

# Antidiabetic Effects of a Tripeptide That Decreases Abundance of Na<sup>+</sup>-D-glucose Cotransporter SGLT1 in the Brush-Border Membrane of the Small Intestine

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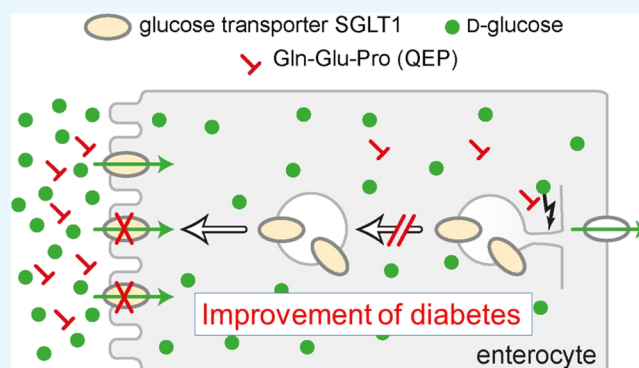


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Supporting Information

**ABSTRACT:** In enterocytes, protein RS1 (*RSC1A1*) mediates an increase of glucose absorption after ingestion of glucose-rich food *via* upregulation of Na<sup>+</sup>-D-glucose cotransporter SGLT1 in the brush-border membrane (BBM). Whereas RS1 decelerates the exocytotic pathway of vesicles containing SGLT1 at low glucose levels between meals, RS1-mediated deceleration is relieved after ingestion of glucose-rich food. Regulation of SGLT1 is mediated by RS1 domain RS1-Reg, in which Gln-Ser-Pro (QSP) is effective. In contrast to QSP and RS1-Reg, Gln-Glu-Pro (QEP) and RS1-Reg with a serine to glutamate exchange in the QSP motif downregulate the abundance of SGLT1 in the BBM at high intracellular glucose concentrations by about 50%. We investigated whether oral application of QEP improves diabetes in db/db mice and affects the induction of diabetes in New Zealand obese (NZO) mice under glucolipotoxic conditions. After 6-day administration of drinking water containing 5 mM QEP to db/db mice, fasting glucose was decreased, increase of blood glucose in the oral glucose tolerance test was blunted, and insulin sensitivity was increased. When QEP was added for several days to a high fat/high carbohydrate diet that induced diabetes in NZO mice, the increase of random plasma glucose was prevented, accompanied by lower plasma insulin levels. QEP is considered a lead compound for development of new antidiabetic drugs with more rapid cellular uptake. In contrast to SGLT1 inhibitors, QEP-based drugs may be applied in combination with insulin for the treatment of type 1 and type 2 diabetes, decreasing the required insulin amount, and thereby may reduce the risk of hypoglycemia.



## INTRODUCTION

Selective inhibitors of SGLT2 that reduce blood glucose by decreasing renal glucose reabsorption have been introduced for treatment of diabetes mellitus.<sup>1–3</sup> These inhibitors have antihypertensive and renoprotective effects and can be administered in combination with other antidiabetic drugs. Inhibition of SGLT1 together with SGLT2 has additional antidiabetic effects but also increases risk of adverse drug effects.<sup>3,4</sup> Studies with SGLT1 knockout mice revealed that removal of SGLT1 decreased small intestinal glucose absorption during the oral glucose tolerance test (OGTT) as well as renal glucose reabsorption.<sup>5,6</sup> Normally, ~97% of glucose filtrated in the glomeruli is reabsorbed *via* SGLT2 and ~3% is reabsorbed *via* SGLT1 in late proximal tubules. However, when SGLT2 is blocked, 30–40% of filtrated glucose is reabsorbed *via* SGLT1.<sup>2,7,8</sup> Hence, full inhibition of both SGLT1 and SGLT2 blocks renal glucose reabsorption almost completely. This increases the risk of hypoglycemia between meals. As SGLT1 is also expressed in heart, brain, and T-lymphocytes, SGLT1 inhibitors may induce adverse drug

effects in these tissues if they become systemically available.<sup>2–4,9</sup>

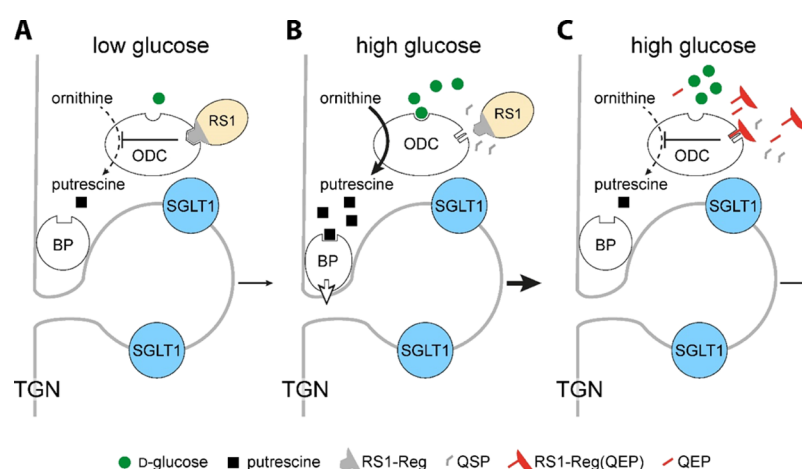
Orally applied inhibitors have been developed that target both SGLT1 and SGLT2 or target SGLT1 in the small intestine selectively.<sup>10</sup> For example, sotagliflozin (LX4221)<sup>11–14</sup> and licogliflozin<sup>15</sup> have been introduced as dual inhibitors of SGLT1 and SGLT2. They decrease small intestinal glucose absorption, enter the blood, and reduce renal glucose reabsorption. GSK-1614235,<sup>16</sup> LX2761,<sup>17</sup> SGLS213,<sup>18,19</sup> and mitagliflozin<sup>20</sup> inhibit SGLT1 only in the small intestine. Whereas GSK-1614235 and mitagliflozin inhibit SGLT1 with much higher efficacy than SGLT2,

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**Figure 1.** Model depicting the presumed RS1-dependent mechanism for upregulation of SGLT1 in the BBM of the small intestine in the presence of high D-glucose concentrations (A,B) and downregulation of SGLT1 by RS1-Reg(QEP) and QEP at high D-glucose (C).<sup>10,21,22,24,25</sup> At high D-glucose, the release of SGLT1 containing vesicles from the TGN is stimulated by putrescine that is generated by ODC at the TGN (B). At low concentrations of D-glucose in the enterocytes between meals, the release of SGLT1 containing vesicles from the TGN is decelerated by RS1 that binds *via* its RS1-Reg domain to ODC and blocks the enzymatic activity of ODC (A). At high concentrations of D-glucose after glucose-rich meals, D-glucose binds to ODC and induces a structural change that prevents binding of RS1-Reg and QSP and cancels the inhibition of ODC activity (B). As a consequence, the concentration of putrescine at the TGN is increased and the release of SGLT1 containing vesicles from the TGN is accelerated, resulting in upregulation of SGLT1 in the BBM. When RS1-Reg(QEP) or QEP is introduced into the enterocytes, ODC activity at high D-glucose is blocked and the release of SGLT1 containing vesicles from the TGN is decelerated, leading to a decrease of SGLT1 in the BBM. BP indicates a postulated budding protein complex containing a putrescine binding site that induces the release of vesicles containing human SGLT1 from the TGN.

LX2761 and SGL5213 inhibit SGLT1 and SGLT2 with similar efficacies but enter the blood slowly.<sup>17,19</sup> GSK-1614235 decreased postprandial elevation of blood glucose and the secretion of glucagon-like peptide 1 (GLP-1) in normal rats,<sup>16–18</sup> whereas LX2761 and SGL5213 decreased postprandial elevation of blood glucose and increased postprandial GLP-1 secretion in normal mice and in rats with a streptozotocin-induced diabetes.<sup>17,18</sup>

A peptide derived from RS1 (*RSC1A1*) provides an alternative possibility for treating diabetes by targeting SGLT1 in the small intestine.<sup>10</sup> RS1 is involved in posttranslational regulation of some plasma membrane transporters including SGLT1, SGLT2, the glucose sensor SGLT3, organic cation transporter OCT1, and concentrative nucleoside transporter 1 (CNT1) by inhibiting the exocytotic pathways of these transporters.<sup>2,21–23</sup> The regulations are mediated by a domain of RS1 (RS1-Reg), in which the selectivity for transporters is controlled by phosphorylation.<sup>22</sup> The downregulation of SGLT1, SGLT2, and SGLT3 depends on the intracellular glucose concentration and is mediated by binding of RS1-Reg to ornithine decarboxylase (ODC), whereas downregulation of CNT1 occurs independent of glucose and ODC.<sup>21,22,24</sup> In Figure 1, the proposed mechanism for glucose-dependent regulation of SGLT1 abundance in the brush-border membrane (BBM) of the small intestine by RS1 is depicted. This mechanism involves interaction of RS1-Reg with ODC. A mutation in RS1-Reg or in a peptide motif of RS1-Reg causes the potential to downregulate SGLT1 at high D-glucose concentrations in the small intestine.<sup>21,22</sup> At low glucose, RS1-Reg downregulates the abundance of SGLT1 in the BBM, decreasing the capacity for glucose absorption between meals (Figure 1A). At high glucose, the RS1-mediated downregulation of SGLT1 in the BBM is blunted, resulting in an increased capacity for glucose absorption (Figure 1B). Of note, the motif Gln-Ser-Pro (QSP) in RS-Reg downregulates

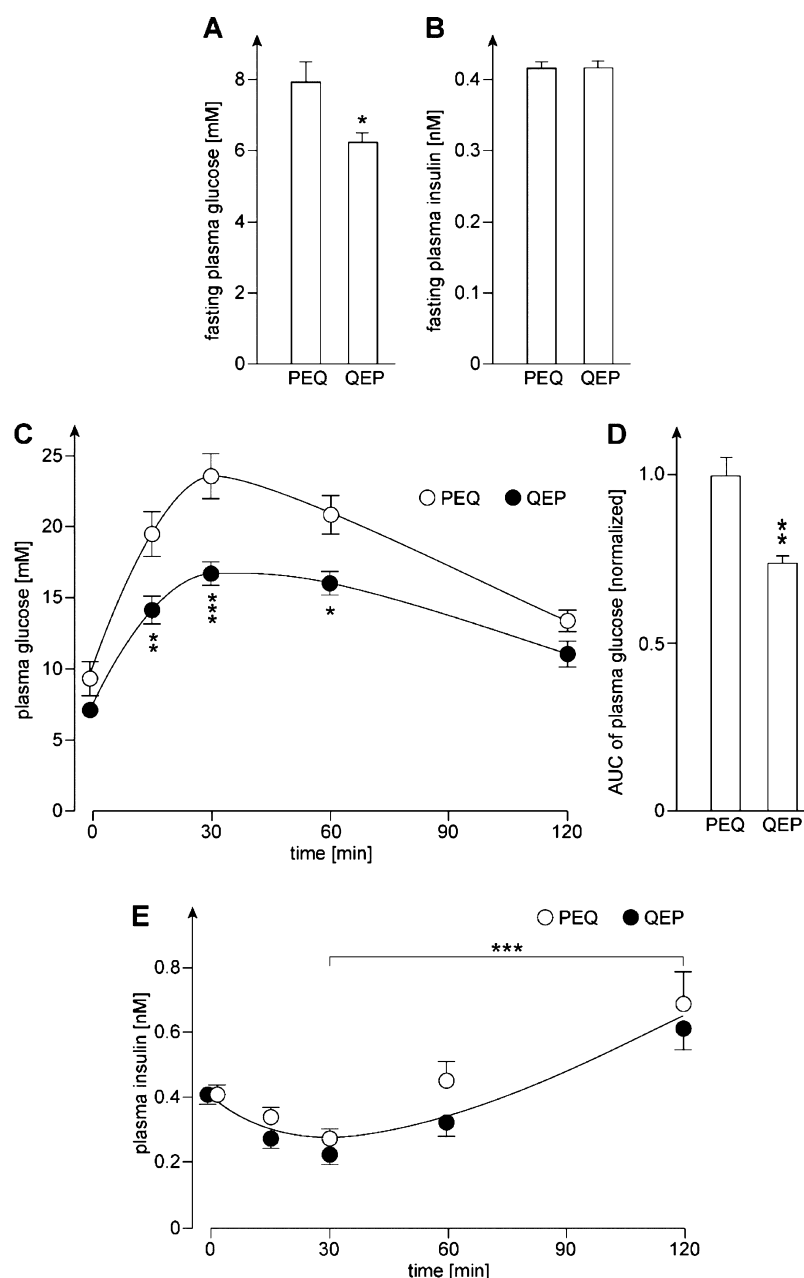
SGLT1 similar to RS1-Reg, being effective at low but not at high glucose concentrations.<sup>25</sup> In contrast, Gln-Glu-Pro (QEP), in which phosphorylation of serine in QSP is mimicked, or RS1-Reg(QEP), in which serine in the QSP motif is replaced [RS1-Reg(QEP)], downregulates SGLT1 in the presence of high intracellular glucose (Figure 1C).<sup>21</sup>

In the present study, we investigated effects of oral application of QEP on diabetic db/db mice and prediabetic New Zealand obese (NZO) mice, in which diabetes can be induced by a carbohydrate-containing high-fat diet.<sup>26</sup> We observed that 6-day administration of QEP to db/db mice improved the diabetes and that administration of QEP to NZO mice decelerated the emergence of diabetes under glucolipotoxic conditions.

## RESULTS

**Plasma Membrane Abundance of SGLT1 and SGLT2 in the Small Intestine and Kidney of db/db Mice Compared to Wildtype.** Choosing db/db mice to investigate the impact of QEP on diabetes, we first evaluated whether the plasma membrane abundance of SGLT1 and SGLT2 in the small intestine and kidney in db/db mice was different from wild-type (WT) mice with the same genetic background (C57BL/6RJ). Employing immunohistochemistry with an SGLT1-specific antibody,<sup>27</sup> we observed that the abundance of SGLT1 in the jejunal BBM of db/db mice was 30% higher than in WT mice (Figure S1). This is consistent with the observation that SGLT1 in small intestinal BBMs of patients with type 2 diabetes mellitus (T2DM) was higher compared to healthy individuals.<sup>28</sup>

In BBMs of renal proximal tubules, we observed a similar SGLT1 abundance in the db/db and WT mice (Figure S2). At variance, in Akita mice, a model for type 1 diabetes, a lower renal BBM abundance of SGLT1 was observed compared to WT, whereas a higher BBM abundance of SGLT1 was



**Figure 2.** Comparison of fasting plasma glucose, fasting plasma insulin, and OGTTs in db/db mice after 6-day administration of drinking water with PEQ vs QEP. 9 week old male db/db mice on standard diet were supplied for 6 days with drinking water containing either 5 mM PEQ or 5 mM QEP. After overnight removal of food maintaining supply with peptide-containing drinking water, plasma glucose (A) and plasma insulin (B) were measured, and OGTTs (C–E) were performed. In the OGTTs, mice were gavaged with 0.4 mL of water containing 0.2 mg D-glucose, and changes of plasma glucose concentration (C), the AUC of plasma glucose (D), and changes of plasma insulin (E) were determined. Mean values  $\pm$  SEM of data from 29 animals (A), 8 animals (B), and 9–10 animals (C–E) are indicated. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  difference analyzed by Student's *t*-test (A,D) or by two-way ANOVA with the posthoc Tukey test (C,E).

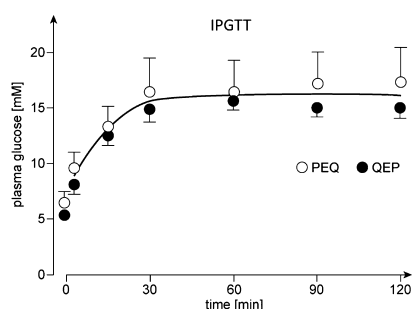
observed in black and tan, brachyuric (BTBR) db/db mice compared to WT mice.<sup>29,30</sup> Concerning SGLT2 in renal proximal tubules, we observed a 160% higher BBM abundance in db/db mice than in WT mice (Figure S3). Similarly, in renal plasma membranes, 38, 60, and 90% higher SGLT2 concentrations compared to WT were observed in Akita mice, BTBR db/db mice, and db/db mice, respectively.<sup>29–31</sup> At variance, in mice with streptozotocin-induced diabetes, the plasma membrane abundance of SGLT2 was 40% lower compared to WT mice.<sup>29,30</sup>

The data suggest complex and tissue-specific changes of the plasma membrane abundance of SGLT1 and SGLT2 in different animal models of diabetes. The db/db mouse is considered an appropriate animal model to study how drug-mediated changes of plasma membrane abundance and/or transport activity of SGLT1 affect T2DM.

**Six-Day Oral Administration of 5 mM QEP Decreased Fasting Plasma Glucose, Improved OGTT, and Increased GLP-1 Secretion in db/db Mice.** To determine whether oral application of QEP can improve diabetes, we added 5 mM QEP or 5 mM Pro-Glu-Gln (PEQ) for 6 days to

the drinking water of 9 week old male db/db mice. The reversed peptide PEQ used as control showed no effects on the regulation of SGLT1 expressed in oocytes.<sup>21</sup> The mice had a body weight of  $44.5 \pm 0.4$  g [mean  $\pm$  standard error of the mean (SEM),  $n = 80$ ], a random plasma glucose concentration of  $17.3 \pm 0.7$  mM (mean  $\pm$  SEM,  $n = 80$ ), and a fasting plasma glucose concentration of  $7.9 \pm 0.6$  mM (mean  $\pm$  SEM,  $n = 29$ ), indicating diabetes. After administration of QEP, fasting plasma glucose was decreased by  $\sim 25\%$ , whereas fasting plasma insulin was not altered (Figure 2A,B). QEP also improved the OGTT (Figures 2C,D and S4). In db/db mice, we performed OGTTs using 0.2 mg D-glucose per animal for gavage because this amount of glucose was sufficient to increase plasma glucose above 20 mM in control db/db mice receiving drinking water without peptides or with PEQ. Whereas in mice treated with PEQ blood glucose increased up to 26 mM (Figures 2C and S4 left panel), in mice treated with QEP blood glucose only increased up to 17 mM (Figures 2C and S4 left panel). After QEP treatment, the areas under the curves (AUCs) of plasma glucose concentrations measured during the OGTTs were decreased by  $\sim 25\%$  (Figures 2D and S4 right panel). Plasma insulin concentrations during the OGTT were similar in animals treated with QEP or PEQ (Figure 2E). In both groups, plasma insulin was decreased by  $\sim 25\%$  30 min after glucose gavage, whereas plasma insulin was increased by  $\sim 60\%$  120 min after glucose gavage. The early decrease of plasma insulin is supposedly caused by experimental stress during gavage,<sup>32</sup> whereas the later observed increase of plasma insulin probably represents a delayed stimulation of insulin secretion.

After 6-day application of 5 mM QEP or 5 mM PEQ with the drinking water, similar intraperitoneal glucose tolerance tests (IPGTTs) were obtained (Figure 3). This indicates that small intestinal functions are required for the improvement of the OGTT by QEP.



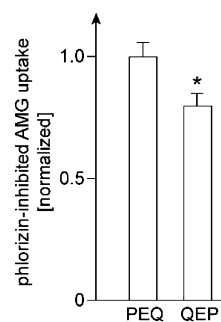
**Figure 3.** Comparison of IPGTTs in db/db mice after 6-day administration of PEQ or QEP in drinking water. 9 week old male db/db mice on standard diet were supplied for 6 days with drinking water containing either 5 mM PEQ or 5 mM QEP. After overnight removal of food maintaining supply with peptide-containing drinking water, IPGTTs were performed in which mice were i.p. injected with 0.2 mL of water containing 10 mg D-glucose. Mean values  $\pm$  SEM of data from 9 or 10 animals are indicated.

Wondering if the QEP treatment leads to an increase in GLP-1 secretion during the OGTT as described for SGLT1 inhibitors,<sup>16,17</sup> we measured GLP-1 in the portal vein 120 min after glucose gavage. GLP-1 concentrations of  $32 \pm 7$  and  $7.8 \pm 4.1$  pM (mean values  $\pm$  SEM,  $n = 4$  each,  $P < 0.05$ ) were obtained after administration of 5 mM QEP and 5 mM PEQ,

respectively, indicating a QEP-induced increase of GLP-1 secretion.

#### Six-Day Oral Administration of 5 mM QEP to db/db Mice Decreased Glucose Uptake across the Small Intestinal BBM and SGLT1-Abundance in the BBM.

Reasoning that the improved OGTT may be explained by a decreased SGLT1-mediated glucose absorption across the small intestinal BBM in the presence of high glucose (Figure 1),<sup>21,22</sup> we measured the phlorizin-inhibited uptake of 1 mM  $\alpha$ -methyl-D-glucopyranoside (AMG) into everted jejunal segments (Figure 4). For these measurements animals, were killed



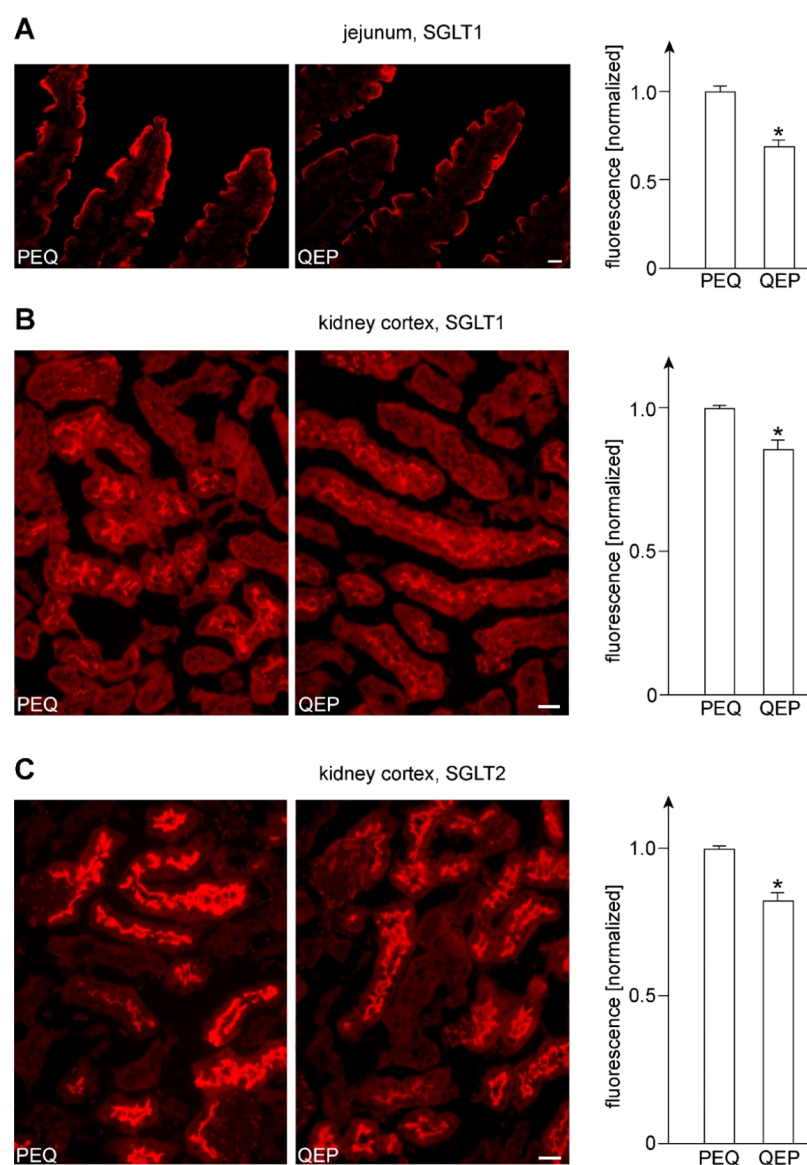
**Figure 4.** Comparison of SGLT1-mediated *ex vivo* AMG uptake in jejunum of db/db mice after 6-day administration of drinking water with QEP or PEQ. After OGTTs described in Figure 2 were finished, mice were killed, and small intestines were removed and kept for 2–3 h in DMEM containing 5 mM D-glucose. For uptake measurements, everted jejunal rings were incubated for 2 min at 37 °C with 1 mM radioactively traced AMG in the absence or presence of 0.2 mM phlorizin and phlorizin-inhibited uptake was calculated. Mean values  $\pm$  SEM of data from 5 animals are indicated. \* $P < 0.05$ , Student's *t*-test.

after OGTTs (described in Figure 2C) had been finished. The small intestines were removed and kept for 2–3 h in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM D-glucose until the uptake measurements were started. In mice with QEP in the drinking water, 20% lower phlorizin-inhibited AMG uptake was determined compared to mice receiving PEQ (Figure 4). The relatively small QEP-induced decrease of AMG uptake is supposed to be due to a decrease of the QEP concentration in the enterocytes during the OGTT and the time that passed until the uptake measurements were performed.

Next, we investigated if the reduced SGLT1-mediated AMG uptake into enterocytes was due to downregulation of SGLT1 in the BBM. Small intestines were obtained when the OGTTs had been finished and fixed for immunohistochemistry. In mice receiving QEP, the abundance of SGLT1 in the BBM of enterocytes in jejunum was 30% lower than in mice receiving PEQ (Figure 5A). The data suggest that the primary effect of orally applied QEP is due to a decrease in postprandial glucose absorption in the small intestine during high glucose concentrations.

#### Six-Day Oral Administration of 5 mM QEP to db/db Mice Downregulated BBM Abundance of SGLT1 and SGLT2 in Kidney and Increased Insulin Sensitivity.

We wondered whether QEP-mediated downregulation of glucose absorption exhibits secondary effects on renal glucose reabsorption and/or insulin sensitivity. After 6-day administration of 5 mM QEP versus 5 mM PEQ in the drinking water, we determined the abundance of SGLT1 and/or



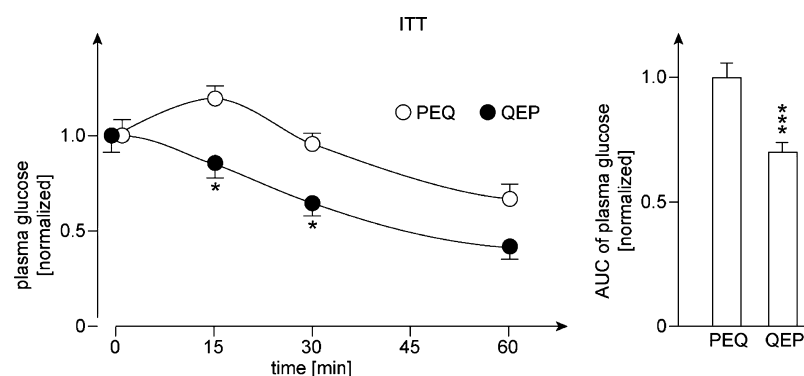
**Figure 5.** Effects of 6-day administration of drinking water with QEP to db/db mice on BBM abundance of SGLT1 in the small intestine and of SGLT1 and SGLT2 in the kidney. Nine week old, male db/db mice on standard diet were supplied with drinking water containing 5 mM PEQ or 5 mM QEP, and OGTTs were performed as described in Figure 2. Thereafter, small intestines and kidneys were removed, fixed, and sectioned for immunohistochemistry. (A left panel) Immunostaining of jejunum with mSgl1-ab and a fluorescent secondary antibody. (A right panel) Quantified fluorescence intensity in the BBM that was corrected for background fluorescence. (B left panel) Immunostaining of proximal tubules in the inner renal cortex with mSgl1-ab and a fluorescent secondary antibody. (B right panel) Quantified intensity of BBM-associated fluorescence corrected for background fluorescence. SGLT1-related immunoreactivity was analyzed in S2 segments of proximal tubules in the inner renal cortex and in S3 segments of proximal tubules in medullary rays.<sup>33</sup> (C left panel) Immunostaining of renal proximal tubules in outer renal cortex with rSgl2-ab and a fluorescent secondary antibody. (C right panel) Densitometric quantification of BBM-associated fluorescence corrected for background fluorescence. SGLT2-related immunoreactivity was analyzed in the S1 and S2 segments of renal proximal tubules.<sup>33</sup> Bars indicate 20  $\mu\text{m}$ . Mean values  $\pm$  SEM of data from three mice are indicated. In each mouse, three sections were analyzed measuring fluorescence intensity in 10 regions per section. The values obtained from each mouse were averaged and used for calculation of the presented mean values and statistics. \* $P < 0.05$ , Student's  $t$ -test.

SGLT2 in BBMs of renal proximal tubules in kidneys by immunohistochemistry. The kidneys were taken from mice that were killed after OGTTs had been finished. QEP decreased the SGLT1 abundance in the BBM of renal proximal tubules by 14% (Figure 5B) and the abundance of SGLT2 in the BBM of renal proximal tubules by 17% (Figure 5C).

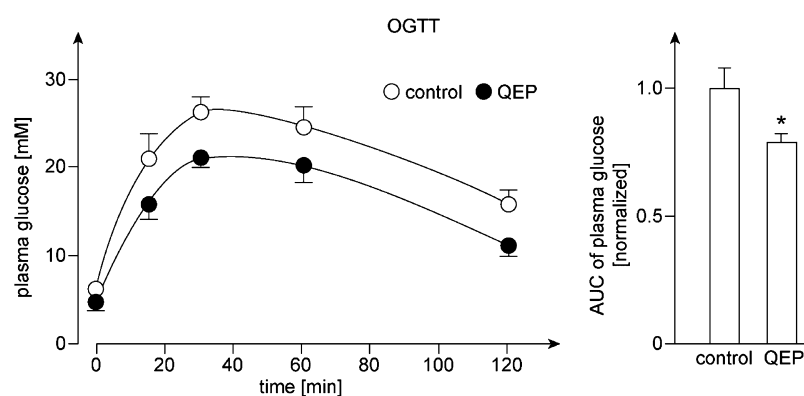
Reasoning if the observed SGLT1 abundance in the BBM could be due to an effect of QEP that may have entered the blood, we investigated if orally applied QEP enters the portal

vein (see Supporting Information). When db/db mice were gavaged with 0.2 mL of 100 mM QEP, only a QEP concentration around 10 nM was determined in the portal vein after 2 h (see Supporting Information). This concentration is much lower than 1 mM QEP that was required for downregulation of SGLT1 in the small intestine in *ex vivo* experiments.<sup>21</sup>

To determine whether the QEP-induced decrease of D-glucose in the blood changes insulin sensitivity or not, we performed an insulin tolerance test (ITT) in mice after 6-day



**Figure 6.** Comparison of ITTs in db/db mice after 6-day administration of PEQ or QEP in drinking water. Nine week old male db/db mice on standard diet were supplied for 6 days with drinking water containing either 5 mM PEQ or 5 mM QEP. Nonstarved animals were injected i.p. with 7.5 I.U. insulin. Blood concentrations were measured after different time intervals (left panel), and AUCs (right panel) were determined. Mean values  $\pm$  SEM of data from 9 or 10 animals are indicated. \* $P < 0.05$ , two-way ANOVA with posthoc Tukey test. \*\*\* $P < 0.001$ , Student's  $t$ -test.



**Figure 7.** Effect of gavage with QEP to NZO mice on plasma glucose concentrations during an OGTT which was performed 3 h later. Eight week old male NZO mice on HF-CH diet which were starved overnight were gavaged with 0.2 mL water (control) or 0.2 mL water containing 100 mM QEP. Three hours later, the mice were gavaged with 2 mg D-glucose per g body weight. Serum concentrations of glucose were measured after different time intervals (left panel) and the AUC of glucose concentrations (right panel) was determined. Mean values  $\pm$  SEM of data from 8 animals are presented. \* $P < 0.05$ , Student's  $t$ -test.

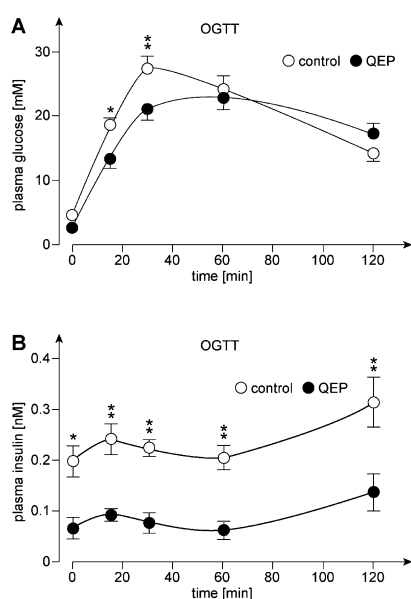
administration of drinking water with 5 mM PEQ or 5 mM QEP. These mice were not starved overnight and showed morning plasma glucose concentrations of  $13.9 \pm 1.2$  and  $10.3 \pm 0.8$  mM after application of PEQ or QEP, respectively ( $n = 9$  each,  $P < 0.05$ ). After i.p. injection of insulin, plasma glucose decreased more rapidly in mice treated with QEP compared to PEQ (Figure 6). This indicated that the insulin sensitivity was increased.

**Six-Day Oral Administration of 1 mM QEP Did Neither Alter Fasting Plasma Glucose Nor Improve the OGTT in db/db Mice.** To determine whether an antidiabetic effect could be obtained with QEP in the drinking water lower than 5 mM, we administered drinking water containing 1 mM QEP or 1 mM PEQ to db/db mice and performed OGTTs as in Figure 2C. We observed that 1 mM QEP did neither decrease the fasting plasma D-glucose concentration nor blunt the increase of plasma D-glucose during the OGTT (Figure S5).

**Acute Effect of QEP on Plasma Glucose Increase during the OGTT in NZO Mice on High-Fat Diet.** To investigate the impact of QEP on the emergence of diabetes, we selected NZO mice on high-fat, carbohydrate-free (HF-CH) diet in which diabetes can be induced by a carbohydrate supplementation (+CH) diet.<sup>34</sup> In an initial experiment, we tried to evaluate if QEP decreases glucose absorption in the small intestine of NZO mice at high luminal glucose, as has

been assumed for WT mice, db/db mice, and humans on the basis of *ex vivo* experiments (see above and Schäfer and co-workers<sup>21</sup>). We gavaged 8 week old NZO mice on HF-CH diet with 0.2 mL water containing 100 mM QEP or 0.2 mL water (as control). Three hours later, we performed OGTTs gavaging the mice with 2 mg D-glucose per g body weight. At this age, the animals have a fasting plasma glucose concentration of around 5 mM and a fasting plasma insulin concentration of around 0.2 nM. After gavage with QEP, the increase of plasma glucose during the OGTT was blunted, showing a 21% reduced AUC (Figure 7). The data suggest that QEP decreases glucose absorption in NZO mice similar to WT mice and db/db mice.

**After 3-Day Administration of QEP to NZO, Mice on HF-CH Diet Glucose Absorption and Plasma Insulin Concentrations during OGTTs Were Decreased.** To 8 week old NZO mice on HF-CH diet, drinking water containing 5 mM QEP was administered for 3 days, and OGTTs were performed employing 2 mg D-glucose per g body weight for gavage. QEP treatment did not change fasting plasma glucose but did decrease the fasting plasma concentration of insulin by 67% (control:  $196 \pm 31$  pM, QEP:  $65 \pm 23$  pM,  $n = 4$ ,  $P < 0.05$ ). In the OGTT, QEP blunted the increase of plasma glucose observed 15 min and 30 min after glucose gavage (Figure 8A). After administration of QEP versus PEQ, the peak of plasma glucose concentration



**Figure 8.** Effect of 3-day administration of QEP with drinking water to NZO mice on an OGTT. Drinking water with 5 mM QEP or normal drinking water (control) was administered to 8 week old, male NZO mice on HF-CH diet. The mice were starved overnight but had access to pure water (controls in A and B) or to water containing 5 mM QEP (QEP in A and B). Thereafter, OGTTs were performed in which the mice were gavaged with 2 mg D-glucose per g body weight. Plasma glucose concentrations (A) and plasma insulin concentrations (B) were measured after different time intervals. Mean values  $\pm$  SEM of data from 4 animals were analyzed. \* $P < 0.05$ , \*\* $P < 0.01$  for difference to control analyzed by two-way ANOVA with the posthoc Tukey test.

during the OGTT decreased more slowly. This is supposed to be due to the reduced level of plasma insulin after QEP administration (Figure 8B). Although the plasma insulin concentrations were much smaller in mice receiving drinking water with QEP compared to pure water, similar changes of plasma insulin were observed during the OGTT (Figure 8B).

**Induction of Diabetes in 18 Week Old NZO Mice by Carbohydrate Containing (+CH) Diet Was Attenuated by Addition of QEP.** In obese, 4 month old NZO mice on HF-CH diet with slightly increased random plasma glucose and increased random plasma insulin, pronounced diabetes is induced by feeding with carbohydrates (+CH diet).<sup>34</sup> To explore if QEP can retard the emergence of diabetes, NZO mice were kept on HF-CH diet until the age of 18 weeks and then fed for 21 days with +CH diet that was supplemented with QEP or PEQ. As previously described for +CH diet, the +CH diet supplemented with PEQ or QEP had no impact on body weight (Figure 9A) and showed a transient drop of food intake after the switch from HF-CH to +CH diet, as has been described for +CH without added peptides<sup>35</sup> (Figure 9B). The +CH-PEQ diet induced a quick increase of random plasma glucose from 8.9 mM to about 12 mM after 2 days which was persistent over the whole experimental period (Figure 9C). Moreover, upon administration of +CH-PEQ diet, elevations in random plasma insulin concentrations were observed (Figure 9D). Random insulin increased from  $1 \pm 0.3$  to  $6 \pm 1.3$  nM after 4 days, remained for 11 days on a similar level, and decreased later on. Supplementation of the +CH diet with QEP instead of PEQ had no different effect on body weight or food intake (Figure 9A,B). However, the increase of random

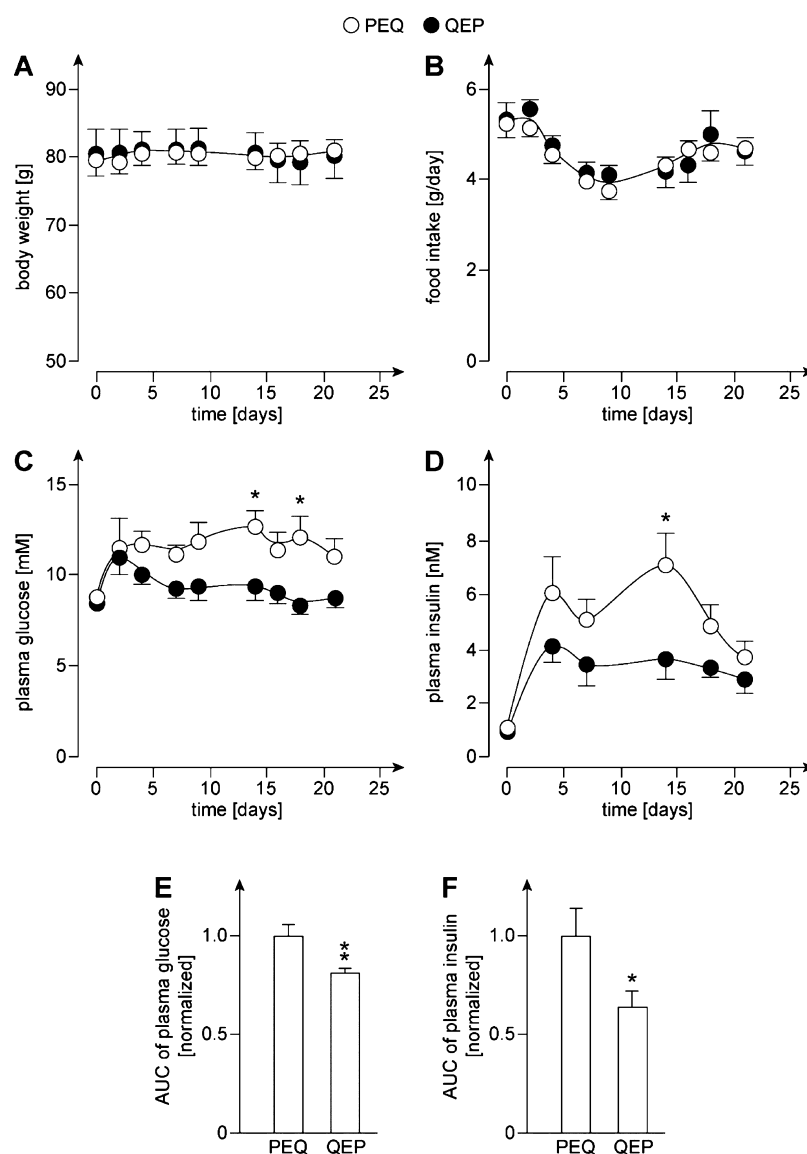
plasma glucose observed after +CH-PEQ diet or +CH diet<sup>34</sup> was blunted 7 days after application of +CH QEP diet (Figure 9C,E). In addition, the increase of random plasma insulin observed after application of +CH-PEQ diet was blunted when the +CH diet was supplemented with QEP (Figure 9D,F). The data indicate a protective effect of QEP on emergence of diabetes under glucolipotoxic conditions by reducing intestinal glucose absorption.

## DISCUSSION

Employing two mouse models, diabetic db/db mice and prediabetic NZO mice in which diabetes can be induced by a carbohydrate-rich diet,<sup>34</sup> we provided evidence that oral application of QEP improves T2DM and retards its emergence. QEP represents a modification of the functional active peptide motif QSP within the regulatory RS1 domain RS1-Reg.<sup>22,25</sup> RS1-Reg or its active tripeptide motif QSP downregulates the abundance of SGLT1 in the plasma membrane, provided that the intracellular glucose concentration is low (Figure 1A,B).<sup>22,25</sup> At low glucose, RS1-Reg or QSP binds to ODC and blocks its enzymatic activity, generating putrescine that promotes release of SGLT1 containing vesicles from the trans-Golgi network (TGN) (Figure 1A). At high intracellular glucose concentrations, D-glucose binds to ODC and promotes a conformational change that prevents binding of RS1-Reg or QSP (Figure 1B). At variance, RS1-Reg, in which serine in the QSP motif is replaced by glutamate [RS1-Reg(QEP)] or QEP, binds to ODC and downregulates the plasma abundance of SGLT1 also at high intracellular glucose concentrations (Figure 1C).<sup>21,22</sup> The glucose-induced abolition of RS1-Reg-mediated downregulation of SGLT1 in the BBM promotes the upregulation of the SGLT1 abundance in the small intestinal BBM after the ingestion of glucose-rich food.<sup>22</sup> This leads to an increased small intestinal glucose absorption (Figure 1C).<sup>6</sup>

We hypothesize that the antidiabetic effects of QEP in db/db and NZO mice are caused by a reduction in small intestinal glucose absorption after glucose ingestion. In normal mice and in obese patients, we previously demonstrated *ex vivo* that SGLT1 abundance in small intestinal BBMs was downregulated after a 30 min incubation with QEP in the presence of 5 mM D-glucose.<sup>21</sup> In the present study, we showed in db/db and NZO mice that the increase of blood glucose during OGTTs was blunted when QEP had been added for 6 or 3 days to the drinking water, respectively, and that abundance and transport activity of SGLT1 in small intestine of db/db mice were decreased. Similar to reported effects of SGLT1 inhibitors on GLP-1 secretion in rats,<sup>18</sup> QEP induced an increase of GLP-1 in the portal vein during the late phase of the OGTT in db/db mice. This effect is supposed to be caused by an increase in the glucose concentration in the distal ileum where most of the GLP-1 secreting L-cells are located.<sup>36</sup> The QEP-induced increase of GLP-1 suggests that QEP does not downregulate the SGLT1-mediated GLP-1 secretion by the L-cells. The reason may be that QEP did not enter the L-cells in a sufficiently high concentration or that QEP does not downregulate SGLT1 in L-cells.

Whereas the observed effects of QEP on glucose absorption and GLP-1 secretion are similar to effects reported for SGLT1 inhibitors,<sup>17,18</sup> additional antidiabetic effects showed up after 6-day administration of QEP to db/db mice. After 6-day treatment of 9 week old db/db mice with QEP, the slightly elevated fasting glucose was normalized and insulin sensitivity



**Figure 9.** Effects of QEP administration in combination with carbohydrate-rich diet on emergence of diabetes in NZO mice. Five week old male NZO mice were kept for 13 weeks on HF-CH diet. Thereafter, the prediabetic mice were fed for 21 days with +CH diet which was supplemented with 14.5 g/kg PEQ or QEP, and body weight (A), food intake (B), plasma glucose concentrations (C), and plasma insulin concentrations (D) were measured. (E) AUC of time course of plasma glucose concentrations shown in (C). (F) AUC of time course of plasma insulin concentrations shown in (D). Mean values  $\pm$  SEM of data from 12 to 14 animals are indicated \* $P < 0.05$ , \*\* $P < 0.01$  determined for difference by two-way ANOVA with the posthoc Tukey test (C,D) or by Student's *t*-test (E,F).

was increased. The QEP-mediated decrease in fasting and postprandial blood glucose causing a decreased glucose concentration in the renal glomerular filtrate is supposed to be the reason for the decreased abundance of SGLT1 and SGLT2 in BBMs of renal proximal tubules. This hypothesis is based on the observations that renal expression of SGLT1 and SGLT2 was increased in diabetic rodents in which the glucose concentration in the blood and in the glomerular filtrate was increased.<sup>29–31,37–39</sup> Direct effects of QEP on renal abundance of SGLT1 and SGLT2 can be excluded because only very small amounts of QEP enter the blood which are too low to promote posttranscriptional downregulation of SGLT1 in the kidney. Thus, 2 h after gavage of WT mice with 0.2 mL of water containing 100 mM QEP, we measured QEP concentrations in the portal blood that were around 10 nM (see Supporting Information). In contrast, an extracellular QEP concentration of 1 mM was required to downregulate SGLT1-mediated

glucose uptake in human and mouse small intestinal mucosa in *ex vivo* experiments.<sup>21</sup> Because the immunohistochemical analysis of SGLT1 and SGLT2 in BBMs after administration of QEP or PEQ were performed with kidneys from animals which were killed after OGTTs, it cannot be distinguished whether the QEP effects on BBM abundance of SGLT1 and SGLT2 in kidney represent short-term postprandial alterations or long-term changes that persist between meals. The mechanism(s) of the effects of QEP on the expression of SGLT1 and SGLT2 after treatment with QEP or QEP related peptides has to be elucidated in future investigations.

We employed NZO mice to investigate the impact of QEP on the emergence of diabetes. Notably, administration of QEP to obese NZO mice on a HF-CH diet with slightly elevated random blood glucose during the application of a diabetogenic high-fat/high-carbohydrate (+CH) diet prevented a further increase in random blood glucose and blunted the increase of



blood insulin. The data suggest that QEP helps to prevent the emergence of diabetes in prediabetic patients on carbohydrate-rich diets.

We regard QEP as a lead compound for the development of novel antidiabetic drugs rather than as a druggable compound by itself because relatively high concentrations of QEP have to be applied to downregulate SGLT1 abundance in the small intestinal BBM and to improve diabetes. Thus, an extracellular concentration of 0.5 mM QEP showed no effect in *ex vivo* experiments with human small intestinal mucosa, whereas a 5000-fold lower intracellular concentration of QEP was effective in *Xenopus* oocytes, in which human SGLT1 was expressed.<sup>21</sup> Because degradation of QEP in the gastrointestinal tract could be excluded (data not shown), the reason is probably an inefficient uptake of QEP into the enterocytes. This may be due to an inefficient passage of the mucus-covered glycocalyx of the microvilli and/or a slow uptake of QEP into the enterocytes which is supposedly mediated by peptide transporter PepT1.<sup>25,40</sup> Hence, for oral application, the QEP motif must be administered in a drug formulation that enables an efficient uptake into the enterocytes.<sup>41</sup> As one possibility, we propose the encapsulation of RS1-Reg(QEP) or a small peptide containing the QEP motif within specific nanohydrogels. In our hands, the encapsulation of human RS1-Reg(QEP) in nanohydrogels consisting of a colloidal hydrophilic polymer network that was cross-linked by disulfide bridges and modified by the cell permeating peptide TAT proved to be highly effective to promote uptake of RS1-Reg(QEP) into enterocytes.<sup>22,42</sup> Gavage of mice with 0.2 mL of water containing 0.2 pmol of RS1-Reg(QEP) that was incorporated into nanohydrogels containing TAT-peptide was sufficient to induce downregulation of SGLT1 in enterocytes in the presence of high glucose. Recent experiments showed that RS1-Reg(QEP) incorporated into the nanohydrogels was taken up by endocytosis, located in endosomes and at the TGN where it was co-located with ODC (T. Keller, A. Ewald, A. Krystyna, H. Koepsell, and J. Groll, unpublished data).

Similar to the effect of SGLT1 inhibitors in enterocytes, the effect of QEP in enterocytes is most probably specific for SGLT1. RS1-Reg downregulates the exocytotic pathways of transporters from different families such as SGLT-transporters of the *SLC5* family, organic cation transporters of the *SLC22* family,<sup>43</sup> and CNT-transporters of the *SLC28* family;<sup>44</sup> however, only downregulation of SGLT-transporters is blunted by D-glucose, and SGLT2 and SGLT3 are not expressed in human enterocytes.<sup>21,22,24,45</sup>

The question arises if formulations of QEP-based drugs that enter enterocytes and downregulate SGLT1 in the BBM at high extracellular glucose have advantages compared to SGLT1 inhibitors which do not enter the blood.<sup>16–19</sup> There are two principal differences between QEP-based drugs and SGLT1 inhibitors. First, in contrast to SGLT1 inhibitors, QEP-based drugs decrease glucose absorption only after ingestion of glucose-rich food. This allows an effective absorption of D-glucose when the glucose concentration in the small intestine is low like in post-meal periods when carbohydrates are released from polysaccharides of ingested vegetables or fruits or after ingestion of food with low carbohydrate content. Second, QEP-based drugs cannot inhibit glucose absorption completely like the SGLT1 inhibitors. In contrast to SGLT1 inhibitors, QEP-based drugs may be combined with insulin during treatment of patients with T1DM or later stages of T2DM. Because treatment with insulin includes the risk of

hypoglycemia between meals or during the night that may acutely impair brain functions and is a risk factor for Alzheimer's disease,<sup>3,46</sup> the blood glucose during insulin treatment is often adjusted to values higher than normal glucose levels to avoid hypoglycemia but with the risk of moderate hyperglycemia that may promote cardiovascular diseases. Coapplication of QEP-based drugs is supposed to decrease the required amount of insulin similar to SGLT1 inhibitors by attenuating the increase of blood glucose after uptake of glucose-rich food. Thereby, QEP-based drugs reduce the risk of hypoglycemia, whereas SGLT1 inhibitors may adversely increase the risk of hypoglycemia because they may inhibit glucose absorption between meals. Because QEP-based drugs reduce glucose absorption by only about 50% independent of dosage, they pose no risk of causing diarrhea, as has been described for the SGLT1 inhibitor mizagliflozin.<sup>20</sup> An additional anticipated advantage of QEP-based drugs versus SGLT1 inhibitors could be a more user-friendly drug administration. Because the reversible SGLT1 inhibitors are only effective as long as they are present in the intestinal lumen, they have to be applied together with glucose-containing food and dosing protocols have to be closely adjusted to individual lifestyles. In contrast, QEP-based drugs could be effective with one or two pills a day. Having entered the enterocytes, QEP-based drugs may be effective for hours because downregulation of SGLT1 in the BBM is supposed to be slowly reversible, and dosing can be adjusted to ensure long-lasting effective intracellular concentrations.

## CONCLUSIONS

Downregulation of SGLT1 in the BBM of the small intestine by QEP-based drugs represents an advantageous alternative to selective inhibition of SGLT1 in the small intestine for therapy of T1DM and late stages of T2DM in combination with insulin. Like SGLT1 inhibitors, QEP-based drugs decrease glucose absorption after glucose-rich meals and thereby reduce the amount of insulin needed. Because QEP-based drugs downregulate SGLT1 by about 50% after ingestion of glucose-rich food and are not effective after ingestion of food with low carbohydrate content they do not pose any risk of inducing hypoglycemia or diarrhea. It is a challenge for the future to develop a QEP-based drug that effectively enters enterocytes, to determine its duration of action, and to optimize an effective user-friendly dosage.

## EXPERIMENTAL SECTION

**Materials.** [<sup>14</sup>C]AMG (11.1 GBq/mmol) was obtained from American Labeled Chemical Inc. (St. Louis, MO). PEQ and QEP were purchased from Xiaia Custom Peptides (Göteborg, Sweden).

**Antibodies.** The characteristics of the employed non-commercial antibodies, affinity-purified rabbit-raised polyclonal anti-mouse SGLT1 (mSglt1-ab) and anti-rat SGLT2 (mSglt2-ab) that cross-reacts with mouse SGLT2 have been described earlier.<sup>6,33,47</sup> The employed secondary antibody, CY3-labeled goat anti-rabbit IgG, was purchased from Jackson ImmunoResearch Laboratories (USA).

**Animals.** All animal experiments were performed in accordance to the national guidelines of animal care and were approved by the Institutional Ethic Committees and the local government representatives, and 9 to 10 week old or 18 week old male mice were used for the experiments. The mice

had free access to water and food and were housed in a temperature-controlled room ( $22 \pm 1$  °C) on a 12/12 h light dark cycle. Eight week old C57BL6/6J WT mice and db/db mice on C57BL6/6J background were obtained from Janvier Labs (Le Genest St., Isle, France). They were fed with standard chow (ssniff V1534-000R/M-HND) supplied by ssniff GmbH (Soest, Germany). To test the effect of QEP on diabetes, QEP or the reverse control peptide PEQ was added for 6 days to the drinking water of the db/db mice. Electron microscopic examinations revealed no significant differences between the small intestinal morphology of db/db mice treated for 6 days with 5 mM QEP versus 5 mM PEQ.

The employed NZO mice were bred in the German Institute of Human Nutrition in Potsdam-Rehbrücke, Germany.<sup>34</sup> After a short post-weaning feeding period with standard diet, mice received an obesity-inducing HF-CH diet containing 20% (w/w) protein and 68% (w/w) fat with 29 kJ/g at the age of 5 weeks.<sup>34</sup> Three weeks later, short-term effects of orally applied QEP on OGTTs were determined (Figures 7 and 8). At the age of 18 weeks, the HF-CH diet was changed to a diabetes-inducing, carbohydrate-rich (+CH) diet containing 20% (w/w) protein, 28% (w/w) fat and 40% (w/w) carbohydrates (10% sucrose, 30% starch) with 21 kJ/g supplemented with PEQ or QEP (14.5 g/kg each). The peptide containing +CH diet was applied for 21 days, and the effects on body weight, food intake blood glucose, and plasma insulin were determined (Figure 9).

**Ex Vivo AMG Uptake Measurements in the Small Intestine.** Uptake measurements were performed in db/db mice as described.<sup>21</sup> The animals were starved overnight with free access to water or peptide-containing water. Between 11 and 12 a.m., the mice were killed. The small intestine was removed, perfused with Krebs–Ringer buffer [25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid pH 7.4, 108 mM NaCl, 4.8 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{CaCl}_2$ ], and stored between 2 and 3 h in DMEM containing 5 mM D-glucose. For uptake measurements, the intestine was everted, eight 1 cm long segments of the proximal jejunum were prepared, and the samples were washed with Krebs–Ringer buffer. They were incubated for 2 min at 37 °C with Krebs–Ringer buffer containing 1 mM AMG traced with [ $^{14}\text{C}$ ]AMG in the absence or presence of 0.2 mM phlorizin. Uptake was stopped and the segments were washed with ice-cold Krebs–Ringer buffer containing 0.2 mM phlorizin. After solubilizing the samples with tissue solubilizer, Soluene-350 (PerkinElmer Inc., Waltham, MA) radioactivity was determined by liquid scintillation counting. In each mouse, eight uptake measurements were performed, four in the absence and four in the presence of phlorizin, and mean values of phlorizin inhibited uptake were calculated.

**OGTT, IPGTT, and ITT.** The tests were started between 10 and 11 a.m. with mice that had been starved overnight with continuous water supply. For OGTT, the animals were gavaged with 0.4 mL of water containing 0.2 mg D-glucose (db/db mice) or 2 mg D-glucose per g body weight (NZO mice). For IPGTT, db/db mice were i.p. injected with 0.2 mL of water containing 10 mg D-glucose. For ITT, nonstarved db/db mice were i.p. injected with 7.5 I.U. of human insulin. Concentrations of glucose and/or insulin in the blood were measured at the indicated time points.

**Analytical Measurements.** Blood was collected from the tail by incision of the dilated tail vein. Determination of blood glucose and plasma insulin was performed as described

previously.<sup>35</sup> Total serum GLP-1 were determined using total GLP-1 enzyme-linked immunosorbent assay (ELISA, Millipore, EZGLP1T-36K, Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Serum insulin was determined using the mouse ultrasensitive insulin ELISA (80-INSMSU-E01) from Alpco Diagnostics (Salem, USA). Serum glucose was measured using a glucose meter (GlucoSmart 2, MSP Bodmann GmbH, Boblingen, Germany).

**Immunohistochemistry.** Three mice were used for each experimental group. After killing by cervical dislocation, abdominal cavities were opened, large blood vessels cut, and mice bled out under cold running water. Kidneys and jejunum were removed, cut into small pieces, and fixed for 24 h with 4% (v/v) *p*-formaldehyde. Immunohistochemical analysis was performed on 4  $\mu\text{m}$  thick cryosections employing antigen retrieval as described.<sup>33</sup> Cryosections were incubated overnight at 4 °C with primary antibodies diluted in phosphate-buffered saline (PBS) (Sglt1-ab 1:200 for kidneys and 1:600 for small intestine, Sglt2-ab 1:500). After rinsing sections were incubated for 60 min at room temperature with CY3-labeled goat anti-rabbit IgG GARCY3 (1.6  $\mu\text{g}/\text{mL}$  in PBS), overlaid with VECTASHIELD (Vector Laboratories, USA), and cover slipped. The immunostaining was examined under the fluorescence microscope Opton III RS (Opton Feintechnik Oberkochen, Germany). Images were taken with the attached digital camera (Spot RT Slider; Diagnostic Instruments, Sterling Heights, MI, USA) and processed with Adobe Photoshop 6.0.

**Staining Quantification of the BBMs for SGLT1 and SGLT2.** The pixel intensity of SGLT1- and SGLT2-related immunofluorescence staining at BBMs of the small intestine and/or renal proximal tubules was measured as described<sup>27,33</sup> using the noncommercial software ImageJ 1.46r (National Institutes of Health, Bethesda, Maryland, USA). From jejunum and kidney of each of the three animals used per experimental group, three cryosections were immunostained. Using a 250-fold magnification, 10 randomly chosen regions at BBMs of proximal tubules (S1/S2 segments for SGLT2 and S2/S3 for SGLT1) and enterocytes were selected, and the pixel intensities were determined. To determine background fluorescence, pixel intensity was also measured in nonstained renal tubular cells and in the nonstained basal regions of enterocytes. The background-corrected fluorescence staining obtained from each mouse was averaged, and this value was used for calculation of the presented mean values and for statistical analysis.

**Statistical Analysis.** Means  $\pm$  SEM are presented. Significant differences between several groups were determined by two-way ANOVA using posthoc Tukey comparison. Unpaired Student's *t*-test was used to determine significance of differences between two groups.  $P < 0.05$  was considered significant.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c03844>.

Immunostaining of SGLT1 and SGLT2 in the small intestine and kidneys of WT and db/db mice and quantification of staining intensities in the BBMs, OGTTs in db/db mice after 6-day administration of drinking water with PEQ or QEP, and determination of

QEP in the portal blood after gavage of mice with QEP (PDF)

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

AMG,  $\alpha$ -methyl-D-glucopyranoside; AUC, area under curve; BBM, brush-border membrane; +CH, carbohydrate-rich; +CH-PEQ, +CH diet supplemented with PEQ; +CH QEP,

+CH diet supplemented with QEP; CNT, concentrative nucleoside transporter; DMEM, Dulbecco's modified Eagle's medium; GLP-1, glucagon like peptide 1; HF-CH, high-fat carbohydrate-free; IPGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; NZO, New Zealand obese; ODC, ornithine decarboxylase; OCT, organic cation transporter; OGTT, oral glucose tolerance test; PBS, phosphate buffered saline; RS1-Reg, regulatory domain of RS1; RS1-Reg(QEP), RS1-Reg in which serine in QSP is replaced by glutamate; SGLT, sodium glucose cotransporter; T2DM, type 2 diabetes mellitus

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