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O-GlcNAcylation regulates hyperglycemia-induced GPX1 activation

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ABSTRACT

Hyperglycemia induces activation of glutathione peroxidase 1 (GPX1), an anti-oxidant enzyme essential for cell survival during oxidative stress. However, the mechanism of GPX1 activation is unclear. Here, we report that hyperglycemia-induced protein glycosylation by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) is crucial for activation of GPX1 and for its binding to c-Abl and Arg kinases. GPX1 itself is modified with *O*-GlcNAc on its C-terminus. We also demonstrate that pharmacological injection of the *O*-GlcNAcase inhibitor NTZ induces GPX1 activation in the mouse liver. Our findings suggest a crucial role for GPX1 and its *O*-GlcNAc modification in hyperglycemia and diabetes mellitus.

Introduction

The anti-oxidant glutathione peroxidase (GPX) is a selenium-containing enzyme that converts H_2O_2 to H_2O by oxidizing glutathione to its disulfide form, resulting in cell protection from oxidative stress [1,2]. Among six glutathione peroxidases (GPX1-6) identified in human, GPX1 is the major isoform and is expressed ubiquitously and most abundantly [1,2]. GPX1 is located in the cytosol and mitochondria [1,2], and deficiency in GPX1 renders mice sensitive to acute oxidative stress. The survival time for GPX1 knockout mice following intraperitoneal injection of the oxidant paraquat decreases by 10-fold compared to wild-type control mice in the presence of an adequate supply of dietary Se [3–5].

GPX1 activity is regulated by oxidative stress-induced posttranslational modifications including phosphorylation. The nonreceptor tyrosine kinases c-Abl and Arg are known to phosphorylate GPX1 on Tyr-96, and these two kinases are essential for protecting cells from apoptosis during oxidative stress [6].

Hyperglycemia leads to various cellular changes, and the following three are of particular interest to us: First, hyperglycemia induces oxidative stress primarily through mitochondrial oxidative phosphorylation, which generates superoxide anions [7,8]. Second, hyperglycemia upregulates GPX1 activity, which may protect cells from free radical-induced cell damage [9,10]. Third, hyperglycemia increases *O*-GlcNAc modification on many nuclear and cytosolic proteins [11,12]. This modification is dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) [11,12], and plays important roles in many processes including transcription, translation, nuclear transport, protein degradation, protein localization, and protein–protein interactions [11,12]. Various anti-oxidant enzymes such as catalase and superoxide dismutase are also modified with *O*-GlcNAc [13]. Based on these findings, we hypothesized that *O*-GlcNAc modification affects GPX1 activity in hyperglycemic conditions. Here, we show that the cellular *O*-GlcNAcylation level indeed affects GPX1 activity. Reducing the cellular *O*-GlcNAcylation level suppresses hyperglycemia-induced activation of GPX1 in rat vascular smooth muscles cells (VSMCs). Conversely, increasing the cellular *O*-GlcNAcylation level enhances GPX1 activity. The cellular *O*-GlcNAcylation level also affects binding of GPX1 to c-Abl or Arg kinases. We also show that injection of the *O*-GlcNAcase inhibitor NTZ increases GPX1 activity in the mouse liver. Finally, we demonstrate that GPX1 is *O*-GlcNAcylated, and this modification occurs on the C-terminal half of the protein.

Material and methods

Cell culture, DNA transfection, and plasmids. Rat VSMCs and HEK293 cells were maintained as previously described [14,15]. Medium was supplemented with 100 nM sodium selenite [16].

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DNA transfection was performed as previously described [14,15]. Expression constructs encoding full-length (1–201) and C-terminal deletion (1–100) versions of human GPX1 (Sec47Cys) for overexpression in HEK293 cells [6] were prepared using PCR and cloned into the pRK5-FLAG mammalian expression vector (Genentech). The human *OGA* gene was cloned into the pRK5-FLAG mammalian expression vector (Genentech), and the human *OGT* gene was cloned into the p3X FLAG-CMV7.1 mammalian expression vector (Sigma–Aldrich).

Preparation of mouse tissues. B6C3F1/J mice were obtained by mating C57BL/6J female mice to C3H/HeJ male mice. 1,2-dideoxy-2′-propyl-alpha-D-glucopyranoso-[2,1-d]-Delta2′-thiazoline (NTZ) (500 mg/kg) or PBS was injected into 5-month-old mice by intraperitoneal injection. Animals were sacrificed 1 day after NTZ injection.

Reagents and antibodies. PUGNAc was purchased from Toronto Research Chemicals, and sodium selenite was purchased from Sigma. NTZ was kindly provided by Dr. Kwan Soo Kim (Yonsei University, Korea). Antibody against GPX1 (LF-MA0206, mouse monoclonal) was purchased from Ab Frontier (Seoul, Korea). Antibodies against c-Abl (K-12, rabbit polyclonal), Arg (H-300, rabbit polyclonal), and actin (C-2, mouse monoclonal) were purchased from Santa Cruz Biotechnology. CTD110.6 was purchased from Covance. Antibodies against OGT (rabbit polyclonal), FLAG (mouse monoclonal), and FLAG-Agarose were purchased from Sigma.

Two-dimensional gel electrophoresis, immunoblotting, and immunoprecipitation. Two-dimensional gel electrophoresis, immunoblotting, and immunoprecipitation were performed as previously described [14,15].

Assays for GPX1 activity. Cell lysates were assayed with a GPX activity assay kit (Sigma) at 25 °C (pH 8.0) following the manufacturer's instructions. For mouse liver lysates, hydrogen peroxide was used as a substrate instead of *tert*-butyl hydro-peroxide, and 1 mM sodium azide was added to the assay buffer.

Results

Hyperglycemia-induced activation of GPX1 is suppressed by OGA

We examined the possible relationship between GPX1 activation and *O*-GlcNAc modification in hyperglycemic conditions. GPX1 activity and *O*-GlcNAcylation are both known to increase significantly in hyperglycemic conditions [9–12]. We confirmed that hyperglycemia increased GPX1 activity in rat VSMCs. When cells were exposed to high glucose (25 mM) for 48 h, GPX1 activity was 2.5-fold greater than in cells grown in normal glucose (5 mM), while the GPX1 expression levels were the same (Fig. 1A and B). High glucose also increased the total *O*-GlcNAcylation level, as reported previously (Fig. 1B, panel 1).

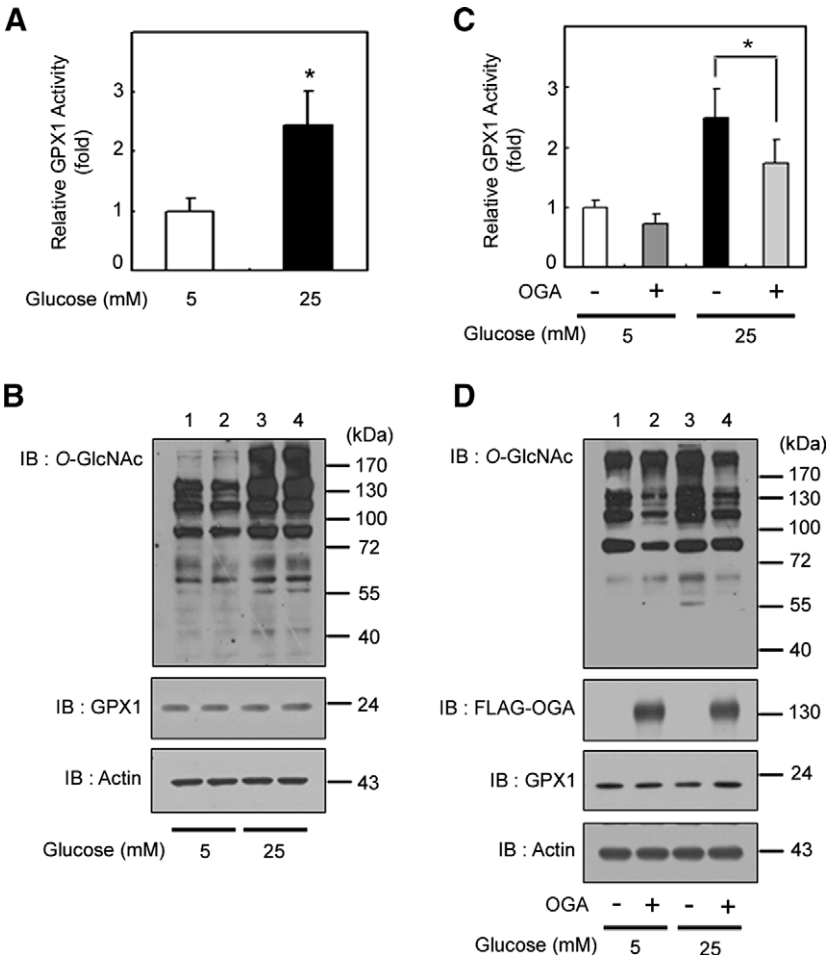

Fig. 1. Hyperglycemia-induced GPX1 activation is suppressed by decreasing cellular *O*-GlcNAcylation. (A) Rat VSMCs were incubated for 48 h in normal glucose (5 mM) or high glucose (25 mM), and GPX1 activity was measured in cell lysates. The data represent the mean ± SD ($n = 3$); * $P < 0.02$ by Students *t*-test. (B) The cellular *O*-GlcNAcylation level and the amount of GPX1 protein were determined in the same cell lysate using immunoblotting (panels 1 and 2). Actin was used as a loading control (panel 3). (C) FLAG-tagged human OGA was expressed, and the cells were incubated for 48 h in normal or high glucose. The changes in GPX1 activity were measured. The values represent the mean ± SD ($n = 3$); * $P < 0.02$ by Students *t*-test. (D) The cellular *O*-GlcNAcylation level and the amount of FLAG-OGA and GPX1 proteins were determined in the same cell lysate using immunoblotting (panels 1–3). Actin was included as a loading control (panel 4).

To examine the role of *O*-GlcNAcylation in hyperglycemia-induced GPX1 activation, we reduced the cellular *O*-GlcNAcylation level by transfecting VSMCs with FLAG-tagged human *OGA* and analyzed GPX1 activity in hyperglycemic conditions. Interestingly, *OGA* overexpression decreased GPX1 activity by 30% in high glucose (Fig. 1C). *OGA* overexpression decreased the total *O*-GlcNAcylation level as expected (Fig. 1D, panel 1), but did not affect the total GPX1 level (Fig. 1D, panel 3). These results indicate that *O*-GlcNAcylation is a crucial factor involved in mediating hyperglycemia-induced GPX1 activation.

GPX1 activity is increased by OGT

To examine whether increasing the cellular *O*-GlcNAcylation level was sufficient for GPX1 activation, we overexpressed FLAG-tagged human *OGT* in VSMCs and measured GPX1 activity. As shown in Fig. 2A, GPX1 activity was increased about 0.5-fold by *OGT*. As expected, *OGT* overexpression substantially increased the cellular *O*-GlcNAcylation level (Fig. 2B, panel 1). However, GPX1 activation in hyperglycemic conditions cannot be solely explained by *O*-GlcNAc modification because GPX1 activity was increased by 2.5-fold in hyperglycemic conditions (Fig. 1A), whereas it was increased by only 0.5-fold by *OGT* overexpression (Fig. 2A). Similarly, hyperglycemia-induced activation of GPX1 was not fully suppressed by *OGA* overexpression (Fig. 1C), suggesting that additional factors are involved besides *O*-GlcNAc in mediating hyperglycemia-induced GPX1 activation.

OGA inhibition enhances GPX1 activation in the mouse liver

We next examined whether *O*-GlcNAcylation increases GPX1 activity *in vivo*. Pharmacological injection of NTZ has been reported to effectively increase the *O*-GlcNAcylation level in the mouse hippocampus [17]. We injected wild-type mice with 500 mg/kg of NTZ intraperitoneally and sacrificed them 24 h later. Interestingly, GPX1 activity in the NTZ-injected mouse liver was increased by 41% compared to that in the PBS-injected mouse liver (Fig. 2C). NTZ treatment increased the total *O*-GlcNAcylation level (Fig. 2D, panel 1), but did not affect the total GPX1 level (Fig. 2D, panel 2).

A change in the cellular O-GlcNAcylation level affects the interaction between GPX1 and the tyrosine kinases c-Abl and Arg

We investigated the mechanism of how *O*-GlcNAcylation affects GPX1 activation. GPX1 binds c-Abl or Arg, an interaction that is regulated by oxidative stress [6]. Therefore, we examined the binding of GPX1 to c-Abl and Arg under normal or high glucose conditions. The glucose concentration in the medium did not affect the amount of c-Abl or Arg protein (Fig. 3A and C, panel 3). We next examined the interaction of GPX1 with the two kinases by analyzing the amount of GPX1 that co-immunoprecipitated with c-Abl or Arg. High glucose enhanced the binding of GPX1 to c-Abl and Arg (Fig. 3A and C, panel 1).

We then investigated whether the cellular *O*-GlcNAcylation level affected the interaction of these proteins. Overexpression of *OGA* did

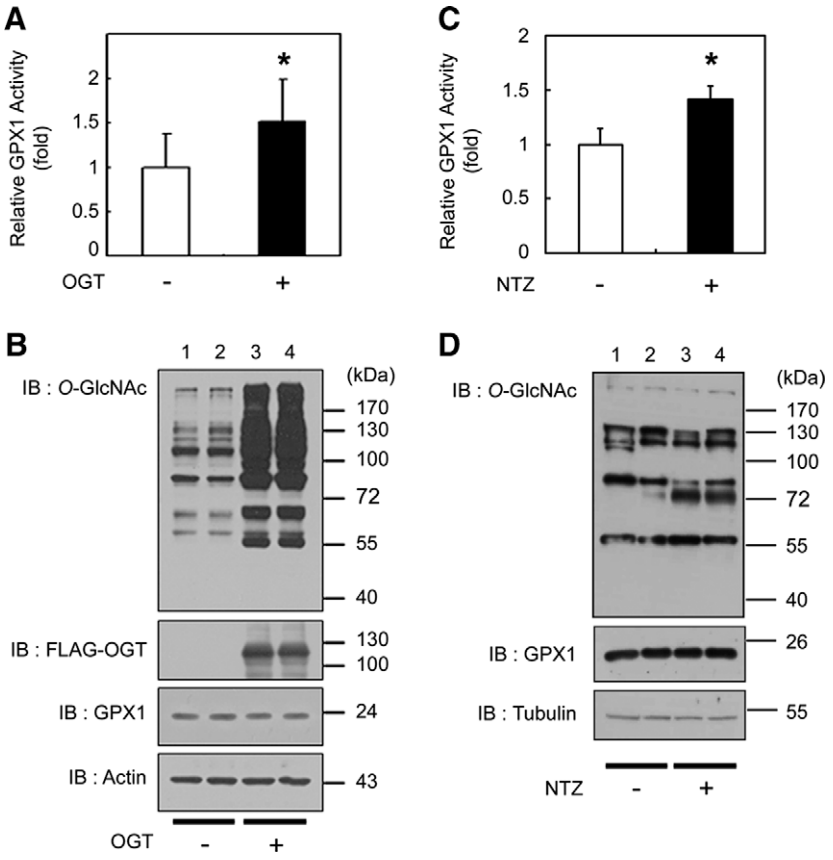

Fig. 2. GPX1 activity is increased by elevating cellular *O*-GlcNAcylation both *in vitro* and *in vivo*. (A) VSMCs were transfected with FLAG-tagged human OGT and incubated for 48 h in normal glucose. GPX1 activity was measured in the cell lysates. The data represent the mean ± SD ($n = 3$); $^{*}P < 0.05$ by Students *t*-test. (B) The same cell lysates were used to measure the *O*-GlcNAcylation level and the amount of FLAG-OGT and GPX1 proteins using immunoblotting (panels 1–3). Actin was used as a loading control (panel 4). (C) GPX1 activity was increased in the mouse liver by pharmacological injection of NTZ. The OGA inhibitor NTZ (500 mg/kg) or the same volume of PBS was injected intraperitoneally into B6C3F1/J mice. Twenty-four hours later, mice were sacrificed, and liver lysates were prepared. The lysates were measured for GPX1 activity. The data represent the mean ± S.D. ($n = 4$); $^{*}P < 0.05$ by Students *t*-test. (D) Liver lysates were prepared separately from two individual mice in each group and were analyzed using immunoblotting with anti-*O*-GlcNAc and anti-GPX1 (panels 1 and 2). Tubulin was used as a loading control (panel 3).

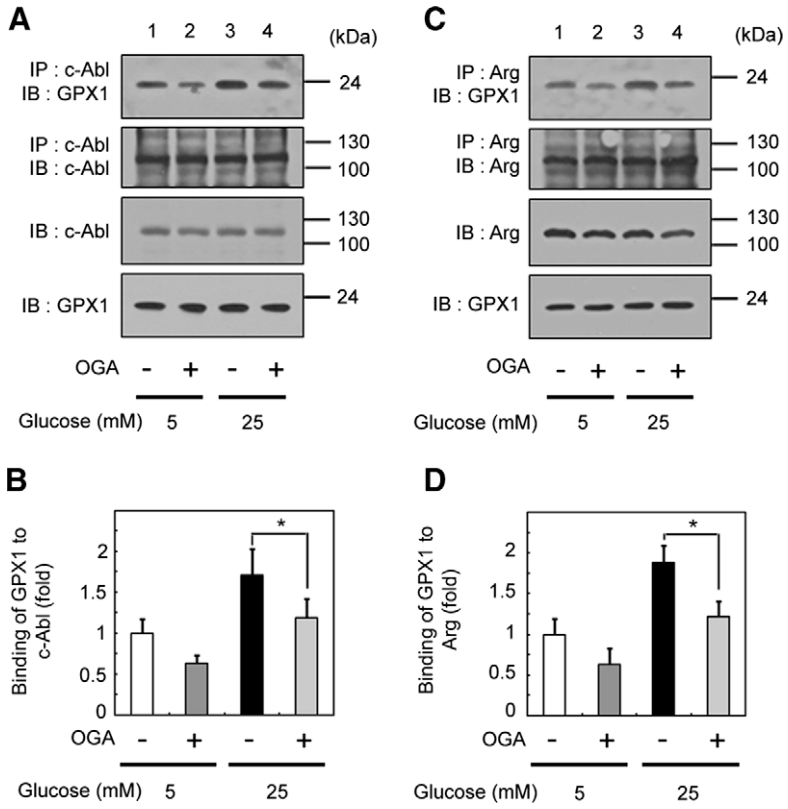

Fig. 3. OGA inhibits binding of GPX1 to c-Abl and Arg. VSMCs were exposed to normal or high glucose for 48 h in the presence or absence of FLAG-OGA. Binding of GPX1 to c-Abl (A and B) or Arg (C and D) tyrosine kinases was measured after coimmunoprecipitation and examined with western blotting. (A) Cell lysates were subjected to coimmunoprecipitation using anti-c-Abl followed by western blotting for GPX1 (panel 1) or c-Abl (for immunoprecipitation control, panel 2). (B) The results in (A) were quantified using densitometry. The values were normalized to total c-Abl levels. The data represent the mean ± SD ($n = 3$); $^{*}P < 0.05$ by Students t-test. (C) Cell lysates were subjected to coimmunoprecipitation using anti-Arg followed by western blotting for GPX1 (panel 1) or Arg (for immunoprecipitation control, panel 2). (D) The results in (C) were quantified using densitometry. The values were normalized to total Arg levels. The data represent the mean ± SD ($n = 3$); $^{*}P < 0.02$ by Students t-test.

not change the total amount of c-Abl or Arg protein (Fig. 3A and C, panel 3), but decreased the interaction of GPX1 with c-Abl or Arg (Fig. 3A and C, panel 1). These results indicate that hyperglycemia increases the association of GPX1 with c-Abl and Arg, and downregulation of *O*-GlcNAcylation suppresses these interactions, which likely results in decreased GPX1 activity (Fig. 3B and D).

GPX1 is modified with O-GlcNAc at its C-terminus

We examined whether GPX1 itself is an *O*-GlcNAcylated protein. Using a whole-cell lysate of VSMCs treated with the *O*-GlcNAcase inhibitor PUGNAc, we observed that GPX1 protein was detected by the GPX1 antibody with a molecular weight of about 22 kDa and a p*I* value of about 6.0 in a two-dimensional gel (Fig. 4A, panel 1). The same band was also detected by the anti-*O*-GlcNAc antibody CTD110.6 when GPX1 was immunoprecipitated from the same lysate (Fig. 4A, panels 2 and 3).

To examine the GPX1-OGT interaction, we generated full-length (1–201) and C-terminal deletion constructs (1–100) of FLAG-tagged *GPX1*. These constructs were individually expressed along with *OGT* in HEK293 cells, and FLAG-GPX1 was immunoprecipitated using the anti-FLAG antibody. As shown in Fig. 4B, the two versions of GPX1 were able to bind OGT with the same affinity, indicating that the interaction occurs at the N-terminal half of GPX1. To identify the *O*-GlcNAc modification region on GPX1, the two GPX1 constructs were expressed in HEK293 cells. Cells were treated with PUGNAc, immunoprecipitated with anti-FLAG, and *O*-GlcNAcylation was examined using anti-*O*-GlcNAc. Full-length FLAG-GPX1 was modified with *O*-GlcNAc, while the truncated FLAG-GPX1 was not, despite the fact that truncated GPX1 was still able to bind OGT (Fig. 4C). Thus, *O*-GlcNAcylation occurs on the C-terminal half of GPX1.

Discussion

Here, we found that *O*-GlcNAcylation plays a critical role in regulating GPX1 activation both *in vitro* and *in vivo*. We showed that association of GPX1 with c-Abl and Arg kinases is regulated by the cellular *O*-GlcNAcylation level. Because GPX1 is modified by *O*-GlcNAc, this modification may regulate the binding of GPX1 to kinases. Alternatively, *O*-GlcNAc modification of kinases or other proteins may be more crucial for regulation of this association. We could not detect *O*-GlcNAcylation on c-Abl or Arg under the same experimental conditions used to analyze GPX1 GlcNAcylation (data not shown). Therefore, we favor the view that increased binding of GPX1 to c-Abl and Arg under hyperglycemic conditions is due to *O*-GlcNAcylation of GPX1.

The mechanism by which *O*-GlcNAcylation modulates protein function is actively being studied by many investigators. One of the best characterized examples is that *O*-GlcNAcylation influences the phosphorylation state of the protein either by competitive occupancy at the same site (Ser or Thr) or at adjacent sites [11,12,15]. In other words, *O*-GlcNAc and *O*-phosphate have an antagonistic relationship. Our findings suggest that *O*-GlcNAc modification of GPX1 increases its phosphorylation. In human neuroblastoma cells, *O*-GlcNAcylation on Akt is not antagonistic with phosphorylation on Thr308 and Ser473, two sites that are important for Akt activation, which is consistent with our findings [18].

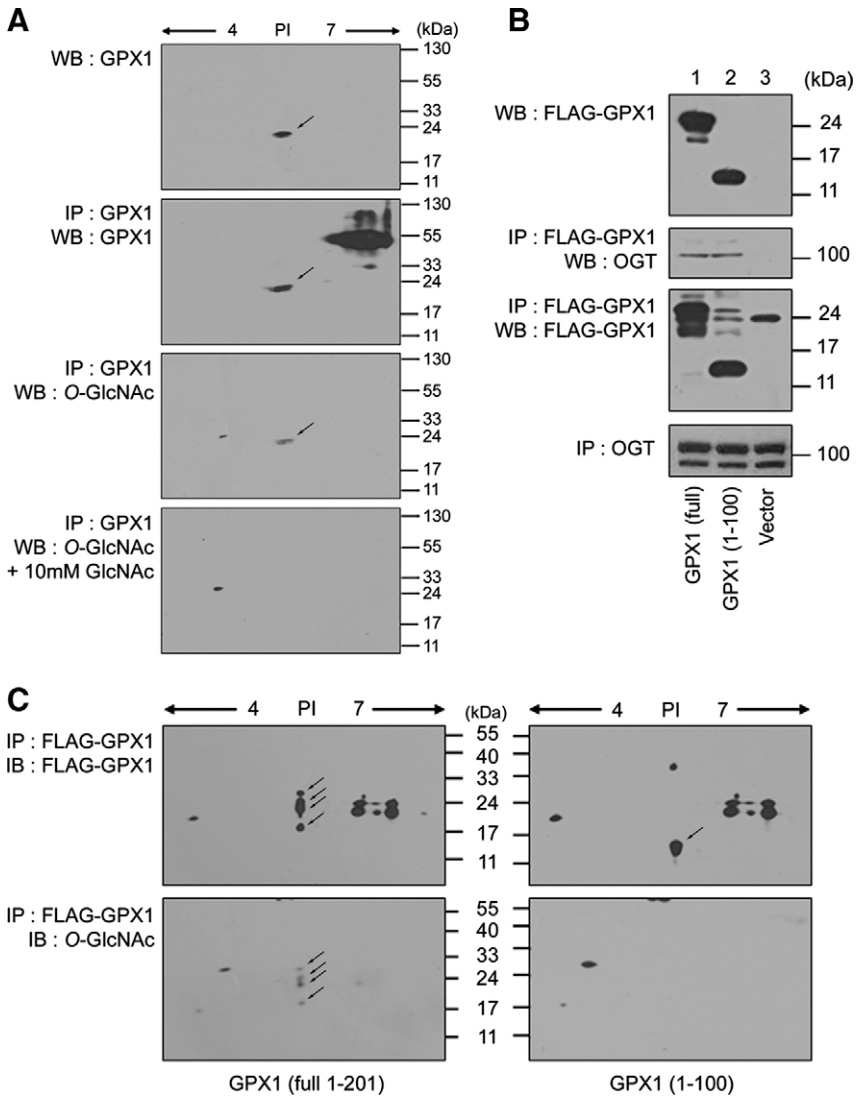

Fig. 4. GPX1 is *O*-GlcNAc modified at the C-terminus. (A) Endogenous GPX1 protein was immunoprecipitated from the lysate of VSMCs previously treated with 100 μM PUGNAc for 12 h. The GPX1 immunoprecipitates were separated with two-dimensional gel electrophoresis to separate the immunoglobulin light chain and were analyzed by immunoblotting for GPX1 (panel 2) or *O*-GlcNAc (panel 3). The specificity of the *O*-GlcNAc antibody was examined by addition of 10 mM free GlcNAc (panel 4). The arrows indicate GPX1. (B) To examine the GPX1-OGT interaction, full-length (1–201) and C-terminal deletion (1–100) mutants of FLAG-tagged GPX1 (Sec47Cys) [6] were expressed in HEK293 cells. The FLAG-GPX1 immunoprecipitates were analyzed by western blotting using anti-OGT (panel 2) or anti-FLAG (panel 3). (C) After PUGNAc treatment, cells were lysed, and FLAG-GPX1 was immunoprecipitated using anti-FLAG. The FLAG-GPX1 immunoprecipitates were separated by two-dimensional gel electrophoresis and were analyzed by immunoblotting with anti-*O*-GlcNAc (lower panels). The arrows indicate FLAG-GPX1.

GPX1 binds c-Abl and Arg kinases at amino acids 132–145 and is phosphorylated on Tyr96 [6]. Our data indicate that the N-terminal half of GPX1 is responsible for the binding to OGT, but GPX1 is GlcNAcylated on its C-terminal half. Therefore, *O*-GlcNAcylation on the C-terminus of GPX1 seems to affect the association of GPX1 with c-Abl and Arg under hyperglycemic conditions. Mapping the GlcNAcylation site on GPX1 will lead to a better understanding of the mechanism of GPX1 activation, including the requirement for *O*-GlcNAcylation for GPX1 activation, phosphorylation, and cell protection.

GPX1-overexpressing mice develop insulin resistance and obesity, which are characteristics of diabetes [19]. *O*-GlcNAcylation is also well known to be closely associated with diabetes. For example, OGT transgenic mice display type 2 diabetic characteristics such as insulin resistance [20]. In addition, PUGNAc treatment in adipocytes induces insulin resistance [21,22]. Elucidating the mechanism of GPX1 activation in hyperglycemia will likely provide important insights into the pathogenesis of diabetes and may suggest a therapeutic approach to this disease.

Acknowledgments

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