with insulin.
The role of the kinase activity present in TRPM6 was excluded by showing that the phosphotransferase-deficient mutant TRPM6(K1584E) was still stimulated by insulin (17). Cross-species sequence alignment of TRPM6 suggested the presence of two potential phosphorylation sites, TRPM6(T1391) and TRPM6(S1585), in the vicinity of the SNPs. Ablation of one of these phosphorylation sites prevented insulin-mediated recruitment of TRPM6 to the cell surface, despite the presence of the other functional second phosphorylation site. In addition, mimicking constitutive phosphorylation at one of the phosphorylation sites did not result in a constitutively active TRPM6 because the other phosphorylation site also needs to be activated by insulin. From these results, we conclude that phosphorylation of both T1391 and S1585 is necessary for insulin-dependent activation of TRPM6, whereas phosphorylation of one of these residues is not sufficient.

The TRPM6 variants V1391I and K1584E are not sensitive to insulin. Importantly, this insulin-insensitivity of TRPM6(V1391I) and TRPM6(K1584E) could be rescued by inserting T1391D or S1585D, into the respective genetic variants. Thus, our combined approach including TIRF microscopy and patch clamp analysis suggested that the identified SNPs interfere with insulin-mediated phosphorylation of TRPM6. However, we could not detect direct phosphorylation of TRPM6 by insulin. These findings suggest that phosphorylation of TRPM6 regions in proximity to the identified SNPs determines the insulin sensitivity of TRPM6.

The CDK5 inhibitor roscovitine abolished insulin-mediated TRPM6 activation, substantiating its functional role. It is possible that TRPM6(V1391I) decreases the phosphorylation probability by CDK5 at T1391. Initially, CDK5 was implied in the regulation of neuronal functions including cytoskeletal remodeling and synaptic transmissions. Recently, an increasing array of evidence established its function and presence in non-neuronal cells as well (24). CDK5 has been shown as a regulator of the association between GLUT4 and the syntaptotagmin homolog, E-syt1, to modulate glucose transport in adipocytes upon insulin stimulation (25).

PI3K is well established as one of the key enzymes, associated with the signaling downstream of the IR (19). For instance, the epidermal growth factor (EGF)-mediated activation of TRPM6 channels involves a PI3K-dependent process (22). Rho-GTPases are involved in cytoskeletal rearrangement, regulation of vesicular trafficking, and membrane processes (26). Here, we show that the inhibition of PI3K using wortmannin and LY294002 abrogates the insulin-mediated activation of TRPM6. Further, the dominant negative mutant of the Rho-GTPase, Rac1 [Rac1(T17N)], abolished insulin-dependent TRPM6 activation, whereas its constitutively active Rac1(G12V) mutant increased basal TRPM6 currents and augmented the channel activation by insulin significantly, compared with that of TRPM6 alone. In the absence of insulin, constitutively active Rac1(G12V) facilitates increased trafficking of TRPM6 to the plasma membrane. These data unequivocally show that Rac1 is a downstream mediator of the insulin-dependent TRPM6 activation. The involvement of PI3K and Rac1 in the TRPM6 activation suggests an altered endomembrane trafficking and their potential redistribution to the plasma membrane. A similar mechanism by insulin was described for the cationic channel TRPV2. In nonstimulated conditions, TRPV2 was mainly observed in the cytoplasm of MIN6 cells. Application of insulin induced translocation and insertion of TRPV2 in the plasma membrane, resulting in an increased calcium influx (27).

Given the fact that insulin activates the TRPM6 whole-cell current together with the increase in TIRF intensity it can be concluded that this hormone augments the number of functional channels at the cell surface (Fig. S5). In vivo, in the DCT, the number of TRPM6 channels on the plasma membrane is rate limiting and the physiological effect of insulin on channel activity will be even more significant. To date, endogenous TRPM6 channel activity has never been measured and is technically demanding due to the very low expression of the channel and the lack of a specific channel blocker.

In the present study, most importantly, we established clinical relevance of these noteworthy findings. Epidemiological research suggests that women who do have GDM bear an increased risk of DM2 in later life (1, 28). It is associated with a higher risk of maternal as well as offspring morbidity and mortality during pregnancy, at birth and later in life (1, 28). The major complications of this situation are preeclampsia, neonatal hypoglycemia, and respiratory distress syndrome (1, 28). Increased concentrations of pregnancy hormones like estrogen and progesterone depicting their relation. By using a combination of electrophysiological, biochemical, and live cell imaging techniques, we demonstrate that insulin stimulates the activity of TRPM6, but not that of TRPM6(V1391I) and TRPM6(K1584E). These SNPs have been predicted to be a risk factor for DM2 in elderly women under low Mg^2+ intake (11). Moreover, we show that pregnant women carrying these SNPs, have higher TGH levels, indicating worsening of insulin resistance compared with those carrying wild-type TRPM6, signifying its role in the hypomagnesemia observed in GDM/DM2 patients.

Our data demonstrate that insulin increases the TRPM6 channel activity specifically by engaging the IR, whereas the channels harboring the SNPs lack insulin-dependent channel modulation. Of note, IR is expressed in the DCT, the site where TRPM6 facilitates transepithelial Mg^2+ transport. Several experiments were conducted to explain the molecular mechanism behind the insensitivity of the TRPM6 variants to insulin. The role of the kinase activity present in TRPM6 was excluded by showing that the phosphotransferase-deficient mutant TRPM6(K1584E) was still stimulated by insulin (17). Cross-species sequence alignment of TRPM6 suggested the presence of two potential phosphorylation sites, TRPM6(T1391) and TRPM6(S1585), in the vicinity of the SNPs. Ablation of one of these phosphorylation sites prevented insulin-mediated recruitment of TRPM6 to the cell surface, despite the presence of the other functional second phosphorylation site. In addition, mimicking constitutive phosphorylation at one of the phosphorylation sites did not result in a constitutively active TRPM6 because the other phosphorylation site also needs to be activated by insulin. From these results, we conclude that phosphorylation of both T1391 and S1585 is necessary for insulin-dependent activation of TRPM6, whereas phosphorylation of one of these residues is not sufficient.

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### Table 1. Maternal TGH of the study population including maternal V1391 and K1584 E genotypes

<table>
<thead>
<tr>
<th>Maternal V1391 genotype</th>
<th>Total</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
<th>Linear</th>
<th>A rec</th>
<th>A dom</th>
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<td>N</td>
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<td>790</td>
<td>189</td>
<td>7</td>
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<tr>
<td>Maternal TGH, %</td>
<td>6.3 ± 0.7</td>
<td>6.3 ± 0.7</td>
<td>6.2 ± 0.7</td>
<td>6.7 ± 0.9</td>
<td>0.09</td>
<td>0.04</td>
<td>0.81</td>
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<td>Maternal TGH ≥ 7.0%</td>
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<td>14.7</td>
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<table>
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<td>294</td>
<td>26</td>
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</tr>
<tr>
<td>Maternal TGH, %</td>
<td>6.3 ± 0.7</td>
<td>6.3 ± 0.7</td>
<td>6.2 ± 0.7</td>
<td>6.8 ± 0.8</td>
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<td>Maternal TGH ≥ 7.0%</td>
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<td>15.5</td>
<td>14.6</td>
<td>38.5</td>
<td>0.002</td>
<td>0.01</td>
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</table>

Data are given as mean ± SEM or %. Odds ratios for TGH ≥ 7.0%: 4.59 (95% confidence intervals: 1.59–13.23) in maternal K1584E genotype G rec. Refer also to Tables S1 and S2.
lead to lower fasting glucose concentrations and deposition of fat, delay in gastric emptying, and increased appetite. As gestation progresses, however, postprandial glucose concentrations steadily increase, indicating a diminished sensitivity to insulin. To maintain proper glucose control in pregnancy, maternal pancreatic β cells have to increase insulin secretion to counteract the corresponding fall in tissue sensitivity to insulin. Apparently, pregnant women who develop GDM are unable to increase their insulin production to compensate for the increased resistance to insulin (1, 28).

Postreceptor defects in the insulin-signaling cascade are implicated in the development of insulin resistance. Mg²⁺ is an essential cofactor for multiple enzymes involved in glucose metabolism including the IR function. Several cohort studies found an inverse association between Mg²⁺ intake and risk for diabetes or insulin resistance (29, 30). Pregnancy represents a unique situation of relative Mg²⁺ deficiency (31). We assume that this condition may synergistically increase the risk for impaired glucose tolerance beside the well-known effects of female sex hormones (1, 28). Our study clearly demonstrates that two genetic variants of the TRPM6 channel are associated with TGH levels. The more frequent genetic variant, TRPM6(K¹⁸⁵⁶E), was associated with a higher likelihood of developing a significant impairment in glycemic control (Table 1).

To conclude, pregnant women carrying TRPM6(V¹³⁹¹I) and TRPM6(K¹⁸⁵⁶E) possibly lack the physiological regulation of TRPM6 by insulin. Because Mg²⁺ is very important for β-cell function (5) and sensitivity of IRs to insulin (6, 12), we speculate that the presence of TRPM6(V¹³⁹¹I) and TRPM6(K¹⁸⁵⁶E) in these women may lead to a hypomagnesemastate resulting in impaired β-cell function and insulin secretion and/or IR sensitivity. Therefore, these women develop an impaired glucose tolerance with a higher risk for GDM. These genetic variants of TRPM6 could be used as potential biomarkers to improve diagnosis and identify those at risk for developing GDM/DM2-induced hypomagnesemia. Importantly, similar to sodium-dependent glucose cotransporters (SGLT) (32, 33) the TRPM6 channel might serve as a renal target for drug development in the field of diabetes.

Materials and Methods

Subjects and Data Collection. A total of 997 women from the previously described Berlin Birth Cohort (BBC) (13, 14) were included in the study. Mean weight and mean blood pressure were calculated every trimester. At delivery, weights of the newborns were measured and maternal blood from a cubital vein was collected (see SI Materials and Methods for details).

TGH. Fetal and maternal blood was analyzed using the HPLC-based variant TGH testing system (Bio-Rad) as described previously (14) (SI Materials and Methods).

Genotyping Using MALDI-TOF MS. Genomic fragments of the TRPM6 gene containing the SNPs V¹³⁹¹I and K¹⁸⁵⁶E were amplified in a multiplex PCR. For SNP detection, purified PCR products were mixed with the extension mix, polymorphism detection primers, and nucleotides to a final volume of 10 mL. The purified products were subjected to MALDI-TOF MS (SI Materials and Methods).

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