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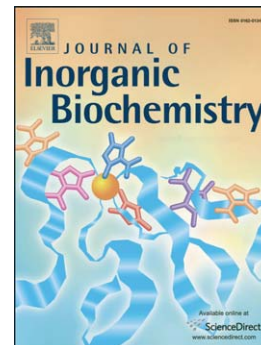
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PII: S0162-0134(13)00316-4
DOI: doi: [10.1016/j.jinorgbio.2013.11.005](https://doi.org/10.1016/j.jinorgbio.2013.11.005)
Reference: JIB 9426

To appear in: *Journal of Inorganic Biochemistry*

Received date: 7 August 2013
Revised date: 15 November 2013
Accepted date: 17 November 2013



Please cite this article as: N. Domingues, J. Pelletier, C.-G. Ostenson, M.M.C.A. Castro, Therapeutic properties of $\text{VO}(\text{dmpp})_2$ as assessed by *in vitro* and *in vivo* studies in type 2 diabetic GK rats, *Journal of Inorganic Biochemistry* (2013), doi: [10.1016/j.jinorgbio.2013.11.005](https://doi.org/10.1016/j.jinorgbio.2013.11.005)

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Therapeutic properties of VO(dmpp)₂ as assessed by *in vitro* and *in vivo* studies in type 2 diabetic GK rats

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Abstract

The bis(1,2-dimethyl-3-hydroxy-4-pyridinonato)oxidovanadium(IV), VO(dmpp)₂, has shown anti-diabetic effects by *in vitro* studies in Wistar (W) rat adipocytes and *in vivo* in obese Zucker rats. The aim of this work is to confirm the therapeutic properties of VO(dmpp)₂ in non-obese type 2 diabetic Goto-Kakizaki (GK) rats. An *in vivo* study was carried out, treating W and GK rats during 21 days with a daily dose of VO(dmpp)₂ (44 µmol/kg). It was shown that VO(dmpp)₂ doesn't affect the normal increase of body weight of both W and GK rats, after 8 days of treatment ameliorates glycaemia in GK rats (8.4 ± 0.3 vs 10.1 ± 0.2 mM in GK control, P<0.001) but doesn't interfere with glucose levels in W rats and, after 21 days of treatment, improves the glucose intolerant profile of GK rats (13.1 ± 0.5 vs 20.6 ± 0.7 mM/min in GK control, P<0.001), despite no increase of plasma insulin levels during glucose tolerance test. Additionally, it was demonstrated that VO(dmpp)₂ significantly enhances [³H]-glucose uptake by W and GK rat adipocytes (non-toxic concentration of 100 µM: respectively 193±20 and 254 ± 21 %, P <0.001, relative to the basal value) showing an efficacy similar to insulin 1.72 nM and better than the same concentration of BMOV (P<0.01). Western blotting revealed that in W and GK rats VO(dmpp)₂ significantly promotes IRS2 (P<0.05) and p-AKT expression (P<0.001 and P<0.05, respectively, relative to the respective controls) and in GK animals reduces the increase of PTP1β expression (P<0.001, relative to GK control).

Keywords: Type 2 diabetes, VO(dmpp)₂, Goto-Kakizaki rats, Glucose uptake by adipocytes, Glucose tolerance, Insulin signaling pathway

Introduction

Type 2 diabetes (T2D) is characterized by hyperglycaemia due to a combination of reduced insulin sensitivity in liver and extra hepatic tissues such as muscle and fat cells and impaired insulin secretion by the pancreatic β -cells [1-5]. Several oral anti-diabetic drugs are available, but suffer from long-term inadequate efficacy and a number of adverse effects [6,7]. Therefore, T2D still remains a major health concern all over the world being of the utmost importance to find new therapeutic approaches to treat diabetes. Intensive research has been carried out to synthesize new, more efficient and less toxic drugs and understand their mechanism of action to ameliorate diabetic features.

In the last two decades vanadium compounds (VCs) have attracted much interest due to their demonstrated pharmacological properties [8,9]. In particular, their potential insulin mimetic capacity has been extensively investigated [10-16]. It has been shown that VCs can be used to mitigate insufficient insulin response in DM thus presenting insulin-mimetic properties *in vitro* [17-20] *in vivo* [21-24] and two of them were tested in clinical trials [25]. To date, the main limitation for the clinical use of VCs in the treatment of diabetes has been their potential toxicity [26]. Most recently, renal changes detected in a three-month preclinical safety program required discontinuation of a VC development program that had already proceeded to early Phase IIa clinical testing [<http://www.medicalnewstoday.com/releases/136363.php>]. However, studies in this area continue to be pursued in an attempt to minimize the likelihood of toxic effects [27].

A large number of vanadium (IV and V) complexes have been synthesized, structurally characterized [28-31] and their biological activity has been tested using adequate cellular models by measuring glucose uptake levels [32,33], inhibition of free fatty acid release [33,34] and *in vivo* studies with diabetic rat models to test their capacity to reverse diabetic features [23,24,35,36]. Among the many vanadium compounds reported in the literature, emphasis is given to the bis(matolato)oxidovanadium(IV) (BMOV) [11,14,37] and the similar one bis(ethylmatolato)oxidovanadium(IV) (BEOV) [25], the only vanadium compounds tested in clinical trials up to date,

but other compounds like bis(1,2-dimethyl-3-hydroxy-4-pyridinonato)oxidovanadium(IV), [VO(dmpp)₂] [31,33,36-41], bis(picolinato)oxidovanadium(IV) [VO(pic)₂] [24,42,43] and bis(allixinato)oxidovanadium(IV) [18,44,45] have also attracted much interest due to the positive results which have been obtained through *in vitro* and *in vivo* studies.

In an attempt to interpret the insulin mimetic activity of VCs, in particular to investigate how they interfere in the insulin pathway and in glucose and lipids metabolism, studies have been carried out to find molecular targets for these compounds [16,29,46,47,48]. Nevertheless, the exact cellular mechanism of action of VCs appears to involve a combination of several post-receptor events in the insulin-signalling cascade. It has been demonstrated that protein tyrosine phosphatases (PTPases) play a complex role in the regulation of glucose-induced insulin secretion and increased expression and/or activity of a specific PTPase may contribute to impaired insulin sensitivity in several biological systems [49-51]. Vanadium salts and VCs have been shown to inhibit these enzymes [49,50,52], particularly some VCs inhibit phosphotyrosine phosphatase 1 β (PTP1 β) [50,52] within the insulin signalling cascade thus maintaining this enzyme in the phosphorylated state [27]. The mechanism of the insulin-mimetic action of VCs has been investigated in detail and some results have ruled out stimulation of phosphotyrosine kinases, important proteins of the insulin cascade [50,53-55]. Stimulation of glucose uptake via GLUT4 transporter has also been demonstrated for BMOV [56].

The VO(dmpp)₂ compound has been extensively studied for a structural characterization [30,31] and to investigate its therapeutic properties using adequate cellular models [33,37-40]. Recently, it was shown that it is able to restore normal glucose and lipid metabolism in an obese pre-diabetic animal model, the Zucker fatty rats [36]. These results demonstrated promising anti-diabetic and anti-obesity activity of this vanadium compound which showed to be more effective than BMOV [33]. The aim of the present work is to study the therapeutic properties of this vanadium compound in an animal model of T2D, the Goto-Kakizaki (GK) rats, to validate previous data and to provide some insights into the potential molecular mechanisms of its anti-diabetic

action. The GK rat is a non-obese substrain of Wistar (W) rat origin, developing T2D early in life. Glucose intolerance is most likely primarily due to impaired β -cell function on the background of a polygenic inheritance. In addition, secondary to chronic hyperglycaemia impaired insulin action may superimpose [57,58]. Adipocytes isolated from GK rats were used to characterize the effects of VO(dmpp)₂ on glucose cell uptake. The obtained results were compared with those with BMOV, using W rat adipocytes as a control. In addition, a chronic treatment of GK rats with VO(dmpp)₂ during 21 days was carried out to investigate the effects on glycaemia, plasma insulin levels and glucose tolerance. Furthermore, Western blotting was used to clarify the mechanism of action at the molecular level, looking for VO(dmpp)₂ targets in the insulin signalling pathway.

2. Materials and Methods

2.1. Animals

Male type 2 diabetic GK rats were bred at the Department of Molecular Medicine and Surgery (Karolinska Institutet, Stockholm, Sweden). Normal male W rats were purchased from a commercial breeder (Charles Rivers, Sulzfeld, Germany) and used as non-diabetic controls. All animals were kept at 22°C on 12/12-hour light/dark cycle with food and water available *ad libitum*. For the *in vitro* study, 10-12-week-old rats were used and for the *in vivo* study, the treatment was initiated in 6-week-old rats.

The present investigation was performed in accordance with the guiding principles in the care and use of animals (Laboratory Animal Ethics Committee of the Karolinska Institutet, N499/11).

2.2. Glucose uptake studies

The experiments were performed with adipocytes isolated from rat epididymal fat, digested during 120 min at 37°C with 0.25 mg/mL of type II collagenase (Sigma-Aldrich) in a Krebs-Ringer medium (139 mM NaCl, 5.4 mM KCl, 1 mM NaH₂PO₄, 1 mM MgSO₄, 2.2 mM CaCl₂, pH 7.4) buffered with 20 mM Hepes, containing 2% BSA (Bovine Serum Albumin) with 7 mM glucose. Isolated cells were obtained by filtration through a coarse nylon mesh (250 µm) before being washed twice with a 2% BSA buffer. After isolation, 1% adipocyte suspension were incubated for 2 h at 37°C with [3-³H]-glucose (1 µCi/mL, Perkin Elmer), 1 mM D-glucose solution (Sigma Aldrich) and VO(dmpp)₂ or BMOV in a range of concentrations from 10 µM to 750 µM. After the incubation, the vials containing the cell suspensions were transferred into ice to stop the reactions and 3 mL of scintillation cocktail (2 M PPO (2,5-diphenyloxazole) and 0.02 M POPOP (1,4-Bis(4-methyl-5-phenyl-2-oxazolyl)benzene), dissolved in toluene, Sigma Aldrich) was added to each vial at room temperature. The glucose uptake was determined by measuring the radioactivity of ³H-glucose incorporated in the *de novo* synthesized lipids, which is proportional to ³H-glucose taken up by the cells, with the Liquid Scintillator Analyzer (Tri-Carb 1900TR, Packard)

[60]. The same experiment was carried out with insulin concentrations ranging from 0.1-172 nM used as control of glucose uptake, as well as with 1.72 nM of insulin and 25 μ M or 250 μ M VO(dmpp)₂, to check the insulin enhancement effect of VO(dmpp)₂.

2.3. *In vivo* VO(dmpp)₂ treatment

The compound bis(1,2-dimethyl-3-hydroxy-4-pyridinonato)oxovanadium(IV), VO(dmpp)₂, was synthesized according to a published procedure [31]. Its purity and structure was confirmed by elemental analysis and spectroscopic data. A 3 mM VO(dmpp)₂ saline solution (0.9% NaCl) at pH 7.4 was prepared. This solution was filtered through a 0.2 μ m membrane and stored at 4 °C. In the chronic treatment, both GK and W rats were treated during 21 days with VO(dmpp)₂ by a once daily intraperitoneal (i.p.) injection at a dose, 44 μ mol/kg body weight [18]. For the acute treatment a single VO(dmpp)₂ dose of 44 μ mol/kg was administered to each animal 30 min before the experiment. A saline solution (0.9% NaCl) was injected as placebo in both GK and W rats to be used as control.

2.4. *Glycemia and body weight*

Blood samples for determination of glucose were taken after small tail incisions. Blood glucose levels were monitored by the glucose dehydrogenase method (Accu-Check Aviva, Roche Diagnostics) [61] every 2 days before injection of VO(dmpp)₂ or placebo. During the experimental period, body weight of all animals was measured daily.

2.5. *OGTT (Oral Glucose Tolerance Test)*

After 21 days of chronic treatment (at day 21), as well as 30 min after the acute treatment, OGTTs were performed in overnight fasted rats. Tail vein blood samples were collected at 0, 15, 30, 60, 90, 120 and 150 min after an oral gavage of glucose (3mg/g body weight, D-glucose, Sigma-Aldrich) and blood glucose levels were immediately measured by the glucose dehydrogenase

method [61]. In addition, blood samples collected at 0, 30 and 120 min after the glucose load were placed into ice-cold heparinised tubes, plasma was immediately separated by centrifugation (8000xg, 10 min, 4°C) and plasma insulin was quantified using a radioimmunoassay [62]. Glucose homeostasis was assessed by calculating the area under the curve (AUC) of plasma glucose levels.

2.6. Western Blotting

Proteins were extracted from 300 mg of frozen adipose tissue collected from the animals under study, using a RIPA lysis buffer containing 1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 1 mM NaF (Sigma-Aldrich), 1x protease inhibitors cocktail (Roche Diagnostics), 1x phosphatase inhibitors cocktail (Roche Diagnostics). Adipose tissue lyses were performed in a homogenizer tissue (PT-2000, Polytron, Kinematica AG). Denaturing samples were separated on SDS-PAGE and blotted onto nitrocellulose membranes (Sigma-Aldrich). After blocking with 5% fat-free milk, membranes were probed for IRS-2, AKT2, phospho-Thr308-AKT, PTP1B and GAPDH protein detection using appropriate antibodies: anti-IRS-2 (sc-8299, Santa Cruz), anti-AKT-2 (3063, Cell signalling), anti-phospho-Thr308-AKT (4056, Cell signalling), anti-PTP1B (sc-1718, Santa Cruz) and anti-GAPDH (3683, Cell signalling). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was used for detection: HRP-conjugated anti-rabbit (7074, Cell signalling). Proteins were visualized using an enhanced chemiluminescence procedure (34080, SuperSignal West Pito Chemiluminescent Substrate, Thermo Scientific) or (p90720, Immobilon Western, Chemulinescent HRP substrate, Millipore). Quantification was carried out using Luminescent Image Analyzer (Image Reader LAS-100 Pro v1.0, Fujifilm) and ImageJ software (v1.47b, National Institute of Health).

2.7. Statistical analysis

Data are expressed as means ± SEM. Student's paired t-test was used to evaluate the significance of changes of glucose uptake within a group. Two-way analysis of variance was followed by the

Student-Bonferroni multiple-range test to estimate the significance of differences between groups for the body weight, glycaemia during treatment and OGTT. One-way analysis of variance was followed by the Student-Bonferroni multiple-range test to estimate the significance of differences between groups for Area Under Curve (AUC) of blood glucose levels during OGTT. One-way analysis of variance was followed by the Student-Newman-Keuls multiple-range test to estimate the significance of differences between groups for Western Blot analysis. A value of $P < 0.05$ was considered as statistically significant. Data were analysed using GraphPad Prism (v5.0, GraphPad Software).

3. Results and Discussion

The anti-diabetic properties of VO(dmpp)₂ were evaluated by *in vitro* studies with primary GK rat adipocytes and *in vivo* studies in GK rats. The effect of this compound on some abnormal parameters indicative of diabetes was tested. Its capacity to improve glucose uptake by adipocytes was measured. Body weight and blood glucose levels of GK rats subjected to a daily administration of this compound during 21 days were periodically determined and at the end of the chronic treatment (day 21), an OGTT was also carried out as well as 30 min after the acute treatment.

3.1. *In vitro* studies with VO(dmpp)₂ in isolated rat adipocytes

The GK rats used in this study had significantly lower body weight than the W rats (286 ± 15 vs 336 ± 11 g, P < 0.001), in agreement with published data which reported that although an increase in the number of adipocytes occurs before weaning, a decreased body weight and lean mass is observed in the GK rats [63] and displayed hyperglycaemia (7.0 ± 0.2 vs 5.1 ± 0.2 mM, P < 0.001). Moreover glucose uptake was significantly impaired in adipocytes from GK rats when compared with W rats (81 ± 4 vs 121 ± 7 DPM/h, P < 0.001, DPM = disintegration per minute). The improvement of glucose uptake by adipocytes is an indication of anti-diabetic properties of a drug [33]. To confirm the therapeutic properties of VO(dmpp)₂ previously demonstrated by *in vitro* studies with W rat adipocytes [33], further experiments with GK rat adipocytes were performed to assess the capacity of this VC to promote glucose uptake. Parallel studies with the promising compound BMOV [14,33], as well as with VO(dmpp)₂ in the presence of insulin [33], were also carried out, with insulin as a control.

To establish the adequate concentrations of insulin, VO(dmpp)₂ and BMOV to be used in these studies, a dose response assay was carried out with different concentrations of these candidate drugs (Figure 1A). GK rats, due to their characteristic insulin resistance, present a significant lower response (P < 0.05) than Wistar rats to glucose uptake when stimulated with increasing insulin

concentrations (Figure 1 A). W and GK adipocytes also respond to increasing concentrations of VO(dmpp)₂ and BMOV, although a better response is obtained with the former compound (Figure 1.A). Moreover, these data indicate that both vanadium compounds decrease the differences in glucose uptake capacity between the diabetic and healthy rats.

Figure 1 (B,C) show the normalized values of glucose uptake percentage for W and GK adipocytes, in the presence of different concentrations of insulin and non-toxic concentrations of the VC [33], compared with the respective baselines (considered as 100%). The results showed that 100 μM VO(dmpp)₂ and 500 μM BMOV had effects on glucose uptake similar to those of 1.72 nM insulin, in adipocytes from both W and GK rats. The *in vivo* physiological concentration of insulin is approximately 1.72 nM and the highest response is achieved with 17.2 nM of insulin [33]. When adipocytes of W rats (Figure 1B) were incubated with VO(dmpp)₂, glucose uptake was enhanced 1.9 (100 μM: 193 ± 20%, P<0.01) to 3.2 times (500 μM: 322 ± 16%, P<0.001) compared to baseline (100 ± 9%), while glucose uptake induced by BMOV was only 1.1 times (100 μM: 111 ± 20%, not significant) to 1.5 times higher (500 μM: 153 ± 23%, P<0.05) than baseline. Thus, the effects of BMOV were significantly lower than those of VO(dmpp)₂ (100 μM P<0.01; 250 and 500 μM P<0.001). Similarly, VO(dmpp)₂ improved glucose uptake in adipocytes of GK rats (Figure 1C) 2.5 (100 μM: 254 ± 21%, P <0.001) to 4.2 times (500 μM: 424 ± 37%, P<0.001), while in the same concentrations, the BMOV improved only 1.5 (100 μM: 145 ± 26%, P <0.05) to 2.2 times (500 μM: 219 ± 37%, P <0.01) the basal glucose uptake (100 ± 8%). These findings demonstrate and confirm the previously published results [33] that VO(dmpp)₂ is more effective than BMOV, concerning induced glucose uptake levels, showing a behavior similar to that of insulin. Since insulin signalling is impaired in T2DM it is of great interest to find compounds acting through mechanisms that regulate glucose uptake in peripheral tissues

Studies of glucose uptake in the presence of VO(dmpp)₂ and insulin were also carried out (data not shown), to check if the effects of VO(dmpp)₂ and insulin are additive in GK rat adipocytes. Glucose uptake in the presence of 25 μM or 250 μM of VO(dmpp)₂, and in the

absence or presence of 1.72 nM insulin, did not show significant difference. These results indicate that VO(dmpp)₂ does not behave as an insulin enhancer, in agreement with previous published data [33].

3.2. Effect of VO(dmpp)₂ treatment on body weight, blood glucose levels and glucose tolerance profile

The *in vivo* experiments have assessed the effects of VO(dmpp)₂ on diabetic (GK) and non-diabetic (W) animals. Each group of animals had its own control group submitted to a placebo treatment. At day 0, GK rats had a significantly lower body weight than W animals (respectively, 148 ± 4 vs 200 ± 6 g, $P < 0.001$) (Figure 2A). After 21 days of treatment the body weight was 353 ± 4 and 233 ± 5 g for control Wistar and GK rats; 342 ± 6 and 225 ± 3 g for VO(dmpp)₂ treated Wistar and GK, respectively. Thus, a similar body weight gain was observed for placebo and VO(dmpp)₂ treated animals of each type, which indicates that VO(dmpp)₂ had no effect on the normal weight development of GK and W rats. VO(dmpp)₂ was shown to induce a decreased body weight gain in obese Zucker rats [36], in contrast with the lack of effect on the body weight of non-obese GK rats.

Chronic hyperglycemia was demonstrated in GK rats compared with non-diabetic W rats (Figure 2B) throughout the whole treatment period. At day 0, blood glucose concentration of GK rats was significantly higher (10.0 ± 0.3 mM, $P < 0.001$) when compared with W rats (7.9 ± 0.3 mM), in agreement with the hyperglycemic state of T2D. There were no significant differences in blood glucose levels between placebo and VO(dmpp)₂ treated W rats throughout the study (6.8 ± 0.2 and 6.6 ± 0.2 mM, respectively, at day 21) (Figure 2B). However, from the 8th day, a significant decrease was observed for VO(dmpp)₂ treated relative to placebo treated GK rats (8.4 ± 0.3 vs 10.1 ± 0.2 mM, $P < 0.001$), which was maintained until the last day of treatment (8.3 ± 0.1 vs 9.4 ± 0.2 mmol/l, $P < 0.05$). The values obtained for VO(dmpp)₂ treated Wistar and GK rats are statistically different ($P < 0.001$), but there is a significant difference between VO(dmpp)₂ treated

and non-treated GK animals from 8th to 21th day. Therefore, this chronic treatment with VO(dmpp)₂ reduced hyperglycemia in the diabetic animals, possibly by improving glucose uptake in adipocytes and other tissues.

The effect of VO(dmpp)₂ on the glucose homeostasis of W and GK rats was also investigated through an OGTT, which is currently used to assess clinical pre-diabetic and diabetic conditions [64,65]. The OGTT assesses glucose tolerance of the animal by measuring blood glucose levels for a period of time after the administration of a glucose load. In insulin sensitive animals, blood glucose concentration will drop after a certain period of time, restoring the normal glucose values. However, in conditions of impaired glucose metabolism, insulin resistance or T2DM, insulin signaling does not function properly and blood glucose concentration remains high for a longer period of time [66-68]. The OGTT results demonstrated that glucose was significantly better tolerated in W than in GK rats (Figure 3A, $P < 0.001$) and that the 21-day treatment with VO(dmpp)₂ improved significantly glucose tolerance in GK rats (Figure 3A, $P < 0.05$ or less; values of area under curve 20.6 ± 0.7 vs 13.1 ± 0.5 mM/min in placebo and VO(dmpp)₂ treated GK rats, respectively, $P < 0.001$, Figure 3B). Interestingly, this glucose-lowering effect by VO(dmpp)₂ was not seen in the W rats (8.7 ± 0.2 mM/min in placebo treated W rats and 8.0 ± 0.4 mM/min in VO(dmpp)₂ treated W rats) (Figure 3B), indicating that the compound does not appear to induce hypoglycemia in non-diabetic rats, but may interact specifically with pathological mechanisms in diabetes.

To further assess the effects of VO(dmpp)₂ on glucose homeostasis, plasma insulin levels in OGTT were evaluated, before glucose load (0 min), at the maximum effect of the overload (30 min) and 120 min after glucose load, to test the ability of the animal to respond to a rapid glucose challenge (Figure 3C-D). In W rats, in which VO(dmpp)₂ treatment did not affect glucose tolerance (Figure 3A-B), plasma insulin levels were significantly lower than in animals treated with placebo throughout the test (respectively, at 0 min: 7.1 ± 1.8 vs 20.7 ± 3.1 μ U/mL, $P < 0.01$; at 30 min: 29.8 ± 3.8 vs 50.9 ± 3.7 μ U/mL, $P < 0.05$; at 120 min: 7.6 ± 1.0 vs 21.2 ± 5.5 μ U/mL, $P < 0.05$) (Figure 3C).

This discrepancy can be explained by the increased glucose uptake induced by VO(dmpp)₂. However, the kinetics of plasma insulin during the OGTT remained the same. After 30 min, plasma insulin levels in W rats treated with placebo or VO(dmpp)₂ were significantly higher than their respective groups before the glucose challenge (20.7 ± 3.1 vs 50.9 ± 3.7 $\mu\text{U}/\text{mL}$, $P < 0.01$; 7.1 ± 1.8 vs 29.8 ± 3.8 $\mu\text{U}/\text{mL}$, $P < 0.001$, respectively) (Figure 3C). Plasma insulin concentrations decreased after 120 min compared to 30 min (50.9 ± 3.7 vs 21.2 ± 5.5 $\mu\text{U}/\text{mL}$, $P < 0.01$; 29.8 ± 3.8 vs 7.6 ± 1.0 $\mu\text{U}/\text{mL}$, $P < 0.001$, respectively) but were not significantly different from basal plasma insulin levels. Indeed, although blood glucose profiles during OGTT were similar in W rats treated with VO(dmpp)₂ or placebo, plasma insulin levels were lower in the former than in the latter group of animals. These results suggest an improvement in glucose uptake which could be by changing signaling pathway of insulin at the storage tissue.

In GK rats, VO(dmpp)₂ did not significantly decrease plasma insulin levels either at basal conditions and after 30 min of the glucose load (Figure 3D), compared with GK rats treated with placebo. A significant decrease in plasma insulin levels was found only at 120 min (Figure 3D, 45.0 ± 5.1 vs 26.9 ± 4.2 $\mu\text{U}/\text{mL}$, $P < 0.05$), suggesting an improvement in glucose tolerance observed during the OGTT (Figure 3A). As for W rats, the glucose overload induced, 30 min later, a significant increase in plasma insulin levels (Figure 3D) in placebo treated (37.8 ± 4.1 vs 53.5 ± 5.9 $\mu\text{U}/\text{mL}$, $P < 0.05$) and VO(dmpp)₂ treated (25.5 ± 3.8 vs 49.2 ± 2.4 $\mu\text{U}/\text{mL}$, $P < 0.001$) GK rats (Figure 3D). After 120 min of the glucose load, plasma insulin levels were not statistically different from the basal ones at 0 min, but the group treated with VO(dmpp)₂ showed a significant decrease when compared to 30 min. Importantly, VO(dmpp)₂ treated GK rats have shown plasma insulin levels similar to placebo treated W rats, demonstrating the efficiency of VO(dmpp)₂ to improve the glucose tolerance with less insulin-resistance in diabetic GK rats [58,59]. These results show that GK rats treated with VO(dmpp)₂ recover from a glucose load with a profile similar to W rats, presenting blood glucose values significantly lower than those from GK control. This provides clear evidence that VO(dmpp)₂ ameliorates the glucose intolerant profile characteristic of GK rats

and this effect occurs without any significant change in plasma insulin concentrations at 30 min of OGTT [69,70].

To evaluate the acute effects of VO(dmpp)₂ treatment in GK rats, an OGTT was carried out in animals which were submitted to a single dose of 44 µmol/kg (animal body weight) of VO(dmpp)₂ administered 30 min before the glucose load and the results were compared with GK rats control and those under a chronic treatment. The obtained data are shown in Figure 3E and demonstrate that a chronic treatment with VO(dmpp)₂ is more efficient than an acute one concerning recovery from a glucose load. The values of blood glucose levels in GK rats control are statistically different from those submitted to a VO(dmpp)₂ treatment ($P < 0.001$) and there is a significant difference between GK rats under an acute and chronic treatment ($P < 0.001$ at 60 min and $P < 0.05$ between 60-120 min after glucose load) although, at 150 min, these values are similar. Several hypothesis can be formulated to explain this finding: the improvement of pancreas function and consequently an increase in blood insulin concentration, as a response to a feeding state, after a long term treatment with VO(dmpp)₂; the increase of glucose uptake and regulation of glucose metabolism in adipocytes, muscle and others peripheral tissues, due to an effect on gene expression of key proteins involved in glucose homeostasis.

3.3. Effect of VO(dmpp)₂ treatment on insulin signaling pathway of adipose tissue

The decrease in plasma insulin levels at 120 min in GK rats treated with VO(dmpp)₂ suggests a more efficient and faster glucose uptake than in GK animals treated with placebo. During hyperglycaemia, the body regulates glucose homeostasis by increasing insulin secretion to improve glucose storage in the target tissues. In T2D, however, insulin-sensitive cells may be resistant to insulin. As shown in this study, GK rats have impaired glucose tolerance associated with the ineffectiveness of insulin stimulated pathway and, thus, decreased glucose internalization [18]. The treatment with VO(dmpp)₂ did not increase plasma insulin levels in GK rats in parallel with improvement of glucose tolerance. In VO(dmpp)₂ treated W rats a normal

glucose tolerance profile was observed despite the significant decrease in plasma insulin levels, suggesting that VO(dmpp)₂ improved glucose uptake at the insulin signalling pathway level.

One of the most important metabolic actions of insulin is to promote glucose uptake into adipocytes, skeletal muscle and others peripheral tissue. This is accomplished *via* activation of the PI3K/AKT signaling pathway and subsequent translocation of GLUT4 from intracellular storage vesicles to the plasma membrane [46,47,53,56,71-73]. Western blotting was used to assess the effect of VO(dmpp)₂ on the insulin signalling cascade in adipocytes, more specifically on four target proteins involved in glucose uptake. This will allow to identify key points of this cascade which are deregulated in insulin resistant cells [71] and may be affected by VO(dmpp)₂.

Insulin receptor substrate 2 (IRS2) is a protein directly involved in insulin cell stimulation, the first event beyond insulin receptor activation by tyrosine kinase that unleashes the transmission of intracellular insulin signals [73,74]. Chronic treatment of W and GK rats with VO(dmpp)₂ increased significantly the IRS2 expression compared with their respective controls treated with placebo (0.72 ± 0.07 vs 0.51 ± 0.04 , for W rats; $P < 0.05$ and 0.66 ± 0.05 vs 0.43 ± 0.03 , in GK rats, $P < 0.05$) (Figure 4A). Therefore, the increased IRS2 protein expression can explain the decrease of chronic hyperglycaemia and improvement of glucose tolerance. At first glance, these results suggest a higher sensitivity of the insulin receptor resulting in increased protein expression in adipose tissue. The protein tyrosine phosphatase PTP1 β inhibits the insulin receptor, preventing the activation of the insulin signalling pathway. Moreover, PTP1 β is known to be overexpressed in insulin resistant tissues and it is inhibited by different vanadium compounds, including BMOV [52]. In GK rats treated with placebo, the level of PTP1 β expression was significantly higher compared to W rats treated with placebo (0.62 ± 0.03 vs 0.47 ± 0.05 , $P < 0.05$) (Figure 4B), characteristics shared with other rodent models of insulin resistance [74, 75-77]. However, in the GK rats treated with VO(dmpp)₂, the PTP1 β expression decreased significantly compared to GK rats treated with placebo (0.34 ± 0.06 vs 0.62 ± 0.03 , $P < 0.001$) and W rats treated with VO(dmpp)₂ (0.47 ± 0.05 , $P < 0.01$) (Figure 4B). Therefore, the VO(dmpp)₂ alike other vanadium

compounds [33,76] inhibited the PTP1 β expression, thereby removing the inhibition of insulin receptor and promoting the activation of the insulin signalling pathway. Thus the reduction of PTP1 β expression may have contributed to improved insulin sensitivity in GK rats treated with VO(dmpp)₂. The ability of VO(dmpp)₂ to inhibit PTPase activity may be one of the mechanisms by which it exerts its insulin-mimetic action [75].

Another target of VO(dmpp)₂ can be the AKT activity [33]. To address the role of the AKT pathway in relation to alternate pathways, it is essential to show that activation of AKT alone is sufficient to mimic the effects of insulin [78] associated with VO(dmpp)₂. In this study, the modifications in the protein expression involved in the insulin signalling pathway did not affect the expression of AKT2. Indeed, the levels of AKT2 expression did not change after the *in vivo* VO(dmpp)₂ treatment and were not significantly different between W and GK rats (Figure 4C). The phosphorylated-AKT2 (p-AKT) has been implicated in insulin-regulated glucose uptake into fat cells by promoting the translocation of GLUT4 to the cell surface [56,78]. For this purpose, a Western blot analysis of the phosphorylated protein was assessed. A significant increase in the p-AKT expression was observed in W and GK rats treated with VO(dmpp)₂ when compared to their respective controls treated with placebo (2.54 ± 0.16 vs 1.55 ± 0.08 , $P < 0.001$ in W rats; 1.81 ± 0.14 vs 1.40 ± 0.07 , $P < 0.05$ in GK rats) (Figure 4D). However, the expression levels of this protein were significantly lower in GK rats treated with placebo (1.40 ± 0.07 , $P < 0.001$) or VO(dmpp)₂ (1.81 ± 0.14 , $P < 0.001$) compared to W rats treated with VO(dmpp)₂ (2.54 ± 0.16) (Figure 4D).

According to these data, VO(dmpp)₂ treatment in GK rats improved glucose tolerance and decreased the chronic hyperglycaemia after 8 days of treatment. These effects may be explained by the increase of the IRS2 expression, thus improving the insulin sensitivity in adipocytes, by the inhibition of the PTP1 β expression which exerts an inhibitory action on IRS2 and by the increase of p-AKT expression thus promoting glucose uptake by GLUT4 [71,72,78].

Conclusions

This work clearly demonstrates the *in vitro* and *in vivo* insulin mimetic effects of VO(dmpp)₂ in the GK rats, a T2D animal model. It is shown by the *in vitro* study that VO(dmpp)₂ improves glucose uptake in adipocytes, more effectively than BMOV. A chronic *in vivo* treatment with VO(dmpp)₂ decreases hyperglycaemia and improves glucose tolerance significantly, although a complete normalization during OGTT was not observed in GK rats. Moreover, there is a normal gain of body weight of both diabetic and control VO(dmpp)₂ treated rats, and this compound does not induce hypoglycaemia in non-diabetic W rats. In addition, a better behavior is observed in GK rats submitted to a chronic treatment than an acute one. All these effects can be explained by the direct action of VO(dmpp)₂ on key proteins of the insulin signaling cascade, by specifically increasing IRS2 expression and AKT phosphorylation and inhibiting PTP1β expression. The combined results here presented demonstrate the beneficial effects of VO(dmpp)₂ in GK rats, corroborating previous published data and showing the insulin mimetic activity of this compound. Thus, taking into account problems of toxicity associated with vanadium compounds, which should be minimized, VO(dmpp)₂ could be a good alternative for the T2D therapy.

Acknowledgements

The authors thank Prof. Dr. João Costa Pessoa and his Post-doc student Somnath Roy at the Technical University of Lisbon, Portugal, for the synthesis of the VO(dmpp)₂ compound.

This work was supported by Swedish Research Council, Swedish Diabetes Association and Karolinska Institutet. Julien Pelletier was supported by a grant from the Föreningen för diabetesforskningens främjande. Neuza Domingues was supported by an Erasmus grant during her stay in Karolinska Institutet.

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Legends

Figure 1: Glucose uptake induced by insulin, VO(dmpp)₂ and BMOV on Wistar and GK adipocytes. (A) Dose response curves of glucose uptake (in DPM/h, disintegration per min/h) on W (○, n=9) and GK (■, n=11) rats for (a) insulin (b) VO(dmpp)₂ and (c) BMOV stimulation. (B-C) Percentage of glucose uptake (relative to basal value of 100%) by W (n=9) and GK (n=11) adipocytes induced by different concentrations of insulin, VO(dmpp)₂ and BMOV. Data are shown as mean values ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs respective basal value; ^sP<0.05, ^{ss}P<0.01, ^{sss}P<0.001 vs respective 1.72 nM of insulin; ^tP<0.05, ^{tt}P<0.01, ^{ttt}P<0.001 vs respective 17.2 nM of insulin; ^{##}P<0.01, ^{###}P<0.001 vs the same concentration of VO(dmpp)₂.

Figure 2: Body weight (A) and blood glucose levels (B) of Wistar and GK rats submitted to placebo or VO(dmpp)₂ treatment for 21 days.

A) Values of rat body weight (g) assessed everyday during 21 days of treatment in W rats with placebo (□) and VO(dmpp)₂ (■) and in GK rats with placebo (△) and VO(dmpp)₂ (▲); B) Blood glucose levels (mM) measured every 2 days during placebo (□) and VO(dmpp)₂ treatment (■) in W rats; placebo (△) and VO(dmpp)₂ treatment (▲) in GK rats. Data are shown as mean values ± SEM (n=8). ^{###}P<0.001 vs GK rats; *P<0.05, **P<0.01, ***P<0.001 vs GK rats treated with placebo.

Figure 3: Glucose tolerance profile (A-B) and plasma insulin levels (C-D) after 21 days of treatment and glucose tolerance profile after an acute treatment (E) of W and GK rats with placebo or VO(dmpp)₂. A) Blood glucose values (mM) during an OGTT after 21 days of treatment in W rats with placebo (□) and VO(dmpp)₂ (■) and in GK rats with placebo (△) and VO(dmpp)₂ (▲); B) Values of Area Under Curve (AUC), expressed as mM/min, obtained from the curves of blood glucose concentration vs time after the glucose load in W and GK rats treated with placebo or VO(dmpp)₂, shown in Fig. 3A; C) Plasma insulin levels during an OGTT in W rats treated with

placebo or VO(dmpp)₂; **D**) Plasma insulin levels during an OGTT in GK rats treated with placebo or VO(dmpp)₂; **E**) Blood glucose values (mM) during an OGTT for the control (Δ , control, n=8), chronic (21 days, \blacktriangle , n=8) and acute (single dose of 44 μ mol/kg administered 30 min before the glucose load, \diamond , n=7) effects of VO(dmpp)₂ in GK rats.

Data are shown as mean values \pm SEM (n=8). ***P<0.001 vs W rats; [§]P<0.05, ^{§§}P<0.01, ^{§§§}P<0.001 vs GK rats treated with VO(dmpp)₂; #P<0.05, ##P<0.01, ###P<0.001 vs W rats (Panel A); ***P<0.001 vs W rats; ###P<0.001 vs respective control (Panel B); *P<0.05, **P<0.01, ***P<0.001 vs 0 min of the respective group; ##P<0.01, ###P<0.001 vs 30 min of the respective group; [§]P<0.05, ^{§§}P<0.01 vs respective control (Panel C-D); * P<0.05, **P<0.01 and ***P<0.001 vs GK control; [§]P<0.05 and ^{§§}P<0.01 vs GK rats under an acute treatment (Panel E).

Figure 4: Effects of VO(dmpp)₂ treatment on the insulin signalling pathway in adipose tissue of W and GK rats. Representative immunoblots and densitometry analysis of IRS-2 (A), PTP1 β (B), AKT-2 (C) and p-AKT (D) protein expression in adipose tissue from W and GK rats treated with placebo or VO(dmpp)₂. Data from densitometry analysis are shown as mean values \pm SEM (n=8). *P<0.05 vs W rats treated with placebo; ^{§§}P<0.01, ^{§§§}P<0.001 vs W rats treated with VO(dmpp)₂; #P<0.05, ###P<0.001 vs respective control.

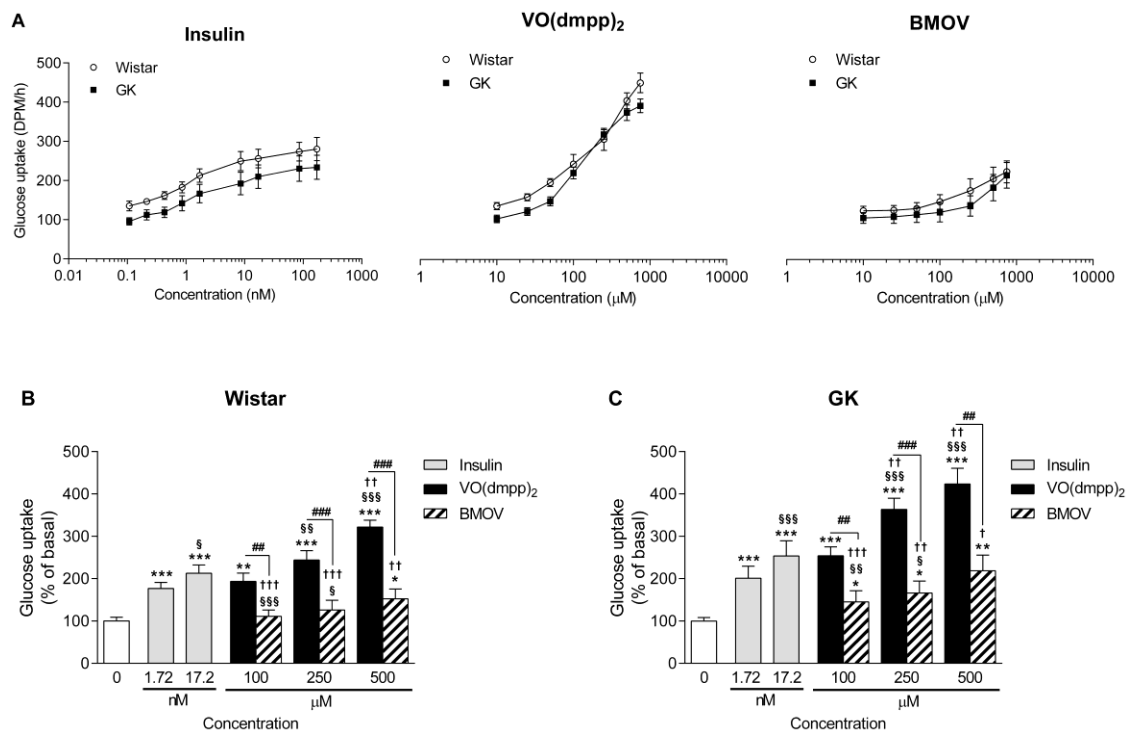


Fig. 1

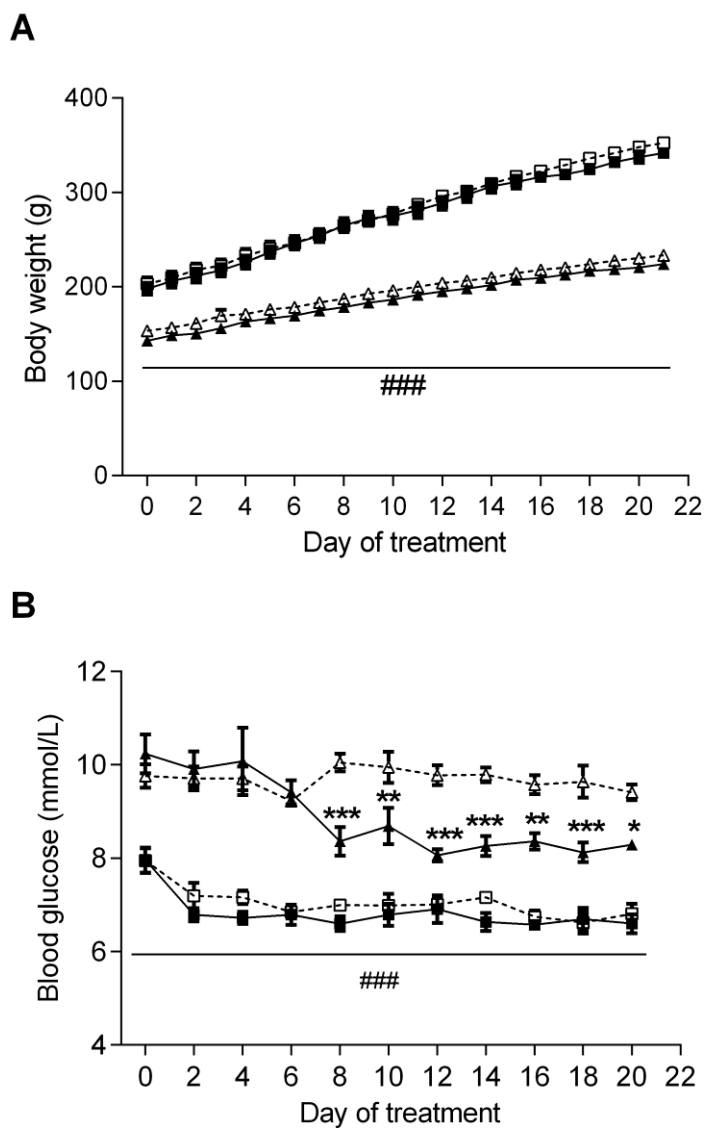


Fig. 2

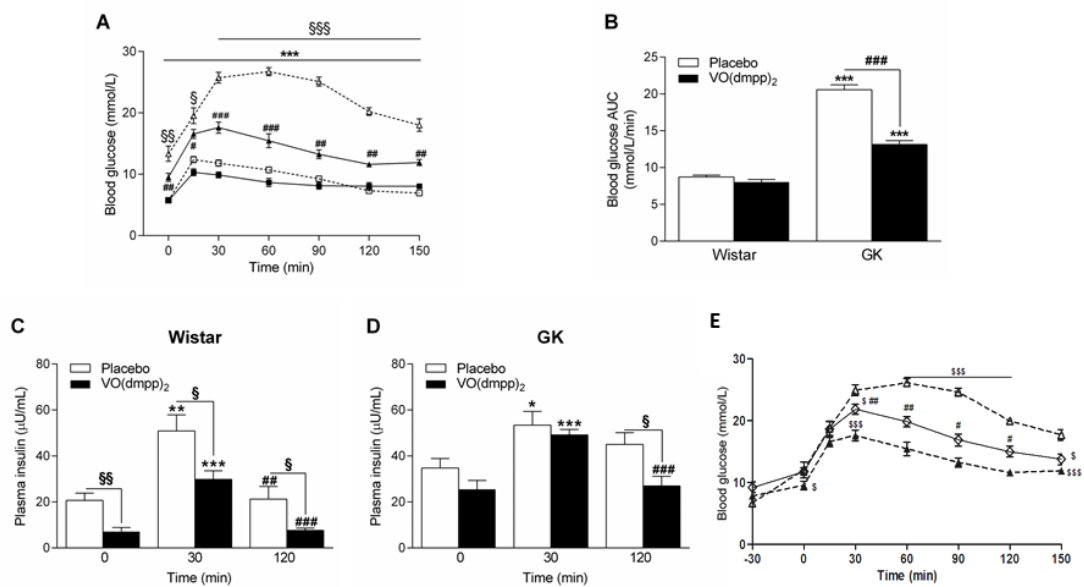


Fig. 3

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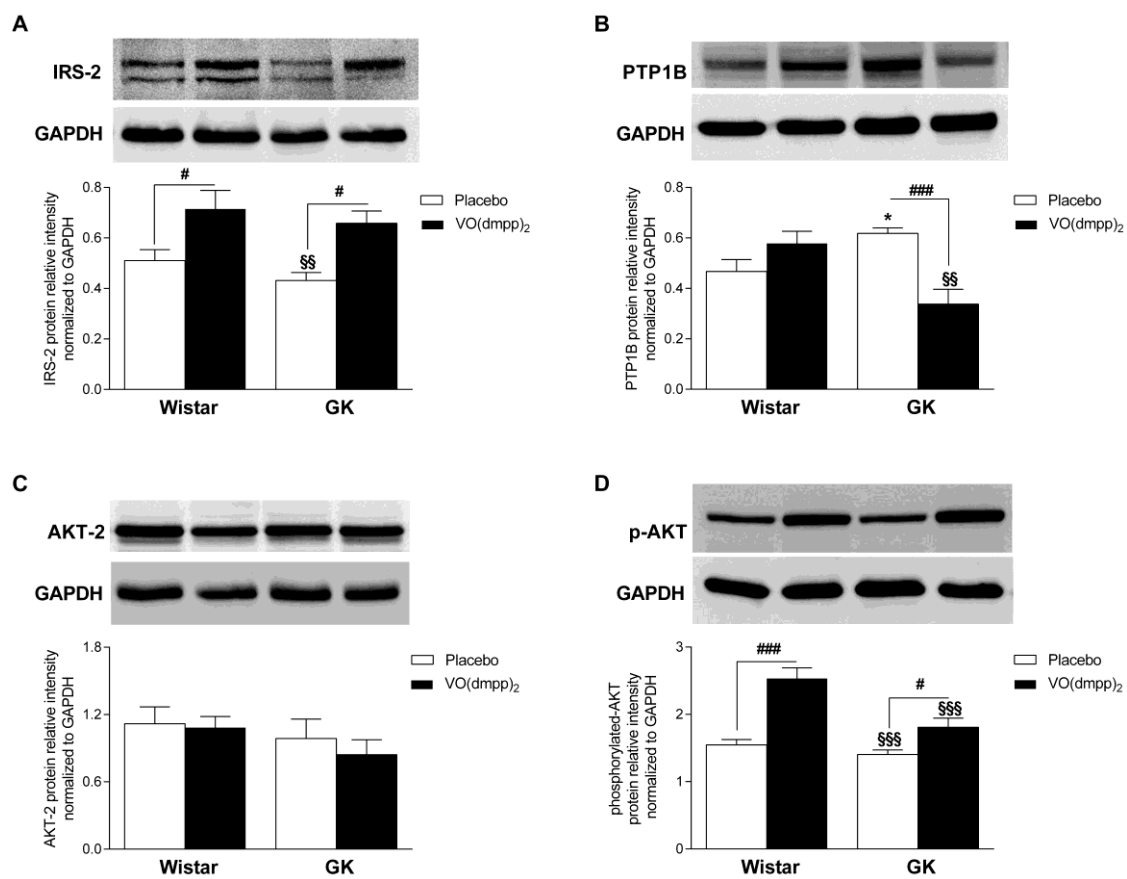
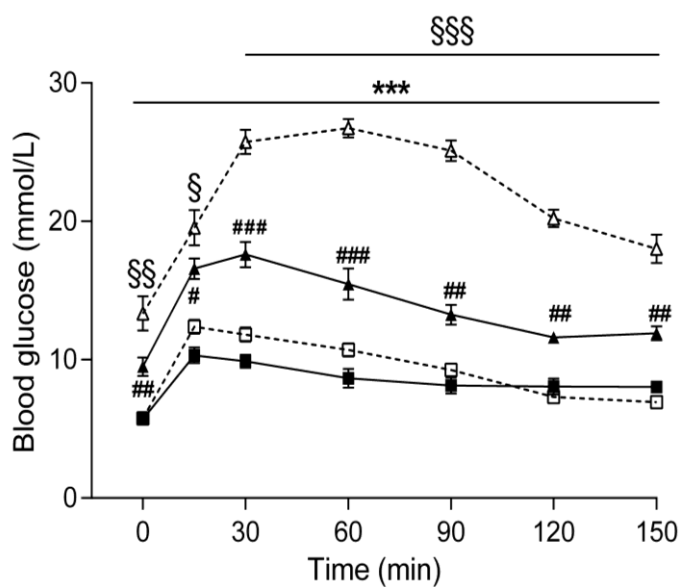
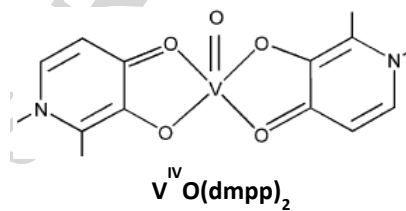


Fig. 4

Graphical abstract



- W rats+placebo
- W rats+VO(dmpp)₂
- △ GK rats+placebo
- ▲ GK rats+VO(dmpp)₂



bis(1,2-dimethyl-3-hydroxy-4-pyridinonato)oxovanadium(IV)

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A VO(dmpp)₂ treatment in the type 2 diabetic GK rats, significantly decreases hyperglycaemia and improves glucose intolerance acting on key proteins of the insulin pathway, thus confirming the anti-diabetic properties of this vanadium compound which may be a promising therapy for diabetes

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Highlights

- VO(dmpp)₂ promotes glucose uptake in GK rat adipocytes, more effectively than insulin or BMOV
- Hyperglycaemia in GK rats significantly decreases with VO(dmpp)₂ treatment
- VO(dmpp)₂ treated GK rats show a significant improvement of glucose intolerance
- VO(dmpp)₂ increases IRS2 and p-Akt expression and inhibits PTP1β expression
- insulin mimetic activity of VO(dmpp)₂ could be of interest for T2D therapy

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