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ALAS1 gene expression is down-regulated by Akt-mediated phosphorylation and nuclear exclusion of FOXO1 by vanadate in diabetic mice

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Porphyrias are diseases caused by partial deficiencies of haem biosynthesis enzymes. Acute porphyrias are characterized by a neuropsychiatric syndrome with intermittent induction of hepatic ALAS1 (δ-aminolaevulinate synthase 1), the first and rate-limiting enzyme of the haem pathway. Acute porphyria attacks are usually treated by the administration of glucose; its effect is apparently related to its ability to inhibit ALAS1 by modulating insulin plasma levels. It has been shown that insulin blunts hepatocyte ALAS1 induction, by disrupting the interaction of FOXO1 (forkhead box O1) and PGC-1α (peroxisome-proliferator-activated receptor γ co-activator 1α). We evaluated the expression of ALAS1 in a murine model of diabetes and determined the effects of the insulinomimetic vanadate on the enzyme regulation to evaluate its potential for the treatment of acute porphyria attacks. Both ALAS1 mRNA and protein content were induced in diabetic animals, accompanied by decreased Akt phosphorylation and increased nuclear FOXO1, PGC-1α and FOXO1–PGC-1α complex levels. Vanadate reversed ALAS1 induction, with a concomitant PI3K (phosphoinositide 3-kinase)/Akt pathway activation and subsequent reduction of nuclear FOXO1, PGC-1α and FOXO1–PGC-1α complex levels. These findings support the notion that the FOXO1–PGC-1α complex is involved in the control of ALAS1 expression and suggest further that a vanadate-based therapy could be beneficial for the treatment of acute porphyria attacks.

Key words: acute porphyria, Akt, δ-aminolaevulinate synthase 1 (ALAS1), forkhead box O1 (FOXO1), insulin, streptozotocin, vanadate.

INTRODUCTION

ALAS1 (δ-aminolaevulinate synthase 1), also called ALAS-N or ALAS-H, is the first and rate-limiting enzyme in the haem biosynthetic pathway in the liver [1]. Porphyrias are disorders of haem biosynthesis caused by inherited partial deficiencies of specific enzymes of the haem pathway. The main clinical manifestations of porphyrias are acute neurovisceral dysfunction and/or skin photosensitivity. The neuropsychiatric syndrome occurs only in porphyrias with intermittent induction of hepatic ALAS1 and consequent accumulation of the precursors δ-aminolaevulinic acid and porphobilinogen, which are believed to be involved in the development of acute porphyria symptoms. Porphyric attacks are often triggered by different agents such as drugs, hormones, diet and fasting [2,3].

Administration of large amounts of glucose induces a clinical improvement and decreased haem precursor excretion in patients with acute porphyria [4,5]. This effect is likely to be related to the observation that, in rodent and chick embryos, glucose inhibits or prevents drug-mediated induction of hepatic ALAS1 [6–8]. However, this response is not observed *in vitro*, probably due to the fact that glucose acts on liver cells by modulating the plasma insulin/glucagon ratio and thereby hepatic cAMP levels [6,8,9].

Furthermore, exposure of cultured hepatocytes to diverse hormones, particularly insulin and glucagon, influences the induction of ALAS1 activity and porphyrin accumulation [6,8]. These data suggest that the effect of glucose on haem synthesis may be hormonally mediated.

Handschin et al. [10] reported that both PGC-1α (peroxisome-proliferator-activated receptor γ co-activator 1α) and ALAS1 are induced in livers from starved mice. Furthermore, ALAS1 induction is controlled by PGC-1α through the interaction of NRF-1 (nuclear respiratory factor 1) and FOXO1 (forkhead box O1), a member of the forkhead transcription factor family, with the promoter of the gene encoding ALAS1 [11]. The authors also showed that insulin blunts ALAS1 induction in hepatocytes by FOXO1 phosphorylation and the subsequent disruption of its binding to PGC-1α [11].

Vanadium is a trace element considered to be important for normal cell function and development. Numerous *in vitro* and *in vivo* studies show that vanadium has insulin-like effects in the liver, skeletal muscle and adipose tissue [12]; however, its role in glucose homoeostasis in humans remains to be fully elucidated. Vanadium stimulates the tyrosine phosphorylation of IRS-1 (insulin receptor substrate 1), leading to the activation of PI3K (phosphoinositide 3-kinase) and its downstream effector Akt/PKB (protein kinase B). In addition, IGF-1R (insulin-like growth factor 1 receptor) transactivation has been proposed as a transducer of vanadium compounds signalling, which induces Akt phosphorylation and its downstream targets through PI3K activation [13].

Although the precise mechanism remains to be established, it has been suggested that vanadium salts, by inhibiting protein tyrosine phosphatase activity, increase the phosphotyrosine content of IRS-1 [14,15], thus activating the insulin signalling pathway. Since Akt mediates the physiological responses of insulin for glucose uptake [16] and glycogen synthesis [17], it is possible that vanadium-induced activation of the PI3K/Akt signalling system may be a mechanism by which insulin-like effects of vanadium compounds are exerted on these processes.

Abbreviations used: ALAS1, δ-aminolaevulinate synthase 1; FOXO1, forkhead box O1; i.p., intraperitoneal; IP, immunoprecipitation; IRS-1, insulin receptor substrate 1; PGC-1α, peroxisome-proliferator-activated receptor γ co-activator 1α; PI3K, phosphoinositide 3-kinase; s.c., subcutaneous; STZ, streptozotocin; TBS-T, Tris-buffered saline with Tween 20.

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Given the relationship between insulin and ALAS1, the aim of the present study was to evaluate the expression of ALAS1 in a murine model of STZ (streptozotocin)-induced diabetes and determine further the effect of vanadate on enzyme regulation to assess its potential as a therapeutic agent for the treatment of acute porphyria attacks.

Findings of the present study show that ALAS1 is strongly induced in diabetes and that both insulin and vanadate abolished the enzyme induction, suggesting that vanadate may have a potential therapeutic role in the treatment of acute porphyria.

EXPERIMENTAL

Animals

Male CF1 mice (18–20 g initial body weight) were housed in a controlled environment with a 12 h light/12 h dark cycle. The mice were supplied with standard pellet diet (Alimento Balanceado Cooperación) and water *ad libitum*. All animals were maintained according to the NIH Care and Use of Laboratory Animals guidelines.

Treatments

Diabetes was induced in mice by a single i.p. (intraperitoneal) injection of STZ (170 mg/kg of body weight; Sigma), freshly dissolved in sodium citrate buffer (0.1 M, pH 4.5). Non-diabetic mice (control group) were injected with an equivalent volume of citrate buffer. Blood glucose levels were measured via tail vein sampling 15 days after STZ injection, and the animals were considered diabetic when their blood glucose levels were greater than 300 mg/dl.

At 16 days after STZ injection, the diabetic animals were randomly subdivided into three groups. One group (STZ) received no treatment. The second group (STZ + Insulin) were administered human insulin Insuman® N (Sanofi-Aventis) [30 units/100 g, s.c. (subcutaneous)] once daily (at 10:00 h) for 16 days. The third group (STZ + Vanadate) received, for the same period of time, sodium metavanadate (0.3 mg/ml) in the drinking water which was supplemented with 80 mM NaCl and the pH was adjusted to neutrality. Vanadate solutions were replaced with freshly prepared solutions every 2 days.

Mice were killed at 16, 20, 24, 32, 36 and 42 days following STZ administration and livers were quickly removed and processed for RNA isolation.

Another group of STZ mice received an i.p. injection with actinomycin D (Fluka) (2.5 μg/g of body weight) dissolved in DMSO on day 32. The corresponding control group received the same volume of vehicle. Animals were killed at 10, 20, 30, 40 and 60 min following actinomycin D administration. Total RNA was prepared and analysed by Northern blotting.

Blood biochemistry

Serum glucose levels were determined by a commercial kit (Wiener Lab). Insulin levels were assessed by RIA (Linco Research).

Preparation of whole-tissue extracts

Animals were killed and livers were quickly frozen in liquid nitrogen. Liver samples were homogenized on ice in buffer A containing 100 mM Tris/HCl (pH 7.4), 1 % Triton X-100, 10 mM $Na_4P_2O_7$, 10 mM NaF, 10 mM EDTA and protease inhibitor cocktail from Sigma. Samples were centrifuged at 17000 ***g*** for 30 min at 4 °C, and supernatants were fractionated and stored at − 70 °C. Total protein concentration was measured using the method of Bradford [18].

Whole-tissue extracts containing equivalent amounts of protein were separated by SDS/PAGE (7.5–8.5 % gels) and transferred for 1 h at 65 V on to a nitrocellulose membrane (GE Healthcare) as described by Towbin et al. [19]. Membranes were then blocked in 5 % (w/v) non-fat dried milk powder or BSA in TBS-T (Tris-buffered saline with Tween 20) A (20 mM Tris/HCl, pH 7.6, 137 mM NaCl and 0.2 % Tween 20) for 1 h, and then incubated with primary antibodies for 1 h at room temperature (22 °C). The dilutions of the primary antibodies were as follows: 1:800 for anti-ALAS1 antibody (custom antiserum), 1:1500 phospho-Akt (Ser^{473}) and 1:1000 for Akt1 (Santa Cruz Biotechnology) diluted in TBS-T A. The blotted membranes were then washed for 30 min with TBS-T A and incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody (1:1000 dilution for rabbit and 1:800 dilution for mouse). The membranes were washed for 30 min with TBS-T A, and the immunoreactive proteins were visualized with an ECL (enhanced chemiluminescence) system, according to the manufacturer's protocol (GE Healthcare) and exposed to film. Protein bands were quantified from scanned images using Scion Image (image-analysis software by Scion) software and results are expressed as means ± S.E.M.

Custom ALAS1 antiserum was generated by immunizing rabbits with a 19-residue synthetic peptide containing a C-terminal ALAS1 sequence conserved across different species: SEREKAYFSGMSKMVSAQA. The antiserum was strongly reactive and detected a protein of the predicted size for mitochondrial ALAS1 (65 kDa) on Western blots.

Nuclear extracts

Livers were cut into small pieces and then homogenized in buffer B (10 mM Tris/HCl, pH 7.6) with protease inhibitor cocktail. The samples were centrifuged at 24 ***g*** for 5 min at 4 °C and supernatants centrifuged further at 855 ***g*** for 5 min at 4 °C. Pellets containing the nuclear extracts were resuspended in buffer A, fractionated and stored at − 70 °C. Total protein in the extracts was measured using the method of Bradford [18].

Immunoassays on nuclear extracts were performed as described above for whole-tissue extracts with the following modifications: primary antibodies against FOXO1 (Cell Signaling Technology L27) and PGC-1α (Santa Cruz Biotechnology SC-13067) were incubated overnight at 4 °C (1:500 and 1:800 dilution respectively). Afterwards, the blotted membranes were washed for 30 min with TBS-T B (5 mM Tris/HCl, pH 7.6, 34.25 mM NaCl and 0.01 % Tween 20) and incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody (1:800 dilution). The membranes were washed for 30 min with TBS-T B. Immunoreactive proteins were visualized and quantified as detailed above. Results are expressed as a percentage of the control.

Northern blot analysis

Liver tissue was homogenized and extracted following the method of Chomczynski and Sacchi [20]. For Northern blot analysis, 18 μg of RNA was size-fractionated on a 1 % (w/v) agarose/formaldehyde gel, blotted and hybridized using standard procedures.

Hybridization products were detected and standardized against 18S bands. The probe for ALAS1 was a rat cDNA, pKRA2cA, kindly provided by Dr Masayuki Yamamoto (Tohoku University School of Medicine, Sendai, Japan) [21]. A probe for 18S

rRNA was generated by PCR using primers as follows: 18S forward, 5′-GGTTGATCCTGCCAGTAGCATA-3′, and reverse, 5′-AATGATCCTTCCGCAGGTTC-3′.

The probes were ^{32}P-labelled with a Random Primer kit (Invitrogen). Bands were visualized on radiographic film, and the resulting images scanned and quantified using Scion Image software.

Co-immunoprecipitation assays

Mice were killed, and livers were excised and washed with PBS. The fresh livers were cut into smaller pieces and placed into PBS/formaldehyde solution [1 % (v/v) final formaldehyde concentration] for 15 min. The reaction was stopped by adding glycine to 125 mM and by subsequent incubation for 5 min. After washing with ice-cold PBS, liver samples were homogenized using a tissue homogenizer for preparation of single cells and centrifuged at 855 ***g*** for 10 min at 4 °C. Pellets were suspended and incubated in lysis buffer (5 mM Tris/HCl (pH 8), 85 mM KCl and 0.5 % Nonidet P40) for 15 min on ice. Samples were centrifuged at 2376 ***g*** for 5 min at 4 °C. Nuclear pellets were suspended and incubated in lysis buffer [50 mM Tris/HCl, pH 8.1, 10 mM EDTA and 1 % (v/v) SDS] on ice for 20 min. The lysate was then sonicated to shear DNA to lengths between 300 and 600 bp. After centrifugation at 18 625 ***g*** for 10 min to remove insoluble material, the protein concentration was measured using the method of Bradford [18]. The supernatant containing 1200 μg of protein was diluted 10-fold with IP (immunoprecipitation) buffer [0.5 % Nonidet P40, 1 % (v/v) sodium deoxycholate and 0.1 % SDS]. The sample solution was incubated with 4 μg of either anti-FOXO1 antibody or normal rabbit GAL4 (galectin 4) (Santa Cruz Biotechnology) at 4 °C overnight. The immunocomplex was captured by incubating samples with 50 μl of Protein A–agarose suspension overnight at 4 °C on a rocking platform. Protein A–agarose beads were collected by centrifugation at 855 ***g*** for 5 min and washed three times with IP buffer. Pellets were then suspended in Laemmli buffer [100 mM Tris/HCl, pH 6.8, 3.2 % (v/v) SDS, 10 % (v/v) glycerol and 8 % (v/v) 2-mercaptoethanol] and boiled for 10 min at 100 °C. The supernatant was subjected to SDS/PAGE (7 % gels) and immunoblotted with an antibody against PGC-1α. All solutions were supplied with protease inhibitor cocktail.

Statistical analysis

All experiments included between three and six mice per group. Experiments were repeated at least three times with similar results. Results are means ± S.E.M.

Statistical significance was evaluated by one-way ANOVA, followed by multiple comparisons among groups or between each group and control using Tukey–Kramer's or Dunnett's test respectively. $P < 0.05$ was considered as statistically significant.

RESULTS

Glucose and insulin levels in STZ-treated mice

STZ is commonly used to induce diabetes in experimental animals since it selectively destroys pancreatic insulin-producing β-cells [22]. The rodent STZ-induced diabetes model is characterized by decreased insulin levels, peripheral insulin resistance and alterations in insulin-dependent signal transduction [23,24].

The efficacy of treatment with STZ was determined by monitoring blood glucose levels (Table 1). The mean glycaemia level in control animals was 165 mg/dl. Blood glucose levels were significantly increased in STZ diabetic mice compared with control mice. STZ treatment increased glycaemia by approximately 400 % (mean level 700 mg/dl) and the diabetic status was detected on day 16 and onwards. In order to confirm the onset of diabetic status, serum insulin levels were measured in both control and STZ-treated animals. The group treated with STZ showed insulin levels significantly lower than non-diabetic controls (Table 1). When diabetic animals were treated for 16 days with either insulin or vanadate, their blood glucose levels were significantly reduced, although only insulin restored glycaemia to normal values.

Table 1 Effect of insulin and vanadate treatment on serum glucose and insulin levels in STZ mice

Mice were made diabetic with a single i.p. injection of STZ and were allowed 15 days to recover before the administration of insulin or vanadate. Glucose and insulin levels were assessed on blood samples drawn at day 32. Results are means ± S.E.M. for each group. *$P < 0.001$; **$P < 0.01$ compared with control; †$P < 0.001$ compared with STZ.

Group	*n*	Body weight (g)	Glucose serum (mg/dl)	Insulin serum (ng/ml)
Control	12	34 ± 3.1	165 ± 36	2.7 ± 1
STZ	18	23 ± 4.2*	717 ± 145*	0.4 ± 0.2**
STZ + Insulin	9	31 ± 5.1†	117 ± 45†	7.7 ± 1*†
STZ + Vanadate	10	20 ± 3.7*	392 ± 100*†	3.8 ± 2.1†

The physical condition of the animals was monitored by measuring body weight. As shown in Table 1, diabetic animals failed in gaining weight compared with controls. Treatment of diabetic mice with insulin caused a significant increase in weight gain, whereas vanadate did not show any effect on this parameter. These findings are in agreement with previous observations that STZ-treated rats are largely catabolic and daily weight gain can be fully corrected by insulin therapy, but not by vanadium therapy [25]. The catabolic nature of the untreated STZ-treated rat model has been attributed to an increased rate in muscle protein degradation, a process that can be arrested by insulin, but not by vanadate [26]. Alternatively, vanadium compounds have been associated with several toxic effects [27,28] such as diarrhoea, decreased fluid and food uptake, and dehydration, which may explain their lack of effect on weight gain [22]. However, we have used vanadium solutions with the addition of NaCl and pH adjusted to neutrality, which have been shown to correct for the abovementioned untoward effects of vanadium solutions [22].

Time course of ALAS1 mRNA expression and protein content in diabetic mice

As insulin and glucose seem to be involved in ALAS1 regulation, as supported by *in vivo* and *in vitro* studies, we assessed ALAS1 expression in STZ-treated mice.

The expression of ALAS1 mRNA in the STZ group was slightly higher than in the control group at day 24 reaching a 100 % increase on day 32 and continued increasing until day 42 (Figures 1A and 1B). In order to determine whether the increase in *ALAS1* mRNA was due to changes in its stability, the half-life of *ALAS1* mRNA in the liver of both diabetic and control animals was calculated. No change was observed in the half-life of *ALAS1* mRNA between diabetic and control animals (Figure 1C). In both groups, the half-life was approximately 25 min, in agreement with the literature [21]. These results suggest that alterations in *ALAS1* mRNA levels observed in diabetic animals reflect changes in the rate of transcription rather than in its processing or degradation.

In order to determine whether changes in *ALAS1* mRNA were reflected in the enzyme protein content, ALAS1 was assessed by Western blotting. As shown in Figure 2, ALAS1 protein content increased in diabetic animals following treatment over the same time as for its mRNA.

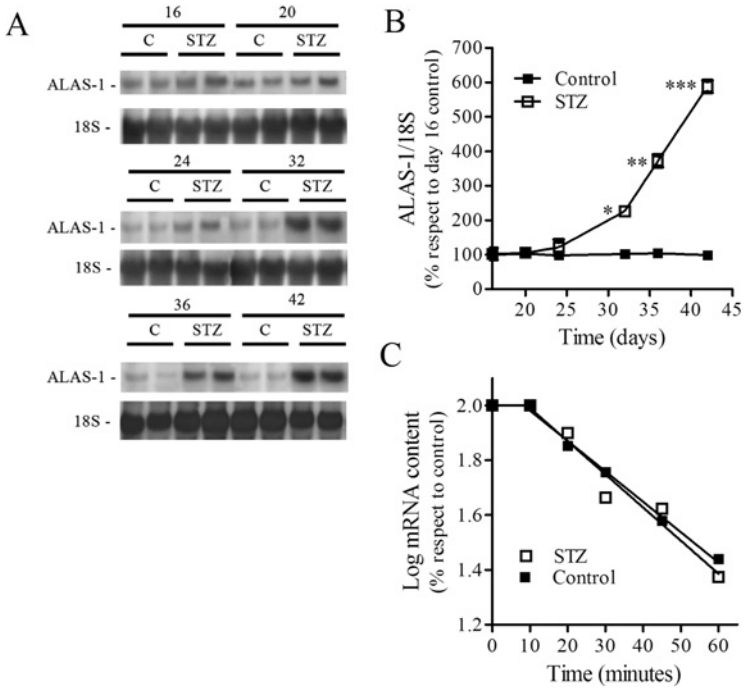

Figure 1 Time course of *ALAS1* mRNA expression in the liver of diabetic animals

(**A**) Northern blot analysis. Starting at day 16 after i.p. injection of STZ, liver mRNA samples were taken at different time points (16, 20, 24, 32, 36 and 42 days) and *ALAS1* mRNA levels were assessed by Northern blot analysis performed using standard procedures, and the membranes were hybridized with a probe specific for *ALAS1* mRNA. As a loading control (C), the same blot was stripped and rehybridized with a probe specific for 18S RNA. (**B**) Densitometric analysis. Results are mean ± S.E.M. percentage values of control animals at day 16 obtained by the Scion Image program ($n \geqslant 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to the control. (**C**) Stability of hepatic *ALAS1* mRNA in control and diabetic animals, 32 days after i.p. injection of STZ. STZ and control animals were treated with an i.p. injection of actinomycin D (2.5 μg/g). The animals were killed at the time points indicated. Total RNA was isolated and assayed for *ALAS1* mRNA. Results are means ± S.E.M. ($n \geqslant 3$). Lines represent the best fits as determined by linear regression analysis of the data ($R^2 > 0.85$), and were used to estimate the half-life of *ALAS1* mRNAs.

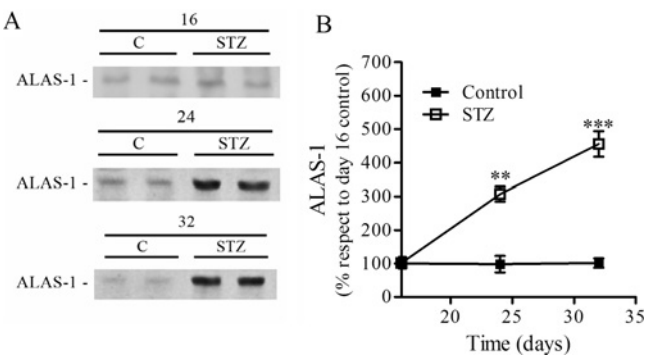

Figure 2 Time course of ALAS1 protein levels in the liver of diabetic and control animals

(**A**) Western blot analysis. Levels of liver ALAS1 protein were assessed at 16, 24 and 32 days after STZ or vehicle i.p. injection. Whole-tissue extracts of liver (50 μg) were resolved by SDS/PAGE (7.5% gels), transferred on to nitrocellulose membranes and immunoblotted. C, control. (**B**) Densitometric analysis. Results are mean ± S.E.M. percentage values of control animals at day 16 obtained by the Scion Image program ($n \geqslant 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to the control.

Effect of insulin and vanadate on ALAS1 in diabetic animals

Insulin has been reported to impair ALAS1 expression in primary mouse hepatocytes and mouse SV40 (simian virus 40)-transformed hepatocytes by inhibiting its transcription [10,29]. Since vanadium and vanadium compounds are responsible for insulin-like activity and can mimic the action of insulin, we decided to study the effect of vanadate and insulin on ALAS1 liver expression in animals treated with STZ.

When diabetic animals received insulin, *ALAS1* mRNA and mitochondrial protein levels returned to control values (Figures 3A and 3B respectively). The effect of vanadate was more dramatic than that of insulin since both ALAS1 mRNA and protein levels fell below those observed in non-diabetic animals (Figures 3A and 3B respectively).

Effect of insulin and vanadate on Akt phosphorylation in STZ mice

In one of its signalling pathways, insulin can activate PI3K, generating PtdInsP_3, which in turn contributes to the activation of protein kinase Akt [30,31]. In addition, vanadate has been reported to increase the activation state of Akt as judged by enhanced Ser^{473} phosphorylation. Moreover, the vanadate-induced Akt activation was shown to depend on PI3K [32]. In the last few years, Akt and its downstream substrates have been implicated in modulating glucoregulatory responses [16,33]. Taking into account that Akt seems to be a common effector in the signalling pathways of both insulin and vanadate, we decided to evaluate their effect on Akt activity in STZ-induced diabetic mice. Akt activity was assessed by inmunoblotting with antibodies against phospho-Akt (Ser^{473}) (Figure 4). A decrease in Akt Ser^{473} phosphorylation was observed in STZ mice with no changes in Akt protein levels. On the other hand, insulin, as well as vanadate, significantly increased phosphorylation of Akt Ser^{473} compared with control animals without any effect on the amount of Akt protein.

Effect of insulin and vanadate on FOXO1 and PGC-1α nuclear levels in liver of STZ mice

FOXO1 has been shown to co-operate with PGC-1α in the activation of gluconeogenic genes such as phosphoenolpyruvate

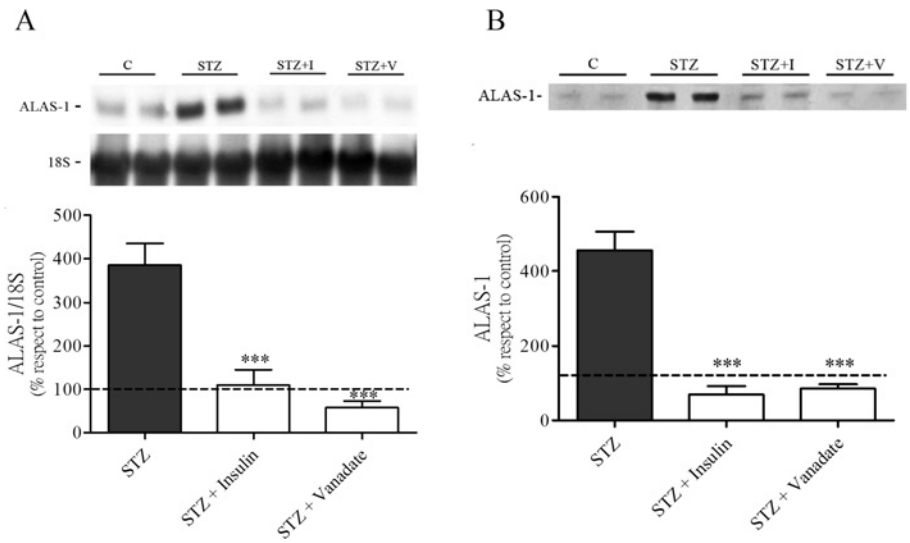

Figure 3 *ALAS1* mRNA overexpression and protein content is down-regulated by vanadate in liver of STZ mice

(**A**) Upper panels: Northern blot analysis. At 16 days after i.p. injection of STZ, the animals were treated for 16 days with a daily s.c. administration of either insulin (STZ + Insulin) or vanadate (STZ + Vanadate) in the drinking water. *ALAS1* mRNA levels were assessed by Northern blot analysis performed using standard procedures and the membranes hybridized with a probe specific for *ALAS1* mRNA. As a loading control, the same blot was stripped and rehybridized with a probe specific for 18S RNA. C, control. (**B**) Upper panel: Western blot analysis. Whole-tissue extracts of livers (50 μg) were resolved by SDS/PAGE (7.5 % gels), transferred on to nitrocellulose membranes, and immunoblotted with an anti-ALAS1 polyclonal antibody as described in the Experimental section. (**A** and **B**) Lower panels: densitometric analysis. Results are mean $\pm$ S.E.M. percentage values of control animals at day 32 obtained by the Scion Image program ($n \geqslant 3$). $^{***}P < 0.001$ with respect to STZ treatment.

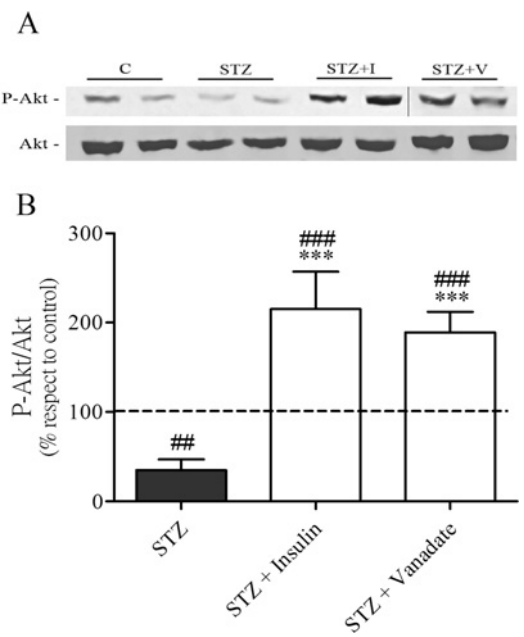

Figure 4 Effect of vanadate treatment on Akt phosphorylation in liver of STZ mice

(**A**) Western blot analysis. At 16 days after i.p. injection of STZ, the animals were treated for 16 days with a daily s.c. administration of either insulin (STZ + Insulin) or vanadate (STZ + Vanadate) in the drinking water. Western immunoblot analysis of phospho-Akt (Ser^{473}) and Akt protein levels were performed. Whole-tissue extracts of livers (60 μg) were resolved by SDS/PAGE (8.5 % gels), transferred on to nitrocellulose membranes and immunoblotted with antibodies against phospho-Akt (Ser^{473}) as described in the Experimental section. Total amounts of Akt protein measured using conventional antibodies remained unchanged in all groups. C, control. (**B**) Densitometric analysis. Results are mean $\pm$ S.E.M. percentage values of control animals at day 32 obtained by the Scion Image program ($n \geqslant 3$). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ with respect to control, $^{***}P < 0.001$ with respect to STZ treatment.

carboxykinase 1 and glucose-6-phosphatase [34–36]. The regulation of these genes by insulin depends on the tight regulation of FOXO1, and is based on the co-operation of FOXO1 with PGC-1α. Moreover, Handschin et al. [10] found that the insulin repression of the *ALAS1* promoter in mouse hepatocytes is controlled by a FOXO1–PGC-1α interaction.

Akt plays a key role in the regulation of FOXO transcriptional activity. Phosphorylation of FOXO1 by Akt leads to disruption of its binding to PGC-1α and translocation of FOXO1 phosphorylated out of the nucleus [36–38].

Since insulin and vanadate were able to activate Akt in our model, we hypothesized that insulin and vanadate suppress the transcription of ALAS1 in the liver of STZ mice by triggering Akt-dependent phosphorylation and subsequent inactivation of FOXO1. Thus we measured the FOXO1 content in hepatic nuclear extracts of all experimental groups.

Nuclear FOXO1 levels increased 8-fold in response to STZ (Figure 5A). On the other hand, and consistent with the augmented phosphorylation of Akt, insulin and vanadate resulted in an almost complete attenuation of FOXO1 nuclear accumulation in diabetic mice (Figure 5A).

Increased levels of hepatic PGC-1α mRNA have been shown in Type 1 and Type 2 diabetes mellitus [39,40]. Furthermore, previous studies have demonstrated that PGC-1α is able to turn on the expression of the *ALAS1* gene in hepatocytes and in liver *in vivo* [10].

To determine whether PGC-1α played a role in the induction of *ALAS1* mRNA in our model of murine diabetes, we assessed PGC-1α levels in the liver of STZ mice. As shown previously, PGC-1α protein levels were induced in the liver of diabetic mice (Figure 5B). Moreover, these values remained significantly above controls when diabetic animals were treated with insulin or vanadate (Figure 5B). These results are in agreement with previous reports indicating that insulin does not have a direct effect on PGC-1α in cultured hepatocytes or liver [36].

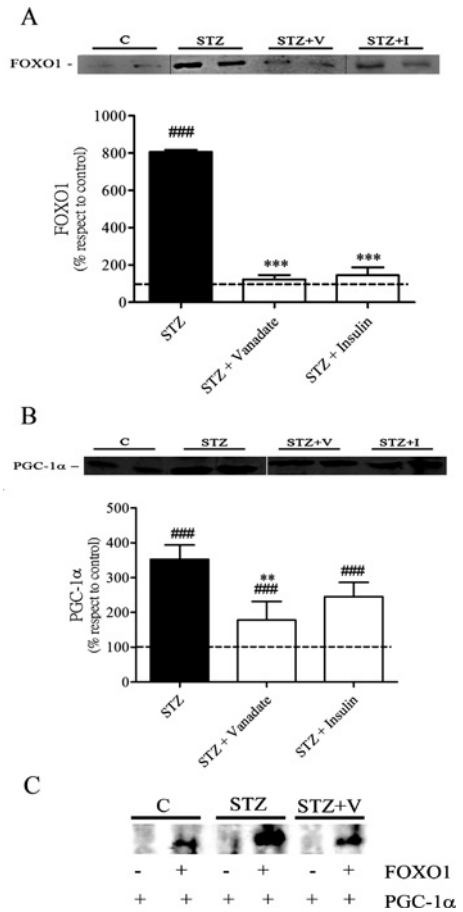

Figure 5 Effect of vanadate on FOXO1 and PGC-1α nuclear expression in liver of STZ mice

(**A** and **B**) Upper panel: Western blot analysis. At 16 days after i.p. injection of STZ, the animals were treated for 16 days with a daily s.c. administration of either insulin (STZ + Insulin) or vanadate (STZ + Vanadate) in the drinking water. (**A**) FOXO1 (80 μg) and (**B**) PGC-1α (60 μg) protein levels were measured on nuclear extracts separated by SDS/PAGE (7 % gels), transferred on to nitrocellulose membranes and immunoblotted with antibodies against FOXO1 and PGC-1α as described in the Experimental section. Lower panels: densitometric analysis. Results are mean $\pm$ S.E.M. percentage values of control animals at day 32 obtained by the Scion Image program ($n \geqslant 3$). ###$P < 0.001$ with respect to control. **$P < 0.01$, ***$P < 0.001$ with respect to STZ treatment. (**C**) Nuclear protein extracts were subjected to immunoprecipitation using anti-FOXO1 antibody. Immunoprecipitates were resolved by SDS/PAGE (7 % gels), transferred on to nitrocellulose membranes and immunoblotted with anti-PGC-1α antibody as described in the Experimental section. The blots are representative of three separate experiments.

Taking into account the role described for the FOXO1–PGC-1α complex in the regulation of the *ALAS1* promoter [10], we carried out a co-immunoprecipitation experiment in order to test the hypothesis that changes observed in nuclear levels of FOXO1 and PGC-1α might result in modification of nuclear levels of the FOXO1–PGC-1α complex which may be directly responsible for the alterations in ALAS1 expression. Nuclear extracts were immunoprecipitated using anti-FOXO1 coupled to Protein A–agarose beads. Immunoprecipitates were then subjected to SDS/PAGE and immunoblotted with anti-PGC-1α antibody. Results depicted in Figure 5(C) show an increase in the nuclear FOXO1–PGC-1α complex in the STZ group which was abolished in the STZ + Vanadate group. These findings correlate with those of *ALAS1* mRNA levels, suggesting that the effect observed in both experimental groups, STZ and STZ + Vanadate, on ALAS1 expression might be mediated by changes in nuclear levels of the FOXO–PGC-1α complex.

DISCUSSION

Although glucose effects on improving porphyria symptoms are well known, the underlying molecular mechanisms remain to be fully elucidated.

Earlier experiments suggest that the therapeutic action of glucose could be mediated by the increase in insulin levels observed *in vivo* as a consequence of glucose intake.

Previous studies performed in cell cultures show that ALAS1 transcription is inhibited by the insulin signalling pathway [10,41]. Present findings support an association between insulin and ALAS1 expression *in vivo* using a murine diabetic model. The diabetic state produced an increase in the levels of both mRNA and hepatic ALAS1 protein expression (Figures 1 and 2). The fact that both insulin and vanadate, an insulinomimetic agent (Figure 3), reverse ALAS1 induction in diabetic animals strengthens the hypothesis that the induction was due to a fall in insulin levels. Furthermore, our results show that a greater expression of ALAS1 in diabetic animals was not associated with changes in the half-life of *ALAS1* mRNA (Figure 1C), suggesting that it resulted from augmented gene transcription. These findings are in agreement with other authors who propose that insulin inhibits ALAS1 transcription [6,8,10,41].

We have observed that the increase in ALAS1 expression levels produced by a diabetic state is accompanied by an increase in nuclear FOXO1. An increase in FOXO1 in our diabetes model is consistent with the observations by Altomonte et al. [42], who reported that *db*/*db* mice exhibit a significant induction of hepatic FOXO1 protein as well as increased nuclear localization, compared with their heterozygous littermates *db*/$^+$. Likewise, the decrease in ALAS1 levels produced by insulin and vanadate was accompanied by PI3K/Akt pathway activation and subsequent reduction of nuclear FOXO1 levels. These findings suggest that the Akt/FOXO1 pathway is involved in the control of ALAS1 expression in our murine model of diabetes [10].

Handschin et al. [10] proposed that the FOXO1–PGC-1α interaction augments ALAS1 activity during acute porphyria attack. Findings of the present study show that increased ALAS1 expression was simultaneous with the increase in both nuclear FOXO1 and PGC-1α.

Furthermore, when diabetic animals were treated with vanadate, ALAS1 expression decreased to control values concurrently with a significant reduction of both FOXO1 and PGC-1α. On the other hand, although insulin also reduced ALAS1 expression, this treatment did not show any effect on PGC-1α levels. These results are in agreement with previous results in murine liver cultures showing that insulin does not have a direct effect on PGC-1α expression [36]. Moreover, co-immunoprecipitation assays revealed that the nuclear FOXO1–PGC-1α complex was augmented in diabetic mice, whereas vanadate treatment reduced it (Figure 5C).

In summary, our findings suggest that ALAS1 expression is regulated by nuclear FOXO1–PGC-1α complex levels, that may in turn be modulated by vanadate treatment. Therefore these findings support the hypothesis that vanadate may have a potential therapeutic role in the treatment of acute porphyria attack through the inhibition of ALAS1 expression.

For over 30 years, haem infusion or carbohydrate intake has been the first-choice treatment for acute porphyric attacks [43,44].

A freeze-dried haematin (hydroxyhaem) preparation is available. It is reconstituted with sterile water. The product is, however, unstable, and degradation products adhere to endothelial cells, platelets and coagulation factors, causing a transient anticoagulant effect and phlebitis at the site of infusion. Reconstitution with human albumin enhances the stability of

haematin and can prevent these adverse effects [45], although a high percentage of patients may have thrombophlebitis [46].

Instead, intravenous administration of haem arginate (Normosang®), rather than haematin, does not cause any significant changes in coagulation and fibrinolysis, and the overall rate of adverse effects is much lower [47].

However, it should be considered that with any of the haem preparations, after repeated infusions, peripheral vein alterations can appear and there is also a risk of progressive iron overload detected by an increase in serum ferritin levels [2,48].

On the other hand, the therapeutic intake of carbohydrates makes patients with hepatic porphyrias gain weight. Furthermore, losing weight can be very difficult for these patients due to the risk of attacks induced by fasting. It would therefore be desirable to identify other molecules that could be alternative strategies for the treatment of acute porphyria. Our findings open the possibility of considering vanadium compounds as one of these alternative treatments.

However, clinical trials have shown that, at the tolerated doses, vanadate does not dramatically improve insulin sensitivity and glycaemic control in humans [49]. Additionally, in patients treated with vanadium salts, gastrointestinal discomfort was a common unwanted side effect, although it could be ameliorated by decreasing the dosage [50,51]. Moreover, it should be recalled that so far, clinical studies have been of short duration (up to 6 weeks) and using lower doses than those administered in animal experiments; thus the long-term toxicity of vanadium in humans remains to be explored. Clearly, at present, there is no consensus on the toxic effects of vanadium compounds, therefore detailed and systematic investigations are needed to evaluate the toxicity of various vanadium compounds before undertaking long-term clinical trials in humans. It should also be noted that the use of chelating agents [27] and organo-vanadium compounds [52] have shown vanadium toxicity significantly lower than that of inorganic vanadium salts.

AUTHOR CONTRIBUTION

Esther Gerez developed the project, designed the experiments, analysed the data and wrote the original draft of the paper. Leda Oliveri performed the experiments, which formed the basis of her Ph.D. thesis, and contributed to the experimental design, data analysis and writing of the paper. Carlos Davio contributed to the preparation of the Figures and preparation of the final paper. Alcira Batlle contributed to the preparation of the final paper. All of the authors discussed the results and commented on the paper.

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