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The purification of reduced β 2-glycoprotein I showed its native activity in vitro

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Abstract

Background: New evidence has shown that reduced β 2-glycoprotein I (β 2GPI) has anti-oxidative stress and anti-inflammatory activity. However, the details are still poorly understood. This study aims to prepare stable reduced β 2GPI with its native bioactivity in vitro.

Methods: Human β 2GPI was purified from plasma first with perchloric acid precipitation and then purified with a series of chromatography methods including Sephadex G-25 desalting, SP HP, AF-heparin HC-650 M, and Sephacryl S-200. The purified human β 2GPI was reduced with thioredoxin-1 (TRX-1) activated by DL-dithiothreitol (DTT). Glutathione (GSH) was selected to block the free thiols in reduced β 2GPI. LC/MS was used to verify the location of free thiols. Western blot analysis was used to detect β 2GPI immunoreactivity. MTS and flow cytometry were conducted to investigate its biological effect on oxidative-stress-induced death of human umbilical vein endothelial cells (HUVECs). The levels of tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12P70 (IL-12P70), interferon-gamma (IFN- γ) and monocyte chemoattractant protein -1 (MCP-1) in mouse serum were quantified to assess its anti-inflammatory activity in lipopolysaccharide (LPS)-mediated systemic inflammation.

Results: We obtained approximately 10 mg β 2GPI (purity 98.7%) from 200 ml plasma. The protein yield was 0.05 mg/ml plasma. β 2GPI was then reduced by TRX-1/DTT in vitro; the free thiols were detected on Cys288 and Cys326 in domain V of β 2GPI. The GSH blockage stabilized the reduced β 2GPI in vitro. This reduced β 2GPI can be recognized by the anti- β 2GPI antibody, can significantly reduce the death of HUVECs after H₂O₂ treatment and can significantly decrease the levels of TNF- α , IL-6, IFN- γ and MCP-1 in mice upon LPS stimulation.

Conclusion: Stable reduced β 2GPI can be obtained in vitro by TRX-1 deoxidation followed by the blockage of thiols with GSH. This reduced β 2GPI maintains the same immunological activity as oxidized β 2GPI and has the ability to counter the oxidative stress induced by H₂O₂ in HUVECs and inflammation in LPS-mediated inflammation in mice.

Keywords: β 2-glycoprotein I, Reduced β 2-glycoprotein I, Thioredoxin-1, GSH, Diabetic vascular disease, Oxidative stress

Background

β 2-glycoprotein I (β 2GPI), which is also known as apolipoprotein H, is a phospholipid-binding plasma protein that circulates at a concentration of approximately 4 μ M. β 2GPI is well known as the major antigen of antiphospholipid syndrome and has a dual function as a procoagulant and anticoagulant [1, 2]. Recent studies have shown that β 2GPI could also play a role in vascular disease [3–6] as well as the innate immune response [7–9].

β 2GPI is composed of five domains and four glycosylation sites connected to its N terminus with a “hook-like” crystal structure [1]. Domains I to IV each have two disulfide bridges, whereas domain V has three disulfide bridges, including a disulfide bridge that incorporates a C-terminal cysteine [1]. Domain V also contains a positively charged lysine-rich region and a hydrophobic flexible loop segment, and both of these regions are required for the binding of β 2GPI to negatively charged macromolecules [1]. The disulfide bond in domain V is susceptible to cleavage by the oxidoreductases thioredoxin-1 (TRX-1) and protein disulfide isomerase (PDI), leading to the generation of free thiols at Cys288 and Cys326 [3, 10, 11].

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This special form of β 2GPI is called reduced β 2GPI. Therefore, β 2GPI exists in two forms (reduced β 2GPI and oxidized β 2GPI) in plasma, and the main form of β 2GPI in plasma is the free thiol form [10]. In recent years, reduced β 2GPI was found to have distinct functions from the oxidized β 2GPI [3, 4, 9]. Reduced β 2GPI has been shown to play a protective role against oxidative stress-induced vascular endothelial cell death, indicating that it has an anti-oxidative stress function. Its anti-oxidative stress function was further confirmed in age-related macular degeneration in patients [12] and hypoxia-induced retinal angiogenesis in mice [13]. Reduced β 2GPI was also found to inhibit macrophage foam cell formation that is induced by oxidized low density lipoprotein (ox-LDL) [4] in vitro and to inhibit vascular lipid deposition and plaque formation by reducing the expression of matrix metalloproteinases/tissue inhibitors of metalloproteinases (MMPs/TIMPs) via the downregulation of the p38 mitogen-activated protein kinase (p38 MAPK) signalling pathway in vivo [14], which suggests that reduced β 2GPI may alleviate vascular lipid toxicity through an anti-inflammatory mechanism. This anti-inflammatory activity was recently confirmed by our previous study in LPS-mediated systemic inflammation in mice [9].

However, the anti-inflammatory mechanism of the reduced β 2GPI is still poorly understood. Although a large proportion of plasma β 2GPI exists in free thiol form, this form is unstable in vitro [3]. Until now, the preparation of stable reduced β 2GPI, whether by isolating the β 2GPI from the plasma or by recombinant methods, has not been reported. Therefore, it is important to obtain a stable product in vitro to understand this special form of β 2GPI.

In this study, we prepared stable reduced β 2GPI in vitro by a deoxidation reaction and the thiol blockage method and investigated its effect on the oxidative stress-induced death of human umbilical vein endothelial cells (HUVECs) and its anti-inflammatory activity in LPS-mediated systemic inflammation in mice.

Methods

Materials

Human plasma was acquired from the Tianjin Blood Centre. The Sephadex G-25 desalting column, SP HP column, AF-heparin HC-650 M column, and Sephacryl S-200 column were purchased from GE Healthcare (USA). Reduced L-glutathione, dithiothreitol, and N-ethylmaleimide were purchased from Sigma (Saint Louis, MO). Recombinant TRX-1 was purchased from R&D Corporation (Minneapolis, MN). The CellTiter 96° Aqueous One Solution Cell Proliferation Assay Kit was purchased from BD Biosciences (Franklin Lakes, NJ). Annexin V-FLUOS staining kit was purchased from Roche (Penzberg, Germany). The other materials

were purchased from The Tianjin Ruentex Science and Technology Development Co. Ltd. LPS from *E. coli*, (serotype 0111:B4) was purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Male C57BL/6 J mice (6 to 8 weeks old) were obtained from the Experimental Animal Center of Peking University Health Science (Beijing, China). Mouse IL-6 and TNF- α ELISA kits were purchased from Sangon Biological Engineering Technology and Services Co., LTD (Shanghai, China). Human plasma was purchased from the Tianjin Municipal Blood Center (Tianjin, China). Rabbit anti-human β 2GPI monoclonal antibody was purchased from Abcam (London, UK). Goat anti-rabbit polyclonal antibody was purchased from Bioworld Technology, Inc. (St. Louis, MO, USA). This study was approved by the Animal Ethics Committee of the Metabolism Disease Hospital of Tianjin Medical University.

Human plasma precipitation and sample desalting

Human plasma was mixed with perchloric acid (70% volume fraction) at a volume ratio of 1:40, stirred for 30 min at 4 °C and then centrifuged at 1000 rpm at 4 °C to recover the supernatant. The supernatant was reserved, and the pH was titrated to 8.0 with a saturated Na₂CO₃ solution.

The cleared supernatant (170 ml) was loaded onto a desalting buffer (50 mM acetate pH 5.2)-preequilibrated G-25 desalting column (XK50 × 20 with 17 cm column height, GE Healthcare, USA) at a flow rate of 20 ml/min. A total of 180 ml of the sample was collected in this step, and the sample was then purified with an SP HP column.

Briefly, the desalted sample was loaded onto a preequilibrated HiTrap SP HP (GE Healthcare, USA) at a flow rate of 3 ml/min in buffer A (50 mM acetate pH 5.2). The column was then washed with buffer A for 2 column volumes (CV), 2% buffer B (50 mM acetate pH 5.2, 1 M NaCl) for 5 CV and eluted with a gradient of 5%–50% buffer B for 20 CV, and 50%–100% buffer B for 5 CV, and then the elution gradient was switched to 100% buffer B for 5 CV. The β 2GPI standard sample and protein fractions of every tube were used for SDS-PAGE. According to SDS-PAGE, the protein fractions with a band showed the same profiles as the standard sample. Some samples of mixed proteins were digested with trypsin PNGase F and analysed by LC/MS to verify β 2GPI-positive fractions. LC/MS analysis was conducted as previously described [13, 14]. Fractions containing β 2GPI were pooled together for AF-heparin HC-650 M column purification.

AF-heparin HC-650 M column purification

An AF-heparin HC-650 M column (Tricorn 10 × 10 with 9 cm column height, USA; the medium-sized column was purchased from TOSHH) was used for the

middle purification step. After the column was equilibrated with equilibration buffer (20 mM Tris 8.0), the pooled samples from the SP HP step were loaded onto the AF-heparin HC-650 M column and washed with buffer A for 2 column volumes (CV), 20% buffer B (50 mM acetate pH 5.2, 1 M NaCl) for 5 CV, 30% buffer B (50 mM acetate 1 M NaCl pH 5.2) for 5 CV, eluted with a gradient of 30%–80% buffer B for 20 CV, and then the elution gradient was switched to 100% buffer B for 5 CV. The fractions were then collected and analysed by SDS-PAGE. The fractions containing the same profile as the standard sample were pooled together.

Protein clean-up with the S-200 column

Fractions containing the target protein from the last step were concentrated by an Amicon Ultra-15 ml concentrator (10 kDa Centrifugal Filter Unit, Merck-Millipore) to 5 ml and then loaded onto a Superdex-200 column (HiLoad 16/60 Superdex 200 prep grade, GE) that was pre-equilibrated with equilibration buffer (20 mM PB, 0.15 M NaCl, 10% glycerol pH 7.2). The fractions collected in this step were also analysed by SDS-PAGE. N-glycosylation was verified by the PNGase F enzyme digestion method [15].

Preparation of reduced β 2GPI and blockage method development

- (a) The following solutions were mixed together: 420 μ l HBS buffer solution, 40 μ l DTT solution (20 mM HBS, 1 mM DTT), and 26 μ l TRX-1 solution (0.78 mg/ml), and then incubated at 3 °C for 45 min.
- (b) Approximately 45 μ l β 2GPI solution (2 mg/ml) was added to 405 μ l HBS buffer solution, and the mixed solution was shaken by brief centrifugation.
- (c) β 2GPI-HBS mixed solution was then added to the DTT-TRX-1-HBS solution, and the reaction solution was incubated at 37 °C for 1 h.
- (d) Thereafter, the reaction solution was divided into three aliquots, and iodoacetamide solution (40 mM), GSH solution (40 mM), or HBS buffer solution was separately added to each aliquot. The solution was placed at 4 °C to end the reduction reaction, and each reaction was analysed by nonreducing SDS-PAGE.

To block the free thiol form of β 2GPI, cysteamine or GSH was added to the 50 μ l reaction with final concentrations of 40 and 80 mM, respectively. The reaction was then incubated at 37 °C for either 2 h or overnight. Aliquots (2 μ g) of samples from each step and time point were analysed by nonreducing SDS-PAGE.

LC/MS analysis of the free thiol form of β 2GPI

To develop an LC/MS-based method to analyse the reduced form of β 2GPI, the purified β 2GPI was pretreated using two different conditions: A) 2 μ g protein was denatured in 50 μ l denaturing buffer (10 mM DTT, 0.1% RapiGest (Waters Corp.), 50 mM NH_4HCO_3) for 30 min at 60 °C; B) 2 μ g protein was denatured as in (A), and iodoacetamide (IAA) was then added to a final concentration of 20 mM and incubated at RT for 30 min to fully alkylate the reduced cysteine. The samples of the two conditions were enzymatically digested for 4 h at 37 °C with trypsin at a 20:1 (*w/w*, protein:enzyme). Trifluoroacetic acid (TFA) was added to each sample to the final of 0.5% to stop the enzyme digestion. The clarified samples were used for LC/MS analysis. LC/MS analysis was conducted as previously reported [16, 17].

The samples from the TRX reduction and alkylation blockage reactions were digested in 0.1% RapiGest, 50 mM NH_4HCO_3 , and the samples from the TRX reduction and GSH reactions were digested with trypsin for 4 h at 37 °C at a 20:1 (*w/w*, protein:enzyme). Trifluoroacetic acid (TFA) was added to each sample to a final concentration of 0.5% to stop the enzyme digestion. The clarified samples were used for LC/MS analysis.

Western blot analysis for the immunoreactivity of reduced β 2GPI

The aliquots (5 ng per lane) of the total protein were resolved on a Nu-PAGE TM 4–12% Bis-Tris gel and blotted onto a nitrocellulose membrane. The membrane was blocked with 2% BSA in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, and 0.01% Tween-20) for 1 h at RT, followed by incubation with anti- β 2GPI antibody overnight. After washing with TBST, the membrane was re-probed with HRP-anti-rabbit IgG (1:1000) in 2% BSA in TBST for 2 h at RT. After exposure in the darkroom, peroxidase activity on PVDF membranes was visualized on X-ray film with an ultraviolet transmission analyser.

Effect of reduced β 2GPI on oxidative stress-induced cell death in human endothelial cells

HUVECs were obtained from freshly delivered umbilical cords after informed consent. Cells were cultured with EGM or M199 plus 20% FBS ECGP and incubated at 37 °C in 5% CO_2 . Endothelial cells were identified by their typical cobble-stone morphology and by immunostaining with anti-CD31 antibody, as previously described [6]. Experiments were conducted using HUVECs between passages 3 and 4. HUVECs were seeded at a density of 1×10^5 cells/mL in 96-well or 24-well plates and grown to confluency over 24–48 h. Cells were grown to confluency and treated with 10 nM H_2O_2 as previously described [4]. After overnight incubation in M199/0.1% FCS, the cells were washed

twice in M199/0.05% BSA; then, 1 μM of β2GPI or reduced β2GPI was added to the medium and cells were incubated at 37 °C for 30 min. The cells were then transferred to a 1.5 ml Eppendorf tube containing H_2O_2 diluted in HBS to a final concentration of 10 mM and incubated at 37 °C for 30 min. H_2O_2 was then deactivated with catalase, cells were incubated overnight at 37 °C with M199/FCS (10%), and cell viability was quantified with MTS as previously described [6]. To investigate the effect of reduced β2GPI on cell proliferation, we used trypan blue dye to count the number of living cells. The cells were transferred to 24-well plates and overgrew the plates. After the cells were collected, 0.4% (*w/v*) trypan blue dye was added, and using a haemocytometer under the optical microscope, the number of living cells (3 wells) was counted. Cell apoptosis was assessed using flow cytometry. Cells were digested with trypsin and collected by centrifugation (2000 rpm, 5 min). Binding buffer (500 μl) was added to cells. Then, 5 μl annexin V-FITC and 5 μl propidium iodide were added to the binding buffer. Samples were incubated in the dark for 10 min at RT, and then flow cytometric analysis was performed. The excitation wavelength was 488 nm. The emission wavelength was 530 nm.

Effect of reduced β2GPI on LPS-mediated systemic inflammation in mice

Sixteen male mice in each group were housed in a specific pathogen-free environment, and their health status was regularly monitored. Purified plasma β2GPI (2 μM) or reduced β2GPI with 1 nM LPS were preincubated for 15 min at 37 °C before injection into mice [7]. Then, male mice were injected with 1 $\mu\text{g/g}$ body weight (gbw) with LPS from *E. coli* (Sigma-Aldrich) in GSH (80 mM) or the same volume of sterile saline through tail vein. Six hours post-injection, mice were euthanized, and approximately 1 ml of blood was collected by cardiac puncture. Serum was collected by centrifugation of clotted blood at 1500 g for 10 min to be used in a cytokine assay. The supernatants were separated, and the levels of IL-6, IL-10, IL-12p70, IFN- γ , TNF- α , and MCP-1 were determined using a cytometric bead array kit for mouse inflammatory cytokines (BD Biosciences) according to the manufacturer's instructions. Briefly, beads with distinct fluorescence intensities are coated with antibodies that specifically react with each of the cytokines and are detected by phycoerythrin-conjugated antibodies, which produces a fluorescent signal in proportion to the amount of bound analyte. The bead populations were mixed together to form the bead array, which was resolved in a FACSAria TM flow cytometer, and the beads were differentiated by their distinct spectral characteristics. Standard curves were determined for each cytokine from 20 to 50,000 pg/ml. The data were acquired on a FACS Aria TM flow cytometer (BD Biosciences) and analysed with FCAP

Array software (Soft Flow Hungary, Kedves, Hungary) by applying the 4-parameter curve fit option.

Statistical analysis

SPSS 11.5 statistical software was used for data processing and analysis. The values for all measurements are expressed as the mean \pm standard error of the mean. One-way ANOVA was used to evaluate significant differences. All *P* values were two-sided and were considered statistically significant if they were less than 0.05 (*P* < 0.05).

Results

Production of β2GPI

In the SP HP chromatography step, β2GPI was eluted as the second main peak (Fig. 1a). The purity of the pooled peak showed a dramatic increase in purity but still contained several impurities (Fig. 1b). After AF-heparin HC-650 M and gel filtration purification, the sample showed a homogenous peak on the S-200 column (Fig. 1c), and the purity was as good as that of the commercial standard (Fig. 1d). Consistent with reports of β2GPI containing several N-glycosylation modifications, the purified protein that was obtained after PNGase F digestion showed smaller bands by SDS-PAGE (lane 3, Fig. 1d). Finally, 10 mg β2GPI was purified from 200 ml human plasma. The purity of β2GPI was approximately 98.7%. The protein yield was 0.05 mg/ml plasma. The purified protein was digested by trypsin and followed by LC/MS-based peptide mapping to confirm the sequence.

Free thiols in reduced β2GPI are induced and blocked by GSH

In the free cysteine blockage step, two different blockage reagents, cysteamine and GSH, were compared at two different time points (2 h and overnight at 37 °C). After blockage for 2 h, the MW was slightly different from the native form, but the difference was not obvious (Fig. 2a). After blockage overnight, the target protein disappeared under the cysteamine-blockage condition. In addition, the GSH-blockage condition resulted in an obvious MW increase (Fig. 2b). The reduced and GSH-blocked forms were compared and analysed by both reducing and non-reducing SDS-PAGE. When analysed by reducing SDS-PAGE, the native and reduced forms had the same MW; however, when analysed by nonreducing SDS-PAGE, the reduced and blocked forms had a higher MW (Fig. 2c).

Based on the protein sequence and the trypsin theoretical digestion, the information for the target peptide (TDASDVKPC) is described in Fig. 3a. The protein sample after reduction contained only the reduced peptide (Fig. 3b & c, upper); however, the protein sample after reduction and alkylation exhibited an alkylated peptide

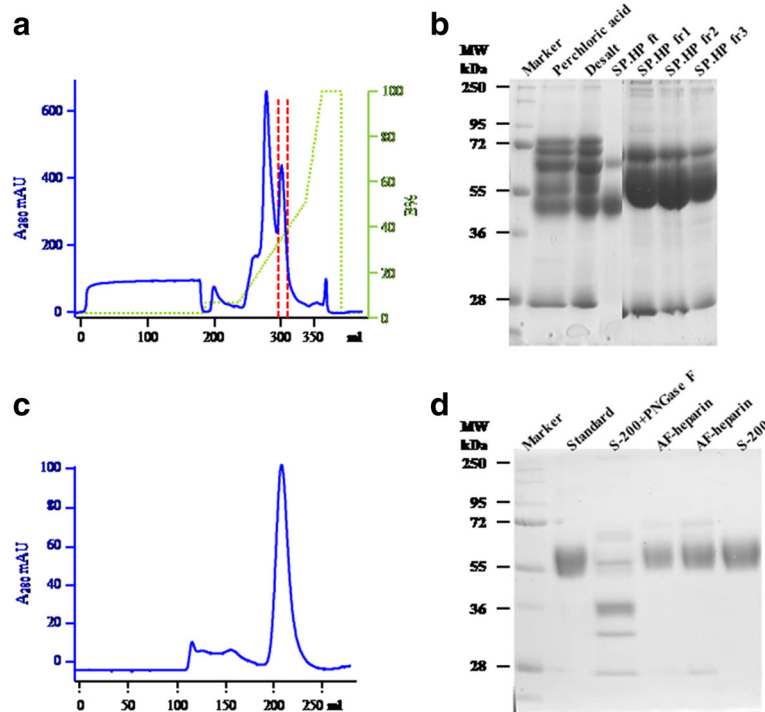


Fig. 1 Purification of β 2GPI. Human plasma was treated with perchloric acid, desalted with G-25 column, and purified with a series of chromatography steps on an SP HP column, AF-heparin HC-650 M column, and S-200 column in sequence. After purification with the SP HP column (a), the sample fractions (b) were analysed by SDS-PAGE. Marker: protein standard marker (Bio-Rad cat. 161-0374), perchloric acid: supernatant after perchloric acid precipitation, Desalt: G-25 desalted sample, SP HP ft.: the flow-through of SP HP, SP HP fr1-3: the pooled elution fractions of SP HP. After further purification with the AF-heparin HC-650 M column and S-200 column (c), the sample fractions were analysed by SDS-PAGE (d). Marker: protein standard marker (Bio-Rad cat. 161-0374), PNGase F: SP digestion, AF-heparin: fractions of AF-heparin chromatography step, S-200: pooled fractions of S-200 step

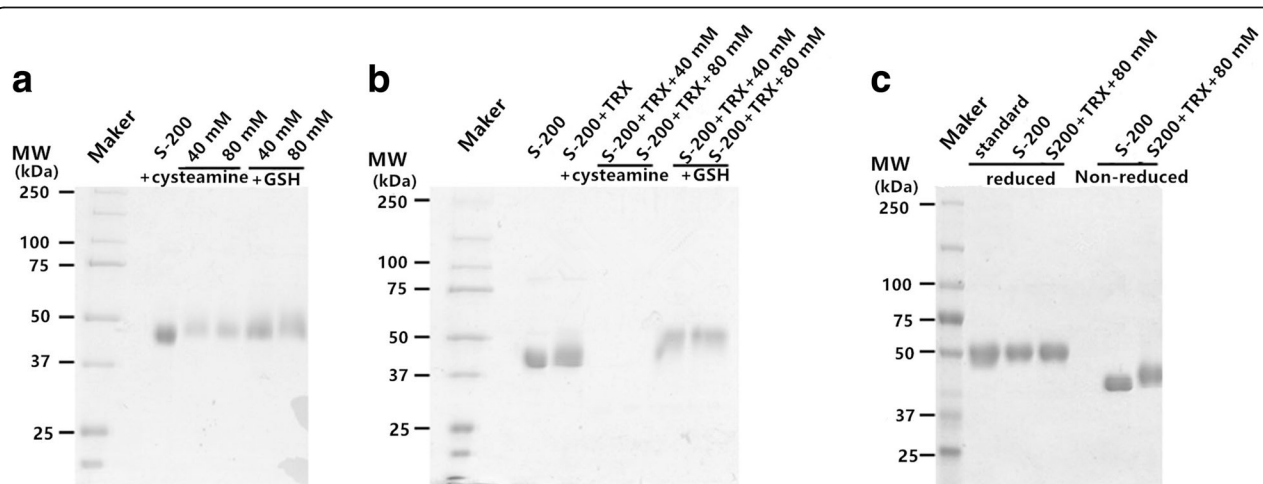
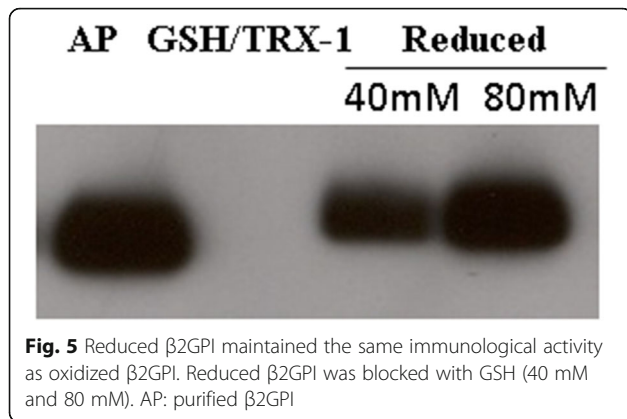


Fig. 2 The method development for reduced from β 2GPI production and its comparison with native β 2GPI. **a** Purified β 2GPI could not reduced by cysteamine or GSH alone. **b** Purified β 2GPI could reduced by thioredoxin-1 (TRX-1). **c** Reduced β 2GPI blocked by GSH under reduced and non-reduced SDS-PAGE conditions. Purified β 2GPI of the S-200 step (lane 2) was reduced by thioredoxin-1 (TRX-1) and then cysteine with cysteamine (lanes 3 and 4 with final concentrations of 40 and 80 mM, respectively, at 37 °C for 2 h) and GSH (lanes 5 and 6 with final concentrations of 40 and 80 mM, respectively, at 37 °C for 2 h) blockage (a). Purified β 2GPI of S-200 step (lane 2) was reduced by thioredoxin-1 (TRX-1) and then cysteine with cysteamine (lanes 3 and 4 with final concentration of 40 and 80 mM, respectively, at 37 °C overnight) and GSH (lanes 5 and 6 with final concentration of 40 and 80 mM, respectively, at 37 °C for 2 h) blockage (b). The final comparison of commercial standard β 2GPI (lane 2) with purified native (lane 3) and GSH blocked (lane 4) under reduced and non-reduced SDS-PAGE conditions. 5 μ g protein were loaded into each lane



experiment as previously reported [4]. Our results showed that reduced β2GPI can significantly improve the activity as well as the number of living cells (Fig. 6a and b) and reduce apoptosis (Fig. 6c and d) in HUVECs after H₂O₂ treatment.

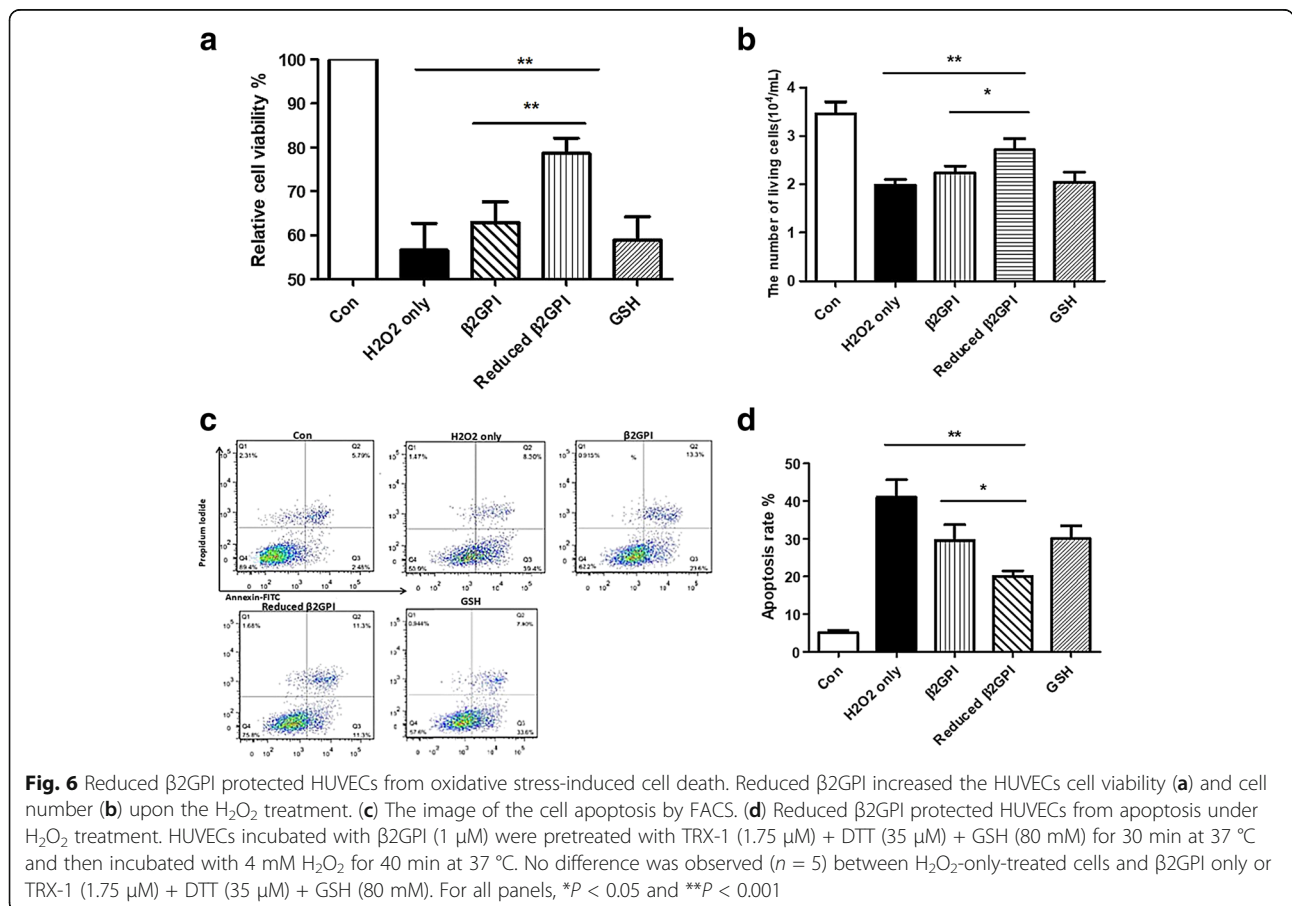
Reduced β2GPI has anti-inflammatory activity in LPS-mediated systemic inflammation

New evidence has revealed that β2GPI plays an important role in the innate immune response [7–9], and reduced β2GPI can relieve LPS-stimulated inflammation

in mice [9]. To assess the anti-inflammatory activity of the reduced β2GPI after GSH blockage, an LPS-mediated inflammation experiment was performed as reported in our previous study [9]. The results showed that only reduced β2GPI could significantly decrease the levels of IL-6, TNF-α, MCP-1 and IFN-γ in mice serum 6 h after LPS injection (Fig. 7).

Discussion

Reduced β2GPI was first reported by Professor Krilis’ team in recent years and was found to be the main form of β2GPI in human plasma [3, 11]. Increasing evidence indicates that this free thiol β2GPI plays a protective role in vascular diseases [3–5] and could also function as a scavenger protein for LPS to attenuate inflammation [7, 9]. These new functions of β2GPI were attributed to the anti-oxidative stress and anti-inflammatory activities of the reduced form of β2GPI. However, the mechanism of action of this special form of β2GPI is poorly understood. Until now, the successful extraction of reduced β2GPI from plasma or serum, which impeded the further study of this special form of β2GPI, had not been accomplished. Therefore, obtaining a stable free thiol β2GPI in vitro is a promising solution.



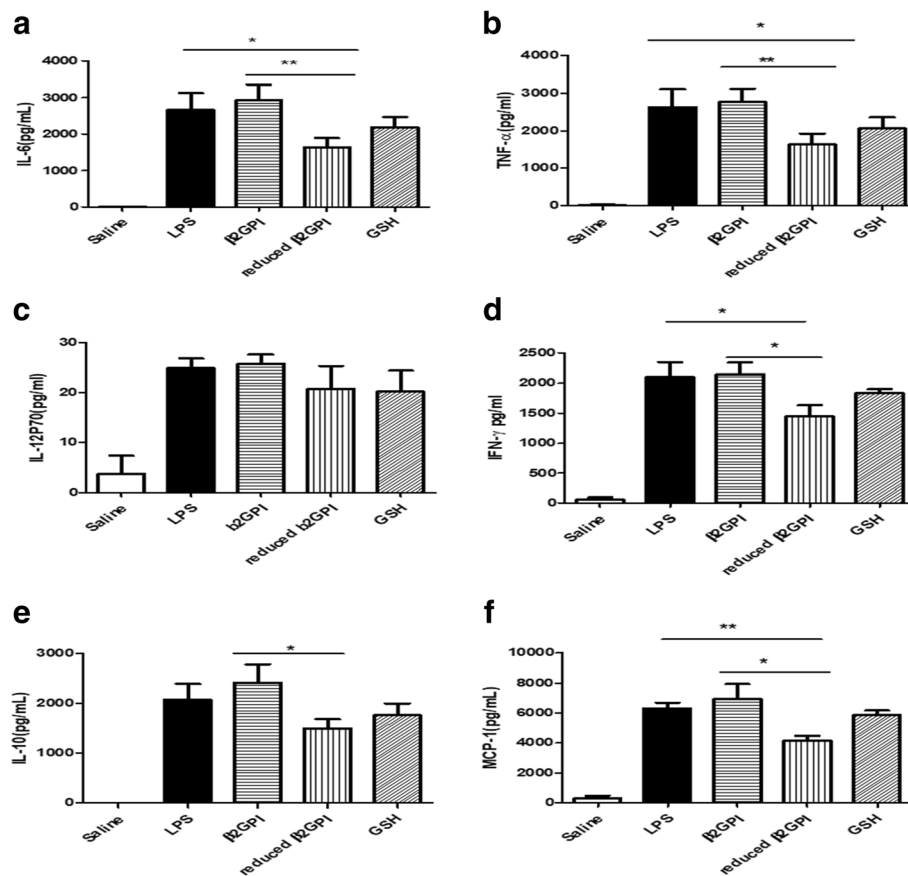


Fig. 7 Reduced β 2GPI inhibited LPS-mediated inflammation in mice. The effect of reduced β 2GPI on the levels of IL-6 (a), TNF- α (b), IL-12P70 (c), IFN- γ (d), IL-10 (e) and MCP-1 (f) in mice serum upon LPS stimulation. Purified plasma β 2GPI (2 μ M) or reduced β 2GPI with 1 nM LPS were preincubated for 15 min at 37 $^{\circ}$ C before injection into mice at 1 μ g/g body weight (gbw) with *E. coli* LPS (Sigma-Aldrich) in sterile saline or the same volume of saline with TRX-1 (1.75 μ M) + DTT (35 μ M) + GSH (80 mM) through the tail vein. At 6 h post injection, mice were euthanized, and approximately 1 ml of blood was collected by cardiac puncture. Serum was collected and the levels of cytokines (IL-6, TNF- α , MCP-1, IFN- γ , IL-10, IL-12P70) were quantified. Reduced β 2GPI significantly reduced the levels of IL-6, TNF- α , MCP-1, and IFN- γ . No difference was observed ($n = 5$) between β 2GPI only or GSH and LPS. For all panels, * $P < 0.05$ and ** $P < 0.001$

β 2GPI is known to be an abundant plasma protein with a molecular weight of approximately 50 kDa, and its concentration in human plasma is approximately 200 μ g/mL [2, 18]. Therefore, the isolation of β 2GPI from plasma is more economical and more efficient than recombinant protein purification. This study established a simple and effective method to purify β 2GPI from human plasma by treating plasma with perchloric acid, followed by a series of chromatography steps, namely, desalting with a G-25 column and purification with an SP HP column, AF-heparin HC-650 M column, and S-200 column. This purification procedure increased the yield of β 2GPI from human plasma. The final yield of β 2GPI was approximately 10 mg from 200 ml plasma. This procedure also yielded a high purity of 98.7%. Thus, this method could effectively isolate β 2GPI with high purity from human plasma.

To prepare the reduced β 2GPI, the DTT-activated oxidoreductase TRX-1 was used to cleave the disulfide bridge

in β 2GPI, as previously reported [3]. A large proportion of plasma β 2GPI exists in free thiol form because thioredoxin (TRX) is expressed widely in prokaryotic cells and eukaryotic cells. β 2GPI is one component of the TRX system, and the other two components are the TRX reductase (TrxR) and reduced nicotinamide adenine dinucleotide phosphate [3]. This system plays an important role in maintaining the stable redox state in vivo. In mammals, three isozymes of TRX exist: TRX-1 (located in the cytoplasm), TRX-2 (located in the mitochondria), and TRX-3 (located specifically in sperm, Sptrx-3) [19, 20]. The thiol exchange reaction was also reported to occur in β 2GPI, with the help of some reductase systems in platelets [21] or vascular endothelial cells [3]. Therefore, using the TRX system to reduce β 2GPI has physiological implications. As such, in the present study, we selected TRX-1 as the reducing agent to simulate the physiological transformation of oxidized β 2GPI to reduced β 2GPI, as

previously reported [3, 11]. LC/MS analysis showed that none of the disulfide bonds in domain I-IV of β 2GPI was disrupted and that only the disulfide bond formed by Cys288 and Cys326 in domain V was disrupted, along with the formation of corresponding thiols at the points of Cys288 and Cys326. This result corresponded to the molecular bioinformatics analysis of the β 2GPI molecular structure that was reported in a previous study [18].

According to the results of molecular bioinformatics analysis, among the 11 disulfide bonds in the molecule, the Cys288-Cys326 disulfide bond in domain V revealed a $-/+$ right-handed hook structure, and TRX-1 specifically interacts with the disulfide bond within this structure [15, 16]. Furthermore, Cys326 is exposed on the surface of this molecule; thus, this disulfide bond easily interacts with TRX-1. Therefore, the TRX-1 reduction method in vitro is an effective way to produce reduced β 2GPI. Other disulfide-reducing agents, such as β -mercaptoethanol, generally reduces most cysteine residues in a non-selective manner. Our results revealed that the β 2GPI sample that reacted with DTT or β -mercaptoethanol had a slower electrophoresis rate than the sample that reacted with TRX-1. Western blot analysis failed to detect its immunological activity (data not shown). Western blot analysis only detected β 2GPI activity in the activated TRX-1-treated samples, which also indicates that the free thiols in DI-DIV of β 2GPI were not disrupted. Thus, neither DTT nor β -mercaptoethanol is able to simulate the physiological thiol conversion reaction in β 2GPI. Meanwhile, TRX-1 is not only a physiological reducing reagent but also an effective reducing agent to produce reduced β 2GPI in vitro.

LC/MS analysis did not detect any free thiols in β 2GPI before treatment with TRX-1/DTT in the present study, thus suggesting that the β 2GPI extracted from plasma existed in the oxidized form. One important reason is that reduced β 2GPI cannot maintain its stability in vitro or under oxidative conditions. Figure 3 shows that reduced β 2GPI, without thiol blockage, was degraded at 37 °C overnight and was unstable.

In this study, we acquired stable reduced β 2GPI in vitro in the following steps: extracting purified β 2GPI from plasma, performing the reduction reaction in vitro, and protecting the free thiols of reduced β 2GPI using GSH. Figure 3 shows that GSH blockage can stabilize reduced β 2GPI compared with cysteamine. GSH is a small physiological molecule that does not cause toxicity and immunogenicity to the human body and could be an ideal thiol blocker for reduced β 2GPI.

With the blockage of GSH, reduced β 2GPI will maintain its immunoreactivity. Western blot analysis showed that both the reduced β 2GPI blocked with GSH and oxidized β 2GPI could detect the same antibody. This finding indicated the existence of a disulfide bond in domain I and

the structural integrity of domain I in reduced β 2GPI because the antibody combined with the specific epitope in domain I of β 2GPI.

Reduced β 2GPI was identified to have anti-oxidative stress and anti-inflammatory activity [3, 9]. To further verify the biological activity of the reduced β 2GPI blocked with GSH, we detected its effect on oxidative stress-(H₂O₂)-induced HUVEC cell death and the cytokine levels in LPS-mediated systemic inflammation in mice. The results confirmed that compared with β 2GPI or GSH, reduced β 2GPI had significantly improved activity, reducing apoptosis and decreasing the IL-6 and TNF- α levels. Therefore, the GSH blocker could not impede the biological activity of reduced β 2GPI, and this activity was independent of GSH.

Conclusion

We prepared stable reduced β 2GPI by TRX-1 deoxidation followed by thiol protection with GSH in vitro. This special form of β 2GPI with GSH-blocked free thiols maintained the immunoreactivity and bioactivity of reduced β 2GPI. This study not only provides an effective way to isolate β 2GPI from plasma but also shows a simple method to maintain the stability of reduced β 2GPI in vitro, which is important to the further study of the physiological function of reduced β 2GPI.

Abbreviations

β 2GPI: β 2-glycoprotein I; CV: Column volume; DTT: DL-dithiothreitol; GSH: Glutathione; HUVECs: Human umbilical vein endothelial cells; IAA: Iodoacetamide; IL-6: Interleukin-6; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MMPs: Matrix metalloproteinase; ox-LDL: Oxidized low density lipoprotein; PDI: Protein disulfide isomerase; Sptrx-3: Sperm-specific TRX-3; TFA: Trifluoroacetic acid; TIMPs: Tissue inhibitors of metalloproteinases; TrxR: TRX reductase

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analysed during the current study.

Authors' contributions

SJZ, ML, JTZ, SHL, XL, RZ and HYL performed the experiments, and SJZ drafted the manuscript. PY and SJZ designed the study. PY coordinated the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were performed in accordance with the institutional guidelines for human and animal research and were approved by the Animal Care and Use Committee of Metabolic Diseases Hospital of Tianjin Medical University, Tianjin, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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