Arch Biol Sci. 2020;72(4):555-565 https://doi.org/10.2298/ABS200928049M

Contribution of O-GlcNAc modification of NF-κB p65 in the attenuation of diabetes-induced haptoglobin expression in rat liver

Vesna I. Martinović, Jelena D. Arambašić Jovanović, Desanka B. Bogojević, Andjelija S. Ivanović, Vesna M. Otašević, Ana T. Stančić and Ilijana I. Grigorov*

Department of Molecular Biology, Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

***Corresponding author**: iligri@ibiss.bg.ac.rs

Received: September 28, 2020; **Revised**: October 28, 2020; **Accepted**: October 30, 2020; **Published online**: October 30, 2020

Abstract: Haptoglobin (Hp) is a hemoglobin-binding protein that prevents free hemoglobin-induced tissue oxidative damage. In streptozotocin-induced diabetic rats, the initial elevation of Hp expression in the serum and liver tends to decrease with diabetes progression, contributing to increased oxidative stress. Glucose toxicity and diabetic complications are closely related to increased modification of nucleocytoplasmic proteins by O-linked-N-acetylglucosamine (O-GlcNAc). We examined the contribution of O-GlcNAcylation of NF-κB p65 to changes in liver Hp expression in diabetic rats. WGA-affinity chromatography revealed a progressive increase in O-GlcNAcylation in nuclear NF-κB p65 during eight weeks of diabetes. DNA-affinity chromatography followed by immunoblot analysis revealed that decreased Hp expression at 4 and 8 weeks of diabetes was accompanied by the absence of Hp gene hormone-responsive element (HRE) occupancy with NF-κB p65, low occupancy with glucocorticoid receptor (GR), and almost no changes in STAT3 occupancy compared to 2 weeks, when Hp expression was highest. Coimmunoprecipitation experiments indicate that these events were the result of impaired NF-κB p65/STAT3 and GR/STAT3 interactions. Results suggest that the attenuation of Hp expression associated with diabetes was at least in part the result of O-GlcNAcylation of NF-κB p65, which prevents the formation of an effective transcription initiation complex on the Hp gene promoter.

Keywords: haptoglobin; gene expression; diabetes; NF-kB p65; O-linked-N-acetylglucosamine modification

Abbreviations and acronyms: CCAAT/enhancer-binding protein β (C/EBPβ); diabetic rats (D); glucocorticoid receptor (GR); haptoglobin (Hp); hemoglobin (Hb); hexosamine biosynthetic pathway (HBP); hormone-responsive element (HRE); horseradish peroxidase (HRP); IkappaB (IκB); interleukin-6, -1 (IL-6, -1); nondiabetic rats (ND); nuclear extract (NE); nuclear factor kappa B (NF-κB); O-linked-N-acetylglucosamine (O-GlcNAc); polyvinylidene difluoride (PVDF); reactive oxygen species (ROS); SDS-polyacrylamide gel electrophoresis (SDS-PAGE); serine (Ser); signal transducer and activator of transcription (STAT); superoxide dismutase (SOD); streptozotocin (STZ); tyrosine (Tyr); threonine (Thr); wheat germ agglutinin (WGA)

INTRODUCTION

Haptoglobin (Hp) is an acute-phase plasma protein with antioxidative properties due to its ability to bind free hemoglobin and prevent oxidative tissue damage caused by hemoglobin [1]. Hp is produced mostly by hepatocytes and its synthesis is significantly increased during acute and chronic inflammatory disorders [2,3]. Decreases in Hp levels are associated with hemolytic anemia, starvation and genetic haptoglobinemia. If the reduction of the Hp does not track the signs of anemia, it can indicate liver damage [3]. Due to the involvement of Hp in states of oxidative stress and inflammation, it is of great interest to elucidate its potential association with tissue injury and dysfunction, especially under conditions of increased glycemic levels. We previously described time-dependent changes in Hp concentrations during the progression of streptozotocin (STZ)-induced diabetes in rats, which correlated with the level of hepatic oxidative stress [4]. The highest level of Hp in the serum and liver was observed during the first two weeks of diabetes, after which it started to decline. Since the serum Hp level reflected the inten-

sity of transcriptional activity of the Hp gene in the liver [5], the decline in Hp expression during diabetes pointed to the existence of diabetes-related changes in activities of transcriptional regulators involved in inducible Hp gene regulation.

The full expression of the rat Hp gene, established during the response to acute stress stimuli, requires the synergistic action of several interacting DNA-binding nuclear proteins within the hormone-responsive promoter element (HRE), also referred to as sequence ABC (-165/-56 bp). HRE/ABC contains elements responsive to glucocorticoids, IL-6 and IL-1 [5]. A core feature of the effective transcription initiation complex working in concert within HRE requires the presence of the glucocorticoid receptor (GR), the STAT3 member of the signal transducer and activator of transcription (STAT) family of transcription factors, C/EBPβ, a member of the CCAAT-enhancer-binding protein (C/EBP) family, and the p65 subunit of the nuclear factor-kappa B (NF-κB) family of proteins [6-8]. GR and NF-κB p65 were recognized as transcriptional coactivators of the Hp gene since crosstalk between STAT3/NF-κB p65 and GR/STAT3 within the IL-6-responsive element B (-146/-88 bp) have been identified as a regulatory step in the elevation of Hp expression [8].

The diversity of functional potentialities of transcription factors is partly explained by the existence of posttranslational modifications. O-GlcNAcylation is recognized as an important modification analogous to phosphorylation, which controls the stability, subcellular localization and DNA-binding activity of transcription factors, and also regulates their protein-protein interactions [9,10]. O-GlcNAcylation occurs in the cytoplasm and nucleus on susceptible target proteins due to a reversible enzymatic transfer of the hexosamine biosynthesis pathway (HPB) metabolite, UDP-N-acetylglucosamine (GlcNAc), on the hydroxyl group of Ser or Thr residues. Since the levels of the substrate UDP-GlcNAc required for O-GlcNAc modification depend on the availability of glucose, the modification of proteins with O-GlcNAc can be regulated in a glucose-dependent manner. An increased production of reactive oxygen species (ROS) during diabetes inhibits glycolysis and increases the concentration of circulating glucose, which activates the HBP and promotes the O-GlcNAc glycosylation of proteins [10,11]. In diabetes, increased O-GlcNAcylation of proteins is associated with the adverse effects of hyperglycemia and is an established pathogenic contributor to glucose toxicity and insulin resistance [12,13]. In the liver, which plays a central role in maintaining glucose homeostasis, a number of transcriptional factors and coactivators were found to be O-GlcNAcylated in diabetes, including forkhead box protein O1 (FOXO1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), CREB regulated transcription coactivator 2 (CRTC2), liver X receptor (LXR) and carbohydrate-responsive element-binding protein (ChREBP) [13]. The O-GlcNAcylation of these proteins is linked to the high glucose-induced expression of gluconeogenic/lipogenic genes and thus may contribute to glucose toxicity.

Signal transduction pathways that operate through free-radical second messengers and/or increased glucose flux rely on changes in O-GlcNAc levels globally, or on specific proteins to modulate cellular behavior [14]. As a glucose-responsive and redox-sensitive transcription factor, NF-κB has been proposed as a critical bridge between oxidant stress and gene expression [15]. In most cells, NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm. In response to various stimuli, NF-κB dissociates from IκB and rapidly enters the nucleus where it activates genes involved in inflammatory and immune responses. The activation of NF-κB requires posttranslational modifications, including phosphorylation and acetylation [16]. Additionally, posttranslational modification of the NF-κB p65 subunit by O-GlcNAc has been reported, but its exact function has not been fully elucidated. In T-lymphocytes, p65 NF-κB has been shown to be O-GlcNAc-modified, which leads to its translocation into the nucleus [17]. NF-κB p65 has also been shown to be O-GlcNAc-modified in mesangial cells and to accumulate in the nucleus after treatment with high glucose or glucosamine [9]. This leads to an increase in its binding to the NF-κB consensus sequence and activation of NF-κB-dependent genes, such as vascular cell adhesion protein 1, also known as vascular cell adhesion molecule 1 (VCAM-1), tumor necrosis factor alpha (TNF-α) and IL-6 [18]. A recent report also demonstrates that NF-κB p65 in liver homogenates of diabetic rats was modified by O-GlcNAcylation [19]. The authors' results pointed to the importance of this modification in the alteration of the functioning of the NF-κB p65 transcription factor involved in the regulation of genes for antioxidant enzymes CuZnSOD and MnSOD, whose expression decrease in diabetic liver.

In addition to our previous study on diabetic rats [4], a study on children with diabetes type 1 has also revealed a time-dependent decrease in the serum level of Hp and increased oxidative stress [20]. The prominent roles of Hp make it an attractive candidate for the development of novel therapies based on molecular drug targeting of certain signaling pathways. In our previous studies we characterized the NF-κB p65 as the principal coactivator of Hp gene upregulation although the NF-κB p65 consensus binding sequence was not found within the HRE/ABC region of the rat Hp gene [8]. However, the molecular mechanisms responsible for the downregulation of Hp gene expression under certain cell-specific conditions are largely unexplored. The data presented above emphasize that O-GlcNAc modification has different effects on the activities of NF-κB transcription factors. This study was conducted to test whether potential O-GlcNAc modification of NFκB p65 during diabetes influences its protein-protein interactions on the Hp gene HRE/ABC sequence, resulting in the attenuation of Hp expression during diabetes progression.

MATERIALS AND METHODS

Animals and treatments

Experiments were performed on 2-month-old male albino Wistar rats weighing 200-220 g. The animals were housed three per cage at a constant temperature (22±2°C), humidity (50±5%), and 12 h dark/light intervals. Tap water and standard laboratory chow were available *ad libitum*. All animal procedures complied with Directive 2010/63/EU on the protection of animals and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković". Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (65 mg/kg; MP Biomedicals, Solon, OH, USA) dissolved in freshly prepared 0.1 M sodium citrate buffer, pH 4.5. Control rats were injected with the vehicle alone. Blood samples for glucose monitoring by a hand-held glucometer (GlucoSure Plus, Apex Biotechnology Corp, Taiwan) were obtained from the tail tip. Rats were considered to have diabetes when the fasting blood glucose level exceeded 20 mmol/L. At the end of the 2nd, 4th and 8th weeks of STZ treatment, age-matched control (nondiabetic) and diabetic rats were weighed and killed by decapitation; the rats were not only used for the described assays but also for biochemical and hormonal analyses. When conducting these analyses in rodents, it is necessary to examine blood samples in the absence of anesthetics, analgesics or other drugs that can influence the accuracy of the assays and obtained data. The serum was obtained after centrifugation at 2000 ×g in an Eppendorf 5415 R centrifuge for 15 min and stored at -20˚C. Livers were removed and frozen in liquid nitrogen and kept at -80°C. The control and diabetic groups were comprised of six rats each. All experiments were replicated three times, each time with new groups of STZ-treated and control rats.

Measurement of serum Hp

The serum Hp levels were measured using a Beckman AU400 Chemistry Analyzer (Beckman Coulter Inc., Brea, CA, USA). The insoluble complex that formed between the anti-Hp antibody and the Hp molecule was measured by optical absorbance at 340 nm using the turbidimetry principle.

Preparation of the whole liver homogenates

Livers (200-400 mg) from nondiabetic and diabetic rats were homogenized in ice-cold homogenization buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA) supplemented with 1× phosphatase inhibitor Mix I and 1× protease inhibitor Mix G (SERVA Electrophoresis GmbH, Heidelberg, Germany). The homogenate was centrifuged at 9700 ×g for 20 min at 4°C, and the resulting supernatant was aliquoted, snap-frozen in liquid nitrogen and stored at -80°C.

Preparation of liver nuclear extracts

Livers from nondiabetic and diabetic rats were homogenized in buffer containing 2 M sucrose, 10 mM HEPES, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 1 mM PMSF, 1 mM DTT and 10% glycerol. After filtering the homogenate through two layers of cheesecloth, the nuclei were pelleted by centrifugation at 72000 ×g in a SW 28 rotor (Beckman L7- 55, Indianapolis, IN, USA) for 30 min at 4°C. The pelleted nuclei were resuspended in lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). To precipitate the chromatin, $(NH_4)_2SO_4$, pH

7.9 was added slowly with constant stirring to a final concentration of 0.36 M. Chromatin was sedimented by centrifugation at 82000 ×g, for 60 min (Beckman Ti 50 rotor, Indianapolis, IN, USA). The nucleoproteins were precipitated from the supernatant after the addition of crystallized $(NH_4)_2SO_4$ to a final concentration of 2.6 M and sedimented by centrifugation at 82000 ×g in a Ti 50 rotor (Beckman, Indianapolis, IN, USA) for 30 min at 4°C. Nuclear extracts were dialyzed overnight against 25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol, resuspended in dialysis buffer and frozen in small aliquots at -80°C.

DNA affinity chromatography

For the DNA affinity column, a nucleotide fragment between -165 and -49 bp from the 5' flanking region of the rat Hp gene [5] was used as a DNA probe. This fragment, functionally identified as a HRE, was obtained from Dr. Heinz Baumann (Roswell Park Memorial Institute, Buffalo, NY). A DNA affinity column with the rat Hp gene HRE was prepared according to Kadonaga and Tjian [21] as previously described [8]. The HRE fragment was annealed and ligated to obtain oligomers and then covalently coupled to CNBr-activated Sepharose CL-2B (Amersham-Pharmacia Biotech, Uppsala, Sweden). The HRE affinity resin was equilibrated in a Bio-Rad Econo-Column with dialysis buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 M KCl. Equal amounts of nuclear extracts (8 mg) prepared from the livers of nondiabetic and diabetic rats were mixed with competitor salmon sperm DNA and incubated for 10 min at room temperature. The protein-DNA mixture was then applied to the HRE affinity column and after 30 min, HRE-bound nucleoproteins were eluted with dialysis buffer containing 1 M KCl. Total eluted proteins were subjected to 12% SDS-PAGE and analyzed by immunoblotting.

Detection of O-GlcNAc glycosylated proteins in liver nuclear extracts

For the wheat germ agglutinin (WGA)-affinity purification of O-GlcNAc glycosylated proteins from the liver nuclear extracts, the Thermo Scientific™ (Waltham, Massachusetts, USA) Glycoprotein Isolation Kit, WGA (Cat. No. 89805) was used. Nuclear extracts (1.5 mg) prepared from the livers of nondiabetic and diabetic rats were diluted with the binding/wash buffer and applied to the WGA resin. Following incubation, the resin was washed and the WGA-bound glycoproteins were eluted. Eluted proteins were separated by 12% SDS-PAGE. The protein gels were subjected to silver staining or analyzed by immunoblot analysis.

Immunoblot analysis

Total amounts of eluted proteins from the HRE affinity column, 50 μg of eluted O-GlcNAc glycoproteins, or 20 μg of the nuclear extracts were separated by 12% SDS-PAGE and transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Blotto Base buffer (0.1% Tween 20, 20 mM Tris–HCl, pH 7.6, 137 mM NaCl) and incubated for an additional 2 h at room temperature in the same buffer containing rabbit monoclonal anti-Hp antibody (Abcam, Cambridge, UK), rabbit polyclonal antibodies specific to NF-κB p65, phosphorylated (p) NF-κB p65, STAT3, p-Tyr STAT3, p-Ser STAT3 and GR (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times with Blotto Base buffer containing 1% non-fat dry milk, the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied for 1 h at room temperature. For re-probing, the membranes were incubated in 2% SDS, 100 mM β-mercaptoethanol and 62.5 mM Tris–HCl, pH 6.8 for 35 min at 50°C, and then rinsed three times, blocked and probed again with another antibody. Immunoreactive bands were identified using an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions. Quantification of immunoreactive bands was performed using TotalLab [Phoretix] electrophoresis software [ver. 1.10], and the protein levels were expressed as relative values.

Immunoprecipitation

Immunoprecipitation was performed with 3 μg of monoclonal anti-GR antibody, clone BuGR 2 (Affinity BioReagents, Golden, CO, USA) or 1 μg of antibodies against NF-κB p65, STAT3 or C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 500 μg of nuclear extract. Normal IgG was used as a negative control. Samples were first precleared with a nonspecific

IgG antibody. Precleared samples were incubated with antibodies overnight and then incubated for 2 h with protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The beads were pelleted and washed five times with TEG buffer (10 mM Tris-HCl, pH 7.6, 4 mM EDTA, 10% glycerol, 50 mM NaCl). The immunoprecipitated proteins were resuspended in sample buffer, separated by 12% SDS-PAGE and subjected to immunoblot analysis. For background elimination of both heavy and light chains during immunoprecipitation, Clean-Blot™ IP Detection Reagent (HRP) was used according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Statistical analysis

All data were expressed as the means±SEM (standard error of mean). Because no differences were found between nondiabetic rats at the different time-points in the studied variables, control data were pooled for statistical analysis. The differences between the corresponding means were verified by one-way analysis of variance (ANOVA) with a post hoc Mann-Whitney U test. The difference was considered statistically significant at $P<0.05$.

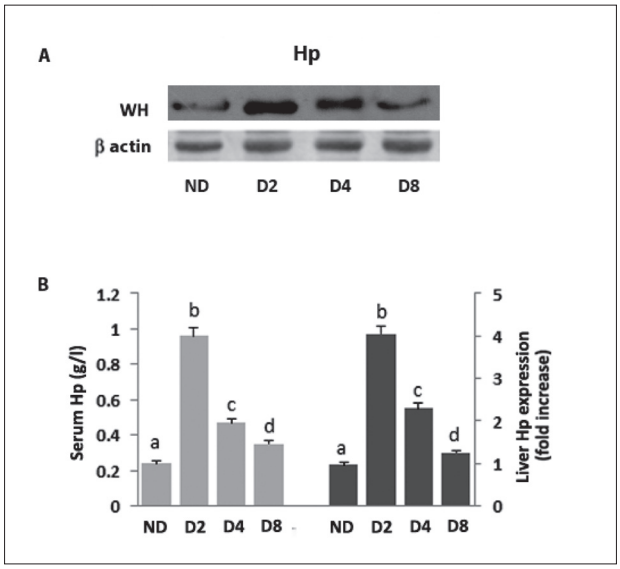

Fig. 1. Changes in the expression level of haptoglobin (Hp) in the liver and serum of diabetic rats. **A** – Immunoblot analysis of whole liver homogenates from nondiabetic (ND) and STZ-induced diabetic (D) rats with an anti-Hp antibody; β-actin was used as loading control. **B** – The concentration of Hp in the serum of ND and STZ-induced D rats determined by the immunoturbidimetric method (left). Immunoblots obtained from three independent experiments were quantified using densitometric analysis and are presented in the graphs (right). The data are expressed as fold-increases with respect to ND rats. ND – control; D2, D4, D8 – 2nd, 4th and 8th week after STZ treatment, respectively. The values are presented as the means±SEM. a,b,c,dMean values with unlike letters were significantly different ($P<0.05$).

RESULTS

Hp protein levels in serum and liver of diabetic rats

As can be seen in Fig. 1, diabetes progression was accompanied by changes in the expression levels of Hp in the serum and liver. The highest increases ($P<0.05$) in Hp protein levels in the serum and liver were measured at the end of the 2nd week after induction of diabetes (3.6-fold and 4-fold, respectively). After the 2nd week, the Hp protein levels declined progressively and at the end of 8th week, the increase was 2.2-fold ($P<0.05$) in the serum and 1.3-fold ($P<0.05$) in the liver when compared to the nondiabetic, control rats.

Nuclear expression profiles and binding activities of NF-κB p65, GR and STAT3 for the Hp gene HRE during diabetes

According to our previous work [8,22], the transcriptional induction of the rat Hp gene in response to a stress stimulus is primarily regulated by the IL-6 responsive element (IL-6 RE) within the *cis*-acting B element (-146/-88 bp) of the Hp gene HRE (Fig. 2A), and with the binding activities of transcription factors STAT3, GR and NF-κB p65. Immunoblot analysis (Fig. 2B) revealed an increased ($P<0.05$) presence of all these proteins in the nuclear extracts from the liver of diabetic rats throughout the 8-week follow-up period. The highest level of NF-κB p65 was observed at the end of the 8th week when it was 2.8-fold higher than in the control group, while the highest levels of GR and STAT3 p91 were detected at the end of the 4th week (2.8-fold and 3-fold, respectively). Only STAT3 p86 exhibited maximal expression level in the 2nd week (a 3.9-fold increase), and after that its protein levels started to decline, and at the end of 8th week it was 2.3-fold higher than in the control group. In contrast to the observed increased presence of the NF-κB p65 in the liver nuclear extracts of diabetic

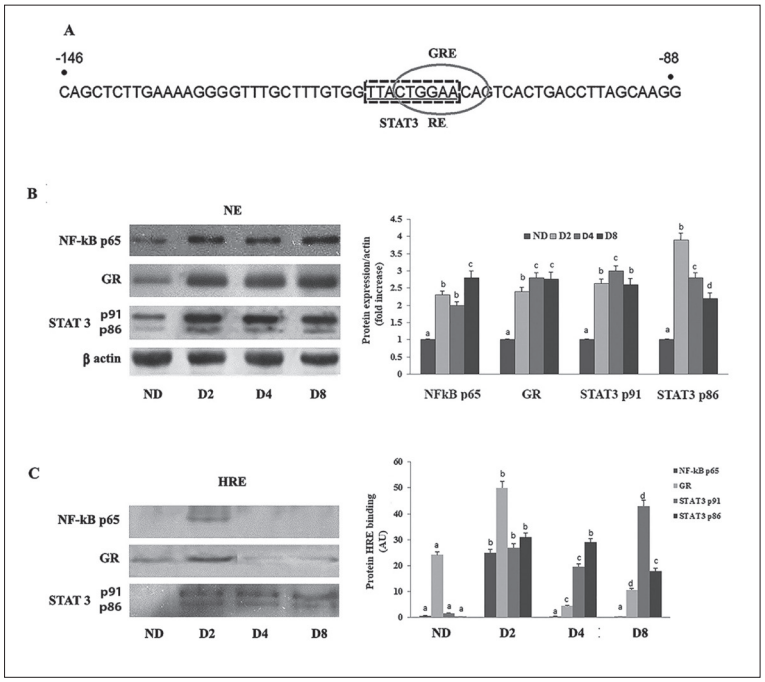

Fig. 2. Diabetes-related changes in nuclear abundance and binding activities of NF-κB p65, GR and STAT3 for the hormone responsive-element (HRE) of the Hp gene promoter. **A** – Nucleotide sequence of the *cis*-acting element B (-88/-146 bp) from the HRE (-165/-56 bp) of the rat Hp gene containing the IL-6 and glucocorticoid-responsive element. Overlapping binding sites for STAT3 and GR are framed. **B** – Immunoblot analysis of liver nuclear extracts (NE) from ND and D rats with anti-NF-κB p65, anti-GR and anti-STAT3 antibodies. **C** – Immunoblot analysis of nuclear proteins (NP) from ND and D rats after DNA-affinity chromatography. Immunoblots obtained from at least three independent experiments were quantified using densitometric analysis and are presented in the graphs. The data are expressed as fold-increases with respect to ND rats. ND – control rats; D2, D4, D8 – 2nd, 4th and 8th week after STZ treatment. The values in the graphs are presented as the means±SEM. [a,b,c,d]Mean values with unlike letters were significantly different (P<0.05).

rats, immunoblot analysis of nuclear proteins eluted from the HRE affinity column (Fig. 2C) revealed the presence of NF-κB p65 in only the HRE-bound nuclear protein fractions obtained from rats in the 2nd week of diabetes when the maximal level of Hp expression was observed. The highest presence of GR was detected in the HRE-bound nuclear protein fractions in the 2nd week of diabetes (Fig. 2C), after which as early as in the 4th week its presence fell below the level detected in nondiabetic rats. Diabetes-related HRE-binding activities of both STAT3 isoforms showed an opposite trend since the highest HRE-binding activity for STAT3 p86 was detected in the 2nd week of diabetes, while the highest HRE-binding activity of STAT3 p91 was detected in the 8th week (Fig. 2C).

Diabetes-related changes in the interplay of STAT3, NF-κB p65 and GR in liver nuclear extracts

Given that the interplay of STAT3, NF-κB p65 and GR represents an additional level required for maximal expression of the rat Hp gene [22], we performed coimmunoprecipitation experiments to examine the possibility that interactions between these proteins are changed during diabetes progression. GR was immunoprecipitated from nuclear extracts prepared from livers of control and diabetic rats and examined by immunoblotting to verify the presence of GR in the immunoprecipitate (Fig. 3A). After stripping, the membrane was re-probed with anti- STAT3 or anti-NF-κB p65 antibody. Analysis of the immunoprecipitates revealed that GR coimmunoprecipitated with STAT3 in the nuclear extracts from nondiabetic rat liver and from the liver of diabetic rats in the 2nd week after diabetes induction. In the precipitates obtained from the nuclear extracts prepared from the livers at 4th and 8th weeks post diabetes induction, interaction between GR and STAT3 was not detected. No relevant changes in the interaction between GR and NF-κB p65 were observed in liver nuclear extracts from the 2nd, 4th and 8th weeks of diabetes.

Analysis of the immunoprecipitates with the anti-STAT3 antibody revealed that STAT3 coimmunoprecipitated with NF-κB p65 (Fig. 3B) in the nuclear extracts from the liver in the 2nd week of diabetes, and weakly in the nuclear extracts on the 4th week. In the precipitates obtained from the liver nuclear extracts of diabetic rats in the 8th week, interaction between STAT3 and NF-κB p65 was not detected. The absence of interactions between STAT3 and GR, and between STAT3 and NF-kB p65 in the 4th and the 8th weeks of diabetes suggest that NF-κB p65 activity could be the crucial component for assembling STAT3 and GR on the HRE of the Hp gene and thereby responsible for the downregulation of the Hp gene.

Activation of STAT3, its translocation into the nucleus and interaction with DNA motifs, other tran-

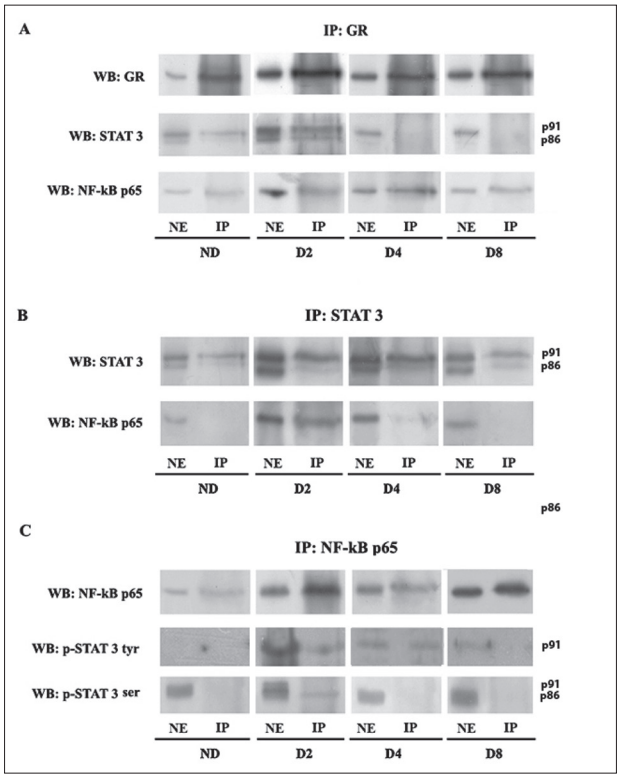

Fig. 3. Coimmunoprecipitation analysis of changes in interaction between STAT3, NF-κB p65 and GR in the liver nuclear extracts during diabetes. Representative immunoblots of immunoprecipitated GR (IP:GR) **(A)**, STAT3 (IP:STAT3) **(B)**, and NF-κB p65 (IP:NF-κB p65) **(C)** from liver nuclear extracts (NE) of ND and D rats with anti-GR, anti-STAT3 and anti-NF-κB p65 antibodies. ND – control rats; D2, D4, D8 – 2nd, 4th and 8th week after STZ treatment, respectively.

scription factors and accessory proteins require phosphorylation on Tyr 705 [23], as well as phosphorylation of Ser 727 [24,25]. Since both types of phosphorylation are present on STAT3 in diabetic liver [26,27], we performed immunoprecipitation experiments with NF-κB p65 from nuclear extracts prepared from livers of control and diabetic rats and examined the presence of Tyr- and Ser-phosphorylated STAT3 in the immunoprecipitates (Fig. 3C). Immunoblotting analysis revealed that NF-κB p65 interacted with both types of phosphorylated STAT3 from liver nuclear extracts of diabetic rats in the 2nd week. Thereafter, only a weak interaction was detected between NF-κB p65 and the Tyr-phosphorylated isoform in the 4th week, and no interaction with Ser-phosphorylated STAT3 in either the 4th or 8th week.

O-linked N-acetylglucosamine modification of NF-κB p65 during diabetes and its relation to phosphorylation

Increased ROS production in diabetes inhibits glycolysis, resulting in increased flux through the HBP, increased O-GlcNAc modification of proteins and changed protein functions [9,28]. To elucidate the mechanisms that change the binding activities of NF-κB p65 with other proteins during diabetes, we examined the levels of O-GlcNAc glycosylation of NF-κB p65 in liver nuclear extracts of nondiabetic rats and rats in the 2nd, 4th and 8th weeks of diabetes. Equal amounts of nuclear extracts

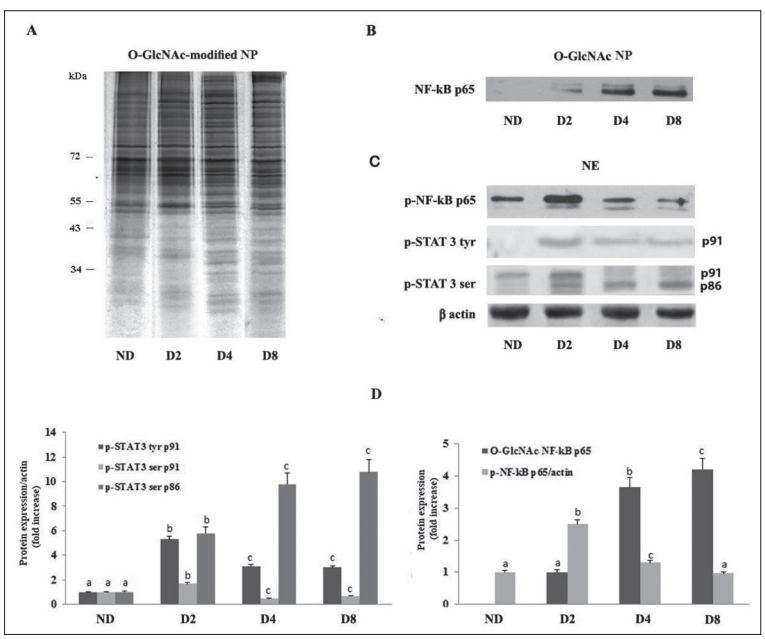

Fig. 4. Diabetes-related changes in O-GlcNAcylation and phosphorylation of nuclear NF-κB p65 and STAT3 proteins. **A** – Electrophoretic profile of liver nuclear proteins (NP) from ND and D rats after elution from the wheat germ agglutinin (WGA) column. **B** – Immunoblot analysis with anti-NF-κB p65 antibody of O-GlcNAc-modified liver nuclear proteins (NP) eluted from the WGA column. **C** – Immunoblot analysis with anti-phosphorylated (p)-NF-κB p65, anti-p- STAT3 (Tyr) and anti-p-STAT3 (Ser) antibodies of liver nuclear extracts. Representative gel and blots from three independent experiments are shown. **D** – Immunoblots obtained from at least three independent experiments were quantified using densitometric analysis and are presented in the graphs. The data are expressed as fold increase with respect to ND rats. ND – control rats; D2, D4, D8 – 2nd, 4th and 8th weeks after STZ treatment. The values in the graphs are presented as the means±SEM. [a,b,c]Mean values with unlike letters were significantly different (P<0.05).

were passed through a WGA column, and total eluted O-GlcNAc-modified nuclear proteins were separated by SDS-PAGE and examined by silver-staining (Fig. 4A) and immunoblotting (Fig. 4B). The observation of profiles of protein fractions after WGA chromatography pointed to a certain increase in O-GlcNAcylation of liver nuclear proteins in the 4th and 8th weeks of diabetes. Immunoblot analysis showed the absence of NF-κB p65 O-GlcNAc glycosylation in nondiabetic rats, the presence of O-GlcNAcylation in the 2nd week of diabetes, and its progressive increase with the duration of diabetes. This increase was 3.6-fold ($P<0.05$) in the 4th week and 4.2-fold ($P<0.05$) in the 8th week when compared to the glycosylation level detected in rats in the 2nd week of diabetes (Fig. 4D, right).

Given that the activation of NF-κB p65 requires phosphorylation and that O-GlcNAcylation can affect the phosphorylation status of a protein by regulating the phosphorylation of adjacent residues or by competing directly with the phosphate for Ser/Thr residues [29], we examined the phosphorylation status of NF-κB p65 in rat liver nuclear extracts during diabetes progression. As can be seen in Fig. 4C and D (right), the phosphorylation of NF-κB p65 exhibited an opposite trend to that observed for O-GlcNAc glycosylation. In the 2nd week of diabetes, NF-κB p65 was observed to be more phosphorylated than O-GlcNAc glycosylated, but in the 4th and 8th weeks of diabetes, a significant increase in its glycosylation was accompanied by a significant decrease in its phosphorylation (48% and 61%, respectively).

According to our previous study [22], STAT3/NF-κB p65 interactions determine the level of inducible Hp expression. For this reason, we also investigated the diabetes-related changes in the phosphorylation status of STAT3 proteins. Immunoblotting with anti-Tyr STAT3 and anti-Ser STAT3 antibodies (Fig. 4C) revealed a 5.3-fold ($P<0.05$) increase in Tyr-phosphorylation, and a 1.7-fold ($P<0.05$) increase in Ser-phosphorylation of STAT3 p91 in the 2nd week of diabetes when compared to matching controls, as well as a 5.8-fold ($P<0.05$) increase in Ser-phosphorylation of STAT3 p86 (Fig. 4D, left). In the 4th and 8th weeks of diabetes, phosphorylation of STAT3 p91 at Tyr residues decreased by about 43% ($P<0.05$) compared to the 2nd week, while STAT3 p91 phosphorylation at Ser residues decreased by 71% and 60%, respectively ($P<0.05$). At the same time, the phosphorylation of STAT3 p86 on Ser increased (1.7- and 1.9-fold, respectively, $P<0.05$).

DISCUSSION

Hp is considered an important antioxidant during the early stage of diabetes development, when it is capable of regulating oxidative stress by lowering the level of free iron, restoring redox balance and delaying the onset of diabetic complications [4]. Also, as a protein with antiinflammatory properties, its presence might be of importance in preventing the increase in diabetes-induced inflammation [27]. The presented study is focused on elucidating the mechanism(s) responsible for attenuating elevated Hp levels in the liver of STZ-induced diabetic rats during diabetes progression. In particular, the changes in the regulatory step of transcriptional activation of the Hp gene involve cross-interactions between regulatory proteins STAT3, NF-κB p65 and GR on the Hp gene IL-6 responsive promoter element. The results presented here show that a progressive increase in inducible O-GlcNAc modification of nuclear NF-κB p65 during diabetes disrupts STAT3/NF-κB p65, and consequently STAT3/GR interactions, contributing, at least in part, to the downregulation of Hp expression presented herein and in our previous study [4].

The addition of O-GlcNAc to cytoplasmic and nuclear proteins is sensitive to the levels of insulin, glucose and cellular stress, which is why O-GlcNAcylation is considered to influence the regulation of cell signaling and transcription in diabetes [30,31]. Due to the dynamic changes in O-GlcNAc patterns which fluctuate with cellular metabolism, the level of O-GlcNAc modification on gene regulatory proteins can modulate their functions in a variety of manners [13,31]. It can increase transcriptional activity of p53 [32], decrease transcriptional activity of cAMP response element-binding protein (CREB) [33], and positively or negatively regulate the transcriptional activities of transcription factor Sp1 [34] and NF-κB p65 [18]. NF-κB p65 is activated by a wide range of stimuli, and in diabetes it plays a significant role in the immune response, inflammation, oxidative stress, cell apoptosis and cell survival, mostly as the result of the regulation of target gene transcription [35]. Since a complete explanation of the molecular mechanisms

that precisely tune NF-κB p65 activity may have great biological and clinical significance, O-GlcNAc modification of NF-κB p65 could be of particular importance. We observed that diabetes leads to an increase in the nuclear presence of NF-κB p65 and its O-GlcNAc modification, which is at a low level during the early phase of diabetes (2nd week) but increases up to the end of the 8 weeks of diabetes. The observed increase in O-GlcNAcylation after the 2nd week is accompanied by a decrease in the level of NF-κB p65 phosphorylation, a disturbance in STAT3/NF-κB p65 interactions and significant depression of the level of Hp.

We previously characterized NF-κB p65 as the main coactivator in the activation of the Hp gene in the liver during the turpentine-induced acute-phase response and dietary restriction [22]. Transcriptional activation of the Hp gene depends on the IL-6-induced STAT3 activation by phosphorylation on the Tyr residue, its translocation to the nucleus and binding with the IL-6 RE of the Hp gene promoter where an enhanceosome is formed with other transcriptional coactivators. In the serum and liver of STZ-induced diabetic rats, the increased presence of cytokines, such as IL-6, and growth factors has been detected [4,27,36]. Beside NF-κB p65, whose interaction with STAT3 contributes to the transcriptional level of the Hp gene, we previously characterized GR as another coactivator of the Hp gene [5,8]. Its contribution to active enhancer formation is based on its interactions with STAT3 bound to the IL-6 RE and C/EBPβ proteins that are bound to the Hp gene HRE. The increased presence of STAT3, NF-κB p65 and GR was detected in liver nuclear extracts of diabetic rats throughout the 8-week follow-up period. However, HRE affinity chromatography revealed the presence of these proteins almost only in the HRE-bound nuclear protein fractions obtained from the rats in the 2nd week of diabetes when the highest expression level of Hp was observed. The decrease in Hp levels in the 4th and 8th weeks of diabetes is characterized by the absence of NF-κB p65 and a slight presence of GR in the HRE-bound fractions. According to the immunoprecipitation analyses, it seems that NF-κB p65 binding to STAT3 is required for the recruitment and maintenance of GR proximal to the bound STAT3 in order for their interaction to occur. This is obvious from the results which show a disruption of NF-κB p65 binding with STAT3 that was followed by the absence of STAT3 binding to GR. These results complement the current knowledge on the mechanisms of regulation of inducible Hp gene expression, and also contribute to the understanding of the transcriptional consequences of NF-κB p65/GR interaction. The crosstalk between GR and NF-κB p65 has been a major focus of research for many years. The association of GR with NF-κB p65 mostly repressed its function to reduce the expression of specific proinflammatory targets [37]. However, our results indicated that the binding of GR to STAT3 and NF-kB p65 contributes to the activation of the Hp gene. Since the binding site for NF-κB p65 is not found in the Hp gene HRE [22], while the binding site for the GR is only half of its typical palindromic sequence [8] and overlaps with the STAT3 binding site, we assumed that its positive transcriptional potential on the Hp gene is realized through the facilitation of the engagement of other coactivators. A similar result was reported for antiinflammatory zinc finger protein A20 (TNFAIP3) where the GR cooperates with NF-κB p65 to increase its gene expression [38].

Although it is known that O-GlcNAcylation of NF-κB p65 is involved in hyperglycemia-induced NF-κB p65 activation [18], the data related to the specific site and function of O-GlcNAcylation of NF-κB p65 remain controversial and are not fully understood. The increased amount of O-GlcNAcylation on NF-κB p65 on different Ser and Thr residues increases its transcriptional activity [39]. O-GlcNAcylation on Thr-352 under hyperglycemic conditions inhibits the interaction between NF-κB p65 and IκB, and suggests that O-GlcNAcylation of NF-κB p65 plays an important role in protein-protein interactions [39]. The phosphorylation of NF-κB p65 on individual Ser, Thr and Tyr also generates a pool of heterogeneously modified NF-κB p65 that transactivates genes and mediates context-dependent functional responses [40]. In our experiments, the level of NF-κB p65 phosphorylation was decreased with diabetes progression, while its O-GlcNAcylation was significantly increased. The activity of some proteins is reciprocally modified with O-GlcNAc and phosphate [41]. Therefore, changes in the O-GlcNAcylation level of NF-κB p65 affect not only its activity but also its phosphorylation level [39], which is in accordance with our results. Aside from NF-κB p65, the transactivation potential of STAT3 was also regulated by phosphorylation. Maximal activation of target genes with STAT3 requires both Tyr and Ser

phosphorylation [24,25]. While phosphorylation on Tyr is required for STAT3 dimerization and DNA binding, Ser phosphorylation antagonizes Tyr phosphorylation and can affect the formation of STAT3 homodimer, its DNA binding [24] and transcriptional activity [42]. We observed that Tyr phosphorylation of the STAT3 p91 isoform decreased with diabetes duration, while the phosphorylation of the STAT3 p86 isoform on Ser increased. In the 4th and 8th weeks of diabetes, Ser phosphorylation of the STAT3 p91 isoform was below basal level. According to the results of immunoprecipitation analysis, NF-κB p65 has the ability to interact with STAT3 phosphorylated on both Tyr and Ser in the 2nd week of diabetes. However, the increase in O-GlcNAcylation levels of NF-κB p65 after this period probably contributed to the observed absence of interactions with both Ser-phosphorylated STAT3 isoforms and Tyr-phosphorylated STAT3. It seems that diabetes-related posttranslational modifications of STAT3 and NF-κB p65, which change their protein-protein interaction either through conformational changes of NF-κB p65 [43] or through their involvement with coregulatory proteins, contribute to altered levels of Hp expression.

An increasing number of studies has demonstrated that the regulation of NF-κB p65 by posttranslational modifications is complex and likely occurs in promoter-specific and stimulus-specific manners. This study sheds light on the functions of O-GlcNAc modification of NF-κB p65 in the context of Hp gene promoter and diabetes-related changes in Hp expression. Considering the antiinflammatory and antioxidative roles of Hp, this study suggests that the O-GlcNAcylation of NF-κB p65 plays a proinflammatory role in diabetes and posits that targeting O-GlcNAc could present a potential therapeutic strategy for treating an inflammatory disease such as diabetes.

Funding: this work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Contract No. 451-03-68/2020-14/200007.

Author contributions: VM and IG designed the study and wrote the manuscript. VM, JA, DB, AI, AS and VO performed the experiments, analyzed and interpreted the results.

Conflict of interest disclosure: The authors declare no conflict of interest

REFERENCES

1. Tseng CF, Lin CC, Huang HY, Liu HC, Mao SJ. Antioxidant role of human haptoglobin. Proteomics. 2004;4(8):2221-8.
2. Wang Y, Kinzie E, Berger FG, Lim SK, Baumann H. Haptoglobin, an inflammation-inducible plasma protein. Redox Rep. 2001;6(6):379-85.
3. Jain S, Gautam V, Naseem S. Acute-phase proteins: As diagnostic tool. J Pharm Bioallied Sci. 2011;3(1):118-27.
4. Arambašić J, Mihailović M, Bogojević D, Ivanović-Matić S, Uskoković A, Poznanović G, Grigorov I. Haptoglobin and the inflammatory and oxidative status in experimental diabetic rats: antioxidant role of haptoglobin. J Physiol Biochem. 2013;69(1):45-58.
5. Marinković S, Baumann H. Structure, hormonal regulation, and identification of the interleukin 6 and dexamethasone responsive element of the rat haptoglobin gene. Mol Cell Biol. 1990;10:1573-83.
6. Grigorov I, Lazić T, Cvetković I, Milosavljević T, Petrović M. STAT3 involvement in the acute phase-related expression of the rat haptoglobin gene. Mol Biol Rep. 2000;27:81–6.
7. Grigorov I, Lazić T, Cvetković I, Milosavljević T, Petrović M. Opposite nuclear level and binding activity of STAT5b and STAT3 proteins with rat haptoglobin gene under normal and turpentine induced acute phase conditions. Mol Biol Rep. 2002;28:217-22.
8. Arambašić J, Poznanović G, Ivanović-Matić S, Bogojević D, Mihailović M, Uskoković A, Grigorov I. Association of the glucocorticoid receptor with STAT3, C/EBPß and the hormone responsive element within the rat haptoglobin gene promoter during the acute phase response. IUBMB Life. 2010;62(3):227-36.
9. Ozcan S, Andrali SS, Cantrell JE. Modulation of transcription factor function by O-GlcNAc modification. Biochim Biophys Acta. 2010;1799(5-6):353-64.
10. Yang X, Qian K. Protein O-GlcNAcylation: emerging mechanisms and functions. Nat Rev Mol Cell Biol. 2017;18(7):452-65.
11. Slawson C, Copeland RJ, Hart GW. O-GlcNAc signaling: a metabolic link between diabetes and cancer? Trends Biochem Sci. 2010;35(10):547-55.
12. Karunakaran U, Jeoung NH. O-GlcNAc modification: Friend or foe in diabetic cardiovascular disease. Korean Diabetes J. 2010;34(4):211-19.
13. Ma J, Hart GW. Protein O-GlcNAcylation in diabetes and diabetic complications. Expert Rev Proteomics. 2013;10(4):365-80.
14. Zachara NE, Hart GW. O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. Biochim Biophys Acta. 2004;1673(1-2):13-28.
15. Lingappan K. NF-κB in oxidative stress. Curr Opin Toxicol. 2018;7:81-6.
16. Huang B, Yang XD, Lamb A, Chen LF. Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway. Cell Signal. 2010;22(9):1282-90.
17. Golks A, Tran TT, Goetschy JF, Guerini D. Requirement for O-linked N-acetylglucosaminyltransferase in lymphocytes activation. EMBO J. 2007;26(20):4368-79.

18. James LR, Tang D, Ingram A, Ly H, Thai K, Cai L, Scholey JW. Flux through the hexosamine pathway is a determinant of nuclear factor kappaB-dependent promoter activation. Diabetes. 2002;51(4):1146-56.
19. Dinić S, Arambašić J, Mihailović M, Uskoković A, Grdović N, Marković J, Karadžić B, Poznanović G, Vidaković M. Decreased O-GlcNAcylation of the key proteins in kinase and redox signalling pathways is a novel mechanism of the beneficial effect of α-lipoic acid in diabetic liver. Br J Nutr. 2013;110(3):401-12.
20. Asayama K, Uchida N, Nakane T, Hayashibe H, Dobashi K, Amemiya S, Kato K, Nakazawa S. Antioxidants in the serum of children with insulin-dependent diabetes mellitus. Free Radic Biol Med. 1993;15(6):597-02.
21. Kadonaga JT, Tijan R. Affinity purification of sequence-specific DNA binding proteins. Proc Natl Acad Sci USA. 1986;83:5889-93.
22. Uskoković A, Dinić S, Mihailović M, Grdović N, Arambašić J, Vidaković M, Bogojević D, Ivanović-Matić S, Martinović V, Petrović M, Poznanović G, Grigorov I. STAT3/NF-κB interactions determine the level of haptoglobin expression in male rats exposed to dietary restriction and/or acute phase stimuli. Mol Biol Rep. 2012;39(1):167-76.
23. Paulson M, Pisharody S, Pan L, Guadagno S, Mui AL, Levy DE. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. J Biol Chem. 1999;274:25343-9.
24. Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell. 1995;82(2):241-50.
25. Decker T, Kovarik P. Serine phosphorylation of STATs. Oncogene. 2000;19(21):2628-37.
26. Kim JH, Yoon MS, Chen J. Signal transducer and activator of transcription 3 (STAT3) mediates amino acid inhibition of insulin signaling through serine 727 phosphorylation. J Biol Chem. 2009;284(51):35425-32.
27. Jovanović Stojanov S, Martinović V, Bogojević D, Poznanović G, Petrović A, Ivanović Matić S, Grigorov I. Modulation of diabetes-related liver injury by the HMGB1/TLR4 inflammatory pathway. J Physiol Biochem.2018;74(2):345-58.
28. Issad T, Masson E, Pagesy P. O-GlcNAc modification, insulin signaling and diabetic complications. Diabetes Metab. 2010;36(6 Pt 1):423-35.
29. Hart GW, Housley MP, Slawson C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. Nature. 2007;446(7139):1017-22.
30. Myslicki JP, Belke DD, Shearer J. Role of O-GlcNAcylation in nutritional sensing, insulin resistance and in mediating the benefits of exercise. Appl Physiol Nutr Metab. 2014;39(11):1205-13.
31. Brimble S, Wollaston-Hayden EE, Teo CF, Morris AC, Wells L. The Role of the O-GlcNAc Modification in Regulating Eukaryotic Gene Expression. Curr Signal Transduct Ther. 2010;5(1):12-24.
32. Shaw P, Freeman J, Bovey R, Iggo R. Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. Oncogene. 1996;12:921–30.
33. Lamarre-Vincent N, Hsieh-Wilson LC. Dynamic glycosylation of the transcription factor CREB: a potential role in gene regulation. J Am Chem Soc. 2003;125:6612–3.
34. Chang WC, Hung JJ. Functional role of post-translational modifications of Sp1 in tumorigenesis. J Biomed Sci. 2012;19(1):94.
35. Patel S, Santani D. Role of NF-kappa B in the pathogenesis of diabetes and its associated complications. Pharmacol Rep. 2009;61(4):595-03.
36. Samarghandian S, Azimi-Nezhad M, Farkhondeh T. Immunomodulatory and antioxidant effects of saffron aqueous extract (Crocus sativus L.) on streptozotocin-induced diabetes in rats. Indian Heart J. 2017;69(2):151-9.
37. Van Bogaert T, De Bosscher K, Libert C. Crosstalk between TNF and glucocorticoid receptor signaling pathways. Cytokine Growth Factor Rev. 2010;21(4):275-86.
38. Altonsy MO, Sasse SK, Phang TL, Gerber AN. Context-dependent cooperation between nuclear factor κB (NF-κB) and the glucocorticoid receptor at a TNFAIP3 intronic enhancer: a mechanism to maintain negative feedback control of inflammation. J Biol Chem. 2014;289(12):8231-9.
39. Yang Won, Park S, Nam H, Kim D, Kang J, Kang E, Kim Y, Lee H, Kim K, Cho J. NFκB Activation Is Associated with Its O-GlcNAcylation State under Hyperglycemic Conditions. PNAS of America 2008;105(45):17345-50.
40. Christian F, Smith EL, Carmody RJ. The Regulation of NF-κB Subunits by Phosphorylation. Cells. 2016;5(1):12.
41. Yang WH, Kim JE, Nam HW, Ju JW, Kim HS, Kim YS, Cho JW. Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability. Nat Cell Biol. 2006;8(10):1074-83.
42. Zhang X, Blenis J, Li HC, Schindler C, Chen-Kiang S. Requirement of serine phosphorylation for formation of STAT-promoter complexes. Science. 1995;267(5206):1990-4.
43. Yu Z, Kone BC. The STAT3 DNA-binding domain mediates interaction with NF-kappaB p65 and inducible nitric oxide synthase transrepression in mesangial cells. J Am Soc Nephrol. 2004;15(3):585-91.