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Regulation of the expression of ferrochelatase in a murine model of diabetes mellitus type I

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ABSTRACT

Background: Diabetes produces changes on cellular hemeprotein metabolism. The last enzyme of heme biosynthetic pathway is ferrochelatase (FECH), an enzyme that catalyzes the insertion of ferrous ion into protoporphyrin IX to produce heme. The aim of this work was to investigate whether FECH expression can be other key point in the regulation of heme biosynthesis in diabetic animals.
Methods: Mice were rendered diabetic with streptozotocin (STZ, 170 mg/kg body weight i.p. for 15 days). Liver FECH protein and mRNA levels were evaluated by Western blot and Northern blot respectively. Vanadate was used as a hypoglycemic agent. The levels of the transcription factor Sp1 bound to the FECH promoter were assessed by chromatin immunoprecipitation (ChIP).
Results: Hyperglycemia caused an increase in FECH mRNA levels but no changes in FECH protein expression. ChIP analysis revealed that the increase in FECH mRNA levels was due to enhanced Sp1 binding to the FECH promoter in diabetic animals, which was reduced by vanadate administration.
Conclusions: In diabetic animals, enhanced binding of Sp1 to the FECH promoter may be responsible for the increase in FECH mRNA levels. However, this increase was not reflected in the amount of FECH protein, which would confirm that FECH could be another control point in heme synthesis.

1. Introduction

Diabetes causes important changes in the cellular hemeprotein metabolism, possibly as a consequence of a biochemical defect in the mitochondrial enzymes involved in heme biosynthesis [1–5]. The first step in the heme biosynthetic pathway in animal cells is catalyzed by the mitochondrial matrix enzyme 5-aminolevulinic acid synthase (ALA-S), which converts glycine and succinyl-CoA to 5-aminolevulinic acid. In the liver and other tissues, except for erythropoietic tissue, this reaction is rate limiting, and ALA-S1, the non-erythropoietic isoenzyme, is regulated by heme levels [6–8]. Some authors have suggested that, in addition to ALA-S1, ferrochelatase (FECH) is also a regulatory enzyme of the heme biosynthetic pathway [9,10]. FECH, the last enzyme of this pathway, catalyzes the insertion of ferrous ion into protoporphyrin IX to produce heme [11]. A single promoter participates in the expression of the two transcripts of 2.2 kb and 2.9 kb of FECH [12–14]. Tephly et al. [9] suggested that the activities of FECH and ALA-S are reciprocally related and that the increase in FECH activity is due to an increase in substrate flux caused by a greater activity of ALA-S1.

Previously, we observed that ALA-S1 was increased in diabetic mice [15]. Furthermore, studies carried out in wild type rats demonstrated that glucose inhibits or prevents drug-mediated induction of hepatic FECH [16–18].

Based on all the above, this work aimed to investigate whether FECH expression can be another key point in the regulation of heme biosynthesis in diabetic animals.

2. Materials and methods

2.1. Animals

Male *CF1* mice weighing 18–20 g were maintained at 24 °C under controlled conditions of light and temperature, free access to water and standard pellet diet (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentina), according to the guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC). Since

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Abbreviations

ALA-S	5-Aminolevulinic acid synthase
ChIP	Chromatin immunoprecipitation
ECL	Enhanced Chemiluminescent System
FECH	Ferrochelatase
i.p.	Intraperitoneal
O-GlcNAc	O-linked N-acetylglucosamine
PCR	Polymerase Chain Reaction
qPCR	Real-time PCR
Sp1	Specificity protein 1
STZ	Streptozotocin
TfR1	Transferrin receptor 1

heme biosynthesis can be altered by several triggering factors, such as hormonal changes, male mice but not female mice were used for the study. The project was approved by the Laboratory Institutional Care and Use Committee (Facultad de Medicina, Universidad de Buenos Aires, Exp-UBA 0061984/2017, code 2371, October 13th, 2017).

2.2. Treatment

Diabetes was induced by administering a single dose of streptozotocin (STZ, Sigma) (170 mg/kg body weight, i.p.) dissolved in a 0.1 M citrate buffer, pH 4.5. The control group received an equivalent volume of 0.1 M citrate buffer, pH 4.5. Blood samples were obtained from the tail vein 15 days after STZ administration. Animals with glucose levels higher than 300 mg/100 mL were considered diabetic. On day 15 of STZ injection, diabetic and control animals received sodium metavanadate (0.2 mg/mL supplemented with 80 mM NaCl, pH 7 in drinking water) for 15 days.

2.3. Northern blot analysis

Total RNA was extracted from the liver immediately after sacrifice and stored frozen until further use [15]. Probes were generated by PCR using the following primers: forward 5′-GACCGAGACCTCATGACACTTC-3′ and reverse 5′-GACAGTTCAGACTCAACTGCGTGGAGC-3′ to quantify FECH mRNA levels, and forward: 5′-GGTTGATCCTGCCAGTAGCATA-3′ and reverse, 5′-AATGATCCTTCCGCAGGTTC-3′ for 18 S rRNA.

2.4. Retrotranscription and real-time PCR

Retrotranscription (RT) was performed using 1 μg of RNA and Thermo-stable M-MLV Reverse Transcriptase (Invitrogen). Real-time PCR (qPCR) was performed using 1X SYBR® Select-Master Mix (Applied Biosystems, Life Technologies, USA) in a StepOne™ Real-Time PCR System (Applied Biosystems). The primers used were: forward 5′-TCATGAGGGAAATCAATGATCGTA-3′ and reverse 5′-GCCCCAGAAGA-TATGTCGGAA-3 for Trf1, and forward 5′-GACGGCCAAGTCATCAC-TATTG-3′ and reverse 5′-CCACAGGATTCCATACCCAAGA-3′ for β-actin [19].

2.5. Measurement of glucose concentration

Glucose levels were determined in serum and urine using the "Glycemia Enzymatic kit" (Wiener lab, Rosario, Santa Fe, Argentina) according to the manufacturer's instructions. Glucose was enzymatically oxidized to gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminophenazone and 4-hydroxybenzoate in a peroxidase-catalyzed reaction, yielding quinoneimine. The determinations were measured in a spectrophotometer at 505 nm against a reagent blank. Glucose concentration was expressed as milligrams per deciliter (mg/dL).

2.6. Immunoblot analysis

For immunoblot analysis, liver samples were processed as previously described by Oliveri et al. [15]. Briefly, liver samples were homogenized in the presence of protease inhibitors (Sigma P8340) and centrifuged at 17,000×*g* for 30 min at 4 °C. The supernatants were fractionated and stored at 70 °C. To perform SDS-PAGE, the same amount of protein from each sample was loaded (usually 50–90 μg) onto each lane of the gel, electrophoresed and then transferred to nitrocellulose membranes (Hybond ECL Amersham, USA). The membranes were blocked and incubated with the corresponding antibodies: FECH antibody, kindly provided by Dr Shigeru Taketani (Department of Biotechnology, Kioto Institute of Technology, Japan); monoclonal anti-*O*-GlcNAc (CTD 110.6), kindly provided by Dr G. Hart (The Johns Hopkins University, Baltimore, MD, USA), and anti-Sp1 from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Monoclonal anti-phosphoserine antibody was produced in mouse clone PSR-45 (P5747, Merck, USA).

Immunoassays of nuclear extracts were performed as described by Oliveri et al. [15]. The membranes were incubated with the secondary antibody conjugated to peroxidase. The choice of the secondary antibody depended on the origin of the primary one. The bands were revealed by using the Enhanced Chemiluminescent (ECL Amersham, USA) Western blot detection system, based on the principle of chemiluminescence, according to the manufacturer's protocol. The plates were exposed to AGFA radiographic plates.

2.7. Chromatin immunoprecipitation assays

For chromatin immunoprecipitation (ChIP) assays, liver samples were processed as previously described by Oliveri et al. [15]. Briefly, livers were excised and washed with PBS 1X, cut into small pieces, and embedded in PBS 1X/formaldehyde 1 % for 15 min at room temperature. The reaction was stopped with 2 M glycine and homogenized with PBS 1X plus protease inhibitors (Sigma P8340). The homogenate was centrifuged at 855×*g* for 10 min at 4 °C. Pellets were resuspended, incubated with lysis buffer (5 mM Tris-HCl, pH 8 plus 85 mM KCl and 0.5 % Nonidet P40) for 15 min on ice, and then centrifuged at 2376×*g* for 5 min at 4 °C. The nuclear fraction obtained was resuspended in the corresponding buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1 % (v/v) SDS) on ice for 20 min. Then, 0.1 g G-5235 glass spheres were added and the samples were sonicated for 5–7 min (10 μm, Soniprep 150) to obtain the DNA (300–600 bp), which was stored at −70 °C.

For immunoprecipitation, the samples were then centrifuged and the supernatants obtained were cleaned with A/G agarose beads (protein A/G Plus-Agarose Santa Cruz Biotechnology) previously blocked with salmon sperm. A fraction of the supernatant was treated with the specific antibody (Sp1), whereas another fraction was used as control to detect the possible non-specific interaction between the chromatin and the A/G agarose beads (mock control).

To recover the complex formed by the antibody, protein and DNA, agarose beads were added again and centrifuged. The pellet obtained (mock control; immunoprecipitated) was washed several times with buffers (a) once with low salinity 20 mM Tris-HCl, pH 8.1 containing 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, and 150 mM NaCl; b) then with high salinity 20 mM Tris-HCl, pH 8.1 containing 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, and 500 mM NaCl; c) then with buffer 10 mM Tris-HCl, pH 8.1 containing 250 mM LiCl, 1 % nonidet P40, 1 % deoxycholate, and 1 mM EDTA; d) and twice with TE buffer (10 mM Tris-HCl, pH 8.1 plus 1 mM EDTA)), and centrifuged at 2000×*g* after each wash. Finally, the pellet was resuspended with elution buffer Tris-HCl, pH 7.5 (1 % SDS, 100 mM $NaHCO_3$). The cross linking was reversed in the presence of sodium chloride and RNAase. DNA purification was carried out by the phenol/chloroform/isoamyl method. The DNA obtained was resuspended in TE buffer and analyzed by PCR.

2.8. DNA amplification from the ChIP

For DNA amplification, a PCR reaction was carried out using 2 μL of DNA from the ChIP. The amplification program was: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 15 s, 72 °C for 30 s, 72 °C for 1 min, and finally 4 °C. The primers used to perform the PCR were: forward 5′-TTTCGACCAGGTTGGCAGAA-3 and reverse 5′-AAGCATTTCCGG-GACGGC-3. The PCR product was a fragment of 339 bp of the FECH promoter area (−241 to +98) [20].

2.9. Protein concentration

Total protein concentration was measured by Bradford method [21].

2.10. Statistical analysis

All experiments included three to six mice for each treatment group. Assays were repeated at least three times with similar results. Data are presented as mean ± S.D. Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by multiple comparisons among groups or between each group and the control using Turkey-Kramer's or Dunnett's test respectively. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characterization of the diabetic mouse model

Table 1 shows the glucose levels in STZ-treated animals (day 32 after STZ injection) and the effect of vanadate administration on this parameter. In the STZ group, blood glucose level was around 300 % ($P < 0.001$) higher (average value: 717 mg/dL) than in control mice (165 mg/dL). After vanadate administration, blood sugar levels in the STZ group decreased 50 %, although they were still above the control value (around 100 %, $P < 0.001$). A similar decrease was observed in control animals receiving vanadate.

In urine, glucose levels reached 2000 mg/dL by effect of STZ. In control mice or in groups receiving vanadate, urine glucose was undetectable.

3.2. FECH mRNA expression levels in diabetic animals as a function of time

In our experimental model of type 1 diabetes mellitus, we evaluated the behavior of FECH mRNA and protein levels. The mRNA levels of the 2.2-kb erythroid FECH transcript showed a significant increase from day 24 after STZ injection (175 %) compared to controls and continued to increase until the end of the trial. The 2.9-kb transcript, found mainly in non-erythroid tissues, increased by approximately 100 % on day 24 but then decreased to 20 % compared to control (Fig. 1).

In contrast, FECH protein levels showed no variations during the time studied (Fig. 2).

Table 1
Glucose levels in diabetic mice.

Groups		Glucose levels (mg/dL)	
	n	Serum	Urine
Control	12	165.0 ± 36.0	ND
STZ	18	717.0 ± 145.0 *	2000
Vanadate	6	79.0 ± 0.7 * ◆	ND
STZ + vanadate	10	392.0 ± 100.0 * ◆	ND

Results are mean ± S.D. for each group. *$P < 0.001$ respect to control.
◆ $P < 0.001$ respect to the STZ group. ND: not detectable, n: number of animals.

3.3. FECH mRNA and protein expression levels in diabetic animals treated with vanadate

Considering that the diabetic status caused a deregulation in the expression levels of FECH mRNA, we next studied the effect of vanadate, an insulin-mimetic agent (Fig. 3). Vanadate reversed the increase in FECH mRNA levels due to the diabetic status. In the case of the 2.2-kb transcript, vanadate caused a decrease below control levels. In normoglycemic animals, FECH mRNA levels were not affected by vanadate.

Regarding FECH protein levels, the treatment with vanadate caused a decrease below controls (Fig. 4).

3.4. Transferrin receptor 1 (TfR1) mRNA expression levels in diabetic animals treated with vanadate

As an indirect assessment of intracellular hepatic Fe levels, TfR1 mRNA expression in diabetic and vanadate-treated animals was determined by qRT-PCR. In STZ mice, hepatic TfR1 mRNA expression was 1.32 times higher than in control animals, while in the vanadate-treated group, the levels were similar to those of the control group (Fig. 5).

3.5. Relationship between phosphorylation and glycosylation of Sp1 in diabetic mice

The effects of the diabetic status and vanadate on Ser phosphorylation levels in the transcription factor Sp1 are shown in Fig. 6. In the liver of diabetic animals, a decrease in Ser phosphorylation was observed compared to control animals. On the other hand, vanadate treatment did not modify phosphorylation levels. These findings confirm that Sp1 glycosylation might be involved in the changes in FECH expression caused by the diabetic status.

3.6. Measurement of the amount of Sp1 binding to the FECH promoter in diabetic animals

To evaluate whether the Sp1 O-GlcNAcylation observed in STZ and STZ + vanadate groups affects its binding to the FECH promoter region and modify mRNA expression, the Sp1 binding to the FECH promoter was analyzed using ChIP.

In diabetic animals, Sp1 binding to the FECH promoter was increased, a fact that correlates with the increase in mRNA levels in these animals. Vanadate treatment reversed this increase in Sp1 binding (Fig. 7).

4. Discussion and conclusions

The last step in the heme biosynthetic pathway occurs in mitochondria and is mediated by FECH [9], an enzyme that catalyzes the insertion of Fe^{+2} into protoporphyrin IX to form heme. Eukaryotic FECH is synthesized as a cytosolic precursor of approximately 47 kDa, being its mature mitochondrial form of around 42 kDa [11,22].

The isolation of mouse and human FECH cDNA has revealed, in both cases, the presence of two mRNAs that differ at their 3′ ends [13,23]. In both species, a single promoter is involved in the expression of constitutive and erythroid FECH [13,20,24]. The FECH promoter contains two Sp1 *cis*-elements and upstream GATA and NF–E2 *cis*-elements [13,25]. Transient transfection assays in non-erythroid and erythroid cells have demonstrated that the minimal promoter (0.150 kb) containing Sp1 binding sites is enough to provide erythroid preferential expression [26].

In mouse tissues such as spleen, liver, brain, kidney, muscle and reticulocytes, as well as in MEL cells, two transcripts of 2.2 and 2.9 kb have been found [12]. This is explained by the fact that two polyadenylation sites produce two mRNAs of different lengths related to the expression of the enzyme in erythroid and non-erythroid cells [11,12,24]. The 2.2-kb transcript is more expressed in erythroid tissues such as

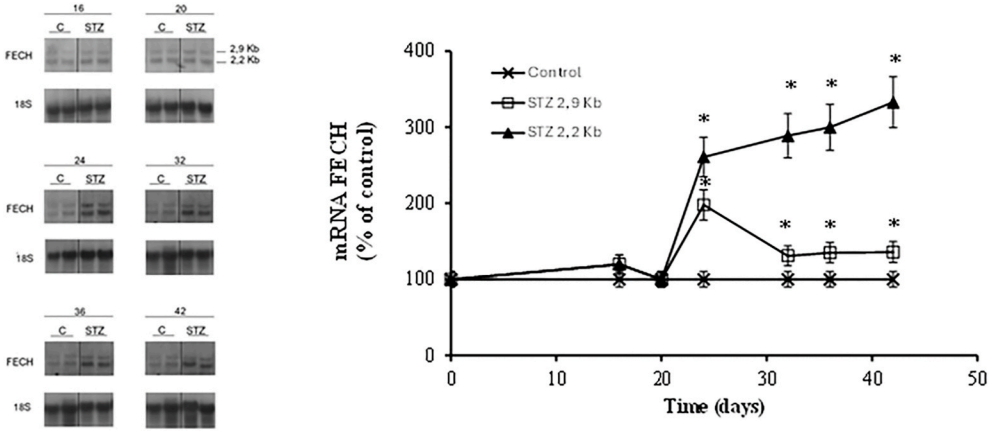

Fig. 1. FECH mRNA expression levels in the liver of diabetic mice as a function of STZ time administration.
Left panel: Northern blot analysis. Samples were obtained at different times after STZ injection (16, 20, 24, 32, 36 and 42 days). Right panel: Quantification of FECH mRNA using 18S rRNA as loading control. Data represent mean values ± SD from three independent experiments and are expressed as a percentage of control values. (*) $P < 0.05$, significant difference as compared to the control group. Other experimental details are described in the text.

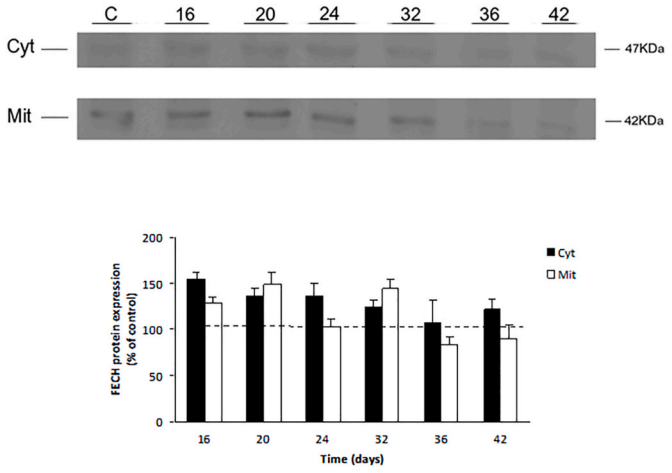

Fig. 2. FECH protein expression levels in liver cytoplasm (Cyt) and mitochondria (Mit) of diabetic mice as a function of STZ time administration.
Upper panel: Western blot analysis. Samples were obtained at different times after STZ injection (16, 20, 24, 32, 36 and 42 days). Bottom panel: Quantification of FECH protein expression. Data represent mean values ± SD from three independent experiments and are expressed as a percentage of control values (——). Other experimental details are described in the text.

spleen and reticulocytes, whereas the 2.9-kb transcript is more expressed in non-erythroid tissues [12,23]. In reticulocytes, the increased stability of the 2.2 kb transcript is due to the absence of sequences rich in A + U in the 3′ UTR of the messenger [12]. This would agree with that observed in the present work, where both FECH messengers were increased; however, the amount of transcripts was different probably due to alterations in their stability. On the other hand, FECH protein levels were unaltered. Other authors have also observed similar findings before the primitive erythroid-cell stage, suggesting the presence of a translational control mechanism [26,27]. This effect has also been observed for other proteins [28–30].

In animals treated with vanadate, FECH protein levels decreased to values lower than basal ones. This decrease could be the result of a decrease in the 2.2-kb mRNA, in its translation and/or due to changes in its half-life.

The decrease observed in the amount of FECH protein caused by vanadate could be due to an inhibition of aconitase enzyme [31], which would prevent the availability of newly formed Fe–S clusters for insertion into the FECH apoprotein and lead to changes in its stability [32]. In

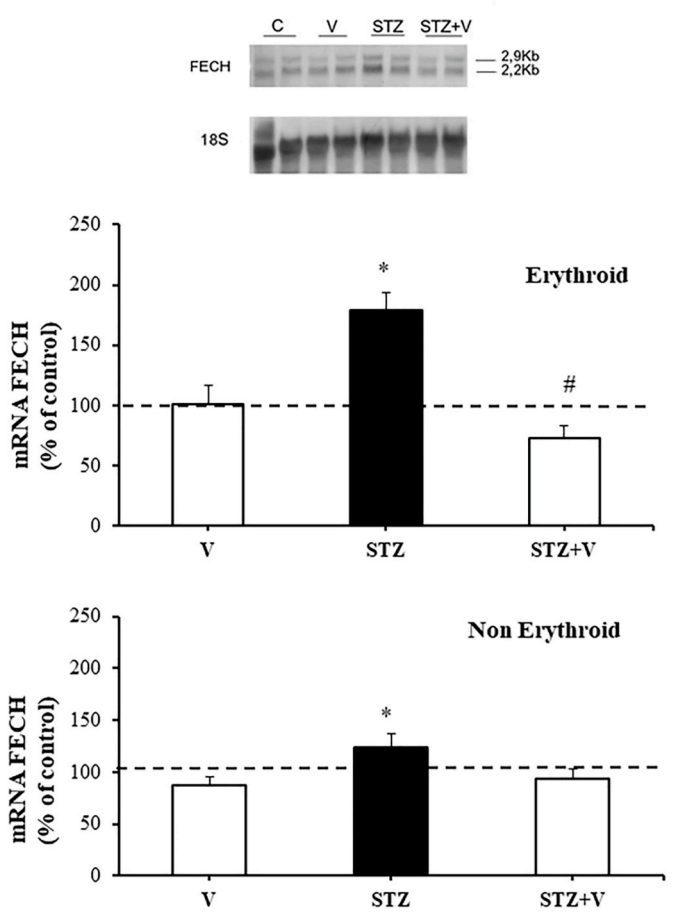

Fig. 3. Effect of vanadate on FECH mRNA expression levels in the liver of diabetic mice.
Upper panel: Northern blot analysis. Bottom panels: Quantification of FECH mRNA using 18S rRNA as loading control. Data represent mean values ± SD from three independent experiments and are expressed as a percentage of control values on day 32. (*) $P < 0.05$, significant difference as compared to the control group (——). (#) $P < 0.01$, significant difference respect to the STZ group. Other experimental details are described in the text.

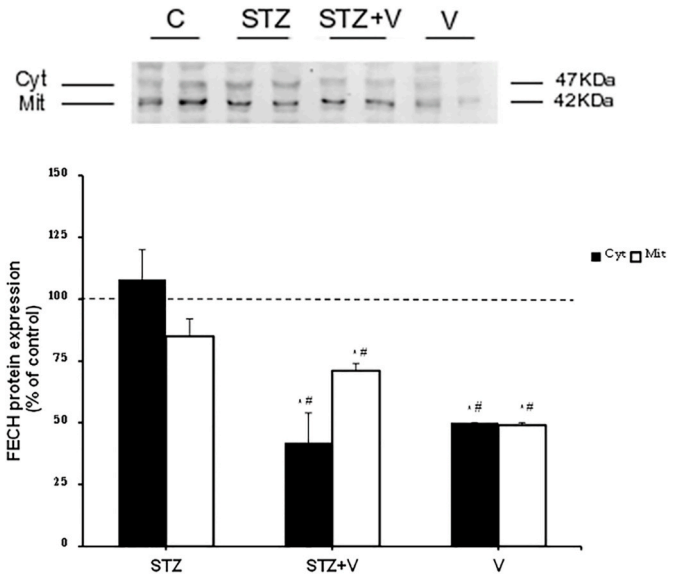

Fig. 4. Effect of vanadate on FECH protein expression levels in liver cytoplasm (Cyt) and mitochondria (Mit) of diabetic mice.
Upper panel: Western blot analysis. Bottom panel: Quantification of FECH protein expression. Data represent mean values ± SD from three independent experiments and are expressed as a percentage of control values on day 32. (*) $P < 0.001$, significant difference as compared to the control group (——). (#) $P < 0.001$, significant difference respect to the STZ group. Control group (——). Other experimental details are described in the text.

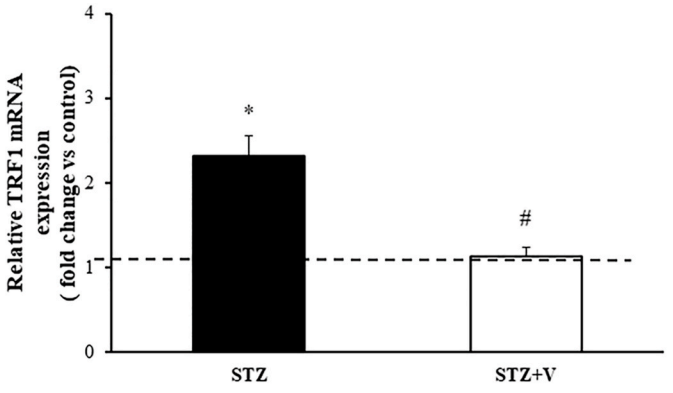

Fig. 5. Effect of vanadate on TfR1 mRNA expression in the liver of diabetic mice.
The expression levels of TfR1 mRNA relative to actin were determined by the qRT-PCR technique. Data represent mean values ± SD from three independent experiments and are expressed as a percentage of control values on day 32. (*) $P < 0.001$, significant difference as compared to the control group (——). (#) $P < 0.001$, significant difference respect to the STZ group. Other experimental details are described in the text.

mammals, the expression and enzymatic activity at the post-translational level of FECH are regulated by the intracellular concentration of Fe through the [2Fe–2S] cluster [33]. When Cos7 cells transfected with a plasmid containing C-terminal region of mammalian FECH were treated with increasing concentrations of iron, a progressive increase in FECH activity was observed [32]. When the level of intracellular iron is low or when nitric oxide is present, the cluster disassembles, resulting in a decrease in heme synthesis [33].

Experiments carried out with MEL cells have shown that the half-life of the *de novo* FECH enzyme decreases from 35 h in normal cells to less than 1 h in iron-deficient cells [34]. These experiments also showed that, after iron depletion, there were no changes in the abundance of the

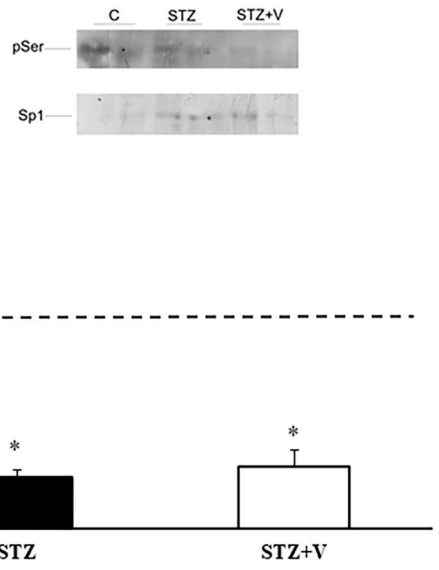

Fig. 6. Effect of the diabetic status and vanadate on the relation between pSer and Sp1 in the liver of diabetic mice. Data represent mean values ± SD from three independent experiments and are expressed as a percentage of control values on day 32. (*) $P < 0.001$, significant difference as compared to the control group (——). Other experimental details are described in the text.

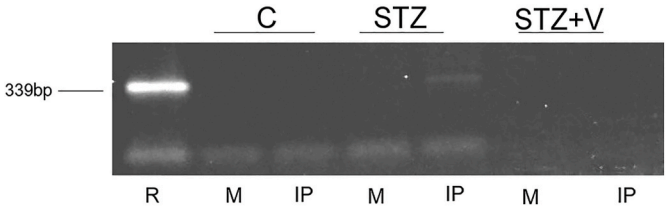

Fig. 7. Sp1 binding to the FECH promoter by the chromatin immunoprecipitation technique.
The arrow indicates the amplified fragment. R: positive control. M: mock. IP: immunoprecipitated.

mature protein. This suggests that, during iron deficiency, the mature mitochondrial FECH containing the [2Fe–2S] cluster is not susceptible to degradation, while the de *novo* synthesized apoprotein is rapidly degraded in the absence of available iron [34].

Regarding TfR1, its expression is regulated post-transcriptionally by the iron regulatory protein system (IRE–IRP) and is inversely correlated with the cellular iron status: TfR1 expression is high during iron deficiency and low when iron levels are high [35–37].

The increase in TfR1 mRNA observed in STZ mice suggests a decrease in intracellular iron levels and agrees with that found by other authors who reported an increase in TfR1 mRNA expression in the liver, kidney and myocardium of STZ diabetic rats compared to control animals [38–40]. These results would support the hypothesis that a decrease in hepatic iron would lead to a decrease in the levels of the [Fe–S] cluster pool and, as a consequence, to the degradation of the FECH protein that is translated *de novo* without modifying the levels of the mature protein. In the present study, the TfR1 levels of the group treated with vanadate were like those of controls; therefore, the decrease in FECH protein levels below the control value would not be due to a lack of hepatic iron, and thus other mechanisms should be considered.

Several studies have shown that a balance between O-glycosylation and phosphorylation may be an important regulatory mechanism for some proteins [41–43]. In this regards, Majumdar et al. [44] demonstrated that hyperglycemia increases O-GlcNAcylation and decreases serine/threonine phosphorylation of Sp1, leading to increased Sp1

transactivation and Sp1-dependent expression of genes like calmodulin.

In diabetic animals, Sp1 protein levels were not modified, while those of its O-glycosylated form were increased compared to control animals [45]. An increase in glucose flux through the hexosamine pathway, as occurs in diabetic patients, is accompanied by an increase in O-GlcNAcylation and in the *in vitro* transcriptional activity of Sp1 [41,46]. In agreement with this, in the present study we observed a decrease in Ser phosphorylation as compared to control animals. On the other hand, vanadate treatment did not modify phosphorylated levels.

These results suggest that the increase observed in FECH mRNA could be due to an increase in Sp1 transcriptional activity and this would agree with *in vitro* studies that have indicated that O-GlcNAcylation can stimulate (up-regulate) the binding of Sp1 to DNA [47].

Some researchers have shown that O-glycosylation activates Sp1-dependent transcription without altering its DNA binding [48–51]. On the other hand, it has also been reported that O-glycosylation of Sp1 inhibits its ability to activate transcription [45,51–53].

In the present study, the immunoprecipitation technique allowed observing that the STZ group showed an increase in the amount of Sp1 bound to the FECH promoter, which was decreased by the administration of vanadate. These results correlate with the mRNA levels of the enzyme found in both experimental groups, suggesting that the effect observed on FECH expression would be modulated by changes in the binding of Sp1 to its promoter.

The increased binding of the transcription factor Sp1 to the FECH promoter in diabetic animals led to an increase in FECH mRNA levels; however, this increase was not reflected in the amount of protein. These results confirm that reported by other authors who have proposed FECH as another control point in heme biosynthesis [9,10], suggesting the presence of a translational suppression mechanism that contributes to maintaining stable intracellular heme levels. These findings are particularly relevant in the light of evidence describing the physiological importance of a precise and robust regulation of the intracellular heme pool since both heme deficiency and excess can be detrimental to cellular metabolism [54–57].

In conclusion, the results here presented show for the first time the involvement of a translational regulation mechanism of FECH *in vivo*. Furthermore, the data on FECH induction in STZ-diabetic animals could support the hypothesis of the existence of molecular mechanisms that establish a connection between carbohydrate homeostasis and the gene expression of key enzymes in the heme biosynthesis pathway [15,58,59].

CRediT authorship contribution statement

Leda María Oliveri: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ana Maria Buzaleh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Esther Noemí Gerez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.101989.

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