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Oxidative Stress Enhances AP-1 and NF-κB-Mediated Regulation of *β2-Glycoprotein I* Gene Expression in Hepatoma Cells

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ABSTRACT

β_2-Glycoprotein I (β_2-GPI), also known as apolipoprotein H, is a plasma glycoprotein with poorly defined gene regulation. The aim of this study was to clarify the role of oxidative stress in *β_2-GPI* gene regulation and determine the essential transcription element regulating *β_2-GPI* expression. We demonstrate that expression of β_2-GPI at the protein and mRNA levels was significantly elevated in Huh7 and HepG2 cells treated with 100 μM hydrogen peroxide (H_2O_2). To address the transcriptional mechanism of H_2O_2-mediated *β_2-GPI* gene regulation, several promoter constructs were cloned and characterized by deletion assays. A region spanning from −2141 to −1419 (relative to the transcription start site), which contains two activator protein-1 (AP-1) sites (AP1-2 and AP1-3) and one nuclear factor-kappaB (NF-κB) site was found to be the main target site for up-regulation of *β_2-GPI* promoter activity by oxidative stress. In addition, we found that H_2O_2 stimulation enhanced the nuclear translocation of AP-1 and NF-κB subunits. Using an electrophoretic mobility shift assay, it was confirmed that nuclear protein binding to the AP1-2, AP1-3, and NF-κB sites was increased in Huh7 cells treated with H_2O_2. Knockdown of the *c-Jun*, *c-Fos*, *p65*, and *p50* genes using small interfering RNAs (siRNAs) further confirmed that AP-1 and NF-κB play an essential role in the H_2O_2-induced β_2-GPI expression. Overall, these findings provide new insight suggesting that multiple *cis*-elements in the *β_2-GPI* promoter work cooperatively to regulate β_2-GPI expression in cells under oxidative stress. J. Cell. Biochem. 111: 988–998, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: OXIDATIVE STRESS; β_2-GLYCOPROTEIN I; H_2O_2; AP-1; NF-κB; HEPATOMA CELLS

β_2-Glycoprotein I (β_2-GPI) is a 50 kDa glycoprotein primarily synthesized in the liver [Wang and Chiang, 2004]. The *β_2-GPI* gene is located on chromosome 17q23-24 spanning 18 kb and consists of eight exons and seven introns [Okkels et al., 1999]. β_2-GPI displays diverse biological functions including involvement in triglyceride metabolism, blood coagulation, and homeostasis [Takeuchi et al., 2000; Yasuda et al., 2000; Miyakis et al., 2004]. Our group has reported on the role of β_2-GPI in anti-LDL oxidation and the prevention of NO-induced apoptosis in macrophages and human coronary artery smooth muscle cells [Lin et al., 2001, 2005]. The role of β_2-GPI in the coagulation system, both in healthy people and in patients with antiphospholipid syndrome (APS), has been increasingly elucidated [Roubey et al., 1992; Miyakis et al., 2004; Shi et al., 2004]. Activation of the coagulation system represents an important facet of immune and inflammatory reactions, which participate in a number of human diseases [Matsuura et al., 2003; Lin et al., 2006; Dunoyer-Geindre et al., 2007].

Although clinical importance has been attributed to β_2-GPI, the molecular basis of *β_2-GPI* gene regulation remains unknown. Previously, we cloned a 4.1 kb genomic fragment corresponding to the 5′-flanking region of the human *β_2-GPI* gene and identified that an atypical TATA box and a hepatic nuclear factor-1 (HNF-1) *cis*-element are crucial for *β_2-GPI* promoter activity [Wang and Chiang, 2004]. An understanding of the transcription machinery associated with the *β_2-GPI* promoter is necessary for developing further insights into the regulation of β_2-GPI expression during various disease processes.

Hydrogen peroxide (H_2O_2), as a major source of reactive oxygen species (ROS), has been extensively used as an inducer of oxidative

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stress in many in vitro models [Zhou et al., 2001; Cunha-Oliveira et al., 2006; Wang et al., 2008; Naziroglu, 2009]. ROS have been shown to play a role in the pathogenesis of several inflammatory diseases including atherosclerosis, rheumatoid arthritis, and ischemia/reperfusion injury [Harrison et al., 2003; Madamanchi et al., 2005; Dalle-Donne et al., 2006]. Activator protein-1 (AP-1) and NF-kappaB (NF-κB) are two redox-sensitive transcription factors that can be activated during various inflammatory disease processes [Karin et al., 2001; Surh et al., 2005]. However, the effect of oxidative stress on β_2-GPI expression and the involvement of AP-1 and NF-κB in *β_2-GPI* gene regulation have not been demonstrated as yet.

The present study was therefore designed to determine whether oxidative stress is able to modulate β_2-GPI expression via activation of its promoter activity in hepatoma cells. In addition, we investigated the contribution of AP-1 and NF-κB to regulation of human *β_2-GPI* gene expression. An investigation of the promoter activities of reporter plasmids containing three AP-1 sites spanning from −2204 to −2068 and one NF-κB site located between −1432 and −1419 in H_2O_2-treated cells was undertaken. This information will help in identifying the regulatory domain of *β_2-GPI* gene associated with oxidative stress. The overall aim of this study was to investigate the regulation of β_2-GPI expression by oxidative stress and identify the specific targets of H_2O_2-mediated *β_2-GPI* gene regulation.

MATERIALS AND METHODS

CELL CULTURE

Huh7 and HepG2 cells were seeded at a density of 5×10^5 cells/cm^2 in tissue culture flasks and grown at 37°C in a humidified 95% air/5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 0.3 mg/ml L-glutamine, and 0.1 mM non-essential amino acids. Fresh medium was added every 2 days and the cells were passaged every 4 days with a 1:4 split ratio.

ANALYSIS OF INTRACELLULAR ROS AND CELL VIABILITY

The levels of intracellular ROS were measured using the dye fluorescence probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma–Aldrich, St. Louis, MO), which passively diffuses into the cells and is cleaved and oxidized to 2,7-dichlorofluorescein (DCF). Huh7 cells (5×10^4 cells/well) were seeded on a poly-L-lysine-coated 96-well plate in DMEM supplemented with 2% FBS for 24 h. Cells were then loaded for 30 min with 20 μM DCFH-DA followed by treated with the indicated concentrations of H_2O_2. Cells were washed with cold PBS twice and the fluorescence of the oxidized dichlorofluorescein was monitored by flow cytometry with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

To measure the cell viability of Huh7 cells undergoing H_2O_2-induced oxidative damage, Huh7 cells (5×10^4 cells/well) were seeded on a poly-L-lysine-coated 96-well plate in DMEM for 24 h. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple formazan [Carmichael et al., 1987]. Huh7 cells were incubated with MTT (0.5%) for 4 h at 37°C. The medium was carefully removed and the formazan crystals were dissolved in isopropanol. The extent of the reduction of MTT was quantified using an ELISA reader at 550 and 690 nm. The net difference of absorbance between 550 and 690 nm was used to express the relative viability as $A_t \times 100/A_c$, where A_t is the absorbance of the H_2O_2-treated cells and A_c is the absorbance of the untreated controls.

WESTERN BLOT ANALYSIS

Total cellular protein was prepared to determine the effect of H_2O_2 at concentrations of 50, 100, and 200 μM on β_2-GPI protein expression in Huh7 and HepG2 cells [Uguz et al., 2009]. Whole-cell extracts were prepared by lysing cells in buffer containing 50 mM HEPES (pH 7.4), 6 mM EDTA, and 1% Triton X-100 supplemented with complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Total protein was separated by centrifugation at 13,200*g* for 20 min at 4°C. Cells were then incubated in cytoplasmic extraction buffer containing 20 mM HEPES (pH 7.4), 10 mM KCl, 1 mM $MgCl_2$, 0.5% NP-40, 0.5 mM dithiothreitol, and complete protease inhibitor cocktail (Roche Applied Science). The cytosolic fraction was separated after centrifugation at 3,000*g* for 5 min at 4°C. The pellet was resuspended in 30 μl of ice-cold nuclear extraction buffer [20 mM HEPES (pH 7.4), 0.4 M NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 20% glycerol supplemented with complete protease inhibitor cocktail (Roche Applied Science)] and centrifuged at 13,200*g* for 20 min at 4°C. The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA) with BSA as the standard. Equal amount of proteins were subjected to 10% SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The immunoblots were then placed in Tris-buffered saline blocking solution (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 5% non-fat milk) for 1 h. Transferred blots were incubated with the primary antibody for 18 h and washed extensively with 1× Tris-buffered saline and 0.05% Tween 20. Following incubation with horseradish peroxidase-conjugated secondary antibody and washes, bound IgG was visualized using an enhanced chemiluminescence (ECL) detection kit system (PerkinElmer, Shelton, CT). The blots were further stripped for probing with actin, α-tubulin, or B23 antibodies (Santa Cruz, CA) as the internal control for total, cytosolic, or nuclear proteins, respectively.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AND REAL-TIME QUANTITATIVE PCR

Expression of *β_2-GPI* mRNA was assessed using RT-PCR and real-time quantitative PCR. Total RNA from 3×10^6 hepatoma cells treated with or without H_2O_2 was extracted with 300 μl of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A sample of 2 μg purified RNA was used to synthesize the first-strand cDNA in a 20 μl volume reaction with oligo(dT) primer using the SuperScript First-Strand Synthesis System (Invitrogen). RT-PCR reactions were then performed in a total volume of 20 μl containing 10 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 μM each dNTP, 1 μM each primer, and 1 U of ExTAQ DNA polymerase. The RT-PCR was performed on a Peltier Thermal Cycler

(Model PTC-200, MJ Research, Inc., Waltham, MA) with a preheating at 95°C for 2 min and 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 45 s. The sequences of the primers were *β_2-GPI* sense 5′-CCAGTGCTCATCTTGTTCTCG-3′ and antisense 5′-GTGTCCCGATAGAGGGAATTG-3′. As an internal control, the porphobilinogen deaminase (PBGD) gene was used and the primers were sense 5′-AGGATGGGCAACTGTACC-3′ and antisense 5′-GTTTTGGCTCCTTTGCTCAG-3′. The PCR products were separated on 2% (w/v) agarose gels using 0.5× TAE (40 mM Tris/acetic acid and 1 mM EDTA) buffer and visualized by ethidium bromide staining.

Real-time quantitative PCR with SYBR Green was performed with LightCycler Carousel-Based System (Roche) according to the manufacturer's protocol. The same designed primers and internal control were used as those in the RT-PCR method. Reaction condition was 10 min at 95°C, and then 20 s at 95°C, 20 s at 50°C, 20 s at 72°C for 40 cycles. In each PCR reaction, a nuclease-free water tube was set as a control. Quantification of *β_2-GPI* mRNA was calculated by the comparative C_t method $(2^{-\Delta\Delta C_t})$, as described by the manufacturer. The *β_2-GPI* mRNA level was normalized to that of PBGD and expressed as folds of control.

CLONING, TRANSIENT TRANSFECTION, AND LUCIFERASE ASSAYS

In order to clone the 5′-flanking region spanning from −2207 to +9 of the human *β_2-GPI* gene, a series of sense primers at varying distances were used to amplify 5′-deleted promoter fragments. The upstream sense primers were 5′-AAACCTGACCCCCATTATCC-3′ (−2207/−2188) for fragment AP1-1, 5′-GTAGCTAAGCTTGTGACACC-3′ (−2152/−2133) for fragment AP1-2, 5′-GAGGCCTTCCTTGTGACTTTT-3′ (−2089/−2069) for fragment AP1-3, and 5′-ACACCCCTACCCCAAGTTTC-3′ (−1734/−1715) for fragment NF-κB. The downstream antisense primer was 5′-ACACTGGCACTACCAAAGTGG-3′ (+9/−12). Various deletions of the human *β_2-GPI* promoter were generated by PCR using high-fidelity ExTAQ polymerase (Takara Shuzo, Kyoto, Japan) with human genomic DNA as template. Reporter constructs were generated by subcloning the human *β_2-GPI* proximal promoter into the promoterless pGL2-basic vector (Promega, Madison, WI), which already carries a firefly luciferase gene. Transient transfections were carried out using Lipofectamine™ 2000 according to the manufacturer's instruction (Invitrogen). Huh7 cells were plated at 4×10^5 per well in 24-well plates 16 h before transfection. For each transfection, 0.5 μg of pGL2 reporter constructs were transfected with 0.1 μg of the internal control cytomegalovirus-β-galactosidase vector (pCMV-β-Gal). After 20 h of transfection, the cells were treated with H_2O_2 for 4 h. Each transfection experiment was performed in triplicate and repeated a minimum of three times. Luciferase and β-galactosidase assays were carried out as described previously [Wang and Chiang, 2004]. Luciferase activity was measured using a luminometer (EG&G Berthold, Oak Ridge, TN) and transfection efficiency was determined by measuring the β-galactosidase activity within the same sample.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Extracts of nuclear proteins were prepared from Huh7 cells in lysis buffer [20 mM HEPES; pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 0.5% NP-40, 0.5 mM dithiothreitol supplemented with complete protease inhibitor cocktail (Roche Applied Science)]. After centrifugation at 3,000*g* for 5 min at 4°C, the nuclear pellet was resuspended in 30 μl of ice-cold extraction buffer [20 mM HEPES; pH 7.4, 0.4 M NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 20% glycerol supplemented with complete protease inhibitor cocktail (Roche Applied Science)]. The concentration of nuclear protein was measured using the Bradford assay (Bio-Rad). The sense sequences of probes for AP1-1, AP1-2, AP1-3, and NF-κB are 5′-GAGAAACCTGACCCCCATTATC-3′, 5′-AGCTAAGCTTGTGACACCATCA-3′, 5′-GGCCTTCCTTGTGACTTTTAGA-3′, 5′-AGCGGAACCTGGGAATTATCCA-3′, respectively. The anti-sense sequences for AP1-1, AP1-2, AP1-3, and NF-κB are 5′-AGGATAATGGGGGTCAGGTTT-3′, 5′-CAGCGTGATGGTGTCACAAGCT-3′, 5′-TCAGTCTAAAAGTCACAAGGAA-3′, 5′-TCTCTGGATAATTCCCAGGTT-3′, respectively. Sense and anti-sense oligonucleotides were heated at 90°C for 5 min and slowly cooled to room temperature. The 3′-end-double-stranded oligonucleotides were labeled with [α-^{32}P]dCTP by fill-in reaction using the Klenow fragment of DNA polymerase I. For each EMSA reaction, 2 μg of nuclear proteins were incubated for 15 min on ice in 30 μl of binding buffer. 1×10^5 cpm of the probe was added to each reaction and the mixtures were incubated at room temperature for 30 min. For the competition assays, cold oligonucleotides were added to the binding reactions. Competitors were generated by annealing the unlabeled sense and antisense oligonucleotides, and were added at 100-fold molar excess for each competition assay. For the supershift assays, 0.4 μg of specific antibodies were incubated with nuclear extracts for 24 h before adding the labeled probe. Anti-c-Jun, c-Fos, Jun-D, activating transcription factor-2 (ATF-2), p65, p50, and control IgG antibodies are obtained from Santa Cruz Biotechnology. The reaction products were size-fractionated on a 5% non-denaturing polyacrylamide gel, then run at 250 V at 4°C for 2 h in Tris/borate/EDTA buffer. The gel was dried and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

ADMINISTRATION OF siRNA TO CELLS

Double-stranded small interfering RNAs (siRNAs) were obtained from the National RNAi Core Facility at the Institute of Molecular Biology (Academia Sinica, Taipei, Taiwan). siRNAs targeting two different regions in *c-Jun* (c-Jun-1, c-Jun-2), *c-Fos* (c-Fos-1, c-Fos-2), *p65* (p65-1, p65-2), and *p50* (p50-1, p50-2) encode small interfering oligonucleotides specific for human *c-Jun*, *c-Fos*, *p65*, and *p50* genes, respectively. VSV-G-pseudotyped lentiviruses were produced by cotransfecting 293 T cells with *c-Jun* siRNA, *c-Fos* siRNA, *p65* siRNA, and *p50* siRNA clones as well as two packaging plasmids: pMD.G and pCMVΔR8.91. Infectious lentiviruses were harvested at 48 and 72 h after transfection. Huh7 cells were then infected with lentiviruses containing *c-Jun* siRNA, *c-Fos* siRNA, *p65* siRNA, *p50* siRNA, and *luciferase* siRNA (control) according to the lentivirus infection protocol provided by the National RNAi Core Facility (Academia Sinica). After 96 h, cells were exposed to 100 μM H_2O_2 for 4 h, the lysates then underwent Western blot analysis.

STATISTICAL ANALYSES

Data are given as means ± SEM. All experiments were repeated at least three times independently. Statistical analysis between two

groups was performed using Student's *t*-test. An analysis of variance (ANOVA), combined with the Tukey's multiple-comparison test, was used to evaluate the statistical significance of differences among cells infected with lentiviruses containing different siRNAs. The value for $P < 0.05$ was considered statistically significant.

RESULTS

EFFECT OF H_2O_2 ON ROS PRODUCTION AND CELL VIABILITY

To explore whether H_2O_2 increases intracellular oxidative stress, ROS were measured in Huh7 cells treated with H_2O_2 at concentrations of 50, 100, and 200 μM. As shown in Figure 1A, ROS were increased at a dose-dependent manner. Since ROS usually result in cellular damage, we next attempted to clarify whether a higher dose of H_2O_2 cause cell toxicity by MTT assay. The data suggested that Huh7 cells were resistant to cell toxicity at 50 and 100 μM H_2O_2. However, 200 μM H_2O_2 significantly reduced the cell viability compared to the controls (Fig. 1B).

EFFECT OF H_2O_2 ON β_2-GPI EXPRESSION AT PROTEIN AND mRNA LEVELS

To investigate the regulation of β_2-GPI expression by oxidative stress, expression of β_2-GPI protein levels was measured by Western blot analysis. The level of β_2-GPI protein was highest in Huh7 and HepG2 cells treated with 100 μM H_2O_2 for 4 h (Fig. 2A). To elucidate whether *β_2-GPI* mRNA level affects the overall β_2-GPI protein level, the *β_2-GPI* mRNA expression level was determined by semi-quantitative RT-PCR (Fig. 2B) and real-time PCR (Fig. 2C). We found that the *β_2-GPI* mRNA level paralleled the β_2-GPI protein level in Huh7 cells, but not in HepG2 cells. Significantly higher levels of *β_2-GPI* mRNA were induced in both Huh7 and HepG2 cells treated with 100 μM H_2O_2 as compared with that in the control group. The stimulatory effect of H_2O_2 on *β_2-GPI* mRNA levels in HepG2 cells was found in a dose-dependent manner.

TRANSCRIPTIONAL ACTIVATION OF *β_2-GPI* PROMOTER ACTIVITY

In order to examine the transcription factors mediating regulation of the *β_2-GPI* gene, Huh7 cells were transiently transfected with pGL2-basic luciferease vector containing the *β_2-GPI* promoter carrying the one NF-κB and three AP-1 *cis*-elements shown in Figure 3A. Cells were harvested after 20 h of transfection and then further incubated with 100 μM H_2O_2 for 4 h. The transcriptional activity in the NF-κB *cis*-element-transfected cells treated with H_2O_2 was 1.24-fold higher compared to the non-H_2O_2 treated cells. In the three AP-1 *cis*-element-transfected cells, the relative promoter activities of the cells with the AP1-1, AP1-2, and AP1-3 reporter constructs were 1.79-, 1.70-, and 1.45-fold higher compared to the same vector-transfected cells without H_2O_2 treatment, respectively (Fig. 3B). To identify the critical *cis*-elements mediating *β_2-GPI* transcription, progressive deletions of 5′-flanking region spanning from −2207 to +9 of the human *β_2-GPI* gene were analyzed in H_2O_2-treated cells. As shown in Figure 3B, a significant induction in promoter activity was found in the transfected cells containing the NF-κB, AP1-2, and AP1-3 reporter plasmids in the presence of 100 μM H_2O_2. However, the extent of the AP1-1 contribution was found to be much less than those of the other two AP1 sites, indicating that the AP1-1 site is not an essential regulatory element for H_2O_2-stimulated *β_2-GPI* transcription. Taken together, NF-κB and AP-1 play independent roles in H_2O_2-stimulated *β_2-GPI* promoter activity, but a synergistic effect of NF-κB and AP-1 was also detected during H_2O_2-induced promoter activation.

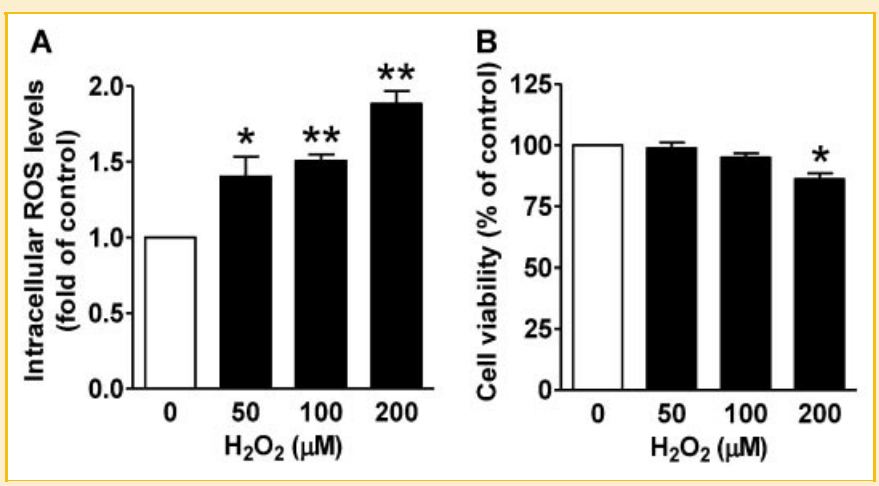

Fig. 1. Effect of H_2O_2 on intracellular ROS production and cell viability. A: Huh7 cells (5×10^4 cells/well) were seeded on a 96-well plate in DMEM containing 10% FBS for 24 h. Cells were then loaded with 20 μM DCFH-DA followed by treatment with various concentrations of H_2O_2 in a range from 50 to 200 μM for 30 min. The fluorescence of the oxidized dichlorofluorescein was monitored by flow cytometry with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The value of control group is set as 1. Values are means ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ represent significant differences compared with the control group. B: Huh7 cells were seeded on a 96-well plate in DMEM for 24 h. Cell viability was measured by MTT assay as described in Materials and Methods Section. The MTT value is expressed as a percentage of the control group, which is set as 100%. Data represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$ versus control group.

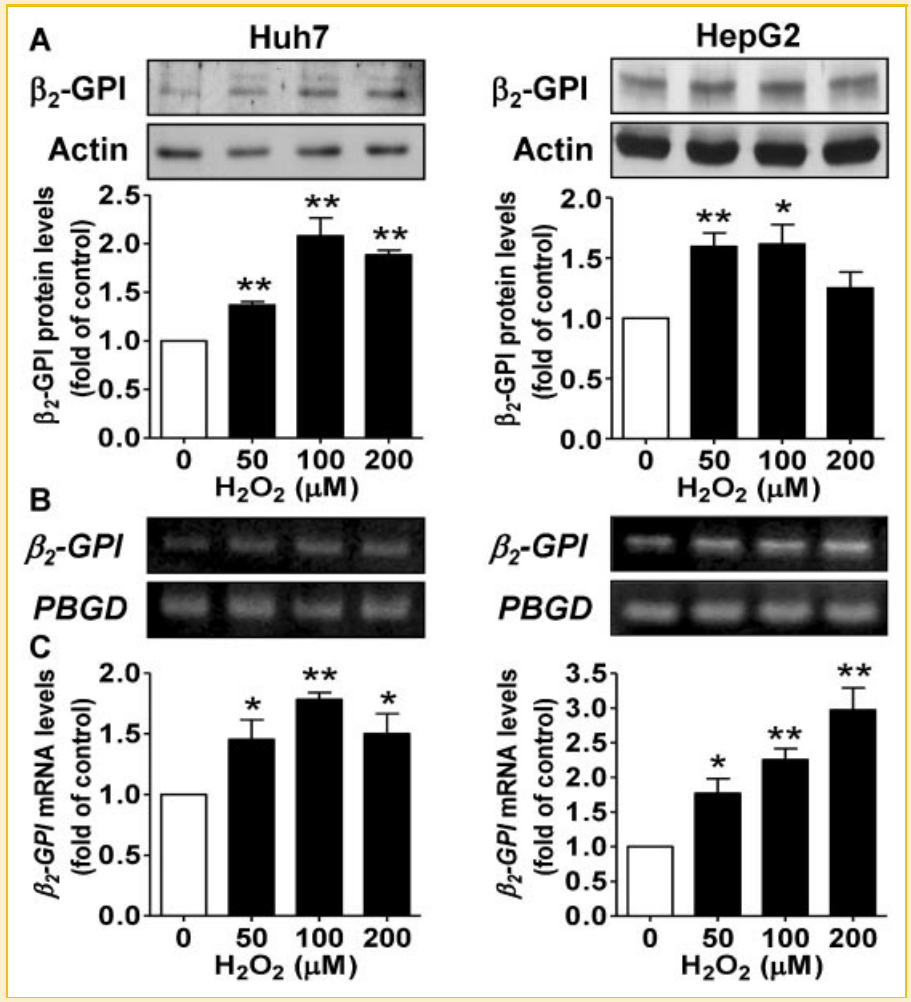

Fig. 2. H_2O_2-induced expression of β_2-GPI protein and mRNA in Huh7 and HepG2 cells. A: Cells were stimulated with H_2O_2 at indicated concentrations for 4 h. Thirty micrograms of protein from the cell lysates were separated by SDS–PAGE and analyzed by Western blot analysis. Representative results from four separate experiments are shown. The blots were stripped and re-probed with actin to show equal loading of the protein. The intensity of protein band normalized by actin was calculated as the fold of controls and then depicted as graph bar. Results are represented by the mean ± SEM of four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control group. B: Cells were stimulated with H_2O_2 at indicated concentrations for 3 h. Total RNA was harvested by using TRIzol reagent (Invitrogen) for semi-quantitative RT-PCR analysis. The expression of PBGD mRNA was used as an internal control. A representative blot from four independent experiments is shown. C: The histogram shows the mRNA levels of *β_2-GPI* in Huh7 or HepG2 stimulated with H_2O_2 determined by real-time PCR. Results are represented by the mean ± SEM of four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

EFFECT OF H_2O_2 ON AP-1 AND NF-κB TRANSLOCATION

Having shown the involvement of AP-1 and NF-κB in the transcriptional regulation of the *β_2-GPI* gene, we next questioned if the H_2O_2-stimulation induced translocation of the AP-1 and NF-κB subunits. To test this possibility, we employed Western blot analysis with antibodies against c-Jun, c-Fos, Jun-D, and ATF-2 as well as p65 and p50 using proteins from nuclear extracts, cytosolic fraction, and whole-cell lysates. As shown in Figure 4A, nuclear translocation of the AP-1 and NF-κB subunits was all significantly increased in the H_2O_2-treated Huh7 cells. However, H_2O_2 did not have remarkable effect on the levels of AP-1 and NF-κB subunits in the cytosolic fraction and whole-cell lysates. Similar to the nuclear translocation changes in Huh7 cells, the levels of c-Jun, c-Fos, Jun-D, as well as p65 and p50 were all increased in the nucleus of HepG2 cells treated with 100 μM H_2O_2 (Fig. 4B).B2-GPI

EVIDENCE THAT AP-1 AND NF-κB BIND THE *β_2-GPI* PROMOTER

Since we had shown that AP-1 and NF-κB are required for H_2O_2-stimulated activation of *β_2-GPI* promoter, we next examined whether subunits of AP-1 (c-Jun, c-Fos, Jun-D, and ATF-2) and NF-κB (p65 and p50) bind to the *cis*-elements of *β_2-GPI* promoter. It was found that there was differential induction of the AP-1 and NF-κB complexes on H_2O_2 treatment. As shown in Figure 5A–C, the binding of nuclear protein to the AP1-2, and AP1-3 sites was stronger than to the AP1-1 site. EMSA also revealed that there was interaction with the responsive element upstream of *β_2-GPI* promoter via the H_2O_2-activated transcription factor NF-κB

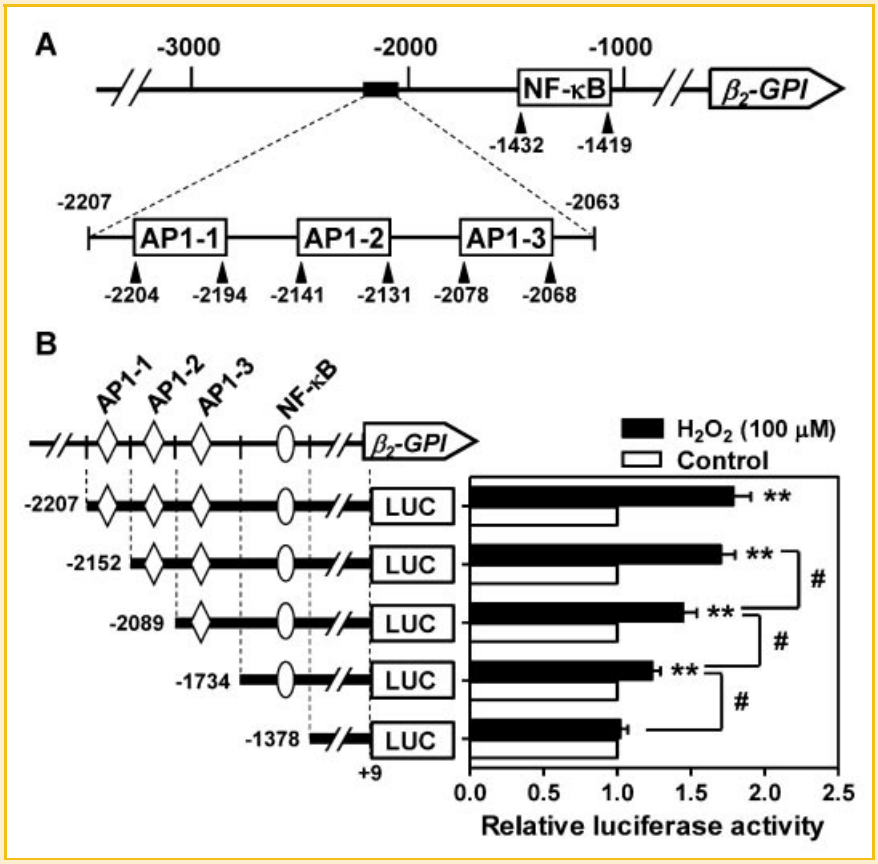

Fig. 3. Effect of H_2O_2 on the promoter activity of *β_2-GPI* reporter constructs containing putative AP-1 and NF-κB *cis*-elements in Huh7 cells. A: Schematic representation of the 5′-flanking region of the human *β_2-GPI* gene. Numbers indicate the position of the putative AP-1 and NF-κB relative to the *β_2-GPI* transcription initiation site (+1). B: Schematic representation of the 5′-flanking deletion fragments cloned into the pGL2-basic luciferase vector in front of the luciferase reporter gene. Huh7 cells were transiently transfected in triplicate with 0.5 μg of the indicated reporter gene construct along with the pCMV-β-Gal expressing β-galactosidase, in the absence (set as control) or presence of 100 μM H_2O_2. The luciferase activity of each reporter construct was normalized with the β-galactosidase activity for correction of transfection efficiency. The relative luciferase activity is indicated as the relative value of the control (set as 1). Results are represented by the mean ± SEM of at least three independent experiments. $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$ versus adjacent group.

(Fig. 5D). Competition assays with unlabeled probes confirmed the specificity of the AP-1 and NF-κB binding. The band intensities were remarkably diminished by preincubation of the nuclear extracts with antibodies against c-Jun, c-Fos, Jun-D, and ATF-2 as well as against p65 and p50, though the presence of supershifted bands was not prominent. Nevertheless, a control IgG antibody failed to attenuate the band intensities or elicit a supershift band. The results from the EMSA indicate that AP-1 and NF-κB are essential elements involved in the control of *β_2-GPI* transcription.

To further identify the involvement of AP-1 and NF-κB in the H_2O_2-activated β_2-GPI protein expression, we generated lentiviral vectors expressing siRNAs for knockdown of the *c-Jun*, *c-Fos*, *p65*, and *p50* genes. The efficacy of siRNAs in gene knockdown and the effect of gene silencing on H_2O_2-mediated induction of β_2-GPI expression are shown in Figure 6. These data showed that silencing either AP-1 (Fig. 6A) or NF-κB (Fig. 6B) resulted in abrogation of the induction of β_2-GPI expression by H_2O_2. These data provide evidence that transcription factors AP-1 and NF-κB were involved in the H_2O_2-induced β_2-GPI expression.

DISCUSSION

β_2-GPI is mainly produced by the liver and behaves as survival factor for hepatocytes [Averna et al., 2004]. There is a wide range of interindividual variation in plasma levels of β_2-GPI that is thought to be under genetic control [Mehdi et al., 2003]. However, gene regulation of *β_2-GPI* expression still remains unknown. Recently, we have reported that increased β_2-GPI expression has been observed in the human atherosclerotic lesions [Lin et al., 2005] and several putative binding sites for AP-1 and NF-κB have been identified upstream of the promoter region of the *β_2-GPI* gene [Wang and Chiang, 2004]. Despite the importance of the proximal promoter in controlling *β_2-GPI* transcription, little information is available on the mechanisms by which transcription factors interact

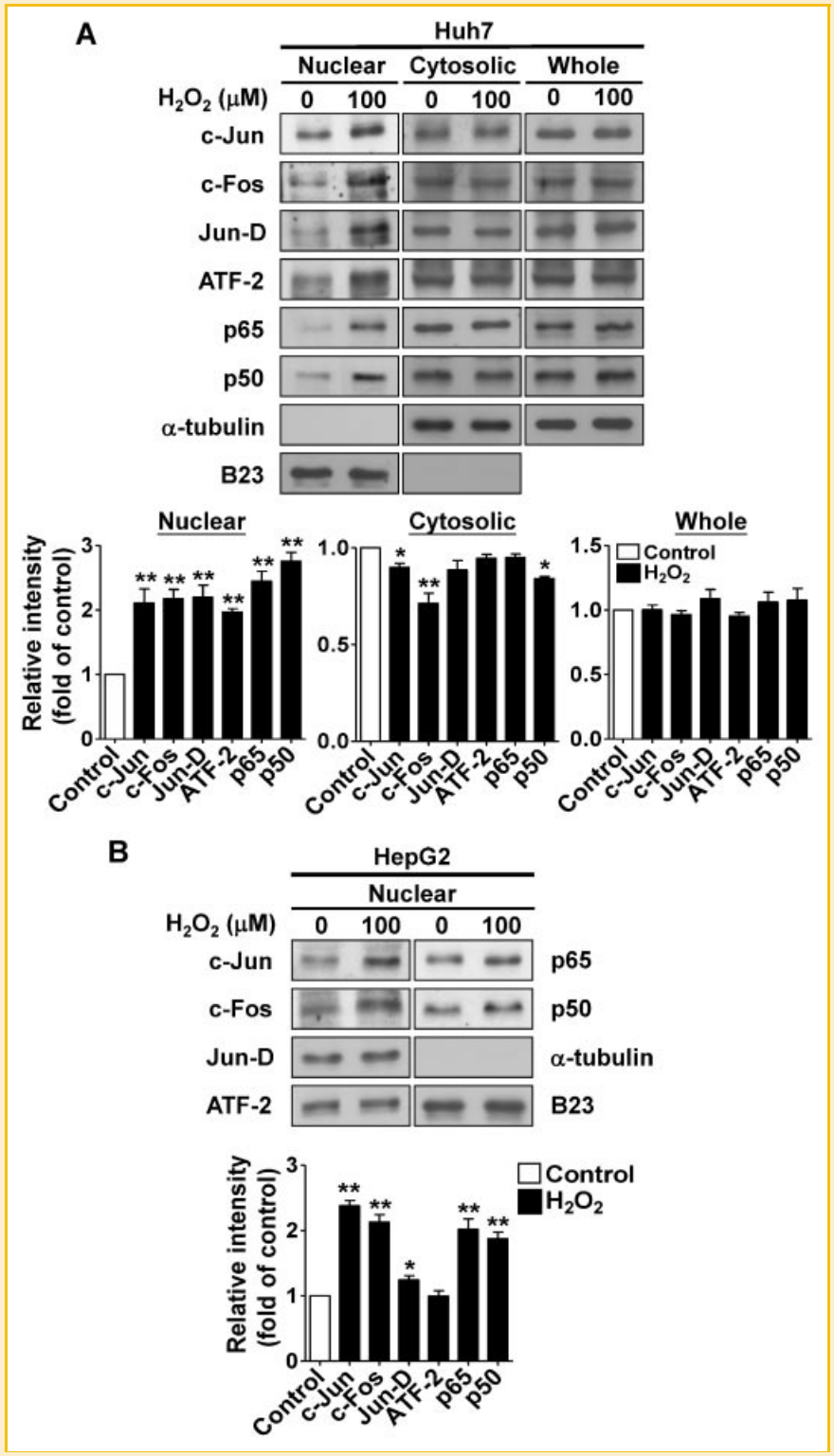

Fig. 4. Effect of H_2O_2 on nuclear translocation of AP-1 and NF-κB subunits in Huh7 (A) and HepG2 (B) cells. Cells treated with 100 μM H_2O_2 for 1 h and the protein levels of AP-1 (c-Jun, c-Fos, Jun-D, and ATF-2) and NF-κB (p65 and p50) subunits were monitored in the nuclear extracts as well as cytosolic fraction or whole-cell lysates by Western blot analysis. The representative results from four separate experiments are shown. The expression of B23 and α-tubulin was used as the internal control for nuclear and cytosolic protein, respectively. The intensity of protein band normalized by the internal control was calculated as the fold of controls and then depicted as graph bar. Results represent means ± SEM of four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

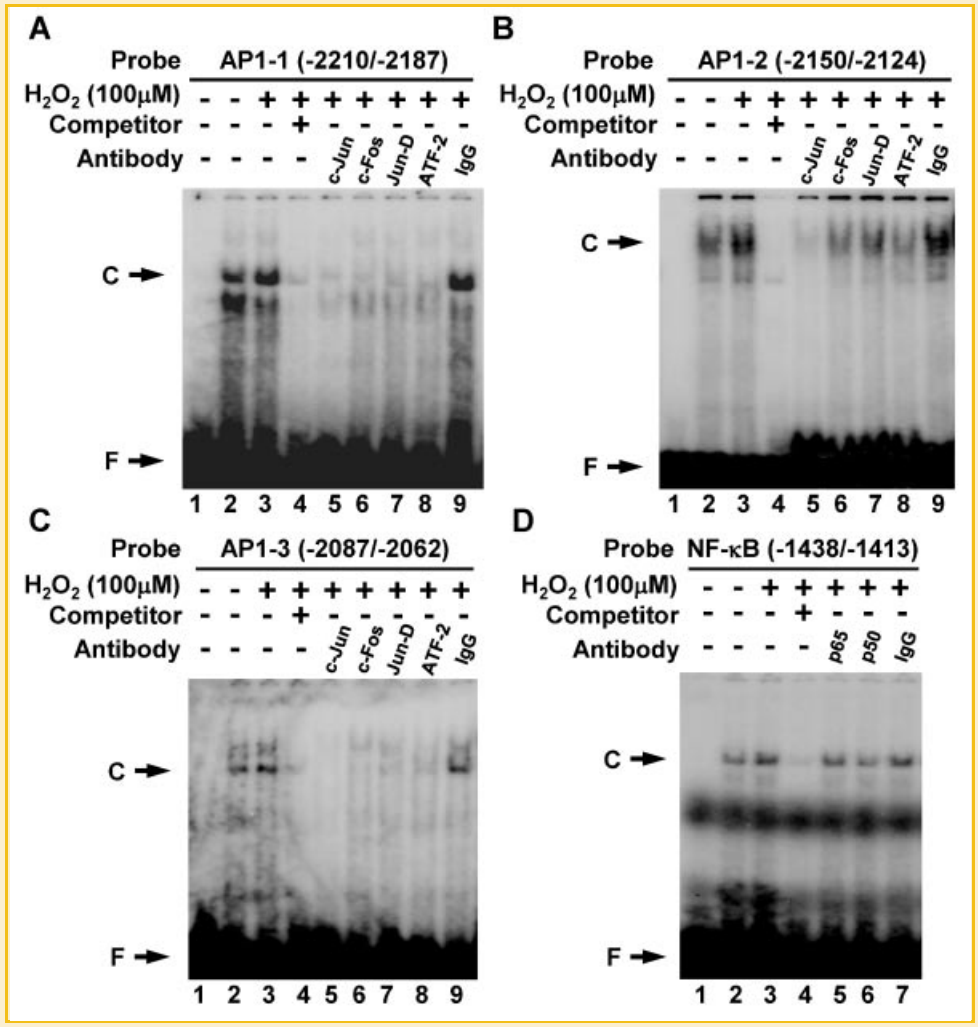

Fig. 5. Electrophoretic mobility shift assay of the putative AP-1 and NF-κB *cis*-elements derived from human β_2-*GPI* gene promoter in Huh7 cells. Cells were treated with 100 μM H_2O_2 for 1 h and nuclear extracts from the treated cells were analyzed by EMSA. The ^{32}P-labeled DNA fragments from −2210 to −2187 (AP1-1) (A), −2150 to −2124 (AP1-2) (B), −2087 to −2062 (AP1-3) (C), and −1438 to −1413 (NF-κB) (D) of the human β_2-*GPI* gene promoter were used as probes. Lane 1: probe alone; Lane 2: nuclear extracts from non-treated cells; Lane 3: nuclear extracts from Huh7 cells treated with 100 μM H_2O_2; Lane 4: nuclear extracts from H_2O_2-treated cells in the presence of unlabeled specific competitors; Lanes 5–9: nuclear extracts from H_2O_2-treated cells in the presence of AP-1, NF-κB, or control IgG antibodies. Shown are representative gels of more than four separate experiments.

this promoter. AP-1 and NF-κB are known as the redox-sensitive transcription factors [Karin et al., 2001; Surh et al., 2005]. In the present study, we disclosed for the first time that AP-1 and NF-κB are involved in the regulation of β_2-*GPI* gene expression in hepatoma cells under oxidative stress.

Oxidative stress is widely recognized as a major risk factor in organ injuries that lead to either acute or chronic disease [Harrison et al., 2003; Madamanchi et al., 2005; Dalle-Donne et al., 2006; Naziroglu, 2009]. Recent studies have suggested that H_2O_2 induces tissue cell damage via oxidative stress [Qamar and Sultana, 2008; Uguz et al., 2009; Xu et al., 2009]. The present study with H_2O_2 as the oxidative stress revealed that H_2O_2 significantly enhances the expression of β_2-GPI both at mRNA and protein levels. These results provide a clue to explain the biological significance of β_2-GPI in the oxidative stress-induced damage in various tissues [Lin et al., 2001, 2005].

The mechanism of β_2-*GPI* gene regulation would seem to be mediated through the response elements in the 5′-flanking region. In this report, we identify several binding sites of AP-1 and NF-κB located between −2207 and −1378 with respect to the transcription start site. AP-1 and NF-κB are transcription factors involved in a wide range of physiological functions, such as apoptosis, cell cycle control, and carcinogenesis [Shen et al., 2005; Zhou et al., 2007]. Cooperation with different signaling pathways, AP-1 and NF-κB play an important role in extensive nucleosome reorganization and regulation of target gene expression [Johnson et al., 2004; Ndlovu et al., 2009]. In the present study, promoter deletion analysis revealed that putative binding sites for AP-1 and NF-κB are essential for up-regulation of β_2-*GPI* gene expression in H_2O_2-stimulated Huh7 cells. The results of luciferase assay indicate that AP-1 and NF-κB play independent roles in H_2O_2-stimulated β_2-*GPI* promoter activation and the effects of AP-1 and NF-κB on β_2-*GPI* transcription are synergistic.

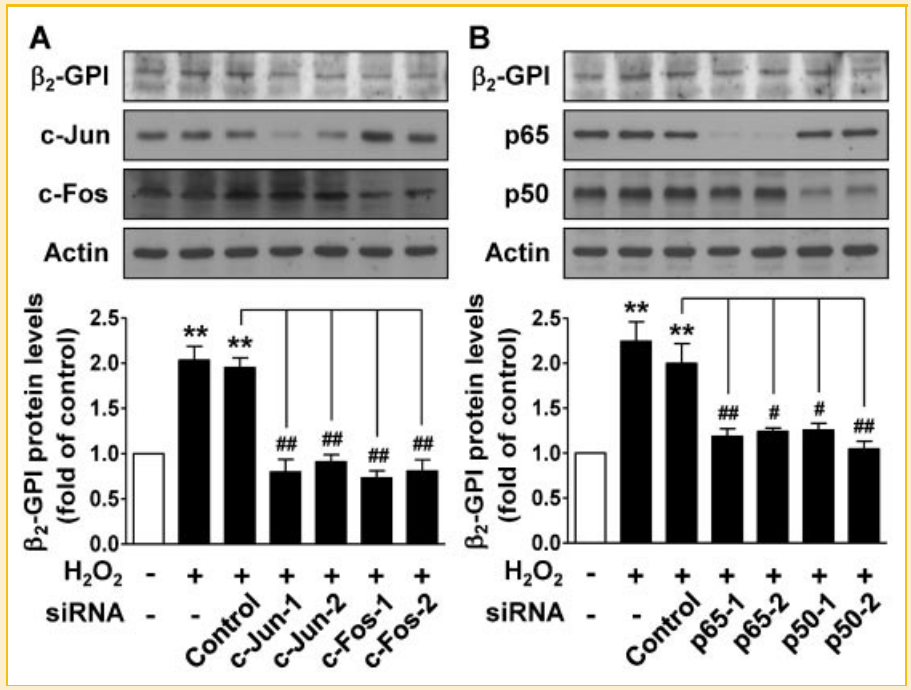

Fig. 6. Effect of siRNAs targeting *AP-1* (A) and *NF-κB* (B) genes on β_2-GPI expression in Huh7 cells. Cells were infected with lentiviruses targeting different regions of *AP-1* and *NF-κB* genes by *c-Jun* siRNA (c-Jun-1, c-Jun-2), *c-Fos* siRNA (c-Fos-1, c-Fos-2), *p65* siRNA (p65-1, p65-2), *p50* siRNA (p50-1, p50-2), and a control siRNA for 4 days. Cellular protein was extracted from siRNA-infected cells treated with or without H_2O_2 for 4 h. The cellular proteins were separated by SDS-PAGE for immunoblot analysis with c-Jun, c-Fos, p65, or p50 antibodies. Results are represented by the mean ± SEM of three independent experiments. $^{**}P < 0.01$ versus non-H_2O_2 treated group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus control siRNA infected group.

AP-1 is a group of transcription factors composed of hetero- or homodimer subunits of protein from the Jun and Fos families as well as the Jun dimerization partner (JDP) and ATF subfamilies [Angel and Karin, 1991; Wisdom, 1999; Hess et al., 2004]. AP-1 is able to be activated by various stimuli, including inflammatory cytokines, growth factors, and oxidative stress [Hsu et al., 2000; Surh et al., 2005]. c-Jun-NH_2-terminal kinase (JNK) is one of the kinases that regulates the activation of AP-1 transcription factor. Upon stimulation, JNK enters the nucleus to induce phosphorylation of AP-1 subunits and result in the regulation of gene expression. Different types of AP-1 complexes are functionally distinct and may regulate different target gene [Karin et al., 1997; Liu et al., 2009]. The NF-κB family comprises five members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) [Hayden and Ghosh, 2004]. The predominant NF-κB dimers are subunits of p50 and p65. NF-κB is maintained in the cytoplasm of non-stimulated cells through interaction with one of the inhibitory IκB proteins (IκBα, IκBβ, and IκBε). Upon phosphorylation of the IκB residues by IκB kinase (IKK), NF-κB subunits are released, allowing nuclear translocation [Hayden and Ghosh, 2004]. Activation of AP-1 or NF-κB in cells may be assessed by measuring increased subunit translocation from the cytoplasm to the nucleus. Reports have indicated that ROS can act as signaling messengers to activate these transcription factors [Griendling et al., 2000; Thannickal and Fanburg, 2000]. We hypothesize that the increase in H_2O_2-stimulated expression of β_2-*GPI* gene may in part be as a result of increased activation of AP-1 and NF-κB.

Our results show that H_2O_2 exposure produced a significant increase in nuclear AP-1 and NF-κB subunits. These results suggest that increases in nuclear AP-1 and NF-κB translocation lead to increases in the physical binding of the activated subunits to their corresponding sites in the upstream of β_2-*GPI* gene promoter. To further clarify the involvements of the three AP-1 sites and one NF-κB site in the β_2-*GPI* gene regulation, EMSA was conducted to compare the binding activities in cells treated and untreated with H_2O_2. We identified the transcription factors of c-Jun, c-Fos, Jun-D, and ATF-2 as well as p65 and p50 which were recruited to the nucleus and enhanced the interaction between the transcriptional factor and the corresponding *cis*-element in the β_2-*GPI* promoter. It was evident that induction of *trans*-factor binding at the AP1-2, and AP1-3 sites displayed greater binding activities than at the AP1-1 site. It seems that physical interaction between AP-1 and AP1-1 site is not essential for the transactivation of β_2-*GPI* gene expression by H_2O_2, but that the cooperative actions of the other *cis*-element binding contribute to the regulation of β_2-*GPI* transcription. Furthermore, we have performed functional approach by siRNA to unravel the involvement of AP-1 and NF-κB in H_2O_2-induced β_2-GPI expression.

In this study, we expand our knowledge of β_2-*GPI* gene regulation in hepatoma cells under oxidative stress. As illustrated in Figure 7, our data show that H_2O_2 increases ROS formation as well as AP-1 and NF-κB activation. We provide evidence that AP-1 and NF-κB subunit translocation, *trans*- and *cis*-element interactions, promoter activity of β_2-*GPI* gene, β_2-GPI expression both at protein

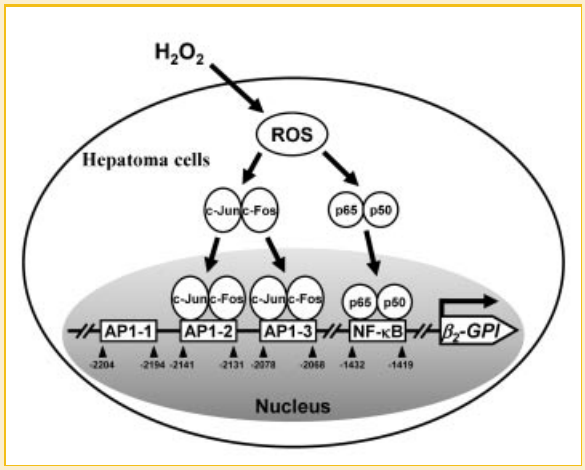

Fig. 7. Hypothetic mechanisms of H_2O_2 in the regulation of β_2-*GPI* gene expression in hepatoma cells.

and mRNA levels could all be enhanced by H_2O_2 treatment. These results reveal a novel molecular mechanism involved in β_2-*GPI* gene regulation under oxidative stress.

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REFERENCES

Angel P, Karin M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129–157.

Averna M, Paravizzini G, Marino G, Emmanuele G, Cefalu AB, Magro G, Bartoloni G, Ragusa M, Noto D, Barbagallo CM, Callari D, Mazzarino MC, Notarbartolo A, Travali S. 2004. Beta-2-glycoprotein I is growth regulated and plays a role as survival factor for hepatocytes. Int J Biochem Cell Biol 36:1297–1305.

Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res 47:936–942.

Cunha-Oliveira T, Rego AC, Morgadinho MT, Macedo T, Oliveira CR. 2006. Differential cytotoxic responses of PC12 cells chronically exposed to psychostimulants or to hydrogen peroxide. Toxicology 217:54–62.

Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. 2006. Biomarkers of oxidative damage in human disease. Clin Chem 52:601–623.

Dunoyer-Geindre S, Kwak BR, Pelli G, Roth I, Satta N, Fish RJ, Reber G, Mach F, Kruithof EK, de Moerloose P. 2007. Immunization of LDL receptor-deficient mice with beta2-glycoprotein 1 or human serum albumin induces a more inflammatory phenotype in atherosclerotic plaques. Thromb Haemost 97:129–138.

Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. 2000. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 20:2175–2183.

Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. 2003. Role of oxidative stress in atherosclerosis. Am J Cardiol 91:7A–11A.

Hayden MS, Ghosh S. 2004. Signaling to NF-kappaB. Genes Dev 18:2195–2224.

Hess J, Angel P, Schorpp-Kistner M. 2004. AP-1 subunits: Quarrel and harmony among siblings. J Cell Sci 117:5965–5973.

Hsu TC, Young MR, Cmarik J, Colburn NH. 2000. Activator protein 1 (AP-1)- and nuclear factor kappaB (NF-kappaB)-dependent transcriptional events in carcinogenesis. Free Radic Biol Med 28:1338–1348.

Johnson BV, Bert AG, Ryan GR, Condina A, Cockerill PN. 2004. Granulocyte-macrophage colony-stimulating factor enhancer activation requires cooperation between NFAT and AP-1 elements and its associated with extensive nucleosome reorganization. Mol Cell Biol 24:7914–7930.

Karin M, Liu Z, Zandi E. 1997. AP-1 function and regulation. Curr Opin Cell Biol 9:240–246.

Karin M, Takahashi T, Kapahi P, Delhase M, Chen Y, Makris C, Rothwarf D, Baud V, Natoli G, Guido F, Li N. 2001. Oxidative stress and gene expression: The AP-1 and NF-kappaB connections. Biofactors 15:87–89.

Lin KY, Pan JP, Yang DL, Huang KT, Chang MS, Ding PY, Chiang AN. 2001. Evidence for inhibition of low density lipoprotein oxidation and cholesterol accumulation by apolipoprotein H (beta2-glycoprotein I). Life Sci 69:707–719.

Lin KY, Wang HH, Lai ST, Pan JP, Chiang AN. 2005. Beta(2)-glycoprotein I protects J774A.1 macrophages and human coronary artery smooth muscle cells against apoptosis. J Cell Biochem 94:485–496.

Lin F, Murphy R, White B, Kelly J, Feighery C, Doyle R, Pittock S, Moroney J, Smith O, Livingstone W, Keenan C, Jackson J. 2006. Circulating levels of beta2-glycoprotein I in thrombotic disorders and in inflammation. Lupus 15:87–93.

Liu H, Zheng H, Duan Z, Hu D, Li M, Liu S, Li Z, Deng X, Wang Z, Tang M, Shi Y, Yi W, Cao Y. 2009. LMP1-augmented kappa intron enhancer activity contributes to upregulation expression of Ig kappa light chain via NF-kappa B and AP-1 pathways in nasopharyngeal carcinoma cells. Mol Cancer 8:92.

Madamanchi NR, Vendrov A, Runge MS. 2005. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 25:29–38.

Matsuura E, Kobayashia K, Koikeb T, Shoenfeld Y, Khamashta MA, Hughes GR. 2003. Atherogenic autoantigen: Oxidized LDL complexes with beta2-glycoprotein I. Immunobiology 207:17–22.

Mehdi H, Manzi S, Desai P, Chen Q, Nestlerode C, Bontempo F, Strom SC, Zarnegar R, Kamboh MI. 2003. A functional polymorphism at the transcriptional initiation site in beta2-glycoprotein I (apolipoprotein H) associated with reduced gene expression and lower plasma levels of beta2-glycoprotein I. Eur J Biochem 270:230–238.

Miyakis S, Giannakopoulos B, Krilis SA. 2004. Beta 2 glycoprotein I—Function in health and disease. Thromb Res 114:335–346.

Naziroglu M. 2009. Role of selenium on calcium signaling and oxidative stress-induced molecular pathways in epilepsy. Neurochem Res 34:2181–2191.

Ndlovu N, Van Lint C, Van Wesemael K, Callebert P, Chalbos D, Haegeman G, Vanden Berghe W. 2009. Hyperactivated NF-κB and AP-1 transcription factors promote highly accessible chromatin and constitutive transcription across the interleukin-6 gene promoter in metastatic breast cancer cells. Mol Cell Biol 29:5488–5504.

Okkels H, Rasmussen TE, Sanghera DK, Kamboh MI, Kristensen T. 1999. Structure of the human beta2-glycoprotein I (apolipoprotein H) gene. Eur J Biochem 259:435–440.

Qamar W, Sultana S. 2008. Farnesol ameliorates massive inflammation, oxidative stress and lung injury induced by intratracheal instillation of cigarette smoke extract in rats: An initial step in lung chemoprevention. Chem Biol Interact 176:79–87.

Roubey RA, Pratt CW, Buyon JP, Winfield JB. 1992. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon beta 2-glycoprotein I. J Clin Invest 90:1100–1104.

Shen G, Jeong WS, Hu R, Kong AN. 2005. Regulation of Nrf2, NF-κB, and AP-1 signaling pathways by chemopreventive agents. Antioxid Redox Signal 7:1648–1663.

Shi T, Iverson GM, Qi JC, Cockerill KA, Linnik MD, Konecny P, Krilis SA. 2004. Beta 2-glycoprotein I binds factor XI and inhibits its activation by thrombin and factor XIIa: Loss of inhibition by clipped beta 2-glycoprotein I. Proc Natl Acad Sci USA 101:3939–3944.

Surh YJ, Kundu JK, Na HK, Lee JS. 2005. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J Nutr 135:2993S–3001S.

Takeuchi R, Atsumi T, Ieko M, Takeya H, Yasuda S, Ichikawa K, Tsutsumi A, Suzuki K, Koike T. 2000. Coagulation and fibrinolytic activities in 2 siblings with beta(2)-glycoprotein I deficiency. Blood 96:1594–1595.

Thannickal VJ, Fanburg BL. 2000. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279:L1005–1028.

Uguz AC, Naziroglu M, Espino J, Bejarano I, Gonzalez D, Rodriguez AB, Pariente JA. 2009. Selenium modulates oxidative stress-induced cell apoptosis in human myeloid HL-60 cells through regulation of calcium release and caspase-3 and -9 activities. J Membr Biol 232:15–23.

Wang HH, Chiang AN. 2004. Cloning and characterization of the human beta2-glycoprotein I (beta2-GPI) gene promoter: Roles of the atypical TATA box and hepatic nuclear factor-1alpha in regulating beta2-GPI promoter activity. Biochem J 380:455–463.

Wang Y, Feinstein SI, Fisher AB. 2008. Peroxiredoxin 6 as an antioxidant enzyme: Protection of lung alveolar epithelial type II cells from H_2O_2-induced oxidative stress. J Cell Biochem 104:1274–1285.

Wisdom R. 1999. AP-1: One switch for many signals. Exp Cell Res 253:180–185.

Xu Y, Szep S, Lu Z. 2009. The antioxidant role of thiocyanate in the pathogenesis of cystic fibrosis and other inflammation-related diseases. Proc Natl Acad Sci USA 106:20515–20519.

Yasuda S, Tsutsumi A, Chiba H, Yanai H, Miyoshi Y, Takeuchi R, Horita T, Atsumi T, Ichikawa K, Matsuura E, Koike T. 2000. Beta(2)-glycoprotein I deficiency: Prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. Atherosclerosis 152:337–346.

Zhou LZ, Johnson AP, Rando TA. 2001. NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. Free Radic Biol Med 31:1405–1416.

Zhou Y, Yau C, Gray JW, Chew K, Dairkee SH, Moore DH, Eppenberger U, Eppenberger-Castori S, Benz CC. 2007. Enhanced NF-κB and AP-1 transcriptional activity associated with antiestrogen resistant breast cancer. BMC Cancer 7:59.