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An increase in *O*-GlcNAcylation of Sp1 down-regulates the gene expression of pi class glutathione *S*-transferase in diabetic mice

Leda María Oliveri [a], Ana María Buzaleh [a,b], Esther Noemí Gerez [a,c,*]

[a] Centro de Investigaciones Sobre Porfirinas y Porfirias (CIPYP), UBA-CONICET, Hospital de Clínicas José de San Martín, Universidad de, Buenos Aires, Argentina
[b] Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de, Buenos Aires, Argentina
[c] Cátedra Bioquímica General Celular y Molecular, Facultad de Ciencias Médicas, Universidad Católica Argentina (UCA), Buenos Aires, Argentina

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ABSTRACT

Oxidative stress is a key factor contributing to the development of diabetes complications. Glutathione *S*-transferases (GSTs) protect against products of oxidative stress by conjugating glutathione to electrophilic substrates, producing compounds that are generally less reactive and more soluble. The expression and activity of GSTs during diabetes have been extensively studied, but little is known about regulation mechanisms of Pi-class GST (GSTP). The aim of the present study was to evaluate how GSTP is regulated in a Streptozotocin (STZ)-induced murine diabetes model. GST activity and GSTP expression were determined in adult male mice diabetized with STZ. Specificity protein 1 (Sp1) expression and O-glycosylation, as well as the role of AP-1 members Jun and Fos in the regulation of GSTP expression, were also assessed. The results showed that GST total activity and GSTP mRNA and protein levels were decreased in the diabetic liver, and returned to normal values after insulin administration. The insulin-mimetic drug vanadate was also able to restore GST activity, but failed to recover GSTP mRNA/protein levels. In diabetic animals, *O*-glycosylated Sp1 levels were increased, whereas, in insulin-treated animals, glycosylation values were similar to those of controls. After vanadate administration, Sp1 expression levels and glycosylation were lower than those of controls. Our results suggest that hyperglycemia could lead to the observed increase in Sp1 O-glycosylation, which would, in turn, lead to a decrease in the expression of Sp1-dependent GSTP in the liver of diabetic mice.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in the secretion and/or action of insulin. This hyperglycemia generates lipid peroxidation induced by free radicals, overactivity of the hexosamine pathway, and protein glycosylation [1–3]. These changes lead to alterations in the synthesis of proteins through modifications in their transcription factors, resulting in a reduction of the endogenous antioxidant defense system [4–6]. Glutathione (GSH), which is the main endogenous antioxidant produced by the cell, participates directly in the neutralization of free radicals [7,8].

Glutathione *S*-transferases (GSTs, EC 2.5.1.18), which are major cytosolic phase II detoxification enzymes highly expressed in the liver, catalyze the conjugation of a number of electrophilic compounds with GSH, acting as a defense mechanism that neutralizes xenobiotics, toxins and carcinogens [9,10].

In mammals, GSTs can be classified into five subclasses, namely Alpha, Pi, Mu, Theta, and microsomal, each of which has different substrate specificity [11,12]. Pi class GST (GSTP) has particularly high affinity for small unsaturated aldehydes generated by lipid peroxidation [13–16]. Previous studies have shown that activator protein-1 (AP-1) and specificity protein 1 (Sp1) recognize sequences that are absolutely required for transcriptional activity of the GSTP promoter [17–19], and that they are influenced by metabolic changes associated with diabetes [20,21]. It has also been found that AP-1 transcriptional complex is stimulated by insulin in several cell types [22,23]. Additionally, other studies have shown that hyperglycemia causes an increase in the *O*-GlcNAcylation of Sp1, which induces activation of the plasminogen activator inhibitor-1 (PAI-1) promoter in vascular smooth muscle cells [24] as well as of TGF-1 and the PAI-1 promoter in arterial endothelial cells [25].

Abbreviations: STZ, Streptozotocin; Erk1/2, extracellular signal-regulated kinase 1/2; *O*-GlcNacylation, O-linked *N*-acetylglucosamine glycosylation.

* Corresponding author. Av. Córdoba, 2351 1120, Buenos Aires, Argentina.
E-mail addresses: esthernoemigerez@uca.org.ar, cuyro@yahoo.com (E.N. Gerez).

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The effects of diabetes or insulin on the activity and expression of GSTs are controversial [26–28] and the regulatory mechanisms underlying GSTP expression in diabetes remain unclear [28]. In diabetic mice, the activity of hepatic GSTs is increased [26], whereas, in rats with chemically induced diabetes, the activity is decreased [27,28].

In the present study, we investigated the changes in GST activity and GSTP expression in a murine model of streptozotocin (STZ)-induced diabetes. We also tested the hypothesis that AP-1 and/or Sp1 are involved in diabetes-induced changes in GSTP expression. We found that both GST activity and GSTP expression were impaired upon the onset of diabetes. The possible role of Sp1 O-glycosylation in the changes of GSTP basal expression is discussed.

2. Materials and methods

2.1. Animals

Adult male mice of the *CF1* strain (18–20 g initial body weight) were maintained in a controlled environment with a 12 h light/12 h dark cycle, with free access to a standard pellet diet (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentina) and water *ad libitum*. All animals were treated in accordance with the guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC). 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. Experimental design

Diabetes was induced by a single dose of STZ (Sigma) (170 mg/kg, of body weight, i.p. Freshly dissolved in 0.1 M Na citrate buffer pH 4.5). Non-diabetic mice (control group) were injected with an equivalent volume of citrate buffer. Sixteen days after STZ injection, glucose levels were measured in a blood sample obtained from the tail. The animals were considered diabetic when their blood glucose levels were greater than 300 mg/dL. These STZ-induced diabetic animals were randomly subdivided into three groups [1]: the STZ group, which did not receive any further treatment after diabetes confirmation [2]; the STZ + I group, which received insulin for 15 days after the confirmation of diabetes (a daily dose of insulin (5 U/100 g, i.p., Sigma) plus a daily dose of Insuman® N human insulin (30 U/100 g, s.c.)), and [3] the STZ + V group, which received the glucose-lowering drug vanadate in the drinking water (0.15 mg/mL) for 16 days, starting immediately after diabetes confirmation.

Animals were sacrificed at different time points, as indicated in the legend of the figures. The livers were removed and quickly frozen.

All experimental procedures included between four and six mice for each treatment group.

2.3. RNA isolation

To evaluate GSTP expression, total RNA was extracted from liver homogenates by using the acid guanidinium thiocyanate-phenol-chloroform method, according to Chomzymski & Sacchi [29]. For Northern blot analysis, 18 μg of RNA was size-fractionated on a 1% agarose/formaldehyde gel, passively blotted to a nylon membrane (Hybond-N+, Pharmacia Biotech), and hybridized according to standard procedures. The probe for GSTP was a rat cDNA, pGP 5, kindly provided by Dr. Masami Muramatsu, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Japan [30]. The probe for the 18 S rRNA gene sequence, used as internal control, was generated by PCR, using the following primers: 18 S forward, 5′- GGTTGATCCTGCCAGTAGCATA-3′ and reverse, 5′-AATGATCCTTCCGCAGGTTC-3'. The probes were P32-labeled with a Random Primer kit (Invitrogen). The bands were visualized on radiographic film, and the resulting images scanned and quantified using Scion Imaging software. The values obtained were graphed.

2.4. Enzymatic activity

GST activity was determined following an adaptation of the method described by Habig et al. [31]. The enzyme solution was the supernatant obtained by centrifugation at 15,000*g* for 20 min of liver whole homogenate with a 100% yield. The reaction mixture, which contained: GSH 1 mM, 0.1 M potassium phosphate buffer pH 6.5, 1-chloro-2,4-dinitrobenzene 1 mM and 0.5 mL of the enzyme solution in a final volume of 2 mL, was incubated at 25 °C for 3 min and the absorbance was read at 340 nm in a HEWLETT PACKARD model HP8452 A (Hewlett Packard Company, San Jose, CA 95122, USA). One enzymatic unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min under assay standard conditions. Specific activity was defined as enzymatic units per mg of protein.

2.5. Immunoblot analysis

For immunoblot analysis, liver samples were homogenized as described previously by Oliveri et al. [32]. Briefly, whole tissue extracts containing equivalent amounts of proteins (45–100 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6.5–12% gels) and electrophoretically transferred to a nitrocellulose membrane (Millipore, Inc.). The specific percentage of gel and the amount of protein loaded in each lane are indicated in the legend of the figures. To measure protein expression, the membranes were incubated with the following primary antibodies: GSTP (BioSystems), Sp1, anti-actin, Erk1/2, *P*-Erk1/2, *c*-Jun, *c*-Fos (Santa Cruz Biotechnology, CA, USA) and monoclonal anti-*O*-linked *N*-acetylglucosamine (anti-*O*-GlcNAc) (CTD 110.6), kindly provided by Dr. G. Hart (The Johns Hopkins University, Baltimore, MD, USA). The membranes were then incubated with the proper secondary antibody, i.e. anti-mouse (Santa Cruz Biotechnology, CA, USA) or horseradish peroxidase-conjugated goat anti-mouse IgM (chain-specific) (Sigma), at 25 °C for 60 min. β-Actin (Santa Cruz Biotechnology, CA, USA) was used as the internal control to normalize the expression levels. Finally, the protein bands were detected with X-ray film. Protein bands were quantified from scanned images using the Scion Image software and the different ratios of the target proteins were calculated. The values obtained were graphed.

2.6. Protein determination

Protein was measured by the procedure of Bradford [33], using bovine serum albumin as the standard.

2.7. Glucose levels

The serum samples were assayed for glucose by using the "Glicemia Enzimática" kit (Wiener lab, Rosario, Santa Fe, Argentina). Glucose concentration was determined following the method described by the manufacturer. The reaction mixture containing glucose oxidase (≥3000 U/L), peroxidase (≥400 U/L), 1.25 mM 4-aminofenazone, 4.6 mM Tris Buffer pH 7.4, and 20 μL of sample in a total volume of 2.02 mL was incubated at 37 °C for 10 min. The absorbance was measured at 505 nm using a HEWLETT PACKARD HP8452 A (Hewlett Packard Company, San Jose, CA 95122, USA). Under the assay conditions, the color of the final reaction was stable for 1 h. The glucose concentration was determined as mg/dL. Glucose was used as a standard.

2.8. Statistical analysis

The results were analyzed using the GraphPad InStat version 2.0 software. All experiments included between four and six mice for each treatment group and were repeated at least three times. Data are

presented as mean ± SD. 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. In all instances, probability values of $p < 0.05$ were considered significant.

3. Results

3.1. Body weight and blood glucose levels

Results corresponding to the assessment of body weight and blood glucose levels of the different experimental groups are shown in Table 1. The average weight of mice in the four groups was 22 ± 2.0 g at the beginning of the study and the mean glycemia level was 165 ± 36 mg/dL. Animals were sacrificed 32 days after the beginning of the treatment. Mice in the control group gained an average of 12 g of body weight and showed normal fasting serum glucose levels. In contrast, diabetic mice were characterized by a significantly lower body weight (68%) and higher blood glucose levels (270%) than the control group. In STZ animals treated with insulin or vanadate for 16 days, blood glucose levels were significantly reduced, although only insulin returned glycemia to control values.

3.2. C: control; I: insulin; V: vanadate

3.2.1. Time course of GST activity and GSTP mRNA expression in diabetic mice

In vivo and *in vitro* results reported by other authors suggest that both diabetes and insulin levels seem to be involved in the regulation of GST [27,28,34]. Therefore, GST activity and GSTP expression in STZ-treated mice were determined at different timepoints after STZ injection (Fig. 1). GST activity decreased continuously throughout the study period from 30% on day 16–60% on day 38. Furthermore, as shown in Fig. 1B, GSTP mRNA expression was significantly reduced at the time of diabetes confirmation (day 16) and remained low throughout the whole period of the experiment (38 days), although with a temporal pattern different from that shown by the activity of GSTs.

Starting on day 16 after STZ i.p. Injection, liver and mRNA samples were obtained at different times (16, 20, 24, 32, 38 days). The data show the mean values ± SD from three independent experiments and are expressed as a percentage of control values. (*) $p < 0.001$ indicates significant differences as compared to the control group (——). Other experimental details are described in the Materials and Methods section. Sp. Act. = specific activity.

Effects of insulin and vanadate on GST activity and GSTP mRNA expression in diabetic animals.

Since the establishment of the diabetic state induces significant changes in the activity and expression of GSTs, we were interested in investigating whether these effects are either the direct consequence of insulin depletion or an indirect action caused by metabolic disorders associated with diabetes. For this purpose, we assessed the effects of the administration of insulin and the insulin-mimetic agent vanadate on the activity of GSTs and the expression of GSTP in diabetic animals (Fig. 2). After diabetes confirmation on day 16 after STZ injection, animals were

Table 1
Body weight and blood glucose levels.

Groups	N	Weight (g)	Glycemia (mg/dL)
C	12	34.0 ± 3.1	174 ± 25
STZ	18	23.0 ± 4.2[a]	643 ± 120[a]
STZ + I	9	31.0 ± 5.1[b]	117 ± 32[b]
STZ + V	10	20.0 ± 3.7[a]	392 ± 100[a,b]

Glucose levels and body weight were determined on day 32.
[a] $p < 0.001$ significant difference as compared to controls.
[b] $p < 0.001$ significant difference as compared to the STZ group.

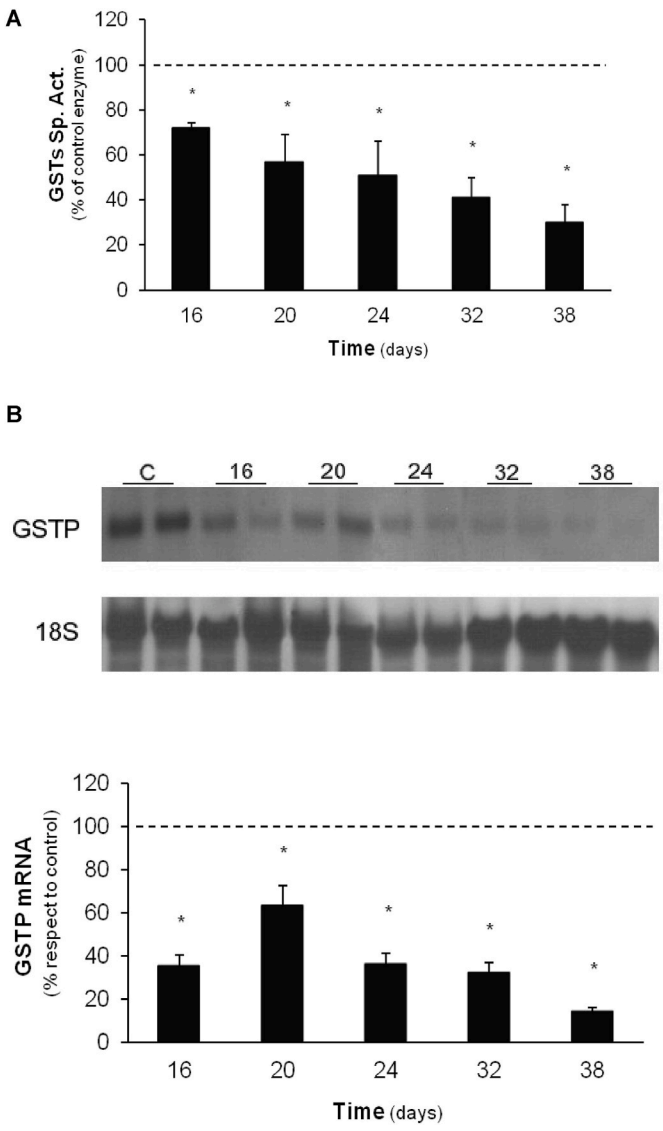

Fig. 1. Time course of GST activity and GSTP mRNA expression in livers from diabetic mice. (A) Hepatic GST activity. (B) GSTP mRNA levels assessed by Northern blot analysis, using 18 S rRNA as loading control. Quantification of liver GSTP mRNA is shown in the bottom panel.

treated for two weeks with daily subcutaneous injections of insulin or vanadate added to the drinking water. When diabetic animals received insulin or vanadate, GST activity levels returned to control values (Fig. 2A). These results are in accordance with that reported by Thomas et al. [35], who found that insulin reversed the effects of diabetes on GST activities. When diabetic animals received insulin, GSTP mRNA levels and protein content were also partially restored (80% of control values) (Fig. 2B and C). On the other hand, vanadate failed to demonstrate the same effect as insulin on both the expression of mRNA and protein content of GSTP (Fig. 2B and C).

Sp1 expression and O-glycosylation in the liver of diabetic mice.

Since the GSTP promoter contains "regulatory elements" for AP-1 and Sp1 that are absolutely required for their transcriptional activity [17–19], we examined the effects of diabetes on these transcriptional factors. Considering that the diabetic status leads to an increase in the O-glycosylation of proteins [5] and that post-translational modifications of Sp1 can modify the activity of this transcription factor [5,36], we decided to study the expression levels of Sp1 and its O-glycosylation (Fig. 3). In the liver of diabetic animals, Sp1 O-glycosylation increased, while the total amount of SP1 protein remained unchanged. The group

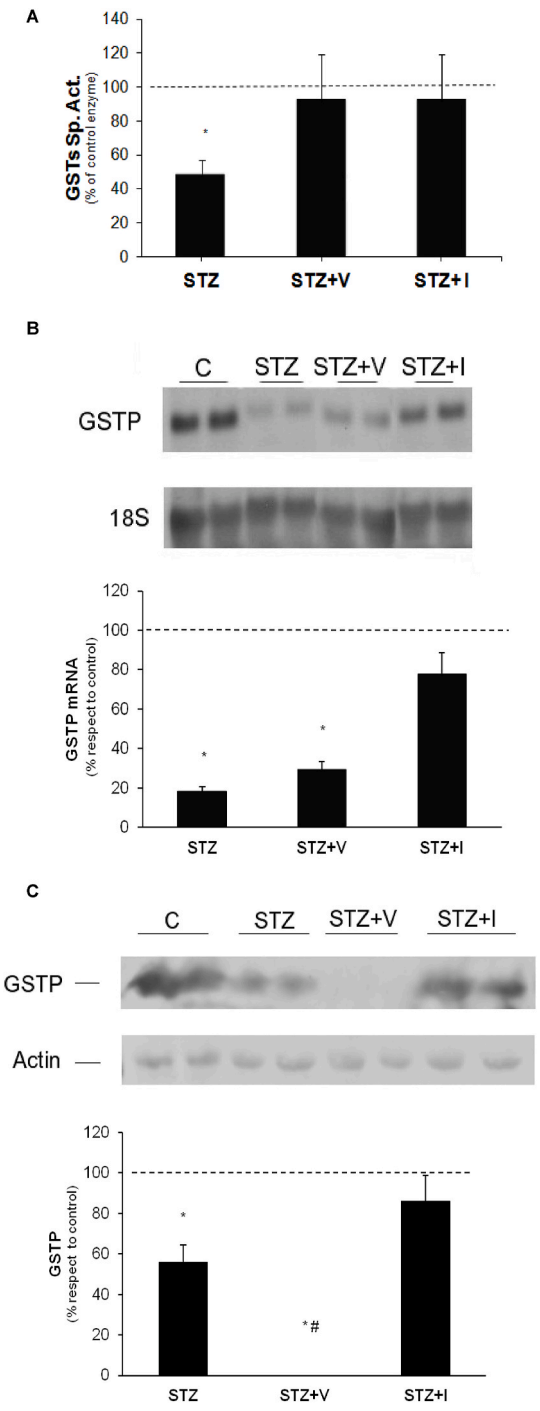

Fig. 2. GST activity and GSTP expression in the liver of diabetic mice treated with insulin or vanadate. (A) Hepatic GST activity. (B) GSTP mRNA levels assessed by Northern blot, using 18 S rRNA as loading control. The quantification of GSTP mRNA is shown in the bottom panel. (C) GSTP protein assessed by immunoblot analysis. Whole tissue extracts of liver (60 μg) were resolved using 12% SDS-PAGE gels. Experiments were performed in the livers of control, STZ, STZ + I and STZ + V mice. The data are expressed as a percentage of control values and represent mean values ± SD from three independent experiments. (*) $p < 0.001$ indicates significant differences as compared to the control group (——). (#) $p < 0.001$ indicates significant differences from the STZ group. Other experimental details are described in the Materials and Methods section. Sp. Act. = specific activity.

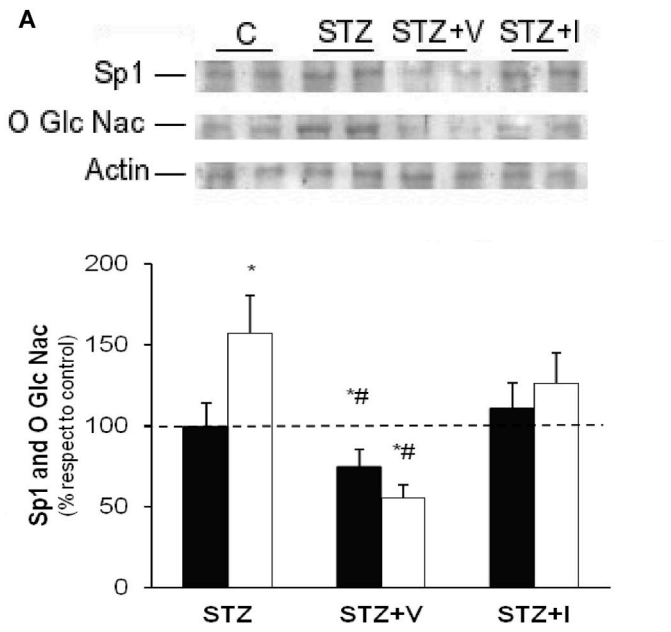

Fig. 3. Levels of Sp1 and its *O*-glycosylated form in diabetic and vanadate and insulin-treated animals. (A) The levels of Sp1 and its *O*-glycosylated form were tested by Western blot (6.5% gel; 90 μg of protein was loaded in each lane); actin was used as loading control. (B) The data are expressed as a percentage of control animals (——) and represent mean values ± SD from three independent experiments. (*) $p < 0.001$ indicates significant differences as compared to controls, (#) $p < 0.001$ indicates significant differences as compared to STZ group. ■ Sp1, □ *O*-GlcNac. Other experimental details are described in the Materials and Methods section.

treated with insulin showed both protein and O-glycosylation values similar to those of the control group. On the other hand, vanadate treatment led to even lower levels of expression and Sp1 O-glycosylation than those of the control group ($p < 0.001$).

Effects of insulin and vanadate on cFos/c-Jun nuclear levels in the liver of diabetic mice.

Since the AP-1 complex also participates in the regulation of GSTP expression, we decided to investigate the changes of this factor in the liver of diabetic mice (Fig. 4).

STZ-induced diabetic animals (STZ group) showed no differences in cFos protein levels compared to the control group, while both the STZ + I and STZ + V groups did exhibit a significant increase in this transcription factor ($p < 0.001$). On the other hand, in the three treatment groups, *c*-Jun protein levels were lower than those of the control group ($p < 0.001$).

The decrease in *c*-Jun levels in diabetic animals could lead to lower levels of AP-1 and, consequently to a reduced expression of GSTP. However, insulin failed to restore the normal levels of *c*-Jun, suggesting that this effect of insulin on the expression of GSTP would not be mediated by AP-1.

Effects of insulin and vanadate on Erk1/2 phosphorylation in diabetic mice.

Since the members of the AP-1 transcription factor family are well-known targets of the Erk1/2 pathway [37,38], the activities of these kinases were next determined. Erk1/2 phosphorylation levels increased in the liver of diabetic mice and remained high when these animals were treated with insulin. In contrast, vanadate treatment caused a decrease in Erk1/2 phosphorylation (Fig. 5) ($p < 0.001$).

These results show no correlation with either the changes observed in GSTP expression or *c*-Jun and *c*-Fos levels, suggesting that the ERK1/2 pathway would not play a significant role in those changes.

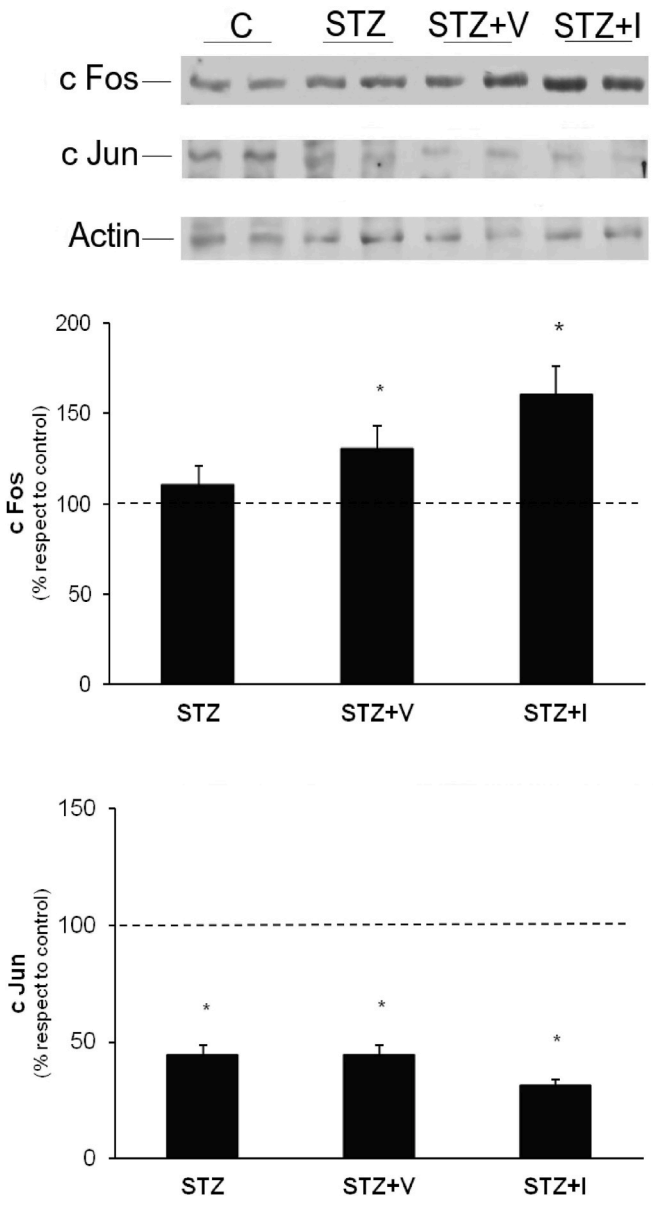

Fig. 4. Effect of vanadate and insulin on the expression of *c*-Fos and *c*-Jun in the liver of control, STZ, STZ + I or STZ + V mice. *c*-Fos and *c*-Jun levels were evaluated by Western blot (10% gel for *c*-Fos and 7% gel for *c*-Jun); 100 μg of protein was loaded in each lane; actin was used as loading control. The data are expressed as percentages of the values of control animals (——) and represent mean values ± SD from three independent experiments. (*) $p < 0.001$ indicates significant differences as compared to the control group. Other experimental details are described in the Materials and Methods section.

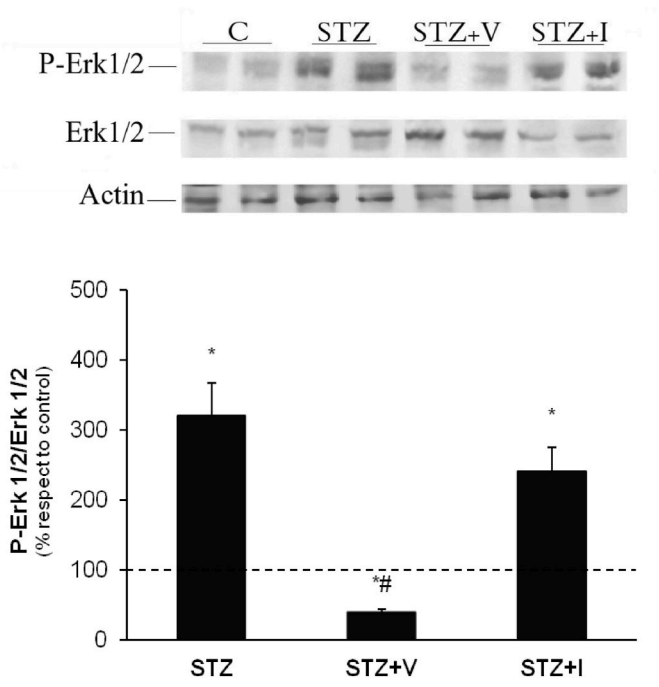

Fig. 5. Effect of vanadate and insulin on the expression of *P*-Erk1/2 and Erk1/2 in the liver of STZ-induced diabetic mice. Westen blot analyses of *P*-Erk1/2 and Erk1/2 levels were performed in the liver of control, STZ, STZ + I and STZ + V animals (45 μg of protein, 12% SDS-PAGE gels). The quantification of *P*-Erk/Erk1/2 is shown in the bottom panel. The data are expressed as percentages of the values of the control animals (——) ± SD from three independent experiments. (#) $p < 0.001$ indicates significant differences as compared to the STZ group. (*) $p < 0.1$ indicates significant differences as compared to the control group. Other experimental details are described in the Materials and Methods section.

4. Discussion

In diabetes mellitus, oxidative stress is increased and is the main contributing factor to the long-term complications of this disease [1,39,40]. The impairment in GSTs activity and GSTP expression observed in our murine model of diabetes could be reflecting the alterations in the drug metabolizing system that ultimately lead to the establishment of the oxidative stress associated with the diabetic state. Although the onset of diabetes affected both GST activity and GSTP expression, the time course of these alterations was different, suggesting that the different isoforms of the GST family would suffer differential regulation, as previously described by other authors [41–45].

The relationship between the diabetic status and the effect of insulin on GST activity has been previously reported [27,28,34]. Similarly, in our diabetic murine model, we found that insulin and vanadate reversed the diminished GST activity.

An explanation for these results could be that the decrease in GSTs would not be related to the low levels of insulin but to secondary metabolic changes that could be restored by vanadate. The same restorative effect of insulin was observed on the expression of GSTP at both mRNA and protein levels. In contrast, vanadate failed to mimic the restorative effect of insulin on GSTP expression, suggesting, as noted above, that the activities of the different GSTs are differentially regulated. Additionally, this discrepancy in the actions of insulin and vanadate could be explained by the differences in the mechanisms of action of both, as already described by Srivastava & Mehdi [46]. Further studies are needed to support these observations, although it is very difficult to know the mechanisms involved due to the widespread action of vanadate [46].

It has been postulated that both O-glycosylation and phosphorylation of Sp1 modulate the expression of several genes in both the normal and diabetic states, with the *O*-glycosylated form of Sp1 being transcriptionally more active than its non-glycosylated form [21]. Previous studies have shown that hyperglycemia increases O-glycosylation and decreases the serine/threonine phosphorylation of Sp1, leading to an induction in Sp1 transactivation and in the expression of Sp1-dependent genes [27]. Furthermore, an increase in Sp1 O-glycosylation is associated with a decrease in its phosphorylation and in the inhibition of its proteosomal degradation [27,47], whereas when Sp1 is hypoglycosylated, it is fast degraded [27,47].

Our results seem to go in the opposite direction since, in our model, the increase in the Sp1 O-glycosylation observed in the diabetic state coincides with a decrease in the expression of GSTP, while the treatment

of diabetic animals with insulin decreased the levels of Sp1 O-glycosylation and reversed the decrease in GSTP expression. Our data are not enough to establish the direct relationship between Sp1 O-glycosylation and GSTP expression; however, other authors have suggested that Sp1 O-glycosylation could also negatively affect its interaction with other transcriptional factors [48–50] If this lack of interaction were occurring, the higher O-glycosylation could be negatively affecting the transcriptional activity of Sp1 on GSTP. Further experimental evidence will be needed to confirm this hypothesis.

On the other hand, the low levels of Sp1 O-glycosylation observed in this work after vanadate administration are consistent with those previously reported *in vitro*, which indicate that vanadate inhibits the activity of the O-ligand b-*N*-acetylglucosamine transferase, an enzyme responsible for Sp1 O-glycosylation [51]. We could argue that, due to hypoglycosylation, Sp1 would be degrading at a higher rate, a fact that would lead to low levels of GSTP expression in this experimental group. However, the significant decrease in GSTP protein expression to undetectable levels can not be explained by the mRNA expression, suggesting that other changes at translational or post-translational level might be occurring.

The mechanism by which insulin activates the AP-1 complex is not clearly understood but it is known that it is dependent on the activation of the Erk1 and Erk 2 isoforms of mitogen-activated protein kinases [23]. Insulin is known to up-regulate *c*-Fos expression and has been reported to promote changes in the phosphorylation states of *c*-Jun and *c*-Fos [22]. The increase in Erk1/2 phosphorylation in both diabetic and insulin-treated animals could potentially lead to an increase in AP-1-mediated GSTP expression. However, in diabetic animals, GSTP expression was decreased. On the other hand, in diabetic animals, *c*-Jun was decreased, which could lead to lower levels of AP-1 complex and therefore to lower expression of GSTP. Although we can not discard this possibility, insulin treatment showed no effect on *c*-Jun levels, suggesting that at least the restorative effect of the hormone would not me mediated by this pathway.

The present work represents the first evidence of how the onset of diabetes negatively affects the baseline expression of GSTP. As insulin treatment normalized GSTP expression and the levels of Sp1 O-glycosylation, we suggest that GSTP expression in the liver of diabetic mice is influenced by changes in Sp1 O-glycosylation. These conclusions are in line with previous results from other authors who have shown that Sp1 plays a central role in regulating the basal levels of GSTP transcription while AP-1 would be more related to the induction of this enzyme [19, 52,53].

These findings provide evidence of some of the possible mechanisms by which the onset of diabetes can lead to the instauration of oxidative stress, which, in turn, opens the doors to numerous metabolic alterations whose ultimate outcome is some of the multiple clinical manifestations of this disease. Additionally, a better understanding of the regulation of GSTP by Sp-1 could facilitate the identification of possible molecular targets and therapeutic strategies that would help to regulate the activity of GSTP. For example, the activation of GSTP could contribute to reduce oxidative stress in the liver, while its inhibition in tumor cells could offer an alternative to eliminate or attenuate drug resistance, which constitutes one of the main escape mechanisms of tumors to treatment with cytotoxic drugs [9].

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Author contribution statement

All authors have made substantial contributions to the manuscript and approved the submitted version. Leda Oliveri made a significant contribution to the acquisition, analysis, and interpretation of the data. Ana Buzaleh made a substantial contribution, she was involved in the drafting and editing of the manuscript. Esther Gerez supervised the project in the conceptualization, development, writing and editing of the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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E. Gerez and A.M. Buzaleh are Associate and Independent Scientific Researchers at the Argentine National Research Council (CONICET). L. Oliveri is a Professional Member at the Technical Career from CONICET. We thank Mrs. V Castillo for technical assistance.

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