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Modulation of Phase II Enzymes by Sulforaphane: Implications for Its Cardioprotective Potential

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Oxidative stress plays a major role in the pathophysiology of cardiac disorders, but the experimental data on the protective effects of exogenous antioxidants are controversial. A promising cardioprotective strategy may be through the induction of the endogenous antioxidants and phase II enzymes by chemical inducers. Sulforaphane is an isothiocyanate derived from cruciferous vegetables, and it has gained attention mainly as a potential chemopreventive agent in part through the induction of detoxifying enzymes. Accordingly, this study was undertaken to investigate the time-dependent induction of gene transcription, protein expression, and enzyme activity of antioxidant and phase II enzymes [glutathione reductase, glutathione-*S*-transferase, glutathione peroxidase, NAD(P)H:quinone oxidoreductase-1, thioredoxin reductase] by sulforaphane in cultured rat neonatal cardiomyocytes. The potential cardioprotective action of sulforaphane was confirmed by the decrease in intracellular reactive oxygen species production, the increase in cell viability, and the decrease in DNA fragmentation after long-term treatment accompanied by the induction of antioxidants and phase II enzymes in cardiomyocytes.

KEYWORDS: Sulforaphane; phase II enzymes; oxidative stress; cardiomyocytes

INTRODUCTION

Cardiovascular diseases remain the leading cause of death in both developed and developing countries, accounting for roughly 20% of all worldwide deaths per year (*1*). Many studies have shown a critical role for oxidative stress in the various forms of cardiovascular disorders, including myocardial ischemia-reperfusion injury, congestive heart failure, atherosclerosis, and chemical-induced cardiotoxicity (*2*−*4*). In this context, administration of exogenous antioxidative compounds has been shown to protect against oxidative cardiovascular disorders in animal models (*1*). However, a major drawback associated with the use of exogenous antioxidants is the short half-life of these molecules in vivo, which may contribute to the inconsistency in protection against oxidative cardiac injury.

It is now widely recognized that another strategy to counteract oxidative cardiac injury may be through the up-regulation of endogenous antioxidants and phase II enzymes in cardiac cells by synthetic and naturally occurring agents (*5*). In this regard, the identification of naturally occurring compounds able to induce enzymes critically involved in electrophilic detoxification, present in edible plants, is of particular interest.

Sulforaphane (SF) [1-isothiocyanate-(4*R*)-(methylsulfinyl)butane] is one of the most promising diet-derived chemopreventive agents and is abundantly found in broccoli. SF and other isothiocyanates have been demonstrated to induce phase II drug metabolizing enzymes in cell lines and in animals (*6*). The induction of these enzymes would result in the detoxification and clearance of potential carcinogens as well as endogenous reactive oxygen species (ROS). SF can also inhibit some isoforms of phase I enzymes (*7*) and induce cell cycle arrest and apoptosis (*8*). In addition, SF has been demonstrated to exert neuroprotective activity. In particular, Zhao et al. (*9*) have shown that SF administration reduced infarct volume following focal cerebral ischemia in rodents, and Kraft et al. (*10*) demonstrated that SF treatment offers robust neuroprotection against oxidative stress to cortical cell cultures.

It appears that very few studies have ever been undertaken to relate SF with cardioprotection. Mukherjee et al. (*11*) have demonstrated that consumption of broccoli triggers cardioprotection by generating a survival signal through the activation of several survival proteins and by redox cycling of thioredoxins. A study conducted on 12 healthy subjects has shown that only 1 week intake of broccoli sprouts improved cholesterol metabolism and decreased oxidative stress markers (*12*). Moreover, broccoli consumption was strongly associated with reduced risk of cardiovascular heart disease death in postmenopausal women (*13*). It is important to emphasize that SF is not the only biologically active compound present in broccoli, so a direct correlation between this compound and cardioprotection cannot be assumed.

In this study, using primary cultures of neonatal rat cardiomyocytes as a model system, we have investigated the cardioprotective effect of SF treatment against oxidative damage. To better understand the mechanism behind SF cardioprotective activity, we have characterized the time-dependent SF effect on gene expression, induction, and activity of a series of endogenous

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Table 1. Primers Used in RT-PCR

gene	RefSeq accession no.[a]	amplicon (bp)
GPX1	NM_030826	156
GR	NM_053906	139
GSTa1	NM_031509	124
NQO1	NM_017000	137
TR	NM_031614	87
GAPD[b]	J04038	390

[a] NCBI Nucleotide Sequence Database accession number. [b] Internal normalizer.

antioxidants and phase II enzymes, namely, glutathione reductase (GR), glutathione-*S*-transferase (GST), glutathione peroxidase (GPX), NAD(P)H:quinone oxidoreductase-1 (NQO1), and thioredoxin reductase (TR).

MATERIALS AND METHODS

Chemicals. CelLytic M, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2, digitonin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, bovine serum albumin, NADP, FAD, dimethyl sulfoxide (DMSO), menadione, monochlorobimane (MCB), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), reduced glutathione (GSH), DMEM-F12, fetal calf serum (FCS), horse serum (HS), gentamicin, amphotericin B, and all other chemicals of the highest analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Primers used in RT-PCR were purchased from Superarray (Frederick, MD) and correspond to the following catalog numbers: GPX-1, PPR45366A; GR, PPR46891B; GSTa1, PPR44866A; NQO1, PPR45314A; TR, PPR51711A; GAPD, PA-022-200. D,L-Sulforaphane (LKT Laboratories, Minneapolis, MN) was dissolved in DMSO and stored at a stock concentration of 10 mmol/L at −20 °C.

Cell Culture and Treatments. Neonatal cardiac myocytes were isolated as previously reported (*14*). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996) and approved by the Ethics Committee of our institution. Briefly, cells, obtained from the ventricles of 2−4-day-old rats, were grown until complete confluence. Cells were treated with 5 μM SF for different times (30 min−48 h), and control cells were treated with equivalent concentrations of DMSO alone. SF concentration utilized in this study is readily achievable in rat plasma (*15*) and is close to that achievable in human plasma after a single dose of broccoli sprout isothiocyanate intake (*16*).

mRNA Expression Analysis with Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Total RNA from control and SF-treated cardiomyocytes was extracted using Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA), following the manufacturer's protocol. cDNA synthesis and subsequent PCR reaction were performed as previously reported (*17*). Briefly, cDNA was synthesized using the ReactionReady First Strand cDNA Synthesis Kit, according to the manufacturer's instructions (Superarray). cDNA was reverse transcribed at 37 °C for 60 min, and the reaction was stopped by heating at 95 °C for 5 min. PCR reaction was carried out in a total volume of 25 μL containing ReactionReady HotStart Sweet PCR master mix, 0.4 μM of each primer (Superarray) and 1 μL of diluted cDNA. Fragment size were predicted on the basis of the mRNA sequence as reported in **Table 1**.

Six microliters of the amplified products was separated by electrophoresis on 10% TBE−polyacrylamide gel (precast gel, Bio-Rad, Hercules, CA). The bands were stained with ethidium bromide and visualized under UV light using a Versa-Doc 4000 Imaging system (Bio-Rad).

Western Immunoblotting. Cardiomyocytes were treated with 5 μM SF for 6−48 h. Cells were incubated on ice in lysis buffer (50 mM Tris, 0.1% Triton X-100, 150 mM NaCl, and 2 mM EGTA/EDTA containing mammalian protease inhibitor mixture), and the resulting whole-cell extracts were boiled for 5 min prior to separation on 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Hybond-C; GE Healthcare, Buckinghamshire, U.K.) in Tris−glycine buffer at 110 V for 90 min. Membranes were then incubated in a blocking buffer containing 5% (w/v) skimmed milk and incubated with anti-GSTa1 (Alpha Diagnostic International, San Antonio, TX), anti-GR (AbFrontier, Seoul, Korea), anti-TR1 (Upstate, Lake Placid, NY), anti-glutathione peroxidase (GPX) 1 (Lab Frontier, Seoul, Korea), anti-NQO1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma) as internal normalizer overnight at 4 °C on a three-dimensional rocking shaker. The results were visualized by chemiluminescence using ECL Advance reagent according to the manufacturer's protocol (GE Healthcare Biosciences). Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer (Bio-Rad Laboratories).

NAD(P)H:Quinone Oxidoreductase Enzymatic Activity Assay. NQO1 enzymatic activity was measured according to the procedure of Prochaska and Santamaria (*18*). Briefly, cardiomyocytes were plated in 96-well plates (2.5×10^3 cells/well) and grown under normal conditions until confluence. Control and SF-treated cells were lysed with a solution containing 0.8% digitonin. Two hundred microliters of reaction mix (0.025 mM Tris-HCl, 0.67 mg/mL bovine serum albumin, 0.01% Tween-20, 5 μM FAD, 1 mM glucose 6-phosphate, 30 μM NADP, 2 U/mL yeast glucose-6-phosphate dehydrogenase, 0.3 mg/mL MTT, 50 μM menadione) was added to each well, and the reaction was arrested after 5 min by the addition of a solution containing 0.3 mM dicoumarol, 0.5% DMSO, and 5 mM potassium phosphate buffer. The plates were then scanned at 610 nm with a microplate spectrophotometer VICTOR3 V Multilabel Counter (Perkin-Elmer, Wellesley, MA). NOQ1 activity was expressed as nanomoles per milligram of protein per minute.

Glutathione-*S*-Transferase Enzymatic Activity Assay. GST activity was assayed using CDNB according to the procedure of Habig et al. (*19*). This assay measures the total GST activity because all of the different GST isoforms catalyze the conjugation of GSH with CDNB (*20*). Control and SF-treated cells were lysed with CelLytic M and centrifuged (10000*g* for 10 min). Ten microliters of supernatant was added to 990 μL of reaction mix (100 mM phosphate buffer, pH 6.5, with 1 mM EDTA, 2 mM GSH, 2 mM CDNB), and absorbance was read at 340 nm at 30 s intervals over 5 min. GST activity was expressed as nanomoles per milligram of protein per minute.

Thioredoxin Reductase Enzymatic Activity Assay. TR activity was assayed by an in vitro reduction of DTNB to 5′-thionitrobenzoic acid (TNB) using a procedure adapted from that of Holmgren and Bjornstedt (*21*). Briefly, cells were lysed with CelLytic M, centrifuged at 10000*g*, and 10 μL of supernatant was added to 990 μL of reaction mix (0.25 mM DTNB, 0.24 mM NADPH, 10 mM EDTA, and 100 mM phosphate buffer, pH 7.5). The conversion of DTNB to TNB was measured spectrophotometrically at 412 nm at 10 s intervals over 1 min. Because several enzymes can reduce DTNB, a specific TR inhibitor was used to determine the reduction of DTNB due only to TR activity. TR activity was expressed as milliunits per milligram of protein. One unit of TR causes an increase in A_{412} of 1.0 per minute per milliliter (when measured in a noncoupled assay containing DTNB alone) at the condition of pH 7.0 and 25 °C.

Glutathione Reductase Enzymatic Activity Assay. GR activity was measured according to the method of Smith et al. (*22*). Briefly, controls and SF-treated cells were lysed with CelLytic M and centrifuged at 10000*g* for 10 min, and 10 μL of supernatant was added to 990 μL of reaction mix (100 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 2 mM NADPH, and 2 mM GSSG). The decrease in absorbance at 340 nm was monitored spectrophotometrically for 1 min at 25 °C. GR activity was expressed as milliunits per milligram of protein. One unit of enzyme activity is defined as the amount of enzyme that causes the oxidation of 1.0 μmol of NADPH at 25 °C at pH 7.5.

Glutathione Peroxidase Enzymatic Activity Assay. GPX activity was assayed spectrophotometrically according to the method described by Flohe et al. (*23*), which is based on the reduction of oxidized glutathione coupled to the oxidation of NADPH. Cells were lysed with 50 mM phosphate buffer (pH 8.0) containing 0.5 mM EDTA. The decrease in absorbance at 340 nm was monitored spectrophotometrically for 1 min at 25 °C. GPX activity was expressed as milliunits per milligram of protein. One unit of GPX activity is defined as the amount of enzyme that catalyzes the reduction of 1.0 μmol of NADPH at the condition of 25 °C and pH 8.0.

Protein Concentration. The protein concentration of the cell lysates was determined by the Bio-Rad Bradford protein assay (Bio-Rad Laboratories).

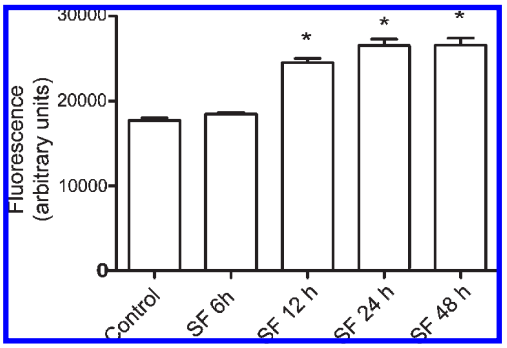

Figure 1. Effect of SF treatment on GSH levels in cardiomyocytes. Cardiomyocytes were treated with 5 μM SF for 6−48 h, and GSH levels were measured using the fluorescence probe MCB. Each bar represents the mean ± SD of four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. ∗, $p < 0.05$ with respect to control.

Reduced Glutathione Levels. GSH levels were determined with a fluorometric assay as previously reported (*17*). Briefly, culture medium was removed, and control and SF-treated cells were washed and incubated for 30 min at 37 °C in 0.1 mL of fresh PBS containing 50 μM MCB. After incubation, fluorescence was measured at 355 nm (excitation) and 460 nm (emission) with a microplate spectrofluorometer VICTOR3 V Multilabel Counter (Perkin-Elmer).

Detection of Intracellular Reactive Oxygen Species. The formation of ROS was evaluated using a fluorescent probe, DCFH-DA, as described by Wang et al. (*24*). Briefly, controls and SF-treated cells were washed with PBS and then incubated with 5 μM DCFH-DA in PBS for 30 min. After DCFH-DA removal, the cells were incubated with 100 μM H_2O_2 for 30 min. Cell fluorescence from each well was measured using a microplate spectrofluorometer ($\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 535 nm). Intracellular antioxidant activity was expressed as the percentage of inhibition of intracellular ROS produced by H_2O_2 exposure.

Cell Viability Measurement. Cardiomyocyte viability in the absence and presence of SF was measured using the MTT assay as previously reported (*17*). Control and SF-treated cells were exposed to 100 μM H_2O_2 for 30 min. After 24 h, MTT was added to the medium and incubated for 1 h at 37 °C. MTT solution was removed, DMSO was added, and the absorbance was measured at 595 nm using a microplate spectrophotometer VICTOR3 V Multilabel Counter (Perkin-Elmer). Data were expressed as the percentage of viable cells with respect to controls.

DNA Fragmentation. The Cell Death Detection ELISA^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) was employed to quantify DNA fragmentation on the basis of antibody detection of histone and fragmented DNA. Briefly, at the end of each experiment cardiomyocytes were washed with PBS, and 200 μL of lysis buffer was added to each well for 30 min, followed by centrifugation at 2200*g* for 10 min. Supernatant was then incubated in a streptavidin-coated 96-well plate with a mixture of two monoclonal antibodies−antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated). After 2 h, 100 μL of peroxidase substrate solution (ABTS) was added to each well for 20 min. The amount of colored product was measured (405 nm) with a microplate spectrophotometer VICTOR3 V Multilabel Counter (Perkin-Elmer). The values were expressed as absorbance at 405 nm.

Statistics. Each experiment was performed at least three times, and all values are represented as means ± SD. One-way analysis of variance (ANOVA) was used to compare differences among groups followed by Dunnett's or Bonferroni's test (Prism 5, GraphPad Software Inc., San Diego, CA). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Effects of SF on GSH Content, GST, GR, and GPX. Because GSH and GSH-related enzymes play a crucial role in the detoxification of ROS and electrophiles and have been suggested to protect cardiac cells against various forms of oxidative injuries, we investigated their inducibility by SF in cardiomyocytes.

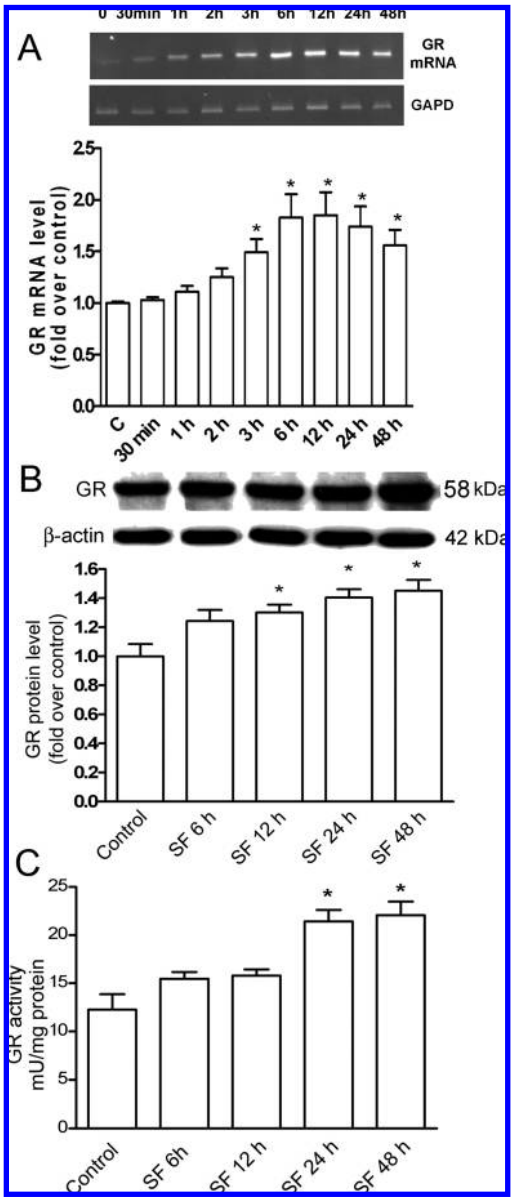

Figure 2. Effect of SF treatment on GR in cardiomyocytes: GR-mRNA level (**A**), protein expression (**B**), and enzymatic activity (**C**) in cardiomyocytes treated with 5 μM SF. In (**A**) and (**B**), the top panels show a representative gel picture of the GR mRNA (**A**) or GR protein (**B**) and the lower panels show quantitative analysis of the GR mRNA (**A**) or GR protein expression level (**B**), normalized with respect to GAPD and β-actin, respectively. Values represent means ± SD ($n = 3$). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. ∗, $p < 0.05$ with respect to control.

Figure 1 represents GSH levels in cultured cardiomyocytes treated with 5 μM SF for 6−48 h. GSH levels significantly increased after 12 h of treatment.

The effects of 5 μM SF treatment on mRNA levels, protein expressions, and activities of GR, GST, and GPX are shown in **Figures 2**, **3**, and **4**, respectively. Incubation of cardiomyocytes with SF led to a marked induction of both GR and GSTa1 mRNA levels from 3 until 48 h of treatment (**Figures 2A** and **3A**). Accordingly, GR and GST protein levels (**Figures 2B** and **3B**) and the corresponding activities (**Figures 2C** and **3C**) were significantly increased with respect to control cells after 12 and 24 h. Protein levels and activities remained significantly higher than in control cells also at 48 h.

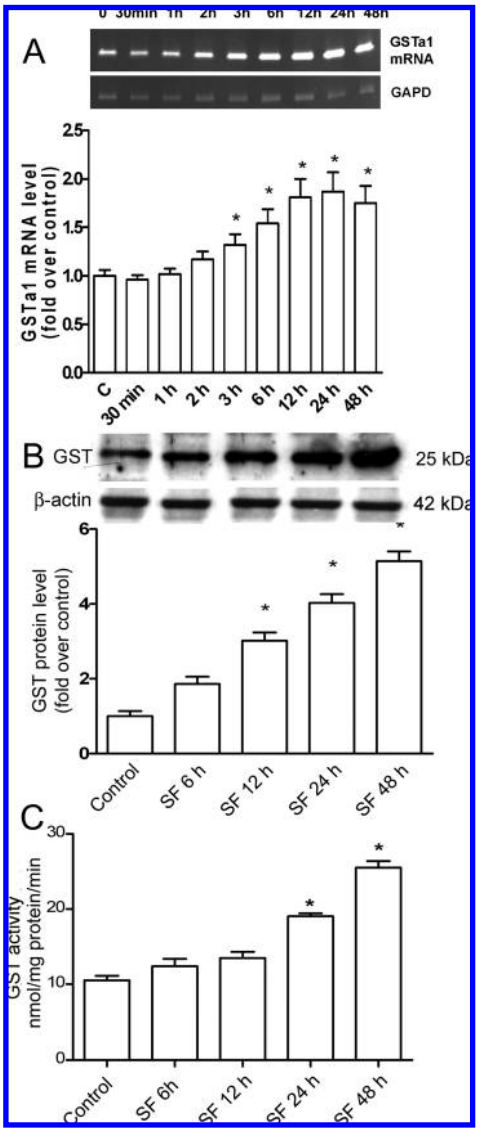

Figure 3. Effect of SF treatment on GST in cardiomyocytes: GST-mRNA level (**A**), protein expression (**B**), and enzymatic activity (**C**) in cardiomyocytes treated with 5 μM SF. In (**A**) and (**B**), the top panels show a representative gel picture of the GST mRNA (**A**) or GST protein (**B**) and the lower panels show quantitative analysis of the GST mRNA (**A**) or GST protein expression level (**B**), normalized with respect to GAPD and β-actin, respectively. Values represent means $\pm$ SD ($n = 3$). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. $*$, $p < 0.05$ with respect to control.

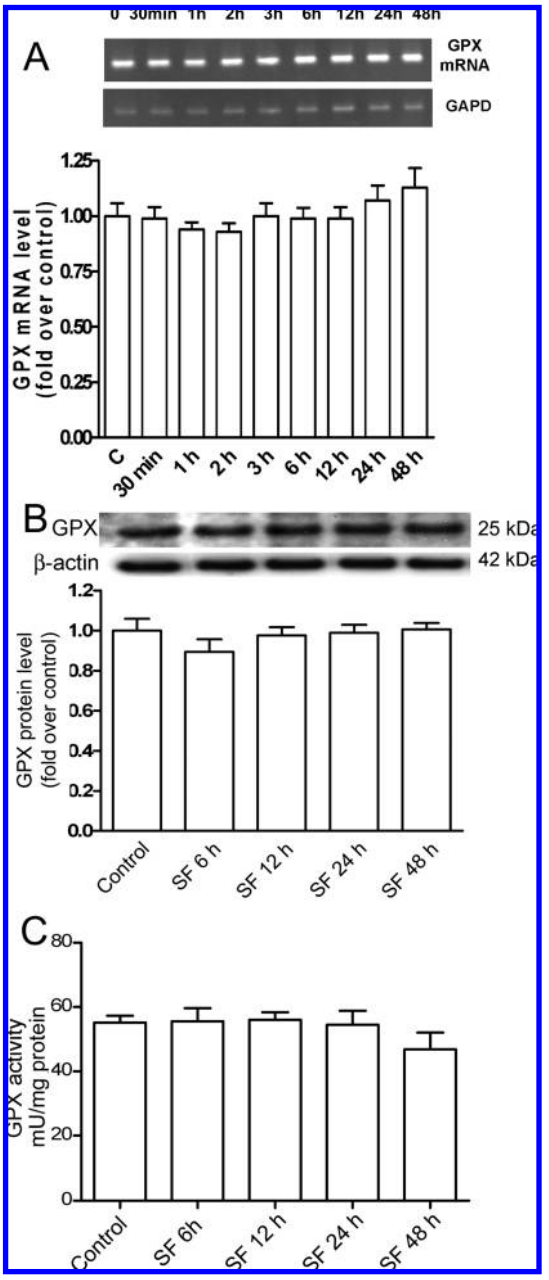

Figure 4. Effect of SF treatment on GPX in cardiomyocytes: GPX-mRNA level (**A**), protein expression (**B**), and enzymatic activity (**C**) in cardiomyocytes treated with 5 μM SF. In (**A**) and (**B**), the top panels show a representative gel picture of the GPX mRNA (**A**) or GPX protein (**B**) and the lower panels show quantitative analysis of the GPX mRNA (**A**) or GPX protein expression level (**B**), normalized with respect to GAPD and β-actin, respectively. Values represent means $\pm$ SD ($n = 3$). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test.

In contrast, GPX mRNA, protein level, and activity were not influenced by SF treatment at any exposure times (**Figure 4**).

GR, GST, and GPX activities were not influenced by vehicle [0.2% (v/v) DMSO] (data not shown).

Effects of SF on TR and NQO1. TR mRNA (**Figure 5A**) was significantly increased with respect to control cells after 2 h of SF treatment, whereas TR protein level (**Figure 5B**) was significantly increased after 6 h of SF treatment. NQO1 mRNA and protein expressions (**Figure 6A**,**B**) exhibited significant induction after 3 and 12 h, respectively. TR and NQO1 activities (**Figures 5C** and **6C**) were significantly increased in cardiomyocytes after 12 h of SF treatment.

TR and NQO1 activities were not influenced by vehicle [0.2% (v/v) DMSO] (data not shown).

Effect of SF on H_2O_2-Mediated Intracellular ROS Production. A significant decrease in ROS production, as detected by DCFH-DA assay, was observed in SF-treated cardiomyocytes following exposure to H_2O_2 (**Figure 7**).

Vehicle controls containing equivalent volumes of DMSO as SF-treated cells [0.2% (v/v)] did not show any significant difference in comparison to cells exposed to H_2O_2. ROS levels were significantly reduced in SF-treated cells after 12−48 h in a time-dependent fashion. At the shortest incubation time, SF did not show any ability to reduce ROS levels.

Effects of SF on Cell Viability. Incubation of cardiomyocytes with 100 μM H_2O_2 for 30 min caused a significant decrease in cell viability, as detected by MTT reduction assay (**Figure 8**).

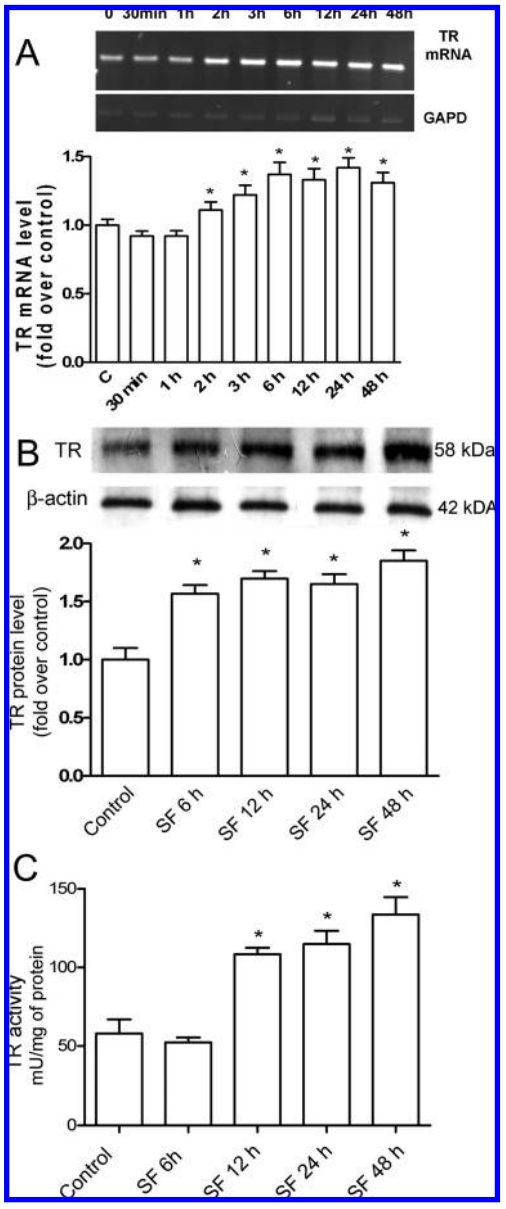

Figure 5. Effect of SF treatment on TR in cardiomyocytes: TR-mRNA level (**A**), protein expression (**B**), and enzymatic activity (**C**) in cardiomyocytes treated with 5 μM SF. In (**A**) and (**B**), the top panels show a representative gel picture of the TR mRNA (**A**) or TR protein (**B**) and the lower panels show quantitative analysis of the TR mRNA (**A**) or TR protein expression level (**B**), normalized iwith respect to GAPD and β-actin, respectively. Values represent means ± SD (n = 3). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. ∗, $p < 0.05$ with respect to control.

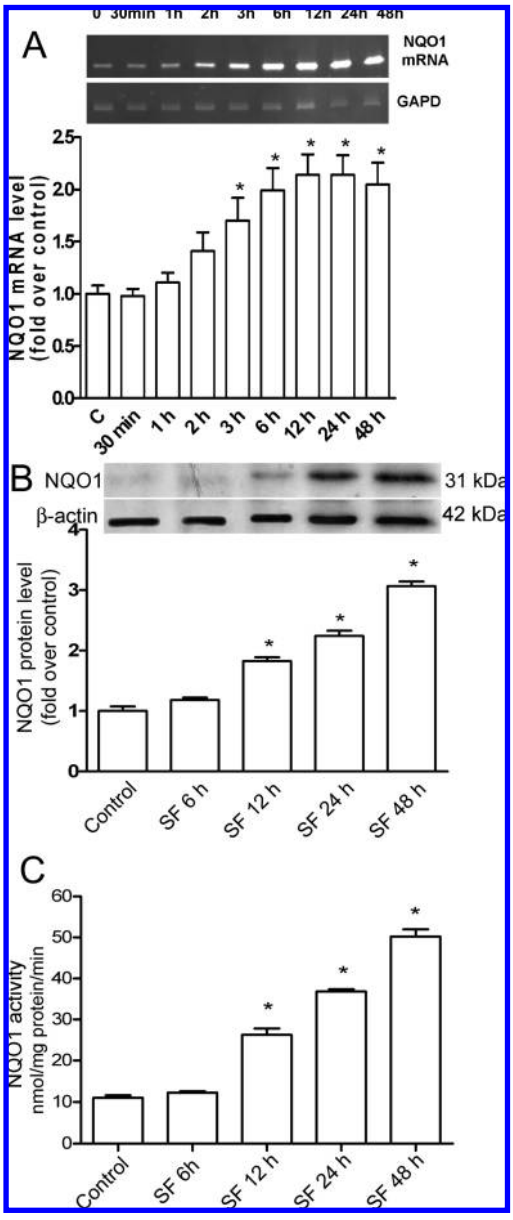

Figure 6. Effect of SF treatment on NQO1 in cardiomyocytes: NQO1-mRNA level (**A**), protein expression (**B**), and enzymatic activity (**C**) in cardiomyocytes treated with 5 μM SF. In (**A**) and (**B**), the top panels show a representative gel picture of the NQO1 mRNA (**A**) or NQO1 protein (**B**) and the lower panels show quantitative analysis of the NQO1 mRNA (**A**) or NQO1 protein expression level (**B**), normalized with respect to GAPD and β-actin, respectively. Values represent means ± SD (n = 3). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. ∗, $p < 0.05$ with respect to control.

Treatment of cardiac cells with SF for 12, 24, and 48 h prior to H_2O_2 exposure led to a marked protection against oxidative damage, as shown by the significant increments in cell viability with respect to H_2O_2-treated cells. Incubation of cardiomyocytes with SF for 48 h resulted in complete protection against peroxide-induced injury. To confirm cell viability data and to clarify that the post-H_2O_2 cell death was due to apoptosis, we used ELISA methods to detect chromosomal and DNA fragmentation, the biochemical hallmark of apoptosis. Consistent with MTT results, H_2O_2 significantly increased DNA fragmentation (**Figure 9**), whereas SF treatment for 48 h was able to maintain DNA fragmentation at levels comparable to those of control cells.

DISCUSSION

This study demonstrates that treatment of cardiac cells with micromolar concentrations of SF results in a significant induction of a panel of key cellular antioxidants and phase II enzymes, including GSH, GR, GST, TR, and NQO1, and in a marked protection against oxidative injury.

Oxidative stress has been suggested to be a contributing factor in the pathophysiology of different cardiovascular diseases (*2*). Moreover, it has been reported that the activity of antioxidant enzymes is low in the heart, so that the susceptibility to oxidative stress is higher in the heart than in other tissues (*25*). It is now widely recognized that the up-regulation of antioxidant and phase II enzymes is a powerful and highly efficient protective strategy

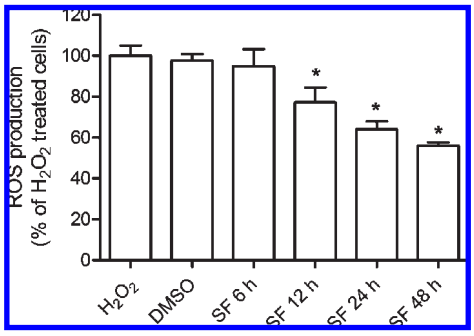

Figure 7. Effect of SF on intracellular ROS production in cardiomyocytes. Cardiomyocytes were treated with 0.2% (v/v) DMSO or 5 μM SF for 6−48 h before the addition of 100 μM H_2O_2, and intracellular ROS were determined using the peroxide-sensitive fluorescent probe DCFH-DA. Data are expressed as percent of H_2O_2-treated cells (=100%). Values represent means ± SD ($n = 4$). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. *, $p < 0.05$ with respect to H_2O_2-treated cells.

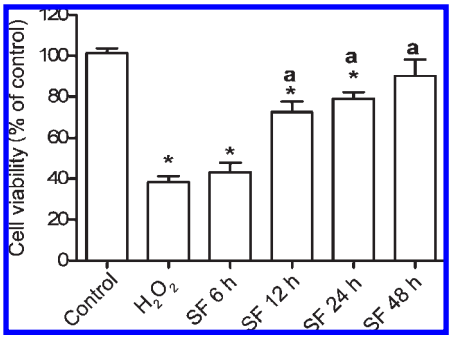

Figure 8. Effect of SF on cell viability in cardiomyocytes exposed to H_2O_2. Cardiomyocytes were treated with 5 μM for 6−48 h before the addition of 100 μM H_2O_2, and cellular damage was assessed by the MTT assay and reported as percent cell viability in comparison to controls. Each bar represents the mean ± SD of four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. *, $p < 0.05$ with respect to control; a, $p < 0.05$ with respect to H_2O_2-treated cells.

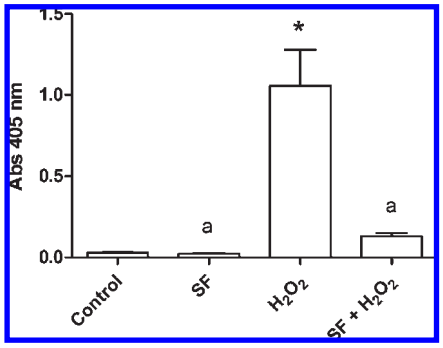

Figure 9. Effect of SF on DNA fragmentation in cardiomyocytes exposed to H_2O_2. Cardiomyocytes were treated with 5 μM for 48 h before the addition of 100 μM H_2O_2, and DNA fragmentation was assessed 24 h after H_2O_2 treatment. Each bar represents the mean ± SD of four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test. *, $p < 0.05$ with respect to control; a, $p < 0.05$ with respect to H_2O_2-treated cells.

against the damaging effects of reactive oxygen intermediates and electrophiles (*26*). Phase II enzymes catalyze diverse reactions that collectively result in a broad protection against electrophiles and oxidants and share common transcription regulation, and their gene expression can be coordinately induced by a variety of synthetic and naturally occurring agents. Among the main phase II enzymes, the thioredoxin (TRX) and the glutathione systems, as well as NQO1, interact with a whole series of important cellular pathways. GSH is the major nonenzymatic regulator of intracellular redox homeostasis, ubiquitously present in all cell types at a millimolar concentration (*27*). GR is important in recycling oxidized glutathione back to GSH. GSH is also a cofactor for GST, an abundant cellular enzyme in mammalian tissues. Several studies have reported that GST plays an important role in protecting cells against ROS-mediated injury, catalyzing the decomposition of lipid hydroperoxides generated from oxidative damage to cellular lipids (*28*). NQO1 is involved in the detoxification of H_2O_2 and superoxide and acts as an antioxidant enzyme via its ability to maintain the cellular levels of ubiquinol and vitamin E (*29*). TR, in conjunction with TRX, is a ubiquitous cellular oxidoreductase system with antioxidant and redox regulatory roles. Yamamoto et al. (*30*) showed that TRX1 is an essential antioxidant in the mouse heart in vivo; furthermore, Turoczi et al. (*31*) showed a cardioprotective role against pathologic insults, such as ischemia-reperfusion.

Especially attractive is the identification of phytochemicals present in the human diet acting as phase II enzyme inducers and eliciting cardioprotective activity. Li et al. (*32*) showed that the red wine polyphenol resveratrol protects cultured aortic muscle smooth cells against oxidative stress through superoxide dismutase, catalase, GSH, GR, GPX, GST, and NQO1 up-regulation. In another study, resveratrol induction of antioxidants and phase II enzymes in cardiomyocytes was accompanied by increased resistance to oxidative stress (*33*). Finally, in a previous study we reported the ability of the flavonol quercetin to up-regulate rat cardiomyocytes phase II enzymes and to markedly increase resistance to ROS-elicited cardiac cell injury (*17*).

In recent years, SF, an isothiocyanate derived from broccoli, has gained great attention mainly for its chemopreventive activity (*34*). Although it has been demonstrated that SF is a strong inducer of endogenous antioxidants and phase II enzymes in various cells and tissues (*35*), the inducibility of the cellular defenses in cardiac cells has not been studied yet. Few studies relate the protection from coronary heart diseases with the consumption of broccoli (*11*−*13*); however, considering that broccoli is also rich in many other antioxidant compounds such as flavonoids and selenium, none of these studies have demonstrated the existence of a direct relationship between SF intake and cardioprotection.

SF treatment led to a significant increase of cellular GSH content and of GR, GST, TR, and NQO1 mRNA and protein expression levels in cultured rat cardiomyocytes after short exposure times. Enzyme activities were significantly increased after SF treatment, and a delay with respect to the corresponding time course of mRNA induction profiles was observed. On the contrary, GPX mRNA levels and protein expression and activity were not affected by SF treatment. This is in agreement with the results reported by Hu et al. (*36*), who did not observe any change in GPX after the treatment of human lung cancer cell A549 with SF. Moreover, Hintze et al. (*37*) observed an increase in TR and not in GPx hepatic expression after dietary SF intake; Campbell et al. (*38*) showed that SF induced only TR activity, whereas SF was able to up-regulate GPX-1 activity only in the presence of 40 nM selenite in human endothelial cell line EA.HY 926. Thus, we can assume that SF selectively activates only one of the Se-containing antioxidant systems in the cell, namely, TR and not GPX.

Because of the ability of SF to up-regulate GSH, GR, GST, NQO1, and TR, we investigated if the induction of the above antioxidant and phase II enzymes led to protection against

ROS-mediated toxicity. Treatment of cardiac cells with SF resulted in a marked protection against H_2O_2-induced cytotoxicity and in a significant decrease in intracellular ROS production. Interestingly, SF is able to protect cardiomyocytes starting from 12 h of treatment and achieving complete protection after 48 h treatment. These results are in agreement with the data on the time course of detoxifying enzyme induction.

Data reported in this paper further highlight the ability of SF to act as an indirect antioxidant, able to provide long-lasting protection against oxidative stress. Efficient detoxification of ROS requires the concerted actions of various cellular antioxidants and phase II enzymes. This study demonstrates that a number of phase II enzymes can be induced in cultured cardiomyocytes already at micromolar concentrations of SF and that this SF-mediated induction of cellular defenses is accompanied by a marked increase in resistance to ROS-induced cardiac cell injury.

The simultaneous induction of a scope of key cellular antioxidants and phase II enzymes by SF in cardiomyocytes may be an important mechanism underlying the protective effect of this nutraceutical from cruciferous vegetables against oxidative stress, a common determinant in many cardiovascular diseases.

ABBREVIATIONS USED

CDNB, 1-chloro-2,4-dinitrobenzene; DCHF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; FCS, fetal calf serum; GAPD, glyceraldehyde-3-phosphate-dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-*S*-transferase; HS, horse serum; MCB, monochlorobimane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO1, NAD(P)H:quinone oxidoreductase-1; ROS, reactive oxygen species; SF, sulforaphane; TR, thioredoxin reductase.

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