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Antidiabetic effect of novel modulating peptides of G-protein-coupled kinase in experimental models of diabetes

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Abstract

Aims/hypothesis. G-protein-coupled receptor kinases (GRKs) play a key role in agonist-induced desensitisation of G-protein-coupled receptors (GPCRs) that are involved in metabolic regulation and glucose homeostasis. Our aim was to examine whether small peptides derived from the catalytic domain of GRK2 and -3 would ameliorate Type 2 diabetes in three separate animal models of diabetes.

Methods. Synthetic peptides derived from a kinase-substrate interaction site in GRK2/3 were initially screened for their effect on in vitro melanogenesis, a GRK-mediated process. The most effective peptides were administered intraperitoneally, utilising a variety of dosing regimens, to *Psammomys obesus* gerbils, Zucker diabetic fatty (ZDF) rats, or *db/db* mice. The metabolic effects of these peptides were assessed by measuring fasting and fed blood glucose levels and glucose tolerance.

Results. Two peptides, KRX-683$_{107}$ and KRX-683$_{124}$, significantly reduced fed-state blood glucose levels in the diabetic *Psammomys obesus*. In animals treated with KRX-683$_{124}$ at a dose of 12.5 mg/kg weekly for 7 weeks, ten of eleven treated animals responded with mean blood glucose significantly lower than controls (4.7±0.4 vs 16.8±0.8 mmol/l, $p \le 0.0001$). Significant reductions in blood glucose compared with controls were also seen in ZDF rats administered KRX-683$_{124}$ and in *db/db* mice, which had significantly reduced fasting and 2-hour postprandial glucose levels after the treatment.

Conclusions/interpretation. Sequence-based peptides derived from GRK2/3 have an antidiabetic effect demonstrated in three different animal models of Type 2 diabetes. By modulating GRK2/3 activity, these peptides enhance GPCR-initiated signal transduction, resulting in improved glucose homeostasis. Sequence-based peptide modulation of GRK could prove useful in the treatment of Type 2 diabetes.

Keywords *Db/db* mice · G-protein-coupled receptor · G-protein-coupled receptor kinase · Insulin resistance · *Psammomys obesus* · Type 2 diabetes · ZDF rats

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Abbreviations: GPCR, G-protein-coupled receptor ·
GRK, G-protein-coupled receptor kinases · MCR, melanocortin
receptor · PBS-A, PBS containing BSA 0.5% · PKC, protein
kinase C

Introduction

Type 2 diabetes is characterised by insulin resistance, impaired insulin secretion by pancreatic beta cells, and hyperglycaemia. Many forms of Type 2 diabetes are part of a potentially reversible metabolic syndrome that is associated with obesity, dyslipidaemia, hypertension, hypercoagulability, and vascular disease [1]. The causes of Type 2 diabetes have not been fully elucidated, and multiple genetic defects have been associated with the diabetic state [2, 3].

A potential way of treating Type 2 diabetes would be to target specific mechanisms leading to a reduction

or abolishment of insulin resistance. Among emerging therapy concepts is stimulation of the G-protein-coupled receptors (GPCRs) associated with Type 2 diabetes, including adrenergic receptors, incretins, and the central melanocortin system [4, 5, 6]. Epidemiological studies have shown that polymorphisms of the β2-adrenergic receptor are associated with diabetes [7, 8]. Additional GPCR targets modulating insulin dynamics include glucose-dependent insulinotropic polypeptide, other incretins, and the GPCR central melanocortin receptors (MCRs) that have been linked to abnormal appetite regulation and dysregulated lipid and/or glucose metabolism [6, 9, 10].

A strategy for enhancing metabolism-related GPCR signalling is to prevent receptor desensitisation. G-protein-coupled receptor kinases (GRK) down-regulate signal transduction initiated by GPCRs via phosphorylation of specific sites located on the receptors [11, 12, 13, 14]. Inhibition of GRK-mediated GPCR desensitisation prolongs receptor activation, resulting in increased ligand-induced GPCR activity. GRK2 plays a major role in the agonist-specific desensitisation of several metabolism-related GPCRs, including the β-adrenergic receptor [7, 8, 9], MCRs, endothelin, and glucose-dependent insulinotropic polypeptide [9, 11, 15, 16]. By interfering with the interaction between GRK2 and its related GPCRs, GRK2 inhibition may restore glucose homeostasis and insulin sensitivity in Type 2 diabetes.

Sequence-based inhibition has been used to modulate the enzymatic activity of GRK2. Utilising a peptide corresponding to the C-terminus of GRK2, several groups have shown that the activities of the β-adrenergic and parathyroid hormone receptors are increased due to endogenous GRK2 inhibition [17, 18]. In previous studies, we have found that small sequence-based peptides taken from a loop (hereafter the HJ loop) that encompasses parts of α helix F and α helix G (subdomains IX-X located in the catalytic domain of the kinase) interfere with kinase-substrate binding among several protein kinases, leading to a decrease in substrate phosphorylation [19].

In this study, short peptides that closely resemble the HJ loop of GRK2/3 were synthesised. The effect of these peptides on glucose regulation in experimental models of Type 2 diabetes was subsequently assessed. Our results indicate that peptide inhibitors derived from the HJ loop of GRK2/3 have a positive effect on glucose metabolism in models of Type 2 diabetes.

Materials and methods

Reagents and peptides

Unless otherwise specified, all chemicals and reagents were purchased from Sigma (Rehovoth, Israel) and CalBiochem (Darmstadt, Germany). The B16F1 murine cells (CRL-6323) used for the melanogenesis assay were obtained from American Type Culture Collection (Manassas, Va., USA). The peptides, listed in Table 1, were synthesised commercially (Novetide, Haifa, Jerusalem, Israel or Multiple Peptide Systems, San Diego, Calif., USA) using FMOC solid phase technology. Peptides were purified by the manufacturer using reverse-phase HPLC. For initial screening, the purity of the peptides was greater than 80%. The peptides KRX-683$_{107}$ and KRX-683$_{124}$, which were extensively studied in animals, were prepared as acetate salts with a purity of more than 98%. Peptides were stored as a powder at 4 °C until immediately prior to use when they were dissolved in specific formulations. For initial screening and melanogenesis assays, peptides were dissolved in an SHS formulation (described below).

For animal studies, specific formulations for KRX-683$_{107}$ and KRX-683$_{124}$ were utilised. The formulations were designed to optimise solubility of the peptides in biological fluids. KRX-683$_{107}$ was administered in a TBI formulation prepared as follows: the peptide was dissolved in DMSO (8% final concentration), and 2 mol/l ammonium bicarbonate (32% v/v). The mixture was boiled for 40 min, after which 2 mol/l HEPES buffer (Sigma-Aldrich, St. Louis, Mo., USA), pH 7.5 (60% v/v) were added. KRX-683$_{124}$ was formulated in SHS as follows. The peptide was first dissolved in DMSO (4% v/v). To this solution, glycerol (6% w/v) and Solutol HS 15 (BASF, Ludwigshafen, Germany) (1.8% w/v) were added. The same formulation was used for KRX-683$_{107}$ in the melanogenesis assays. The peptides, once formulated, were stored at 4 °C until use.

Melanogenesis assay

Previously described methods for measuring in vitro melanogenesis using a B16F1 murine melanoma cell line were used [20]. B16F1 cells were grown in DMEM supplemented with L-glutamine 2 mmol/l, glucose 4.5 g/l, fetal calf serum 10%, penicillin 100 units/ml and streptomycin 0.1 mg/ml (Biologocial Industries, Bet HaEmek, Israel) and incubated in a 5% CO_2 humidified environment. We seeded 96-well plates with approximately 5000 cells per well and incubated them for 24 h prior to melanogenesis induction. For melanogenesis induction, serial dilutions ranging from 25 μmol/l to 0.15 μmol/l of each peptide tested were prepared from stock solutions using PBS 0.1% BSA and subsequently diluted 1:10 in the B16F1 cell cultures.

NDP-αMSH 0.5 mol/l (Sigma, Rehovoth, Israel) in 16% acetic acid was further diluted with the culture medium to final concentrations ranging from 6 to 50 pmol/l. NDP-αMSH was added to B16F1 cells at the same time as the tested peptides. Cells were lysed after 4 days with 70 μl 1 N NaOH, and melanin was measured by optical density at a wavelength of 405 nm using an ELX 800 (Bio-Tek, Winooski, Vt., USA) ELISA reader. Assays were done in six replicates and the results averaged. Cells with medium containing no peptide and/or NDP-αMSH were used as blanks.

The protein kinase inhibitors AG1478, Genistein, GF109203X, LY294002 and PD169316 were obtained from CalBiochem. The final concentration of these inhibitors in cell culture was 10 μmol/l except for Genistein, which was 30 μmol/l. Inhibitors were added together with either KRX-683$_{124}$ (2.5 μmol/l) or NDP-αMSH (0.1 nmol/l). The melanogenesis assays were performed as described above.

Intracellular uptake assay

B16F1 melanoma cells were grown in the same culture medium as used for melanogenesis, and then seeded into each chamber of a 4-chamber slide at a concentration of 30,000

Table 1. Synthetic GRK-683 peptides derived from GRK 2/3 and their relative activity

Peptide	Fatty acid			L$_{384}$	L	R	G	H	S	P$_{390}$	F		OD$_{max}$	EC$_{50}$
KRX-683$_{103}$	Myristyl	G		L	L	R	k	H	S			NH$_2$	0.7	0.4
KRX-683$_{162}$	Myristyl	G		L	L	R	K	H	S			NH$_2$	0	–
KRX-683$_{109}$	Myristyl	G		L	L	R	k	H	S	P		NH$_2$	0.18	–
KRX-683$_{107}$	Myristyl	G		L	L	R	r	H	S			NH$_2$	0.79	0.42
KRX-683$_{112}$	Myristyl	G		L	L	R	r	H	S	I		NH$_2$	0.8	0.4
KRX-683$_{912}$	Stearyl	G		L	L	R	r	H	S	I		NH$_2$	0	–
KRX-683$_{124}$	Lauryl	G		L	L	R	r	H	S	I		NH$_2$	0.8	0.3
KRX-683$_{108}$	Myristyl	G		L	L	R	r	H	S	K^a		NH$_2$	0.74	0.56
KRX-123$_{302}$	Myristyl	G	L	V	T	Y^c	k	K	I	K^b		NH$_2$	0	–
KRX-702$_{105}$	Myristyl	G		R	A	G	N	Q	Y	L		NH$_2$	0	–

Amino acid residues in lower case are dextrorotary enantiomers. ^{a} Biotinylated lysine; ^{b} Nε(benzylamide)lysine; ^{c} 3,5-diiodo-tyrosine; EC$_{50}$, median effective concentration; OD$_{max}$, optimum maximum dose

cells/ml (21,600 cells/well) for 24 h. Biotinylated KRX-683$_{108}$ (10 mmol/l) was dissolved in a solution containing DMSO 10%, NaSCN 45%, and n-butanol 45%. This solution was further diluted 1:1000 in culture media to a final peptide concentration of 10 µmol/l, and incubated with B16F1 cells for 3 h. The cells were washed three times with PBS containing BSA 0.5% (PBS-A), and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilised by incubating them for 20 min in a solution of 0.2% Triton X100 and PBS-A. Cells were again washed three times with PBS-A and incubated with a 1:1000 dilution of Avidin-FITC in PBS-A. Slide wells were covered with a special cover slip (Vectashield, Vector Laboratory, Burlingame, Calif., USA) and then visualised with a confocal fluorescent microscope to determine intracellular fluorescence. Photo-documentation was done by digital imaging. As controls, incubation medium without peptide and a non-biotinylated peptide with the same sequence as KRX-683$_{107}$ were utilised (Table 1). Experiments were done in duplicate.

Selection of KRX-68, a modulator of GRK2/3 signalling

Short peptides resembling a segment of the HJ loop sequence, a potential kinase-substrate interaction region located between amino acids 384-389 of GRK2/3, were synthesised (Table 1). All peptides had a glycine attached to a fatty acid moiety added to the N-terminus to facilitate intracellular penetration (Fig. 1). In order to quantify the effect of a putative GRK2/3 modulator at the cellular level, we used the MSH receptors system, a GPCR family that mediates the induction of pigmentation in the periphery and controls energy homeostasis in the central nervous system. Melanocytic melanin production is stimulated by MSH and other proteins in the melanogenic transduction pathway. By inhibiting desensitisation of the MSH receptors, melanin accumulation is enhanced. The efficacy of GRK2/3-derived peptides was based on the level of melanin produced by B16F1 cells in the absence of exogenous NDP-αMSH.

In initial experiments, the glycine at position 387 was changed to D-lysine (KRX-683$_{103}$ and KRX-683$_{109}$). It was found that in cell-based assays the change to L-lysine enhanced the activity of peptides derived from the HJ loop, possibly by improving peptide stability (e.g., enzymatic proteolytic degradation), as there was minimal melanogenesis when L-lysine was subs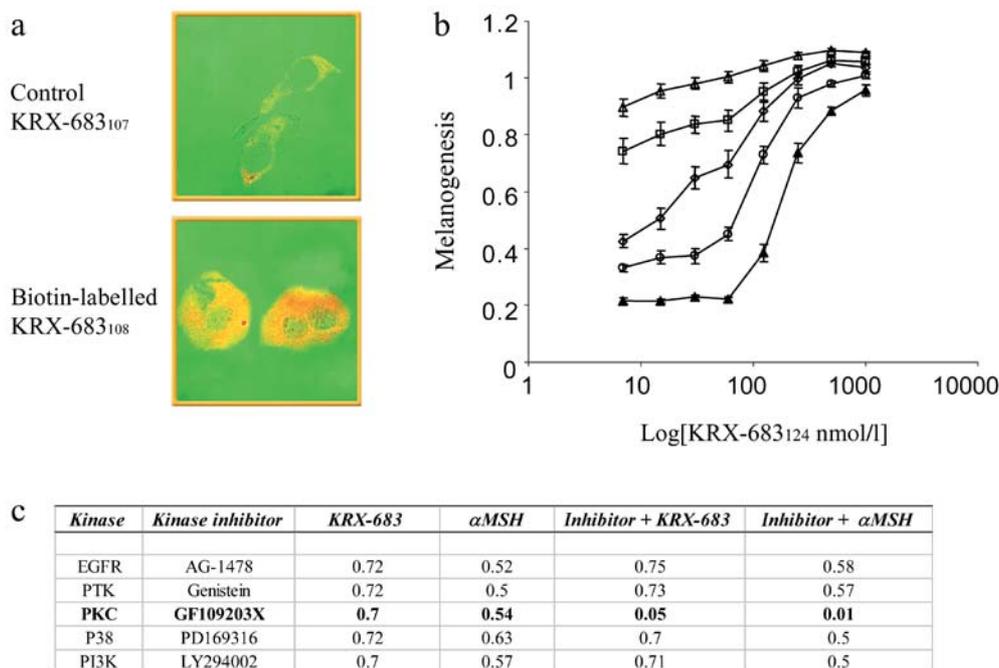tituted for D-lysine (Table 1, KRX-683$_{162}$). As shown in Table 1, KRX-683$_{103}$ stimulated significant melanogenesis (EC$_{50}$=0.4 µmol/l). When Pro390 was included in the peptide sequence, melanin production decreased, possibly due to conformational limitations introduced by the fixed structure of proline (KRX-683$_{109}$).

For subsequent peptide syntheses, D-arginine, another positively charged amino acid residue, was substituted for Gly387 with no loss of melanogenic activity (Table 1, peptides KRX-683$_{107}$, KRX-683$_{112}$, and KRX-683$_{124}$). The length of the fatty acid moiety was seen to have an effect, as there was a significant decrease in melanogenesis when a stearyl group was substituted for myristyl or lauryl (KRX-683$_{112}$ and KRX-683$_{124}$ vs KRX-683$_{912}$). To test for specificity, a myristoylated peptide targeting the same sequence in Lyn kinase (Table 1, KRX-123$_{302}$) and an unrelated myristoylated peptide derived from the serine/threonine kinase PDK1 (Table 1, KRX-702$_{105}$) were evaluated for their effects on melanogenesis. Neither of these peptides had any biological effect.

Animal models of diabetes

To test the effect of KRX peptides in a wide spectrum of syndromes similar to Type 2 diabetes, we used three animal models. The gerbil *Psammomys obesus* is normoglycaemic without genetic mutation [21] but prone to insulin resistance and diabetes on a high-energy diet. Zucker diabetic fatty (ZDF) rats are devoid of leptin receptor, obese, hyperglycaemic and hyperlipidaemic in their basal state. The other animal model used, *db/db* mice, is also devoid of leptin receptor and highly insulin-resistant. Specific experimental protocols are described in the Results. Dosages and frequency of administration were established after initial pilot studies. All experimental procedures were approved by the Hebrew University-Hadassah Medical School Institutional Animal Care Committee. All animals had free access to water and food.

Psammomys obesus. Female *Psammomys* gerbils were bred at the Hebrew University-Hadassah Medical School Animal Farm and housed in individual polypropylene cages. Animals from the diabetes-prone line [22] were weaned at 3 weeks of age and started on a low-energy diet (2.4 kcal/g, 16.7% protein; Koffolk, Petach Tikva, Israel). Animals were subsequently randomised to a high-energy diet (3.0 kcal/g, 23.4% protein; Tekled Global, Madison, Wis., USA) or kept on the low-energy diet. Animals on the high-energy diet with blood glucose

Kinase	Kinase inhibitor	KRX-683	αMSH	Inhibitor + KRX-683	Inhibitor + αMSH
EGFR	AG-1478	0.72	0.52	0.75	0.58
PTK	Genistein	0.72	0.5	0.73	0.57
PKC	**GF109203X**	**0.7**	**0.54**	**0.05**	**0.01**
P38	PD169316	0.72	0.63	0.7	0.5
PI3K	LY294002	0.7	0.57	0.71	0.5

Fig. 1. Intracellular penetration of KRX-683 and its effect on melanogenesis. **a.** B16F1 cells after 2 h incubation with biotinylated KRX-683$_{108}$ and staining with Avidin-FITC (top panel). The lower panel is a control for non-specific staining using non-biotinylated KRX-683. **b.** Melanin production (optical density) after incubating B16F1 cells for 3 days in various concentrations of KRX-683$_{124}$ and NDP-αMSH. Open triangle: 50 pmol/l MSH; open square: 25 pmol/l MSH; open diamond: 12.5 pmol/l MSH; open circle: 6 mol/l MSH; closed triangle: 0 pmol/l MSH. **c.** Effect of protein kinase inhibitors on melanogenesis stimulated by KRX-683$_{124}$ and NDP-αMSH. B16F1 cells were incubated with a kinase inhibitor and either KRX-683$_{124}$ (2.5 μmol/l) or NDP-αMSH (0.1 nmol/l) for 3 days

levels between 11.1 and 20 mmol/l during the course of a week (blood glucose levels tested at least three times) were included in the study.

Zucker diabetic fatty rats. Male homozygous ZDF rats were purchased from Charles River Laboratories (Wilmington, Mass., USA) and housed, two per cage, in polypropylene cages under specific pathogen-free conditions. The rats received a high-energy diet (same as for *Psammomys*). ZDF rats on the high-energy diet developed hyperglycaemia (>11.1 mmol/l) by 9 to 10 weeks of age [23].

C57 BL/Ks lepr^{db} (db/db) mice. Male homozygous *db/db* mice were purchased from Harlan, UK. The mice were housed five to ten per polypropylene cage under specific pathogen-free conditions and had free access to the high-energy diet. They developed hyperglycaemia (>11.1 mmol/l) by 12 to 15 weeks of age [24].

Animal treatments. All animals used in these experiments had their blood glucose and body weight monitored two to four times a week. They were injected intraperitoneally with either KRX-683$_{107}$ or KRX-683$_{124}$ at doses ranging from 5 to 10 mg/kg (see Results). The concentrations of the injected solutions were adjusted so that each *Psammomys* and ZDF rodent received 1 ml per injection and each *db/db* mouse 0.2 ml per injection.

Glucose was determined on blood taken from the tail vein using Glucometer Elite (Bayer, Elkhart, Ind., USA). Animals on the high-energy diet were considered diabetic if their non-fasting blood glucose was greater than 200 mg/dl. Triglycerides (fasting) were measured in ZDF rats one week before the end of the experiment using a commercial kit (Roche, Basel, Switzerland). Plasma insulin was radio-immunoassayed using anti-human insulin antibodies (DiaSorin, Saluggia, Italy) and human insulin as a standard.

Glucose tolerance tests were performed on animals after an overnight fast. Glucose at a dose of 1 g/kg was dissolved in 1 ml doubly distilled H$_2$O and administered intraperitoneally to *Psammomys* and ZDF rats. For *db/db* mice, 2 g/kg dissolved in 0.3 ml was injected. Blood glucose levels were monitored at 0, 0.25, 0.5, 1, 2, and 3 h after injection. At the end of the experiment, the animals were anaesthetised by diethylether and bled to death by abdominal aortic puncture. Immediately after death, liver, heart, kidneys and epididymal or white superscapular fat tissues were excised and weighed.

Statistical analysis

All values are given as means ± SE. Group comparisons were made by non-paired two-tailed Student's *t* test . A *p* value of 0.05 or less was considered significant. As the serum glucose values in ZDF rats at the beginning of the experiment were wide-ranging (11–22 nmol/l), the initial glucose levels at the onset of the experiment were normalised (100%), and the percentage differences between initial glucose and final glucose at the end of the experiment were calculated for each animal.

Results

Preliminaries. Studies were done on KRX-683$_{124}$ to determine the relationship between concentration and effect using melanin production by B16F1 cells as a measure of efficacy. In the absence of NDP-αMSH, a classic concentration–response curve was generated

Table 2. Effect of KRX-683 treatment on blood glucose and body weight in *Psammomys obesus*

Cohort	n	Body weight gain (g)	Responding animals, n	Mean blood glucose at start (mmol/l)	Mean blood glucose at end (mmol/l)	Decrease in glucose (%)
KRX-683$_{107}$	10	10±2	6	19.2±0.6	12.0±2.5	39 *
Responders	6	9.9±4		18.0±0.7	6.9±2.2	62 *
Non-responders	4	12±2		20.7±0.7	19.8±1.8	4
TBI Vehicle	12	19±1	0	18.4±1.2	17.8±0.9	3
Non-treated	5	14±2	0	19.2±1.3	20.0±2.1	−4
Unrelated peptide	8	17±6	0	19.1±1.2	18.3±0.9	4
KRX-683	11[a]	32±5[b]	10	16.8±0.8	4.7±0.4	72 *
SHS Vehicle	7	54±5[b]	0	17.6±0.7	17.5±1.1	0

[a] One of the animals, which became insulinopaenic, was not included in the calculations. [b] These groups of animals were in a stage of rapid growth. * $p<0.05$ compared with vehicle-treated control animals

(Fig. 1a). When increasing concentrations of NDP-αMSH were added to the culture media, basal levels of melanogenesis increased as expected (Fig. 1b). Subsequent addition of KRX-683$_{124}$ increased melanin production in an additive manner with a decrease in the EC$_{50}$. At high levels of NDP-αMSH, the effect of KRX-683$_{124}$ is diminished with only minimal increases in melanin production. The most potent peptides, KRX-683$_{124}$ and KRX-683$_{107}$, were selected for our further studies of the antidiabetic effect of a modulator of GRK2/3 signalling.

Protein kinase C inhibition reverses the effect of KRX-683. To determine the signal transduction pathway that is involved in mediating the effect of KRX-683$_{124}$, a variety of specific kinase inhibitors were incubated in the presence of either KRX-683$_{124}$ or NDP-αMSH and the melanogenic response was measured. GF109203X, an inhibitor of protein kinase C (PKC), abolished melanogenesis stimulated by KRX-683$_{124}$ or NDP-αMSH (Fig. 1c). Taken together, the data indicate that PKC is required for KRX-683$_{124}$ and NDP-αMSH to induce melanogenesis, and that the site of PKC signal transduction in melanogenesis is downstream of GRK2/3 and the MCR.

Effect of KRX-683$_{107}$ on hyperglycaemic Psammomys. To determine whether GRK2/3-derived peptides have an effect on Type 2 diabetes, we tested two of the more active peptides, KRX-683$_{107}$ and KRX-683$_{124}$. Diabetic *Psammomys* were administered KRX-683$_{107}$, 12.5 mg/kg i.p. for a period of 4 weeks (four injections). At the end of the treatment period, the average blood glucose of the KRX-683$_{107}$-treated group had declined by 35% ($p\leq0.027$) (Table 2) in comparison with the vehicle-treated control group, in which the mean blood glucose level did not change during the treatment period. When individual animals were analysed, the animals clustered into one of two discrete groups as shown in Table 2 and Figure 2. A majority of diabetic animals (6 of 10; 60%) had a rapid drop (within first week) in blood glucose to near normal

levels (6.9 mmol/l). The second subgroup (4 of 10; 40%) did not respond throughout the trial and remained diabetic. The effect of this peptide appears to be specific, as none of the untreated animals (vehicle injection, unrelated peptide injection, or no treatment) had a reduction in blood glucose level (Fig. 2a, b). This experiment was repeated and identical response rates were obtained (6 of 11 treated vs 0 of 9 control animals after 3 weeks treatment).

The diabetic phenotype of *Psammomys* is associated with insulin resistance that is characterised by hyperinsulinaemic hyperglycaemia and an abnormal glucose tolerance profile. To test whether KRX-683$_{107}$ affected these parameters, we performed glucose tolerance tests before and after treatment with KRX-683$_{107}$ and measured non-fasting glucose levels. Prior to treatment, all diabetic animals had abnormal blood glucose levels after a glucose load, with an average blood glucose after 3 h of about 1.7 mmol/l for all animal groups. After treatment, the mean blood glucose of the animals treated with KRX-683$_{107}$ was significantly lower than that of the different control groups both at 2 and at 3 hours after the glucose load.

Analysis of individual animals in the vehicle-treated control group showed that seven out of ten had normal glucose levels after 3 h vs two out of ten of the vehicle-treated control and none of the animals of the other control groups. The same KRX-683$_{107}$-treated animals with normal random glucose levels after four weekly injections also had normal results in a 3-h post-glucose load glucose tolerance test (Fig. 3a). With regard to insulin levels, six out of ten of the KRX683$_{107}$-treated animals had insulin levels similar to non-treated, non-diabetic controls (Fig. 3b).

To determine whether KRX-683$_{107}$ affects the obesity phenotype in *Psammomys* fed on the high-energy diet, the change in total weight during the course of the experiment and organ weights at the end of treatment were measured. Total weight gain in *Psammomys* during the weekly KRX-683$_{107}$ peptide administration compared with animals treated with the TBI vehicle is shown in Table 2. Weight gain was slower
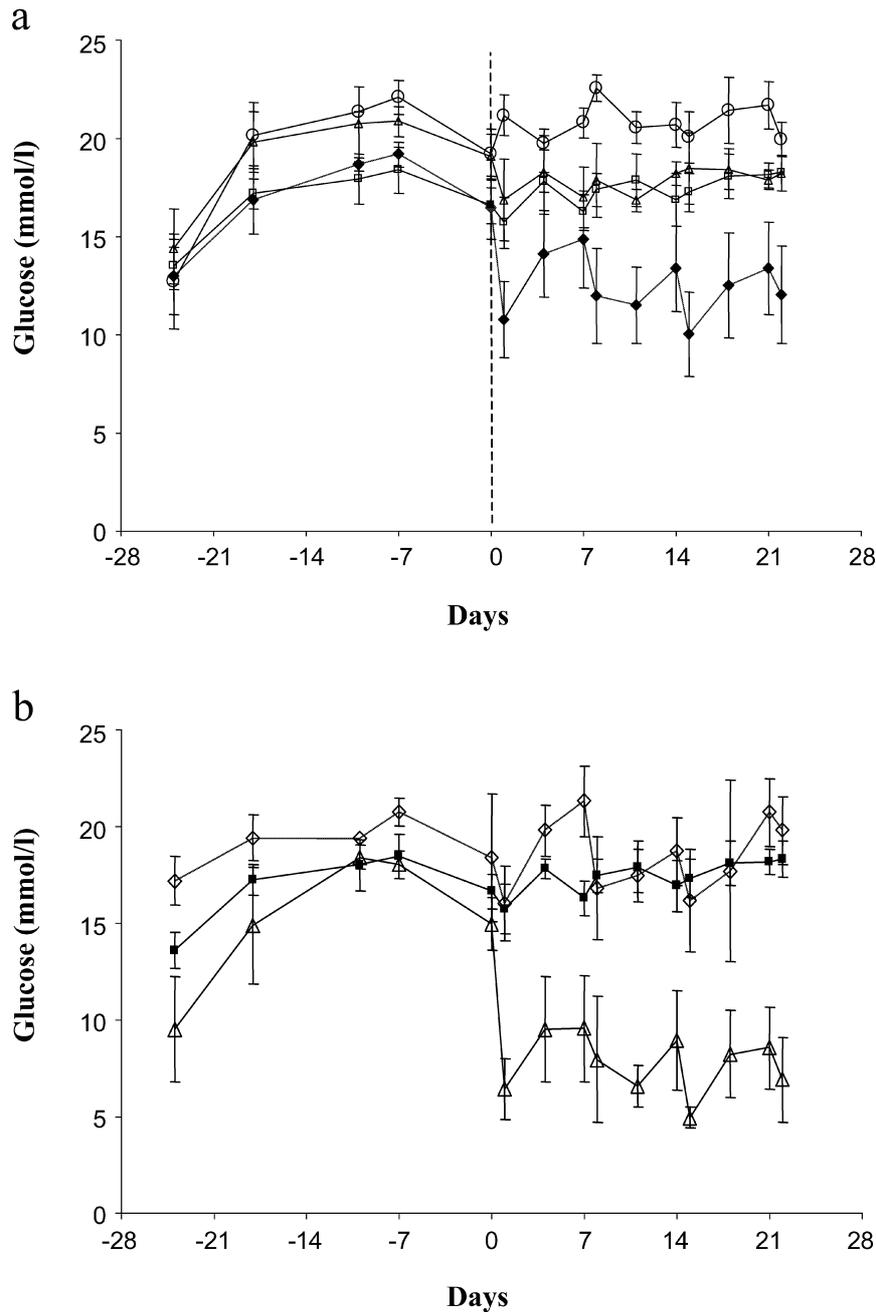
Fig. 2. KRX-683$_{107}$ reduces mean blood glucose level in diabetic *Psammomys*. **a.** Diabetic *Psammomys* treated with either four weekly injections of KRX-683$_{107}$ 12.5 mg/kg (filled diamond), vehicle control (open square), or an unrelated peptide KRX-123$_{302}$ (open triangle). A fourth group received no treatment (open circle). **b.** KRX-683$_{107}$ responders (open triangle), non-responders (open diamond) vs control vehicle (filled square) during four weeks of treatment

Table 3. Adipose tissue weight (g) following KRX-683 treatment

Adipose tissue	Treatment				
	KRX-683$_{124}$	Control SHS	KRX-683$_{107}$	Control TBI	KRX-123$_{302}$
Psammomys, superscapular	1.35±0.07[a]	2.00±0.18	1.58±0.16[a]	2.11±0.11	2.17±0.09
ZDF rats, epididymal	2.34±0.12	2.35±0.04	2.16±0.30	2.20±0.09	
db/db mice, superscapular	5.16±0.50	6.10±0.20			

[a] $p \leq 0.05$ compared with vehicle-treated controls

a

Glucose 3 h post GTT onset

b
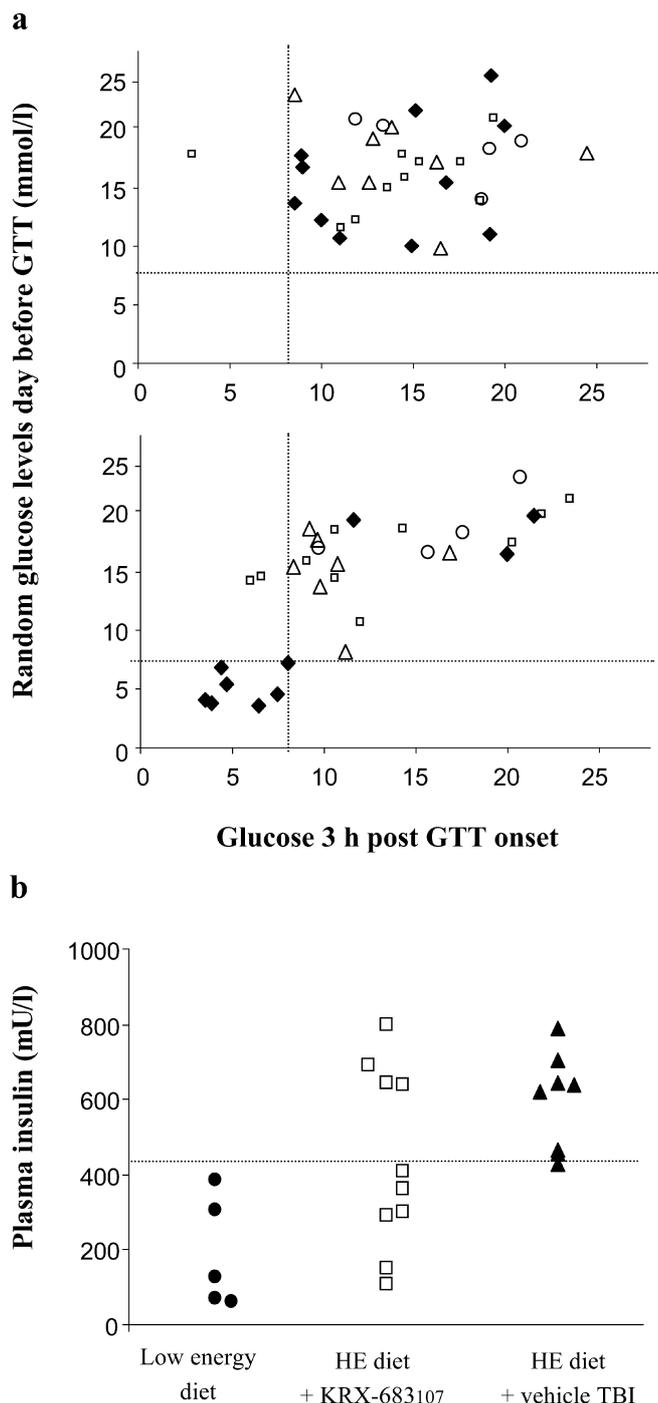
Fig. 3. Relationship (**a**) between random and 3-h glucose tolerance tests (GTT) in diabetic *Psammomys* before (above) and after four weeks of treatment with KRX-683$_{107}$ (below). Filled diamond: KRX-683$_{107}$-treated animals (*n*=11); open square: vehicle-treated animals (*n*=10); open circle, non-treated animals (*n*=5); open triangle: animals treated with the non-related peptide, KRX-123$_{302}$. (*n*=7). **b.** Insulin levels after treatment with KRX-683$_{107}$ in the fed state comparing KRX-683$_{107}$-treated animals (open squares, high-energy diet, *n*=10) to vehicle-treated diabetic animals (filled triangles, high-energy diet, *n*=8) and non-diabetic animals (filled circles, low-energy diet, *n*=5). The vertical and horizontal dotted lines represent the upper limit of normoinsulinaemic *Psammomys* in the fed state. HE, high-energy

in the KRX-683$_{107}$-treated *Psammomys* during the experiment than in animals treated with vehicle. At the end of the experiment, the mean weight gain of the treated animals was 52% lower than that of controls (*p*≤0.012). There was a significant decrease in the mean weight of the superscapular fat pad in the treated animals after treatment with KRX-683$_{107}$ (Table 3). There were no significant differences in the average weight of the heart, kidney and liver between the two groups (data not shown), indicating that the decrease in overall and fat pad weight in the treated group was not related to an anorectic effect of KRX-683$_{107}$.

Effect of KRX-683$_{124}$ on hyperglycaemic Psammomys. Changing the amino acid and fatty acid composition of KRX-683 alters its physiochemical characteristics. To test whether these changes were also associated with a physiological effect on glucose homeostasis, we tested KRX-683$_{124}$ (which is one amino acid longer and has a shorter fatty acid moiety) in a similar way to the way that KRX-683$_{107}$ was tested. *Psammomys* on a high-energy diet received KRX-683$_{124}$, 12.5 mg/kg once weekly or 6.25 mg/kg twice weekly for 7 weeks. As shown in Figure 4 and Table 2, there was a significant reduction in average non-fasting blood glucose in animals treated once weekly with 12.5 mg/kg KRX-683$_{124}$, as compared with pre-treatment glucose values (4.7±0.4 vs 16.8±0.8 mmol/l, *p*≤0.0001). The vehicle-treated group maintained the same mean blood glucose throughout the experiment. Of the eleven treated animals, ten had glucose values restored to near-normal, whereas 100% of the vehicle-treated group remained diabetic (mean glucose 17.5±1.1 mmol/l). The single treated animal that did not respond had evidence of insulinopaenic diabetes (e.g. extreme weight loss, high fasting glucose). The hyperglycaemic response was less impressive in animals receiving 6.25 mg/kg twice weekly. Only four of nine treated animals responded, indicating that a higher dose, even when administered less frequently, was more effective (Fig. 4). The response to KRX-683$_{124}$ was not uniform as some animals responding promptly, and others did not respond until 4 to 6 weeks after treatment began.

Effect of KRX-683 peptides on hyperglycaemic ZDF rats. We tested whether KRX-683$_{124}$ is effective in treating other forms of experimental Type 2 diabetes in ZDF rats. The results of treatment of ZDF diabetic rats with KRX-683$_{124}$ are presented in Figure 5a. Blood glucose concentrations in untreated ZDF rats on a high-energy diet increased from a mean of 10 mmol/l at the age of 10 to 11 weeks to 16.7 mmol/l at the age of 17 to 18 weeks. Same-aged ZDF rats that received KRX-683$_{124}$ peptide for a period of 5 weeks at doses increasing from 10 to 30 mg/kg did not show the same increase (Fig. 5a). As glucose levels in these animals at the start of the experiment were markedly
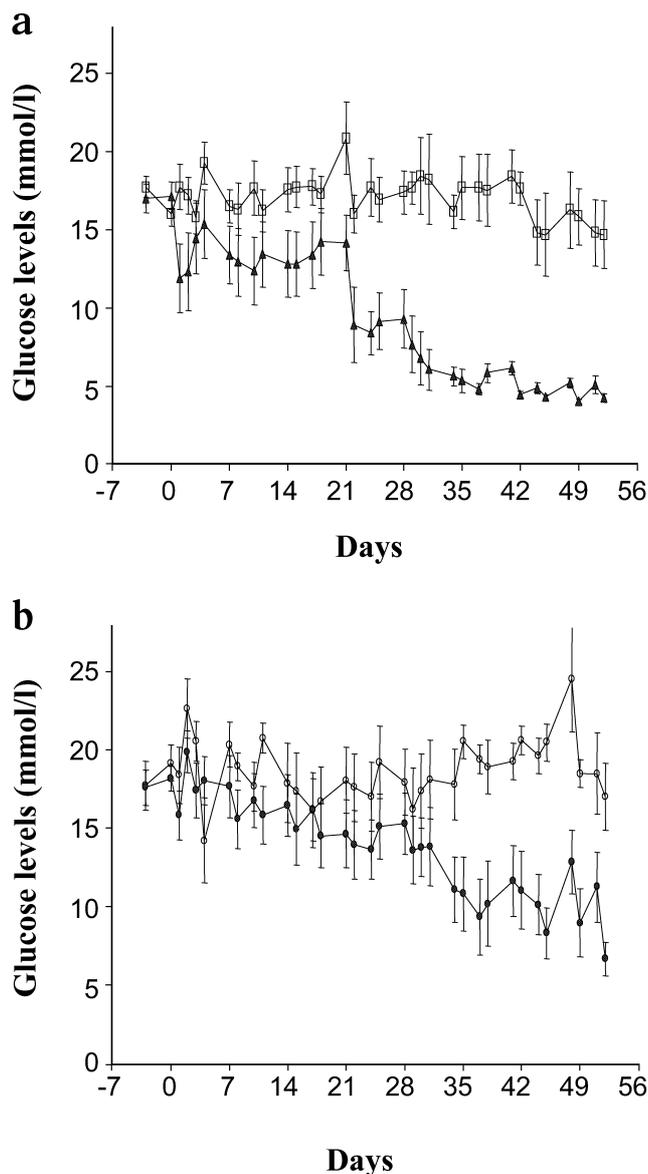
Fig. 4. Effect of different KRX-683$_{124}$ dosing regimens on glucose levels in diabetic *Psammomys*. **a.** Animals received either KRX-683$_{124}$, 12.5 mg/kg weekly, for 7 weeks (filled triangles, n=11) or vehicle control (open squares, n=11). **b.** *Psammomys* treated either with KRX-683$_{124}$, 6.25 mg/kg twice weekly, for 7 weeks (filled circles) or with vehicle (open circles)

heterogeneous, the percent increase in glucose for each animal during the trial was assessed. The non-fasting blood glucose levels of the KRX-683$_{124}$ treatment group was 31% lower than the control group (p≤0.048) 30 days after treatment began. Howerver, no acute drop to normal occurred as in *Psammomys* (Fig. 2b). Animal weights at the end of the protocol did not differ between the treated and untreated group (data not shown), suggesting that the improvement in blood glucose was not related to decreased food intake or altered metabolic rates. In contrast to *Psammomy*s, there was also no change in the superscapular fat pad between treated and non-treated ZDF rats (Table 3).

Other parameters related to diabetes also changed after administration of KRX-683$_{124}$. There was a significant reduction in fasting triglyceride levels after 4 weeks of KRX-683$_{124}$ treatment, compared with the control group (4.9±0.6 vs 6.4±0.4 mmol/l respectively, p≤0.012) (Fig. 5b). Both fasting and the 3-h blood glucose levels after glucose tolerance tests were significantly lower in the KRX-683$_{124}$ animals (38% for fasting blood glucose, p≤0.028; 30%, p≤0.027, for the 3-h post-glucose tolerance tests) (Fig. 5c).

KRX-683$_{124}$ improves fasting and post-prandial glucose levels in hyperglycaemic db/db mice. KRX-683$_{124}$ was also tested in *db/db* mice receiving 20 mg/kg of KRX-683$_{124}$ for 5 weeks. While there was no difference in random non-fasting blood glucose levels between KRX-683$_{124}$-treated and vehicle-treated animals, there were significant differences between the two groups in the glucose tolerance tests. Both the fasting (4.0±0.2 vs 5.9±0.5 mmol/l respectively, p≤0.001) and 2-h blood glucose levels (9.2±1.2 vs 17.0±1.4 mmol/l, p≤0.0004) were lower in the KRX-683$_{124}$-treated animals (Fig. 6). As with the ZDF rats, KRX-683$_{124}$ had no effect on the overall weight gain or the epididymal fat pad mass of these mice (Table 3).

Discussion

Overview. We evaluated the physiological effect of short peptides derived from GRK2/3 on glucose homeostasis. The rationale for using GRK2/3-derived peptides is that this kinase is involved in multiple metabolic processes including those involved in energy balance. These peptides could potentially modulate the activity of GRK2/3 by interfering with kinase-substrate interactions. Two of the peptides, KRX-683$_{107}$ and KRX-683$_{124}$, were shown to stimulate the allegedly GRK-controlled response of melanogenesis, in a defined cell-based assay, via a PKC-dependent pathway. In three models of Type 2 diabetes the two KRX-683 peptides fully or partially reversed the diabetic phenotype in most of the treated subjects, reducing non-fasting blood glucose levels and improving insulin and triglyceride levels associated with Type 2 diabetes.

Role of GRK2/3 in glucose homeostasis. The GRK family of protein kinases plays an important role in regulating the activity of GPCRs. GRK2 and GRK3 regulate the activity of numerous GPCRs primarily by uncoupling the receptor from its associated G proteins. These kinases phosphorylate the serine/threonine located within the cytosolic carboxy terminal portion of GPCRs, when the latter are in their active conformation [25]. The role of GRK2 in pathophysiological processes such as congestive heart failure and

a
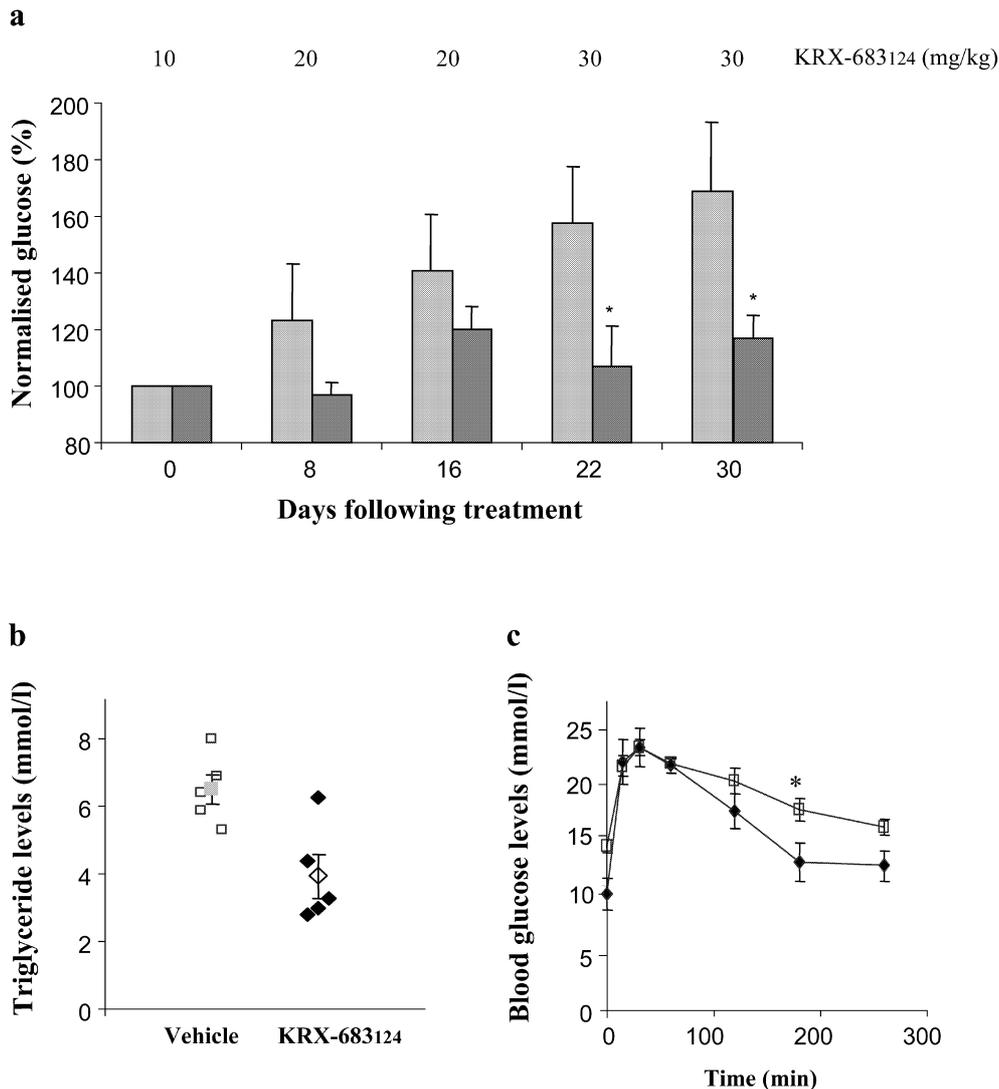
KRX-683₁₂₄ (mg/kg)

Fig. 5. Antidiabetic effect of KRX-683₁₂₄ in Zucker diabetic fatty (ZDF) rats. **a.** Reduced fed glucose levels in ZDF rats treated with increasing doses of KRX-683₁₂₄ for 1 month vs vehicle-treated control rats. Glucose normalisation is based on pre-treatment levels (see text). **b.** Triglyceride levels after 30 days of treatment. KRX-683₁₂₄ treatment (filled diamonds); control animals (open squares). **c.** Glucose tolerance tests curves of treated (filled diamonds) and control animals (open squares)
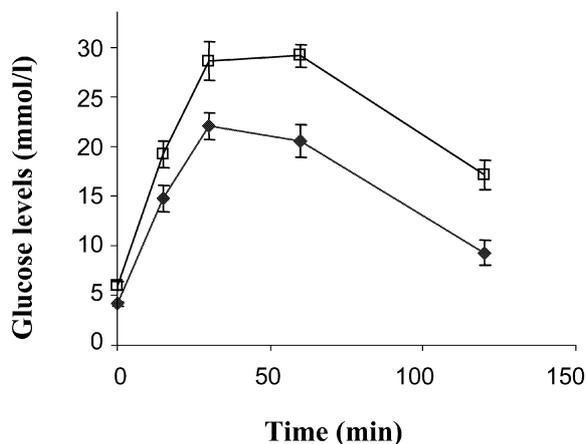
Fig. 6. Effect of KRX-683₁₂₄ on fasting and 3-h glucose tolerance tests in *db/db* mice which received KRX-683₁₂₄, 20 mg/kg weekly, for 5 weeks (filled diamond) or vehicle control (open square)

hypertension has been identified [11]. In the heart, up-regulation of GRK2 contributes to heart failure which is reversible when GRK2 is inhibited [17]. In mice lacking GRK3, airway smooth muscle were hyper-responsive to cholinergic agonists and baroreflex-induced tachycardia was accentuated [26].

The role of GRK2 and GRK3 in glucose homeostasis has not been extensively studied. It is known that the β2-adrenergic receptor is down-regulated by GRK2 and this receptor subtype has a significant role in regulating lipid metabolism in adipocytes [27, 28, 29]. In people with insulin resistance, there is a decrease in lipolysis due to a reduction in β2-adrenergic receptor in fat cells. Alterations in the function of β2-adrenergic receptor correlated with glucose intolerance and insulin resistance [30]. Polymorphisms located within β2-adrenergic receptor have been associ-

ated with obesity in various ethnic groups [31]. One mutation known to decrease receptor density and efficiency is *Arg16Gly*, which is associated with longitudinal increases in weight gain in a large cohort of male children [32]. In other studies, the same mutation has been associated with a greater relative risk for developing Type 2 diabetes [33]. The alteration in fatty acid metabolism associated with polymorphisms of the β2-adrenergic receptor makes this gene a possible "thrifty gene" candidate. One of the fundamental tenets of the "thrifty gene" hypothesis is that in energy-poor environments, organisms that have a lower threshold for insulin secretion and a lower capacity for insulin clearance and are therefore more efficient in storing fat will have a greater chance of survival. These advantages turn into a disadvantage in energy-rich environments, in which excess lipogenesis leads to insulin resistance and Type 2 diabetes [34, 35].

GRK2 may also regulate appetite and regulators of fat metabolism. In the central nervous system, α-MSH, via its interaction with MCR-4, has an anorectic effect and regulates insulin secretion in response to glucose levels. When the interaction between α-MSH and MCR-4 is blocked, as occurs in the agouti mouse phenotype, the animals have obesity and glucose intolerance [36]. The same phenotype is also seen in the MCR-4 knock-out model [37]. A recent study has shown that GRK2 is one of the mediators of MCR-4 desensitisation. This study also suggested that abnormally elevated MCR-4 desensitisation by GRK2 may be associated with obesity and appetite dysregulation [16].

Sequence-based inhibition of GRK2/3. A method for modulating the activity of a protein kinase is to use peptides that contain sequences that are identical or complementary to specific regions within the target protein [38, 39, 40]. The presumed mechanism of action of these sequence-based peptides is to interfere with the interaction between the kinase and its specific substrate [19]. In the case of GRK2, relatively long peptides complementary to the C terminus of the kinase have been successfully introduced into myocytes using viral vectors containing DNA sequences encoding for the peptide. This peptide inhibits the endogenous activity of GRK2 and reverses GRK2-mediated heart failure [41]. To avoid the need for a relatively long peptide or the requirement of transfection, we developed an alternative approach of coupling fatty acid moieties to short synthetic peptides derived from the HJ-loop of GRK2/3, a kinase-substrate interaction site that has been identified in protein kinases [19].

To test efficacy of specific peptides derived from the HJ loop of GRK2/3, a melanogenesis assay was used. Melanogenesis is mediated by GPCRs, including MCR-1 and endothelin-1 receptors [42, 43]. The endothelin receptor is known to be regulated by GRK2, and at least two of the closely related MCRs (MCR-2, MCR-4) are modulated by phosphorylation mediated by GRK2 [15, 16, 44]. In addition, PKC, which also plays a role in melanogenesis, is inactivated by GRK2 in specific tissues [45]. KRX-683$_{124}$ stimulated melanin production in the absence of either α-MSH or endothelin in the culture media. In the presence of α-MSH, the effects of KRX-683$_{124}$ were additive. Both the effect of KRX-683$_{124}$ and α-MSH on melanogenesis were abolished when a PKC inhibitor, GF109203X, was added, suggesting that KRX-683 acts upstream to PKC in terms of its effects on melanogenesis. It is probable that KRX-683$_{124}$ disturbs the interaction of GRK2 with PKC, thereby attenuating the inhibitory effect of GRK2 on PKC-mediated melanogenesis. Alternatively, KRX-683$_{124}$ may enhance the basal activation of the endothelin and MCR receptors by blocking their interaction with GRK.

In vivo effects of GRK2/3 inhibition. The nutritionally induced diabetes in *Psammomys obesus* is analogous to forms of Type 2 diabetes seen in several human population groups such as the Native American Pima Indians and the Australian Aborigines [22]. The course of diabetes in those animals is characterised by an initial stage of hyperinsulinaemia, followed by hyperinsulinaemic hyperglycaemia and a hypoinsulinaemic phase associated with ketosis and ultimate death. Diabetes evolving after provision of a high-energy diet in *Psammomys* has been associated with the "thrifty metabolism" which utilises innate insulin resistance to survive in a nutrient-poor desert environment. Treatment of *Psammomys* with KRX-683 resulted in a reduction of non-fasting blood glucose and insulin levels, and also of weight (fat pad and total animal mass). The fact that both insulin levels and fat pad were reduced suggests that the mechanism of action of KRX-683 is to increase insulin sensitivity leading to enhanced glucose uptake by muscle, and reduced lipogenesis in adipocytes. Such an effect would be consistent with up-regulation of β2-adrenergic receptor which induces lipolysis and reduces insulin resistance.

The antidiabetic effect of the two KRX-683 peptides could be non-specific, related to a non-selective appetite suppression. Such an effect would be consistent with the lower weight of the *Psammomys*. However, if the effect of the drug was only on appetite, organ weight, e.g. the heart, would also be expected to be reduced in ZDF rats and *db/db* mice. This was not the case, for no such weight loss was seen in ZDF rats or *db/db* mice. In addition, related peptides of a similar size had no effect on glucose homeostasis or weight, suggesting that the effect of the KRX-683 peptides were sequence-specific.

The lack of response in some of the *Psammomys* animals to the KRX-683 peptide raises the issue of in-

dividual animal sensitivity to the effects of the drug, which occurred with all compounds tested. As *Psammomys* are not a completely uniform population, it is possible that the level of insulin resistance varied among animals, and that some animals would require higher doses of the drug for an overt effect. Similarly, some of the *Psammomys* may have progressed further towards developing hypoinsulinaemic hyperglycaemia, and KRX-683 would not be expected to have an effect on glucose homeostasis in the absence of endogenous insulin. In this regard, further studies are necessary to determine the dose-response effect of KRX-683 as it relates to the level of insulin resistance and the endogenous insulin reserve.

Recently, it has been found that diabetes prone *Psammomys* overexpress PKC-ε even before the onset of overt hyperglycaemia [46]. PKC-ε, which is activated by diacylglycerols, is associated with muscle insulin resistance in several models of Type 2 diabetes, and it has been postulated that both the high glucose and high triglyceride levels contribute to the activation of this PKC isoenzyme [47]. Even in the pre-diabetic stage there are physiological alterations in energy metabolism that are unique to the *Psammomys* ("thrifty-gene" hypothesis) and that cause a rise in PKC-ε when these animals are provided with a caloric load inappropriate for their metabolic capacity [21, 46].

KRX-683 also had an antidiabetic effect on the ZDF and *db/db* models of diabetes. In the case of ZDF rats, treatment with KRX-683$_{124}$ resulted in a lowering of fasting and post-prandial blood glucose, associated with reduced triglyceride levels. However, animal weight in these hyperphagic animals was not reduced. It should be noted that the glucose response in these genetic mutants was not as impressive as in the *Psammomys*, and the glucose levels did not return to normal. Possible explanations for this may be related to the natural history of diabetes in these animals. Like the *Psammomys*, these animals experience beta cell apoptosis which is mediated by elevated lipid and/or glucose levels [46, 48]. In the experiments described above, it is possible that extensive apoptosis had already occurred, so that even though triglyceride levels improved markedly, damage to the pancreas was already considerable. Such a phenomenon was also described in ZDF rats treated with the peroxisome proliferator-activated receptor-γ agonist, rosiglitazone, which did not reverse diabetes in ZDF rats with established hyperglycaemia, but did prevent progression from insulin resistance to overt diabetes [49]. Thus, it may be necessary to initiate treatment with KRX-683 at an earlier stage, before the start of the high-energy diet.

KRX-683$_{124}$'s antidiabetic effect in *db/db* mice is demonstrated by an improved glucose tolerance tests profile (Fig. 6). In the *db/db* model of diabetes, the genetic defect is associated with a mutation in the leptin receptor [50]. These animals have severe insulin resistance, which is accompanied by insulin hypersecretion leading to obesity. Interestingly, KRX-683 partially reversed the diabetic phenotype induced by a defect in leptin-stimulated signal transduction. One possibility is that KRX-683 compensates for the loss of leptin-mediated stimulation of central MCRs that control both satiety and insulin secretion in response to glucose levels. However, there was no weight difference between the groups treated with KRX-683 and control groups treated with vehicle, which would be expected if KRX-683 did exert an effect on melanocortin-stimulated anorexia. It is more likely that KRX-683 affects insulin-mediated glucose uptake without affecting appetite or caloric intake.

Conclusion. Small peptides based on the kinase-substrate interaction site in GRK2/3 have an anti-diabetogenic effect in three different animal models of diabetes. The data presented suggest that GRK2/3 plays a significant role in glucose homeostasis, and modulation of this kinase can partially reverse the diabetic phenotype. Further research will determine the exact mechanism by which KRX-683 peptides inhibit GRK2/3, and also identify the specific GRK2/3-mediated pathways that regulate glucose metabolism.

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