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β_2-Glycoprotein I Protects J774A.1 Macrophages and Human Coronary Artery Smooth Muscle Cells Against Apoptosis

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Abstract β_2-Glycoprotein I (β_2-GPI) is a plasma glycoprotein with multifactorial relevance to clinical consequences. It was previously indicated that β_2-GPI can selectively bind to apoptotic cells. This study was designed to determine the role of β_2-GPI in apoptosis. Using an immunohistochemical study, we observed that β_2-GPI was co-localized with the apoptotic macrophages and smooth muscle cells (SMCs) of human coronary arteries. The contribution of β_2-GPI to apoptotic death was then investigated in vascular cells. Two nitric oxide (NO) donors, *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl penicillamine (SNAP) were used in this study to trigger apoptosis in J774A.1 macrophages and human coronary artery smooth muscle cells (HCASMC). Cell viability was significantly improved in β_2-GPI-treated cells. It was also possible to detect a remarkable inhibitory effect by β_2-GPI on the NO-induced apoptosis by preventing nuclear shrinkage. Furthermore, the NO-induced apoptosis was associated with increase in caspase-3 activity and in the protein levels of caspase-3, c-Fos, and c-Jun. However, all these apoptosis-related events were inhibited in vascular cells treated with 200 μg/ml β_2-GPI. This is the first study to show that β_2-GPI may be important in the prevention of apoptosis in vascular cells. J. Cell. Biochem. 94: 485–496, 2005.

Key words: β_2-glycoprotein I; apoptosis; macrophages; coronary artery smooth muscle cells; nitric oxide

β_2-Glycoprotein I (β_2-GPI), also known as apolipoprotein H, is a glycoprotein with a molecular weight of 50 kDa, which is composed of 326 amino acids and consists of 5 homologous domains [Kato and Enjyoji, 1991]. Four of them are composed of approximately 60 amino acids and the fifth domain contains 82 amino acid residues. β_2-GPI binds to negatively charged substances such as phospholipids [Sanghera et al., 1997], heparin [Polz, 1988], platelets [Vazquez-Mellado et al., 1994], and apoptotic cells [Cocca et al., 2001]. In the last decade, β_2-GPI has become a subject of increasing interest because it has been described as the actual target antigen for autoimmune antiphospholipid antibodies (aPL) [McNeil et al., 1990; Shoenfeld et al., 1998]. Patients with high levels of aPL are prone to fetal loss, autoimmune thrombocytopenia, and thrombotic events [Roubey and Hoffman, 1997; Kandiah et al., 1998].

β_2-GPI has been implicated in a variety of physiological pathway, including triglyceride metabolism, blood coagulation, and hemostasis [Takeuchi et al., 2000; Yasuda et al., 2000]. β_2-GPI-deficient plasma is unable to inhibit the contact activation of blood coagulation [Takeuchi et al., 2000] and this therefore raises the possibility that people deficient in β_2-GPI may be more susceptible to thrombosis. However, the role of β_2-GPI-deficiency in thrombosis is

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controversial [Bancsi et al., 1992; Sheng et al., 2001]. We recently reported that β_2-GPI could inhibit Cu^{2+}-induced LDL oxidation and cholesterol influx in J774A.1 macrophages [Lin et al., 2001], suggesting that β_2-GPI may play a protective role against atherosclerosis.

Oxidized LDL (ox-LDL) and nitric oxide (NO) are both cytotoxic and can induce apoptosis of vascular cells [Chung et al., 2001; Ramachandran et al., 2002; Salvayre et al., 2002]. Abnormal regulation of apoptosis can lead to disorders of the cardiovascular system such as idiopathic dilated cardiomyopathies, myocardial infarction, arrhythmogenic right ventricular dysplasia, restenosis, and plaque rupture [Cheng et al., 1996; James et al., 1996; Mallat et al., 1996; Yao et al., 1996; Walsh et al., 2000]. Although increasing evidence suggests that apoptosis occurs in many cardiovascular disorders, the mechanism of the control of such programmed cell death in these diverse pathological conditions is still unclear. The aim of this study is to investigate the role of β_2-GPI in the regulation of apoptosis in atherosclerotic vessels and vascular cells.

When a cell receives a signal to undergo apoptosis, it goes through a series of changes including shrinkage of the nuclear membrane and activation of various caspase superfamilies [Haunstetter and Izumo, 1998; Stennicke and Salvesen, 1998]. It has been found that caspases-3, -8, and -9 are activated in apoptotic vascular smooth muscle cells (SMCs) [Li et al., 2002]. Mallat et al. [1997] have demonstrated that caspase-3 is localized within apoptotic cells of human atherosclerotic plaques and this phenomenon is closely involved in apoptosis. In addition, some members of the immediate early genes, such as c-*fos* and c-*jun*, and their encoded proteins have been found to function as intracellular regulators of apoptosis [Preston et al., 1996; Wang et al., 1999; Teng, 2000].

An important clue linking β_2-GPI and apoptosis is provided by the structural basis for the selective binding between β_2-GPI and apoptotic cells [Cocca et al., 2001]. In this study, the distribution of β_2-GPI in normal and atherosclerotic human coronary arteries were compared. We also examined the co-localization of β_2-GPI with apoptotic cells in macrophages and SMCs of the vessel wall to clarify the involvement of β_2-GPI in apoptosis within atherosclerotic lesions. In addition, J774A.1 macrophages and human coronary artery smooth muscle cells (HCASMCs) were used as the cellular models to further clarify the significance of β_2-GPI in NO-induced apoptosis.

MATERIALS AND METHODS

Purification of β_2-GPI and Production of Antibodies

β_2-GPI was purified from plasma samples by methods previously used [Lin et al., 2001]. All blood samples were obtained from healthy donors by venepuncture. Isolation of β_2-GPI from human plasma was done by 3% perchloric acid precipitation and heparin–Sepharose affinity chromatography (HiTrap Heparin; Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of β_2-GPI was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% mini-gel and N-terminal amino acid sequence were analyzed. The pure β_2-GPI showed a single band in gel electrophoresis, approximately 50 kDa band corresponding to the protein marker. The N-terminal amino acid sequence of β_2-GPI was determined using an Applied Biosystems 477A sequencer (Applied Biosystems, Inc., Foster City, CA). The first 15 amino acid residues showed complete identity with human β_2-GPI. Sequence similarity searches were performed using the BLAST facility of the National Center for Biotechnology Information (NCBI). Polyclonal antiserum against β_2-GPI was raised in New Zealand White rabbits using the multiple subcutaneous injection technique and anti-β_2-GPI IgG was purified by protein G-Sepharose chromatography (HiTrap Protein G; Amersham Pharmacia). A titration of anti-β_2-GPI IgG was carried out by Western blot analysis. Preimmune antibodies were used as controls. The anti-β_2-GPI IgG yielded a single band of complete identity with both purified β_2-GPI and normal plasma by Western blot analysis, suggesting that anti-β_2-GPI IgG was selective for plasma β_2-GPI.

Immunohistochemistry

Segments of the coronary arteries were obtained from 30 patients, aged 20–75 years at the time of cardiac transplantation ($n = 13$) or bypass vascular surgery ($n = 17$) for ischemic heart disease. The study was approved by the ethics committee of Veterans General Hospital-Taipei in accordance with institu-

tional guidelines. Arterial segments without intimal thickening were classified as normal and segments containing intimal thickening were classified as atherosclerotic. All specimens of tissues were fixed in 4% paraformaldehyde and embedded in paraffin; sections of 5 μm were cut and mounted on slides. After being deparaffinized, rehydrated, and washed, sections were incubated with the rabbit anti-human β_2-GPI antibody previously described. Next, a biotinylated anti-rabbit IgG secondary antibody and an avidin–peroxidase conjugate were incubated on the same sections (Vectastain ABC-peroxidase kit, Vector Laboratories, Burlingame, CA). Negative controls were performed by incubating the sections with preimmune primary antibodies. A positive reaction was visualized as a brown color product after treatment with 3,3′-diaminobenzidine (DAB; 0.5 mg/ml). All sections were counterstained with hematoxylin.

To identify the cell types immunoreactive for β_2-GPI, triple staining for β_2-GPI, apoptotic cells, and macrophages/SMCs was performed on the same section in the following manner. Section was first immunostained for β_2-GPI with rabbit anti-human β_2-GPI antibody, followed by the alkaline phosphatase-conjugated secondary antibody. After three washes in phosphate-buffered saline (PBS), a positive reaction was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as the substrate of alkaline phosphatase [Ravalli et al., 1996]. Monoclonal antibodies against human SMCs α-actin (Zymed Laboratories, Inc., San Francisco, CA) and human macrophages (Sigma, St. Louis, MO) were used as specific markers for SMCs and macrophages, respectively. Sections were then immunostained by fluorescein isothiocyanate (FITC)-conjugated secondary antibody and visualized by fluorescence microscopy. Negative control with preimmune IgG was conducted in parallel with immunostaining procedure. The specificity of immunoreaction was evaluated in comparison with a negative control specimen. Finally, in situ detection of apoptotic cells was performed using terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) with an in situ cell death detection kit according to the manufacturer's instructions [Gavrieli et al., 1992]. The in situ cell death detection kit (TdT-FragEl DNA Fragmentation Detection Kit) was obtained from Oncogene Research Products (Darmstadt, Germany) and the staining was visualized by use of DAB.

Cell Culture

J774A.1 macrophages were purchased from American Type Culture Collection (Rockville, MD) and were grown at a density of 3.75×10^5 cells per ml in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HCASMCs were purchased from Cascade Biologics (Portland, OR) and plated at a density of 2×10^5 cells per ml in their optimal medium (Medium 231) supplemented with SMCs growth supplement (Cascade Biologics). Passages 6–8 of the HCASMCs were used for experiments. The vascular cells were grown at 37°C with 5% CO_2 and used at 90% confluence. One millimolar *S*-nitrosoglutathione (GSNO) or *S*-nitroso-*N*-acetyl penicillamine (SNAP) was treated for 10 h to induce apoptosis when required [Messmer et al., 1995].

Assessment of Apoptosis

Cells stimulated with GSNO were treated with or without 200 μg/ml β_2-GPI (the physiological concentration in human plasma) to assess the influence of β_2-GPI on NO-induced apoptosis. Treatment of 270 μg/ml bovine serum albumin (BSA) was performed to validate the specific effect of β_2-GPI against apoptosis. Cultured cells were fixed twice with methanol/acetic acid (3:1) for 10 min and stained with Hoechst 33258 (0.5 μg/ml) for 30 min at room temperature [Li et al., 1997]. After being washed with distill water, the morphological feature of apoptosis (nuclear shrinkage) was visualized by fluorescence microscopy. To quantify the apoptotic event, the percentage of apoptotic cells relative to the total cell population was calculated by counting at least 1,000 cells in 10 microscopic fields at the magnifications of 400× for J774A.1 macrophages and 100× for HCASMCs.

Cell viability was measured using the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [Mathews and Kerr, 1993]. J774A.1 macrophages and HCASMCs were grown in 96-well plates and exposed to NO donors or control medium for 10 h. After being washed, cells were incubated with MTT solution (5 mg/ml) for 4 h at 37°C, and then lysed with isopropanol. The formed formazan was determined spectrophotometrically at 550 nm.

Caspase-3 activity was measured according to the method of Koglin et al. [1999]. NO donor-treated and untreated cells were collected, and lysed in 50 mM HEPES, pH 7.5, containing 0.2% Triton X-100 and 10% sucrose. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected. Cell proteins (100 μg) were incubated with reaction buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP-40) and 100 μM Ac-DEVD-*p*-nitroaniline (pNA) substrate for 2 h at 37°C. Digestion of the peptide substrate, Ac-DEVD-pNA, due to caspase-3 activity was assayed by the change in the absorbance of chromophore pNA at 405 nm. Concentration of cytosolic protein was determined using the Bradford assay (Bio-Rad, Hercules, CA) with BSA as the standard. Caspase-3 activity was normalized to equal the amount of cytosolic protein.

Preparation of Nuclear Proteins and Western Blot Analysis

Nuclear proteins were prepared using a modified version of the technique described by Lassar et al. [1991]. NO donor-treated and untreated cells were washed and allowed to swell on ice in a hypotonic buffer (20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 0.8 μM aprotinin, 50 μM bestatin, 20 μM leupeptin, 10 μM pepstatin A) for 10 min at 4°C. The nuclei were pelleted and the cytosol fraction was collected. Nuclear proteins were extracted in a high-salt buffer (identical to hypotonic buffer with 500 mM NaCl) and stored at −20°C until use. The protein content of nuclear extract was determined using the Bio-Rad protein assay kit. For equal protein loading, 50 μg of protein was resolved on a 10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and incubated with primary antibodies that recognize caspase-3 (Calbiochem-Novabiochem Co., San Diego, CA), c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA), c-Jun (Santa Cruz Biotechnology), or α-tubulin (Zymed Laboratories, Inc.). The transferred membrane was incubated with the horseradish peroxidase-conjugated secondary antibody followed by detection using an enhanced chemiluminescence (ECL) detection method.

Statistical Analysis

Results are means ± SD. Each experiment was performed at least three times and differences between two groups were identified with the use of two-tailed Student's *t*-tests. For multiple groups, one-way analysis of variance (ANOVA) and Student–Newman–Keuls tests were used to identify differences. Significance was defined as $P < 0.05$.

RESULTS

Expression of β_2-GPI in Atherosclerotic Lesions

To examine the expression of β_2-GPI in atherosclerotic lesions, we performed immunohistochemical analysis using sections of human coronary arteries. In normal arterial samples, no positive labeling for β_2-GPI was observed with the antibody against β_2-GPI (Fig. 1A, left). In contrast, β_2-GPI was distributed abundantly in the subendothelial and medial regions of atherosclerotic vessels (Fig. 1A, right). To further investigate whether the distribution of β_2-GPI co-localized with apoptotic macrophages and SMCs, triple staining was performed on the sections of atherosclerotic lesions. As shown in Figure 1, some of the β_2-GPI deposition overlapped those of apoptotic macrophages (Fig. 1B) and apoptotic SMCs (Fig. 1C). J774A.1 macrophages and HCASMCs were thus selected as the cellular models to study the role of β_2-GPI in apoptosis in the following studies.

Effect of β_2-GPI on Apoptosis in Macrophages and HCASMCs

The nuclear staining with Hoechst 33258 provided a morphological discrimination between normal and apoptotic cells. J774A.1 macrophages exhibited shrinkage of nuclear membranes (Fig. 2A, left), whereas HCASMCs appeared to be more resistant (Fig. 2B, left) under GSNO treatment ($16 \pm 3\%$ vs. $4 \pm 1\%$ of the population, respectively). In addition to the morphological changes in the nuclear membranes, GSNO treatment caused the cytoplasmic membranes of HCASMCs to become unclear. Quantitative analysis showed that percentage of apoptotic cells decreased significantly from $16 \pm 3.9\%$ to $4 \pm 1.9\%$ ($P < 0.001$) when GSNO-exposed macrophages were treated with 200 μg/ml β_2-GPI (Fig. 2A, right), whereas this anti-apoptotic effect was not apparently in the HCASMCs (Fig. 2B, right). Albumin, a control protein had no effect on GSNO-induced apoptosis.

The effect of β_2-GPI on NO-induced apoptosis in J774A.1 macrophages and HCASMCs was

also determined by cell viability, another indicator of apoptotic cell death (Fig. 3). The MTT value of cells without treatment with GSNO or SNAP was taken as 100% (control cells). J774A.1 macrophages exhibited a remarkable cytotoxic effect under the treatment with 1 mM GSNO for 10 h ($61 \pm 5\%$ of control cells). Addition of 200 μg/ml β_2-GPI significantly reduced the GSNO-induced cytotoxicity ($86 \pm 5\%$ vs. $61 \pm 5\%$; $P < 0.001$). The cell viability of HCASMCs exposed to 1 mM GSNO ($85 \pm 6\%$ of control cells) was higher than that of J774A.1 macrophages. Addition of 200 μg/ml β_2-GPI also significantly reduced the cytotoxicity in GSNO-treated HCASMCs ($97 \pm 4\%$ vs. $85 \pm 6\%$; $P < 0.05$). SNAP was less toxic than GSNO in both J774A.1 macrophages and HCASMCs. The cell viability of J774A.1 macrophages and HCASMCs exposed to SNAP was $78 \pm 4\%$ and $89 \pm 5\%$ relative to control cells, respectively. After treatment of cells with 200 μg/ml β_2-GPI, the cell viability of J774A.1 macrophages and HCASMCs was elevated to $93 \pm 5\%$ and $99 \pm 3\%$, respectively. Figure 3 indicates that β_2-GPI was able to reverse the inhibitory effect of NO donors on the cell viability in a dose-dependent manner.

Furthermore, we examined the activity and expression of caspase-3 after treatment with NO donors for 10 h. The activity of caspase-3 was significantly induced by GSNO and SNAP in J774A.1 macrophages but less in HCASMCs. Co-incubation of cells with β_2-GPI suppressed the induction of caspase-3 activity in a dose-dependent manner and 200 μg/ml β_2-GPI resulted in the most significant reduction (Fig. 4A). The protein levels of caspase-3 in J774A.1 macrophages and HCASMCs under GSNO and SNAP treatment in the absence or presence of β_2-GPI were examined by Western blot analysis. Both GSNO and SNAP significantly increased the expression of caspase-3 in J774A.1 macrophages and HCASMCs (Fig. 4B). β_2-GPI also suppressed the expression of caspase-3 in a dose-dependent manner and the addition

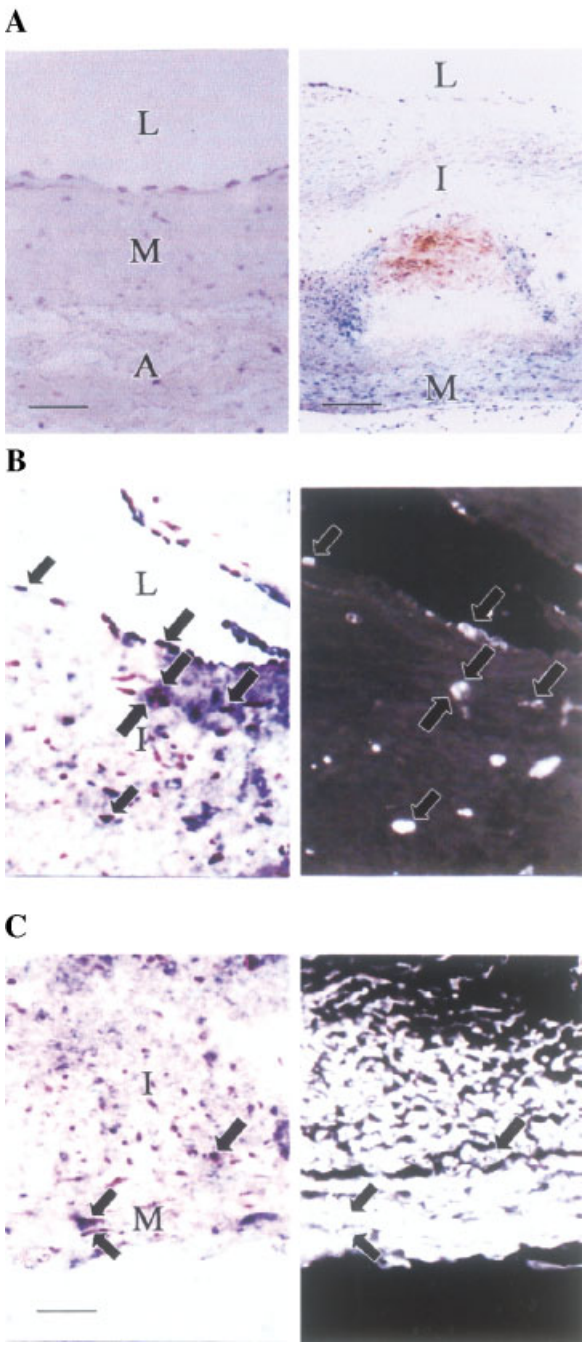

Fig. 1. Immunohistochemistry for β_2-glycoprotein I (β_2-GPI) on sections of normal and atherosclerotic human coronary arteries. Abbreviations for all figures: A, adventitia; M, media; I, intima; L, lumen. **A**: Immunostaining was performed using anti-human β_2-GPI antibody. The positive reaction was visualized as the brown color product after treatment with 3,3′-diaminobenzidine (DAB). **Left**, normal coronary arteries; **right**, atherosclerotic coronary arteries. The bar represents 250 μm. **B**: Triple staining for β_2-GPI, apoptotic cells, and macrophages. Staining for β_2-GPI (purple) and apoptotic cells (brown) is shown in **left panel**. Macrophages were observed by immunofluorescence and are shown in **right panel**. Some apoptotic cells showed co-localized of β_2-GPI and macrophages (arrows). **C**: Triple staining for β_2-GPI, apoptotic cells, and smooth muscle cells (SMCs). Staining for β_2-GPI (purple) and apoptotic cells (brown) is shown in **left panel**. SMCs were detected by immunofluorescence and are shown in **right panel**. Some apoptotic cells showed co-localized of β_2-GPI and SMCs (arrows). The bar represents 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

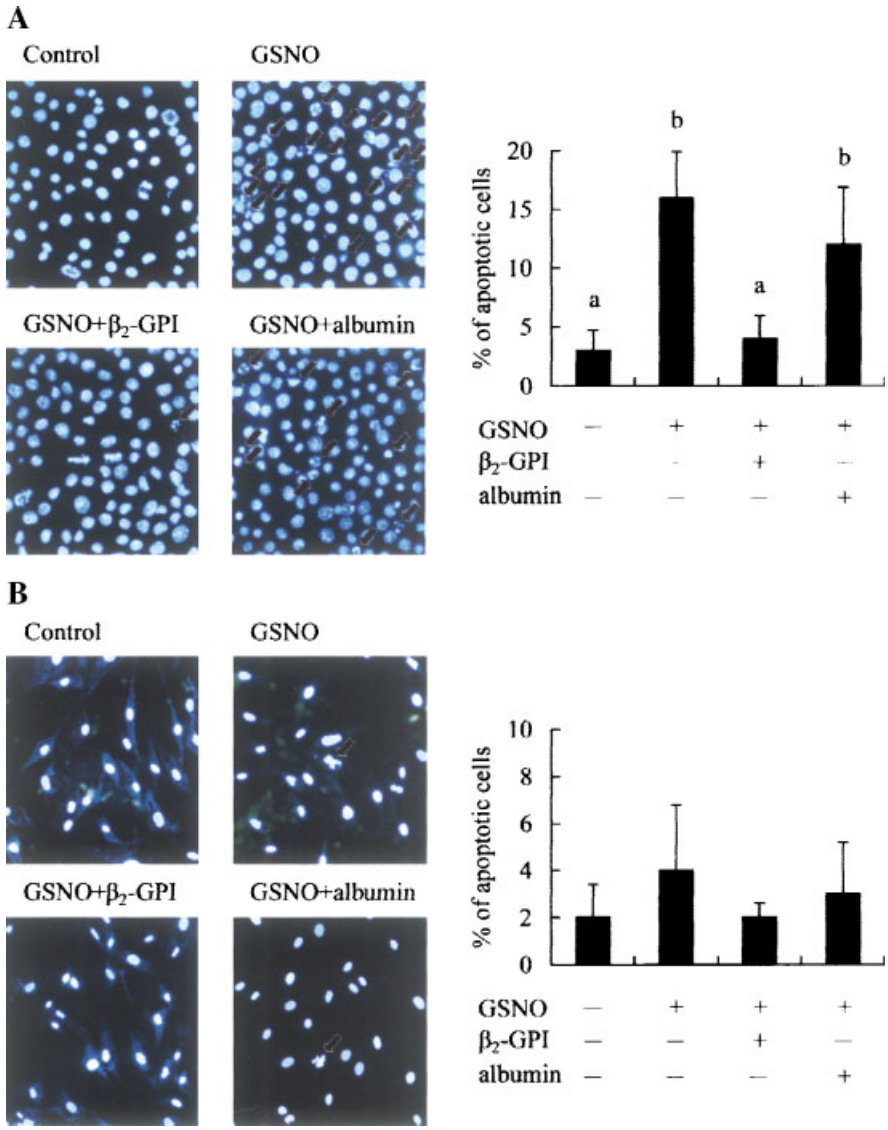

Fig. 2. Detection of apoptotic cells in J774A.1 macrophages (**A**) and human coronary artery smooth muscle cells (HCASMCs) (**B**). Cells cultured in absence of *S*-nitrosoglutathione (GSNO) and β_2-GPI were of the control group. Apoptosis was induced by treatment of cells with 1 mM GSNO for 10 h. Cells were also incubated with 200 μg/ml β_2-GPI (GSNO + β_2-GPI) or 270 μg/ml bovine serum albumin (BSA) (GSNO + albumin), respectively. Apoptotic cells were detected after staining with Hoechst 33258 and examined by fluorescence microscopy. The photographs in the **left panel** are representative of four separate experiments. Arrows point to the apoptotic cells. Quantitative data presented in the **right panel** are means ± SD of four experiments. The percentage of apoptotic cells in the total population was calculated by counting at least 1,000 cells for each experiment. Columns with different letters are significantly different ($P < 0.05$ by ANOVA and Student–Newman–Keuls test). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of 200 μg/ml β_2-GPI reduced the expression of caspase-3 to near basal level.

To confirm that the inhibitory effect of caspase-3 activity as well as its protein expression shown in Figure 4 was mediated through the anti-apoptotic effect of β_2-GPI, anti-β_2-GPI antibody was used to carry out similar experiments. The results indicate that the protective effect of β_2-GPI observed from caspase-3 activity (Fig. 5A) and caspase-3 expression (Fig. 5B) in GSNO- and SNAP-treated cells were diminished by addition of anti-β_2-GPI antibody in a concentration-dependent manner.

Influence of β_2-GPI on the Expression of c-Fos and c-Jun in Macrophages and HCASMCs

c-*fos* and c-*jun* encoded proteins have been found to function as intracellular regulators of apoptosis [Preston et al., 1996; Wang et al., 1999; Teng, 2000]. To elucidate the possible

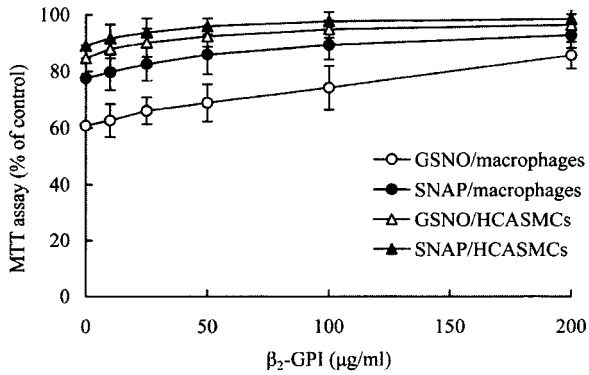

Fig. 3. The effect of β_2-GPI on cell viability of GSNO- or *S*-nitroso-*N*-acetyl penicillamine (SNAP)-treated J774A.1 macrophages and HCASMCs. Each point was expressed as a percentage of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) value of control cells, which was taken as 100%. Results are shown with mean ± SD from four separate experiments.

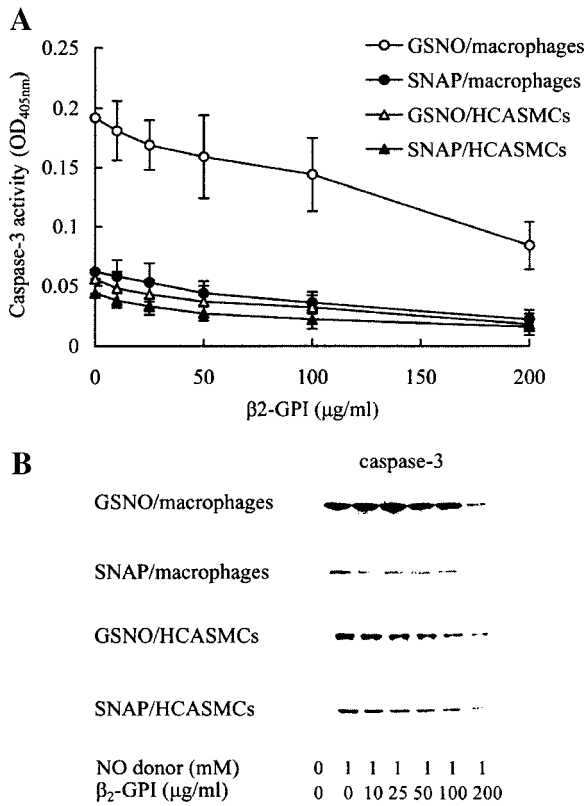

Fig. 4. The effect of β_2-GPI on caspase-3 activity and its protein expression in J774A.1 macrophages and HCASMCs. **A**: Each point is expressed as a value of caspase-3 activity by measuring the release of *p*-nitroaniline (pNA) at $OD_{405\ nm}$. Data are the results of four separate experiments and presented as means ± SD. **B**: The expression of caspase-3 was detected by Western blot analysis. Shown are representative immunoblots of three separate experiments.

association between these immediate early gene expression and NO-induced apoptosis in macrophages and HCASMCs, we examined the expression of c-Fos and c-Jun by Western blot analysis. The expression of c-Fos and c-Jun increased by 5.6- and 5.4-fold, respectively, when J774A.1 macrophages were exposed to 1 mM GSNO for 1 h (Fig. 6A). Addition of 200 μg/ml β_2-GPI brought the induction of c-Fos and c-Jun in GSNO-treated J774A.1 macrophages down to only 2.0- and 1.4-fold, respectively. c-Fos and c-Jun were less induced in SNAP-treated J774A.1 macrophages (Fig. 6B). Addition of 200 μg/ml β_2-GPI significantly reduced the expression of c-Jun, but not c-Fos in SNAP-treated macrophages. Moreover, only c-Jun was induced in GSNO-treated HCASMCs and again addition of β_2-GPI significantly reduced its induction (Fig. 6C). Both c-Fos and c-Jun were not induced in SNAP-treated HCASMCs and addition of β_2-GPI did not affect the expression of c-Fos and c-Jun (Fig. 6D).

DISCUSSION

Increasing lines of evidence from both animal models and human specimens suggest that apoptosis is an important event in the pathophysiology of atherosclerosis [Kockx and Herman, 2000; Kockx and Knaapen, 2000; Kolodgie et al., 2001]. The control of the apoptotic mechanism may thus have significant implications for atherogenesis. Only a few studies about the relationship between apolipoproteins and apoptosis have ever been undertaken. Suc et al. [1997] showed that de-lipidated apolipoprotein A, the main protein from high density lipoprotein (HDL), could inhibit ox-LDL-induced apoptosis in endothelial cells by decreasing the cytosolic Ca^{2+} concentration. Studies also found that various isoforms of apolipoprotein E (apoE) could protect neurons from apoptosis through binding to the lipid peroxidation product 4-hydroxynonenal [Pedersen et al., 2000]. Drouet et al. [2001] demonstrated that the apoptosis of neurons could also be inhibited by the interaction between the C-terminal domain of amyloid beta peptide and apoE. Furthermore, induction of apolipoprotein J (apoJ) by heat-shock or oxidative-stress could prevent A431 human epidermoid cancer cells from apoptosis [Viard et al., 1999]. β_2-GPI is also known as an apolipoprotein that exists in chylomicron, very low density lipoprotein (VLDL),

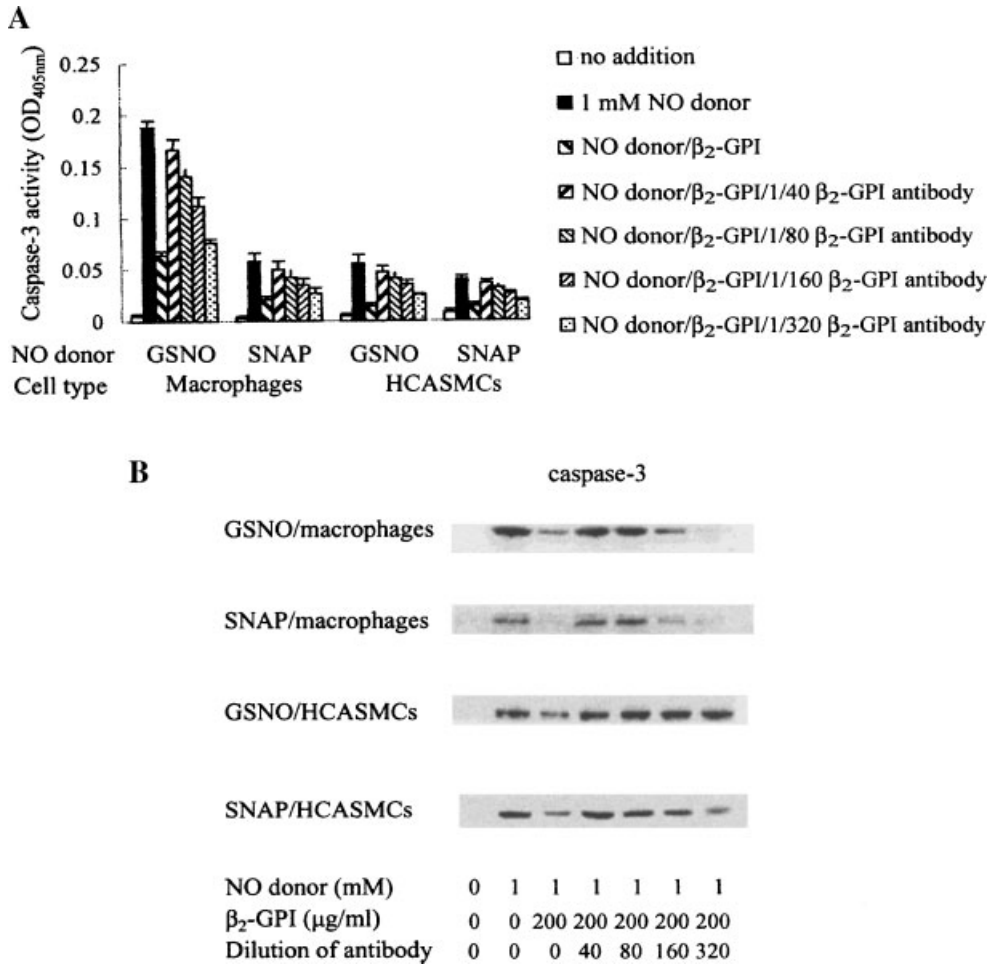

Fig. 5. The effect of rabbit anti-human β_2-GPI antibody on the inhibition of caspase-3 induction mediated by β_2-GPI in J774A.1 macrophages and HCASMCs. Cells were treated with 1 mM nitric oxide (NO) donors and 200 μg/ml β_2-GPI in the absence or presence of antibody at dilutions 1:40, 1:80, 1:160, and 1:320 for 10 h. **A**: The activity of caspase-3 was measured and results of three independent experiments are shown with mean ± SD. **B**: The expression of caspase-3 was detected by Western blot analysis. Shown are representative immunoblots of three separate experiments.

and HDL [Polz et al., 1981; Lee et al., 1983]. This is the first study that shows the involvement of β_2-GPI in the regulation of apoptosis.

Re-distribution of membrane phosphatidylserine from intracellular portion to the outer membrane is a general feature of cells undergoing apoptosis [Schlegel and Williamson, 2001]. Negatively charged phosphatidylserine exposed on the outer membrane of apoptotic cells might be bound by cationic β_2-GPI [Manfredi et al., 1998; Brighton et al., 1999]. This could explain the phenomenon shown in our immunohistochemical study, where β_2-GPI was co-localized with the apoptotic macrophages and SMCs. Since the death of macrophages and SMCs probably promotes the rupture of the fibrous cap [Liby et al., 1996; Haunstetter and Izumo, 1998; Kockx and Herman, 2000], it is suggested that β_2-GPI may have the potential to maintain the stability of atherosclerotic plaque through rescuing vascular cells from apoptosis.

By using GSNO and SNAP as NO donors, our result showed that GSNO had a stronger potency to promote apoptosis in vascular cells. This may point to the different chemical natures and pathway of NO action in response to GSNO and SNAP treatment. GSNO is a physiological *S*-nitrosothiol releasing NO and may produce a persistent increase in tissue *S*-NO content [Alencar et al., 2003]. Modulation of mitogen-activated protein kinases (MAPK) activity is correlated to the occurrence of GSNO-evoked apoptotic parameters such as morphological changes, caspase activation, p53 accumulation, and DNA fragmentation [Callsen and Brüne,

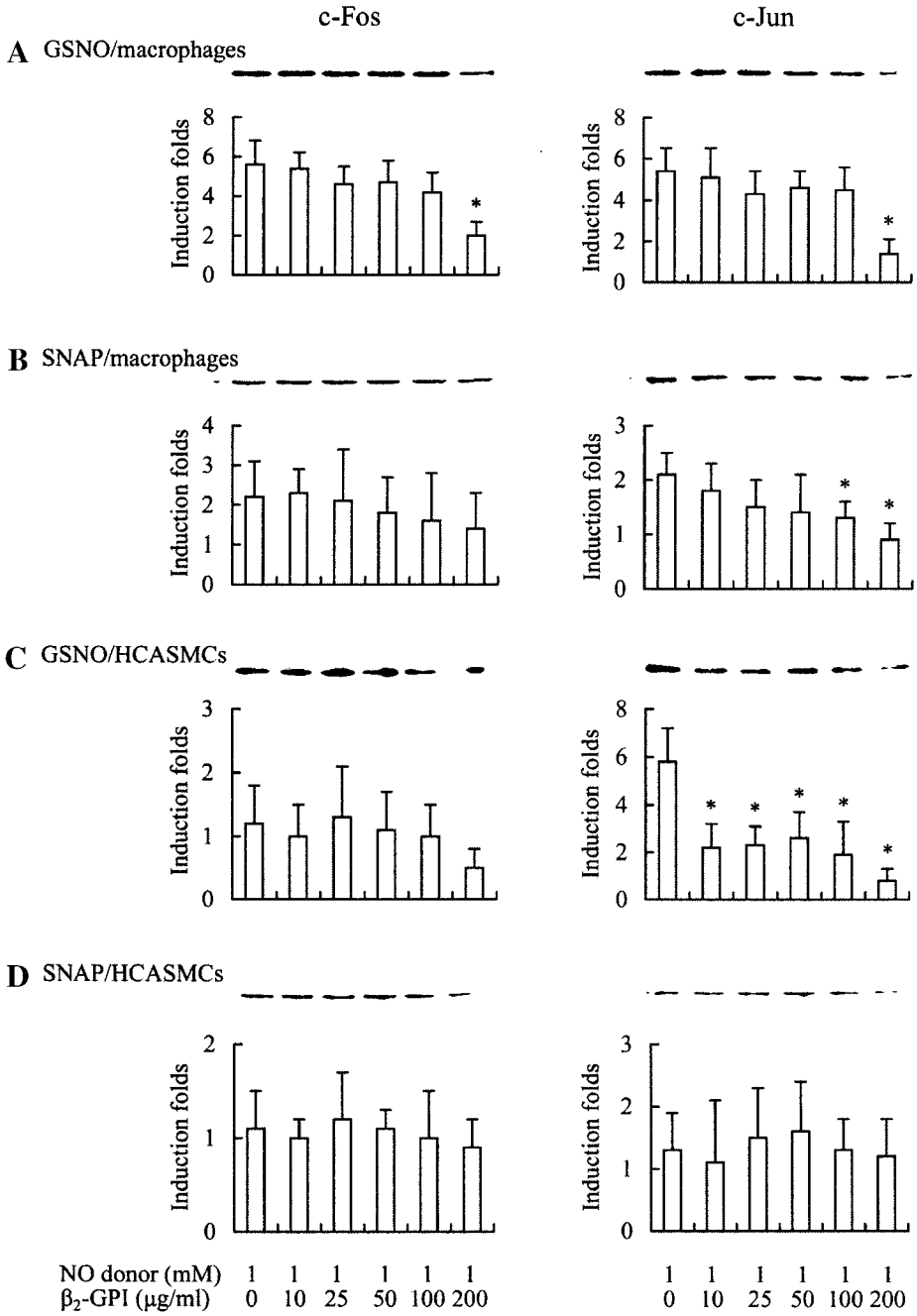

Fig. 6. The effect of β_2-GPI on c-Fos and c-Jun expression in J774A.1 macrophages and HCASMCs. The expression of c-Fos and c-Jun was detected by Western blot analysis and quantified by densitometry. The amounts of c-Fos and c-Jun were normalized to α-tubulin in the cytosol fraction. The value of basal group without NO donor and β_2-GPI treatment was taken as 1. Data are expressed as fold increase of the basal value. Mean values (±SD) of three separate experiments are given. $^*P < 0.05$ compared with the control.

1999]. The persistent NO generation by GSNO may explain its potent and long-lasting apoptotic effect in macrophages and HCASMCs in this study. In contrast, both J774A.1 macrophages and HCASMCs were more resistant to NO-induced apoptosis mediated by the exposure to SNAP. Recently, self-defense mechanisms have been shown for the NO-induced apoptosis [Kroncke et al., 1997]. Okada et al. [1998] demonstrated that SNAP-mediated apoptosis could be suppressed by over expression of Bcl-x_L, though molecules other than Bcl-x_L may also

be able to regulate susceptibility of vascular cells against NO toxicity [Kim et al., 1995; Brüne et al., 1996; Hirvonen et al., 1996]. The mechanisms that regulate NO-induced apoptosis have not been fully elucidated, whereas there are marked differences in the regulation of sensitivity to apoptosis by different NO donors [Brüne et al., 1996; Mohr et al., 1998; Callsen and Brüne, 1999]. In this study, we wished to investigate the role of β_2-GPI in the modulation of apoptotic pathways by different NO donors.

High concentrations of NO are known to induce apoptotic cell death in several cell types, but the sensitivity of cells to NO-induced apoptosis are different [Messmer et al., 1996; Heneka et al., 1998; Chung et al., 2001; Ramachandran et al., 2002]. HCASMCs were more resistant to GSNO- and SNAP-induced apoptosis than macrophages in this study. It is possible that NO elicited different mechanisms to induce apoptosis in HCASMCs and macrophages. In the apoptotic cells, caspase-3 modulates plasma membrane alterations [Rudel and Bokoch, 1997] and cleaves DNA repair enzymes such as poly(ADP ribose) polymerase (PARP) [Tewari et al., 1995], DNA-dependent protein kinase [Song et al., 1996], and the 70 kDa protein component of spicing factor U1 snRNP [Casciola-Rosen et al., 1994]. Thus, higher induction of caspase-3 activity by NO in macrophages might explain the reason why macrophages showed a lower value of cell viability as compared to that in HCASMCs. The other reason for the different responses in NO-treated cells is that there is differential regulation of endogenous NO synthesis in different cells. Zhang et al. [2001] showed that NO donors could differently regulate the expression of inducible NO synthase (iNOS) in rat aortic SMCs versus macrophages, possibly through the activation of NF-κB. However, whether NF-κB was activated by NO donors in the vascular cells in this study remains to be elucidated.

NO-induced apoptotic pathways include induction of cytochrome c and p53 as well as activation of c-Jun N-terminal kinase (JNK)/stress activated protein kinase (SAPK) and p38 kinase [Chung et al., 2001]. All these pathways cause cytosolic cytochrome c-activated caspase-dependent apoptosis. Nishio and Watanabe [1998] showed that activation of caspase-3 was involved in NO-induced apoptosis of vascular SMCs. In this study, β_2-GPI protected the cells from apoptosis through the inhibition of caspase-3 activity and the suppression of its protein levels. The other part of this study focused on the expression of the immediate early genes, c-*fos* and c-*jun*, to investigate another possible route for the anti-apoptotic effect. It has been reported that expression of immediate early genes is associated with apoptosis induced by various agents [Estus et al., 1994; Muthukkumar et al., 1995; Pang and Chau, 1999]. The present study clearly demonstrated that NO-donors are capable of inducing c-Fos and c-Jun expression in macrophages. Nevertheless, only c-Jun was significantly induced in GSNO-treated HCASMCs. All of the induction was inhibited by treatment of cells with 200 μg/ml β_2-GPI. These results suggest that β_2-GPI might play a role in the regulation of c-Fos and c-Jun expression during the anti-apoptotic process in vascular cells.

In conclusion, the present study demonstrates for the first time that β_2-GPI can suppress NO-induced apoptosis in vascular cells. Apoptosis is known to be detrimental to the stability of fibrous cap in the late stage of atherosclerosis [Liby et al., 1996; Kockx and Herman, 2000]. If the induction of β_2-GPI occurs during atherogenesis, we propose that β_2-GPI is beneficial and may protect atherosclerotic vessels from plaque rupture and thrombosis.

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