The RGD sequence present in IGFBP-2 is required for reduced glucose clearance after oral glucose administration in female transgenic mice

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Reyer A, Schindler N, Ohde D, Walz C, Kunze M, Tuchscherer A, Wirthgen E, Brenmoehl J, Hoeflich A. The RGD sequence present in IGFBP-2 is required for reduced glucose clearance after oral glucose administration in female transgenic mice. Am J Physiol Endocrinol Metab 309: E409–E417, 2015. First published June 23, 2015; doi:10.1152/ajpendo.00168.2015.—Recent studies suggest that insulin-like growth factor-binding protein-2 (IGFBP-2) affects both growth and metabolism. Whereas negative growth effects are primarily due to negative interference with IGF-I, the mechanisms for metabolic interference of IGFBP-2 are less clear. As we demonstrate, overexpression of IGFBP-2 in transgenic mice is correlated with a decelerated clearance of blood glucose after oral administration. IGFBP-2 carries an integrin-binding domain (RGD motif), which has been shown to also mediate IGF-independent effects. We thus asked if higher serum levels of IGFBP-2 without an intact RGD motif would also partially block blood glucose clearance after oral glucose application. In fact, transgenic mice overexpressing mutated IGFBP-2 with higher levels of IGFBP-2 carrying an RGE motif instead of an RGD were not characterized by decelerated glucose clearance. Impaired glucose tolerance was correlated with lower levels of GLUT4 present in plasma membranes isolated from muscle tissues after glucose challenge. At the same time, activation of TBC1D1 was depressed in mice overexpressing wild-type but not mutated IGFBP-2. Although we do not have reason to assume altered activation of IGF-I receptor or PDK1/Akt activation in both models, we have identified increased levels of integrin-linked kinase and focal adhesion kinase dependent on the presence of the RGD motif. From our results we conclude that impaired glucose clearance in female IGFBP-2 transgenic mice is dependent on the presence of the RGD motif and that translocation of GLUT4 in the muscle may be regulated by IGFBP-2 via RGD-dependent mechanisms.


MATERIALS AND METHODS

Animals. IGFBP-2 transgenic mice (D-mice) were generated by microinjection of an expression vector containing the murine IGFBP-2 complementary DNA (cDNA) and the CMV promoter for the transcriptional control of the transgene, as described before (13). RGE transgenic mice (E-mice) were generated by microinjection of an expression vector coding for mouse IGFBP-2 containing an RGE domain (Arg-Gly-Glu) instead of a RGD sequence (Arg-Gly-Asp), as described previously (14). Both transgenic lines were identified by real-time PCR analysis (13). Nontransgenic mice were used as controls (C-mice). All mice were maintained under standard (non-barrier) conditions and had free access to a standard diet (Breeding Diet 1314; Altromin, Lage, Germany) and water ad libitum. All procedures were in accordance with the guidelines set by the Animal Care Committee of Mecklenburg-Western Pomerania, Germany, based on the Law of Animal Protection.

Western immunoblot analysis. Tissue samples of quadriceps muscles were homogenized in lysis buffer (no. 9803, New England Biolabs, Frankfurt, Germany) with complete Mini (Roche, Mannheim, Germany) using the Precellys 24 (Peqlab Biotechnologie, Erlangen, Germany). After 20 min on ice, Laemmli’s sample buffer was added, and samples were incubated for 10 min at 94°C. Cell debris was removed by centrifugation (10,000 g, 2 min, 4°C). Protein content of whole cell lysate was quantified using the bicinchoninic acid method as previously described (3). For each sample, 15 μg of protein were separated on 7.5% or 12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Eschborn, Germany). Equal loading of the gels and proper transfer of the proteins to the membranes were verified by Coomassie blue staining. Membranes were blocked in 5% dry milk and 1% Tween 20 dissolved in Tris-buffered saline-TBST (pH 7.4). After three washings in TBST buffer, membranes were incubated with primary antibodies overnight at 4°C and shaking. We analyzed protein abundance and phosphorylation by using a set of different antibodies (Table 1). All antibodies were purchased from Cell Signaling Technology (New England Biolabs, Frankfurt, Germany) or Santa Cruz Biotechnology (Heidelberg, Germany) and were used according to the manufacturer’s instructions. After three washings in TBST, the membranes were incubated with horseradish peroxidase anti-rabbit IgG (1:2,000, no. 7074) for 1 h at room temperature (RT). Finally, membranes were analyzed on a Kodak Image Station 4000MM (Stuttgart, Germany) using an ECL Advance Western Blotting Detection Kit (GE Healthcare, Freiburg, Germany). Band intensities were quantified using the ImageQuant software package (GE Healthcare). To estimate the specific activation for distinct signaling molecules, signals from phosphospecific antibodies were normalized to total protein expression signals (phospho-total protein). Therefore, the membranes were incubated with total antibodies for GLUT4 (rabbit, no. sc-7938), AS160 (rabbit, no. 2670), TBC1D1 (rabbit, no. 4629), PKCζ (rabbit, no. sc-7985-R), Akt (rabbit, no. 9272), PDK1 (rabbit, no. 3062), PTEN (rabbit, no. 9552), IGFRβ (rabbit, no. 3018), ILK (rabbit mAb, no. 3856), and FAK (rabbit, no. 3285). Results were presented in percent phospho-total protein or total protein/loading control.

Measurement of PIP3 and PIP2 levels. PIP3 and PIP2 concentrations in the muscle tissue were measured by sandwich ELISA (Echelon Biosciences, Salt Lake City, UT). Total phosphoinositides were extracted from the muscle tissue homogenate following the manufacturer’s guidelines. Frozen muscle tissue (~50 mg) was homogenized in 50 mM potassium phosphate buffer (pH 6.9) containing 1 mM EDTA and protease inhibitor cocktail (Complete Mini; Roche, Mannheim, Germany) followed by centrifugation at 14,000 rpm for 30 min at 4°C. Proteins and lipids of the resulting supernatant were precipitated by adding of 0.5 ml of ice-cold 0.5 M trichloroacetic acid (TCA) and incubating on ice (5 min) for phosphoinositide extraction. After incubation on ice for 5 min, the precipitate was pelleted by centrifugation at 5,000 rpm for 5 min at 4°C. Two wash steps with 5% TCA 1 mM EDTA followed. Neutral lipids were extracted first from the pellet with methanol–chloroform (2:1) by vortexing three to four times over a period of 10 min at RT followed by centrifugation at 5,000 rpm for 5 min at 4°C. This extraction was repeated, and the supernatants were discarded. The acidic lipids were then extracted with methanol–chloroform–12 M HCl (80:40:1) by vortexing several times over a period of 15 min at RT followed by centrifugation (5,000 rpm, 5 min, 4°C). This extraction was repeated, and the received supernatants were collected. A phase split was established by the addition of chloroform and 0.1 M HCl followed by centrifugation to separate the organic from the aqueous phase at 5,000 rpm for 5 min at 4°C. The organic phase was transferred into a clean tube and dried in a vacuum pump (Concentrator Plus, Hamburg, Germany) enriching lipids which were resuspended in specific assay buffer, vortexed, and sonicated briefly to dissolve the phosphoinositides. The masses of the phosphoinositides were estimated using the respective ELISA kits (PIP3 mass ELISA kit no. K-2500s and PIP2 mass ELISA kit no. K-4500s). Appropriate controls and standards (specified by each manufacturer’s kit) were included in each experiment.

Separation of cell fractions. Muscle samples were homogenized two times for 30 s using the Precellys 24 (Peqlab Biotechnologie) as described above. Cell debris and nuclei were separated by centrifugation (1,000 g, 10 min, 4°C). The supernatant was transferred into a new tube and labeled as the cytoplasmic fraction. The remaining pellet was resuspended in Laemmli’s sample buffer and again separated via centrifugation (1,000 g, 10 min, 4°C), receiving a pellet and a second supernatant. The pellet was resolved 1:1 in lysis buffer and Laemmli’s sample buffer and incubated on ice for 30 min and for 10 min at 94°C. A high-speed centrifugation (21,000 g, 2 min, 4°C) followed to enrich proteins of cell nuclei in the supernatant. The earlier second supernatant containing plasma membranes and cytosolic fraction was centrifuged for 30 min at 21,000 g and 4°C. The remaining pellet contained the plasma membranes and was resolved in Laemmli’s sample buffer, while the supernatant included the cytosolic fraction. The efficiency of membrane and nucleus isolation was proofed by use of special marker proteins that are representative for both fractions (Na+,K+-ATPase for membrane and nucleoporin p62 for the nucleus fraction; data not shown). The protein content of the different fractions was quantified using the biocinchoninic acid method as described previously (3).

Oral glucose tolerance test. Oral glucose tolerance tests (OGTT) were performed in 10-wk-old female mice. Starting the day before the OGTT, mice were fasted between 4 PM and 8 AM. Oral glucose dissolved in tap water was administered (1 g/kg body wt) by use of a curved feeding needle with a ball tip. Concentrations of blood glucose during the OGTT were assessed before and 10, 30, 60, 120, and 180 min post-glucose.

Table 1. Antibodies used in the study

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<tr>
<th>Source</th>
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See text for definitions.
min after the oral glucose bolus. A single drop of blood from the tail tip was used for measurement of glucose concentrations using a commercial glucometer (Roche, Penzberg, Germany). Fasted insulin levels were measured with the Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden). One week after the OGTT, mice were euthanized 20 min after a second oral glucose bolus, as described above. Isolated muscles and liver were weighed. Tissues and serum samples were stored at $-70^\circ$C.

Data analysis and statistics. The data analysis was generated using SAS software (version 9.4 for Windows. SAS Institute, Cary, NC). Descriptive statistics and tests for normality were calculated with the UNIVARIATE procedure of Base SAS software. Data that could be considered approximately normal were analyzed by ANOVA or repeated-measures ANOVA with the MIXED procedure of SAS/STAT software. The ANOVA model contained the fixed factor genotype (levels: C, D, and E). The repeated-measures ANOVA model for blood glucose consisted of the fixed factor genotype (levels: C, D, and E), the repeated factor time (levels: 0, 10, 30, 60, 90, 120, 180 min), and the interaction genotype $\times$ time. For repeated measures an unstructured type of the block diagonal residual covariance matrix was used. In addition least square means (LSM) and their standard errors (SE) corresponding to the fixed effects in the models above

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**Fig. 1.** Decelerated normalization of blood glucose level after oral glucose administration in RGD transgenic mice (D-mice) overexpressing wild-type IGFBP-2 (A) but not in mice overexpressing mutated IGFBP-2 lacking the RGD sequence (E-mice; B) compared with nontransgenic control mice (C-mice) mice. For all data points, $n > 22$. *$P < 0.05$.

**Fig. 2.** Analysis of GLUT4 expression in muscle lysates from 10-wk-old female untreated (A and C) and glucose-treated (B and D) IGFBP-2 transgenic mice. Western blot identified the total expression of the respective signaling molecule ($n = 10$). Coomassie staining was used as loading control. A and B: cytosolic fraction; C and D: membrane fraction. GA, after glucose administration (B and D). **$P < 0.01$, ***$P < 0.005$.  

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were calculated and pairwise tested (Tukey-Kramer test). Effects and differences were considered significant if $P < 0.05$. If not stated differently, data are presented as means $\pm$ SD. For the direct comparison of isolated parameters (translocation of GLUT4), the Welch-test was used.

RESULTS

Decelerated normalization of blood glucose level after oral glucose administration in D-mice but not in E-mice. To determine the role of the RGD sequence of IGFBP-2 on glucose metabolism, we performed OGTTs. Before or 15 min after glucose administration, glucose levels were similar in D-mice and nontransgenic C-mice (Fig. 1A). Also, insulin concentrations were similar in all genetic groups before glucose stimulation (data not shown). At later time points (30, 60, and 90 min after oral glucose administration) blood glucose concentrations were higher in D-mice than in C-mice ($P < 0.05$). While blood glucose concentrations have significantly lower levels in E-mice shortly before glucose administration compared with nontransgenic C-mice at later time points, no differences were present in C- vs. E-mice (Fig. 1B).

GLUT4 levels in the cytosol and plasma membrane. Insulin-dependent glucose uptake in muscle is mediated by translocation of GLUT4 to the plasma membrane from the cytosol (28). We thus studied levels of GLUT4 in both compartments of muscle. Before glucose application, higher levels of GLUT4 were detectable in the cytosolic fraction of D-mice but not of E-mice compared with control mice (Fig. 2A). In the membrane fraction, GLUT4 levels were close to the limit of detection in a number of animals for all genetic groups (Fig. 2C). After glucose administration, cytosolic and plasma membrane levels of GLUT4 were lower ($P < 0.005$) in D-mice than in C-mice (Fig. 2, B and D).

Control of GLUT4 translocation by AS160, TBC1D1, and PKCζ. GLUT4 translocation is stimulated by two Rab-GTPase-activating proteins, TBC1D1 and AS160 (20, 24). Before glucose administration, both transgenic lines showed significantly higher levels of specific activation at Thr$^{590}$ for membrane-associated TBC1D1 compared with control mice (Fig. 3A). In response to glucose administration, specific activation of TBC1D1 present in the plasma membrane was significantly reduced in D-mice vs. C- or E-mice (Fig. 3B).

Before glucose administration, E-mice had by trend an increased level of activation at Thr$^{642}$ AS160 present in the membrane fraction compared with nontransgenic C-mice (Fig. 3C).

After glucose administration, E-mice had significantly increased levels of activation at Thr$^{642}$ AS160 present in the

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 3.** Analysis of TBC1D1 activation at Thr$^{590}$ and of AS160 activation at Thr$^{642}$ in muscle lysates from 10-wk-old female untreated (A and C) and glucose treated (B and D) IGFBP-2 transgenic mice in the membrane fraction. Western blot identified the phosphorylated and total expressions of the respective signaling molecule. Specific activation was calculated from the ratios of phosphorylated vs. total protein (A and C; $n = 4$; B and D; $n = 10$). Coomassie staining was used as loading control. GA, glucose administration (B and D). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. 

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membrane fraction compared with D-mice and C-mice (Fig. 3D). The levels of cytosolic TBC1D1 and AS160 were close to the limits of detection (data not shown). Glucose treatment had no effect on specific activity of PKCζ (data not shown).

**Activation of Akt, PDK1, and IGF-I receptor.** Akt, as an effector of TBC1D1 or AS160, is regulated through the levels of PIP3, PDK1, and the IGF-I receptor. Before glucose stimulation, no differences were present for protein phosphorylation of Akt or PDK1 (Fig. 4, A, C, and E). Membrane fraction of activated Akt at Ser473 was significantly increased in both transgenic lines compared with controls in response to glucose treatment (Fig. 4B). A similar pattern was found for membrane-associated Akt phosphorylated at Thr308, but only in E-mice was phosphorylation significantly increased 20 min after glucose application (Fig. 4D). PDK phosphorylation was increased in E-mice without statistical significance (Fig. 4F).

Conditional activation of Akt was in line with reduced levels of active PTEN (P < 0.05) after oral glucose application in E-mice (Fig. 5B). Again, before glucose administration, no significant difference was detectable (Fig. 5A). If results from Fig. 5A for both transgenic lines were combined, the level of significance was increased (P < 0.01). Further support for higher activation of PDK1 and Akt was derived from the assessment of PIP3 and PIP2 (Fig. 6). Concentrations of PIP3 were significantly reduced in D- and E-mice (Fig. 6A; P < 0.05), whereas those of PIP2 seemed to be increased in both transgenic lines but without statistical significance due to high variability of the PIP2 data (Fig. 6B). IGFBP-2 is known to coregulate activity of the IGF-I receptor and thus also subsequent activation of PI3K/Akt signaling (19). However, no differences were observed between our three mouse lines before and after glucose treatment (data not shown).

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**Fig. 4.** Analysis of Akt activation at Ser473 (A and B) and Thr308 (C and D) and further PDK1 (E and F) activation in muscle lysates from 10-wk-old female untreated (A, C, and E) and glucose treated (B, D, and F) IGFBP-2 transgenic mice and nontransgenic controls. Western blot identified the phosphorylated and total expressions of the respective signaling molecule. Specific activation was calculated from the ratios of phosphorylated vs. total protein (n = 10). Coomassie staining was used as loading control. A and B: Akt at Ser473 in the membrane fraction. C and D: Akt at Thr308 in the membrane fraction. E and F: PDK1 in the cytosolic fraction. GA, glucose administration (B, D, and F). *P < 0.05, ***P < 0.005.

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ILK and FAK. IGFBP-2 can bind to α5β1-integrin via the RGD sequence and initiate the activation of downstream effectors including ILK and FAK (18, 29). In the cytosolic fraction, the abundance of ILK and FAK was independent of glucose administration (data not shown). However, the genotype had a significant effect on cytosolic FAK (Fig. 7A) or ILK levels (Fig. 7B) with higher concentration in D-mice compared with C- and E-mice.

DISCUSSION

In the present study, we discuss the functional role of the RGD sequence of IGFBP-2 for glucose metabolism. This RGD sequence is responsible for IGF-independent effects of IGFBP-1 and IGFBP-2 through binding to α5β1-integrin (29). Integrin ligand binding induces signaling pathways that lead to the activation of ILK and the downstream effectors PIP3 and Akt (5). Akt activation contributes to insulin-dependent regulation of GLUT4 traffic and glucose uptake (33). GLUT4 is the principal glucose transporter protein that mediates the insulin-stimulated glucose uptake into skeletal muscle and furthermore is the major mediator of glucose removal from the circulation and a key regulator of whole body homeostasis (17). GLUT4 has high affinity for glucose and appears mainly in muscle cells and adipocytes to supply these tissues with energy in response to insulin (1). In the absence of insulin, the majority of GLUT4 is located in special GLUT4 storage vesicles in the cytoplasm, whereas only 5% of total GLUT4 is located at the cell surface (21, 31). In fact, before glucose treatment, GLUT4 is present on the cell surface to a similar extent in nontransgenic and transgenic mice. By contrast, in the cytoplasm, D-mice store considerably more GLUT4 than the control mice and also E-mice. Interestingly, higher levels of cytosolic GLUT4 did not make their way to the plasma membrane compared with C- or E-mice, since after glucose stimulation D-mice had lower levels of GLUT4 present on the cell surface than C-mice. In addition, in the cytoplasm, lower levels of GLUT4 were detected by specific antibodies compared with C- or E-mice. While lower plasma membrane levels of GLUT4 were in line with decelerated normalization of glucose in the blood circulation, the reason for the acute disappearance of cytosolic GLUT4 is unclear. Thus, our results raised two major questions: what happens to cytosolic GLUT4 in D-mice; and what are the mechanisms for RGD-dependent control of GLUT4 levels and glucose metabolism? Via its RGD domain, binary IGFBP-2 complexes containing IGF-I can bind to the cell surface, providing a possible mechanism for delivery of IGF-I to target tissues (2, 27). IGF-I can bind to IGF-I receptor and initiate the IGF-I receptor kinase activity and subsequent activate PI3K/Akt pathways (19). We thus analyzed activation of the PI3K pathway.

In muscles from male IGFBP-2 transgenic mice (C57BL/6xNMRI), we were able to demonstrate higher activation of Akt at Ser^473 (23), whereas Western blot analysis revealed higher levels of Akt phosphorylated at Ser^473 only after glu-
IGF-I receptor contributes to insulin regulation of GLUT4 traffic and glucose uptake (33). PKCζ is expressed at quite heterogeneous levels in our experimental system, and a differential effect of the RGD motif could not be derived before or after glucose application.

Several studies have demonstrated that TBC1D1 and AS160 play critical roles in glucose and lipid metabolism as well as in whole body energy homeostasis and depend on Akt (20, 24). Akt-mediated phosphorylation of TBC1D1 as well as AS160 is the link between the insulin signaling and vesicle trafficking and GLUT4 translocation (20). Thus, we also studied localization of both Rab-GTPase-activating proteins in muscles before and after glucose administration. The levels of phosphorylated AS160 present in the plasma membranes were increased before and after glucose bolus and thus cannot easily be discussed in context with Akt, which was phosphorylated only after glucose bolus to a higher extent. Interestingly, activation of TBC1D1 was significantly reduced in D-mice after glucose application. TBC1D1 deficiency in mice results in impaired insulin-stimulated glucose transport that goes along with a 50% reduction in protein abundance of GLUT4 in glycolytic muscle (32). TBC1D1 thus may be relevant in our experimental system characterized by differential glucose metabolism. A direct link between integrins and TBC1D1 has so far not been established. However, such a link also has not been excluded as possibly being mediated by Akt. Finally, we have no reason to assume that the effects of IGFBP-2 on GLUT4 are mediated by insulin in our experimental system, since fasted insulin levels were similar in all genetic groups, which is in agreement with previous reports in D- and C-mice (15). Also, refed insulin levels are independent of the genotype in D- vs. C-mice (15).

To summarize, we have shown that glucose clearance is decelerated in D-mice and that this effect requires the presence of the RGD domain of IGFBP-2. Decelerated glucose clearance was correlated with impaired translocation of GLUT4 to the cell surface in muscle in response to oral glucose applica-

cose application. This discrepancy may be due to the fact that the present study was performed in a different sex and on a different genetic background (C57BL/6). However, by the assessment of IGF-I receptor, PI3K, Akt, or PDK1 in both transgenic mouse lines, a permissive function of the RGD motif present in IGFBP-2 cannot be assumed in our experimental system. Instead, our results may argue for an effect of IGFBP-2 on glucose clearance via the RGD motif, which may be independent of IGF-I receptor or PI3K signaling (Fig. 8). We cannot exclude that one or more heparin-binding domains (HBD) present in IGFBP-2 are related with altered PTEN phosphorylation patterns and/or PDK1/Akt activation in both transgenic lines. Shen et al. (30) demonstrated that IGFBP-2 enhances PTEN phosphorylation and thus increases Akt activation. Akt is also activated by integrins, ILK, and FAK (33). A direct link between integrin signaling and glucose metabolism has been established particularly for the skeletal muscle e.g., by FAK (16). In fact, our results support an effect of the RGD sequence motif for the control of ILK and FAK, since in the cytosol from D- but not from C- or E-mice higher levels of both proteins were detected. However, selective effects of the RGD domain on the expression of ILK or FAK were not consistent with activation patterns of PDK1/Akt. We thus do not have reasons to discuss the effect of ILK in a context with altered PDK1/Akt. Also, PKCζ is activated via PDK1 and contributes to insulin regulation of GLUT4 traffic and glucose translocation (20).
tion. Although we do not have evidence for an involvement of IGF-I receptor- or PTEN-dependent control of PKD1 or Akt activity as the primary cause of differential glucose metabolism of C-, D-, and E-mice, we may speculate on a potential function of the RGD motif present in IGFBP-2, which directly or indirectly may act on phosphorylation of TBC1D1 and thus impair translocation of GLUT4 in response to glucose administration.

GRANTS
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DISCLOSURES
A. Hoeflich and E. Wirthgen are part of Ligandis GbR. This relation has no conflict of interest.

AUTHOR CONTRIBUTIONS

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E416 IGFBP-2 BLOCKS GLUCOSE CLEARANCE VIA THE RGD MOTIF
