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Broccoli: A Unique Vegetable That Protects Mammalian Hearts through the Redox Cycling of the Thioredoxin Superfamily

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Epidemiological evidence indicates several health benefits of the consumption of broccoli, especially related to chemoprevention. Because broccoli contains high amounts of selenium and glucosinolates (particularly glucoraphanin and isothiocyanate sulforaphane), which can produce redox-regulated cardioprotective protein thioredoxin (Trx), it was reasoned that consumption of broccoli could be beneficial to the heart. To test this hypothesis, a group of rats were fed broccoli (slurry made with water) through gavaging; control animals were gavaged water only. After 30 days, the rats were sacrificed; isolated hearts perfused via working mode were made ischemic for 30 min followed by 2 h of reperfusion. The results demonstrated significant cardioprotection with broccoli as evidenced by improved postischemic ventricular function, reduced myocardial infarct size, and decreased cardiomyocyte apoptosis accompanied by reduced cytochrome *c* release and increased pro-caspase 3 activities. Ischemia/reperfusion reduced both RNA transcripts and protein levels of the thioredoxin superfamily including Trx1, Trx2, glutaredoxin Grx1, Grx2, and peroxiredoxin (Prdx), which were either restored or enhanced with broccoli. Broccoli enhanced the expression of Nrf2, a cytosolic suppressor of Keap1, suggesting a role of antioxidant response element (ARE) in the induction of Trx. Additionally, broccoli induced the expression of another cardioprotective protein, heme oxygenase (HO)-1, which could be transactivated during the activation of Trx. Examination of the survival signal revealed that broccoli caused the phosphorylation of Akt and the induction of Bcl2 in concert with the activation of redox-sensitive transcription factor NFκB and Src kinase, indicating a role of Akt, Bcl2, and cSrc in the generation of survival signal. Taken together, the results of the present study indicate that the consumption of broccoli triggers cardioprotection by generating a survival signal through the activation of several survival proteins and by redox cycling of thioredoxins.

KEYWORDS: Broccoli; heart; cardioprotection; ischemia/reperfusion; thioredoxin; glutaredoxin; peroxiredoxin; Nrf2; redox signaling; survival signaling

INTRODUCTION

Epidemiological studies have identified specific phytochemicals in *Brassica* vegetables that may confer protection against certain degenerative diseases such as cancer (*1, 2*). Certain cruciferous vegetables (mustard family) of the genus *Brassica* including cauliflower, broccoli, cabbage, and Brussels sprouts have been studied extensively because of their high nutritional values as they are rich sources of antioxidants, vitamins, and fiber but contain little fat and energy. These properties make them suitable for chemoprevention. In addition, recent studies have indicated that broccoli sprouts can induce carcinogen-detoxifying enzymes (*3*).

Among other chemicals, broccoli contains high concentrations of selenium and glucosinolates, particularly glucoraphanin and isothiocyanate sulforaphane (*4*), Sulforaphane can induce phase II enzymes through the activation of the Keap1/Nrf2 antioxidant response pathway (*5*). Most importantly, sulforaphane can also induce redox-regulated protein thioredoxin through the antioxidant-responsive element (*6*). Because thioredoxin has recently been implicated in cardioprotection, it seems likely that broccoli, in addition to serving as a chemoprotective vegetable, may also function to protect the cardiovascular system. Indeed, a recent clinical study involving 12 healthy subjects has demonstrated that consumption of fresh broccoli sprouts (100 g/day) for a week reduced low-density lipoprotein (LDL) and total cholesterol and increased high-denisty lipoprotein (HDL) cholesterol (*7*). Another related prospective study of 34492 postmenopausal women in Iowa showed that broccoli was strongly associated with reduced risk of coronary heart disease (*8*).

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It appears that very few studies have ever been undertaken to relate the consumption of broccoli with coronary heart disease, and most importantly, the mechanisms of action remain largely unknown. To fill this gap, we fed a group of rats fresh broccoli for 1 month. The results of our study revealed significant cardioprotection with broccoli, which induced the expression of several genes and proteins of the thioredoxin (Trx) superfamily including Trx1, Trx2, thioredoxin reductase, Grx1, Grx2, and peroxiredoxin (Prdx) as well as caused the activation of the antioxidant response pathway and several antideath proteins of the survival pathway.

MATERIALS AND METHODS

Animals. All animals used in this study received humane care in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals; the care adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Sprague–Dawley male rats weighing between 250 and 300 g were used for the experiment. The rats were randomly assigned to two groups: control and broccoli treated. The rats were fed ad libitum regular rat chow with free access to water. The broccoli-treated rats were fed 1 mL of broccoli extract [1.5 g/kg of body weight (BW)] for 30 days, whereas the control group of rats were gavaged 1 mL of water for the same period of time.

Analysis of Sulforaphane and Selenium Contents of the Broccoli Used in Our Study. For sulforaphane analysis, the homogenized broccoli, left at room temperature for about 39 min, was extracted with methylene chloride, dried at 30 °C under vacuum, dissolved in acetonitrile, and filtered through a 0.22 μm membrane filter prior to injection onto HPLC (*9*). Selenium was estimated by atomic absorption spectrometry as described previously (*10*).

Isolated Working Heart Preparation. At the end of 30 days, the rats were anesthetized with sodium pentobarbital (80 mg/kg of BW, ip) (Abbott Laboratories, North Chicago, IL) and anticoagulated with heparin sodium (500 IU/kg of BW, iv) (Elkins-Sinn Inc., Cherry Hill, NJ) injection. After a sufficient depth of anesthesia had been attained, a thoracotomy was performed, and the heart was perfused in the retrograde Langendorff mode at 37 °C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5 min washout period (*11*). The perfusion buffer used in this study consisted of a modified Krebs−Henseleit bicarbonate buffer (KHB): sodium chloride, 118 mM; potassium chloride, 4.7 mM; calcium chloride, 1.7 mM; sodium bicarbonate, 25 mM; potassium biphosphate, 0.36 mM; magnesium sulfate, 1.2 mM; and glucose, 10 mM. The Langendorff preparation was switched to the working mode following the washout period.

At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded. The circuit was then switched back to the retrograde mode, and the hearts were perfused for 15 min with KHB for stabilization (*9*). The hearts were then subjected to 30 min of global ischemia followed by 2 h of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for postischemic stabilization and, thereafter, in the antegrade working mode to allow for assessment of functional parameters, which were recorded at 10, 30, 60, 90, and 120 min of reperfusion.

Cardiac Function Assessment. Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH) connected to a sidearm of the aortic cannula; the signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA) (*11*). Heart rate (HR), left ventricular developed pressure (LVDP) (defined as the difference of the maximum systolic and diastolic aortic pressures), and the first derivative of developed pressure (dp/dt) were all derived or calculated from the continuously obtained pressure signal. Aortic flow (AF) was measured using a calibrated flow meter (Gilmont Instrument Inc., Barrington, IL), and coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart.

Infarct Size Estimation. At the end of reperfusion, a 10% (w/v) solution of triphenyl tetrazolium in phosphate buffer was infused into aortic cannula for 20 min at 37 °C (*12*). The hearts were excised, and the sections (0.8 mm) of the heart were fixed in 2% paraformaldehyde, placed between two coverslips, and digitally imaged using a Microtek ScanMaker 600z. To quantify the areas of interest in pixels, a NIH image 5.1 (a public domain software package) was used. The infarct size was quantified and expressed in pixels.

TUNEL Assay for Assessment of Apoptotic Cell Death. Immunohistochemical detection of apoptotic cells was carried out using the TUNEL method (*12*) (Promega, Madison, WI). The heart tissues were immediately put in 10% formalin and fixed in an automatic tissue-fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic molds, and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed, and tissues became embedded in paraffin on the plastic molds. Prior to analysis of tissues for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 85%, 70%, 50%). Then the TUNEL staining was performed according to the manufacturer's instructions. The fluorescence staining was viewed with a fluorescence microscope (AXIOPLAN2 IMAGING) (Carl Zeiss Microimaging Inc., New York). The number of apoptotic cells was counted and expressed as a percent of total myocyte population.

Preparation of Subcellular Fractions. Tissues were homogenized in 1 mL of buffer A (25 mM Tris-HCl, pH 8, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1× protease inhibitor cocktail) in a Polytron homogenizer. Homogenates were centrifuged at 3000 rpm at 4 °C for 10 min, and the nuclear pellet was resuspended in 500 μL of buffer A with 0.1% Triton X-100. Supernatant from the above centrifugation was further centrifuged at 10000 rpm at 4 °C for 20 min, and the resultant supernatant was used as cytosolic extract. The mitochondrial pellet was resuspended in 200–300 μL of buffer A with 0.1% Triton X-100. The nuclei pellet and mitochondrial pellet were lysed by incubation for 1 h on ice with intermittent tapping. Homogenates were then centrifuged at 14000 rpm at 4 °C for 10 min, and the supernatant was used as nuclear lysate and mitochondrial lysate, respectively. Cytosolic, nuclear, and mitochondrial extracts were aliquoted, snap frozen, and stored at −80 °C until use. Total protein concentrations in cytosolic, nuclear, and mitochondrial extracts were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Western Blot Analysis. Either cytosolic, nuclear, or mitochondrial proteins were separated in SDS-PAGE and transferred to nitrocellulose filters. Filters were blocked in 5% nonfat dry milk and probed with primary antibody overnight (*13*). Primary antibodies such as Nrf2, Bcl2, Trx (total Trx), Trx1, Trx2, Grx2, TrxR, Akt, Phospho-Akt. Src, Phospho-Src, IkB, P-IkB, heme oxygenase, cytochrome *c*, caspase 3, Prdx, Nrf2, Akt, phospho-Akt (Ser 473), and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA; whereas IkB, Phospho-IkB (Ser 32), Src, phospho-Src (Tyr527), cytochrome *c*, and caspase 3 were obtained from Cell Signaling Technology, Beverly, MA. All primary antibodies were used at the dilution of 1:1000. Protein bands were identified with horseradish peroxidase conjugated secondary antibody (1:2000 dilution) and Western blotting luminol reagent (Santa Cruz Biotechnology). GAPDH was used as loading control. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

Total RNA Isolation and RT-PCR. Total RNA was extracted from left ventricular tissues with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and dissolved in 50 μL of DEPC-treated water. Reverse transcription and Polymerase Chain Reaction (RT-PCR) were performed with RETROscript (Ambion, Austin, TX), according to the manufacturer's instructions. The following primers were used in our study.

Thioredoxin-1: forward, 5′-GCCAAAATGGTGAAGCTGA-3′; reverse, 5′-CTGGCAGTCATCCACGTCT-3′.

Thioredoxin-2: forward, 5′-GCATCCTGAGCACCTCCTAC-3′; reverse, 5′-GAGCCACATGTGTGTGTGTG-3′.

Glutaredoxin-1: forward, 5′-ACGTGGTCTCCTGGAATTTG-3′; reverse, 5′-GCAGAGCTCCAATCTGCTTC-3′.

Table 1. Sulforaphane and Selenium Contents of Broccoli

	content per gram of fresh broccoli
sulforaphane	23.6 ± 1.5 μg
selenium	65.0 ± 2.8 ng

Three different samples of broccoli were analyzed; each analysis was run in duplicate. Results are expressed as means ± SEM.

Glutaredoxin-2: forward, 5′-TGGCCAAGAAGATTTTCCAT-3′; reverse, 5′-AGGCAGCAATTTCCCTTCTT-3′.

TrxR1: forward, 5′-TCATGGCTGGACTATCCACA-3′; reverse, 5′-GCCAAAGTGACCATGGTTTT-3′.

GAPDH: forward, 5′-AGACAGCCGCATCTTCTTGT-3′; reverse, 5′-CTTGCCGTGGGTAGAGTCAT-3′.

The PCR products were visualized on a UV-transilluminator and digitalized after electrophoresis on 2% agarose gel containing ethidium bromide.

Statistical Analysis. The values for myocardial functional parameters, total and infarct volumes, infarct sizes, and cardiomyocyte apoptosis are all expressed as the mean ± standard error of the mean (SEM). Analysis of variance test followed by Bonferroni's correction was first carried out to test for any differences between the mean values of all groups. If differences between groups were established, the values of the treated groups were compared with those of the control group by a modified *t* test. The results were considered to be significant if $p < 0.05$.

RESULTS

Sulforaphane and Selenium Contents of the Broccoli Used in Our Study. Because we sought to examine redox regulation by broccoli, we determined sulforaphane and selenium contents of the broccoli used in our study. The results are shown in **Table 1**.

Effects of Broccoli on Left Ventricular Function. We first determined if the hearts of broccoli-treated animals displayed improved ventricular performance compared to those of control animals. As shown in **Figure 1**, aortic flow, LVDP, and LV_{max} d*p*/d*t* of broccoli-treated hearts consistently displayed improved performance compared to the control group during postischemic reperfusion. The heart rate did not vary significantly between the two groups. Coronary flow of the broccoli group was higher compared to the control group only at the end of 90 min and 2 h of reperfusion.

Effects of Broccoli on Myocardial Infarct. Myocardial infarct size at the end of 30 min of ischemia and 2 h of reperfusion as determined by TTC staining method was about 39 ± 1.17% normalized to area of risk (**Figure 2**). There was no infarction when the hearts were perfused with the KHB buffer without subjection to ischemia and reperfusion (control) (data not shown). Broccoli significantly reduced myocardial infarct size to about 15 ± 2.64%.

Effects of Broccoli on Cardiomyocyte Apoptosis. As shown in **Figure 3**, cardiomyocyte apoptosis determined by TUNEL method was about 40 ± 2% at the end of reperfusion. There were no apoptotic cells in the hearts perfused with the KHB buffer without subjection to ischemia and reperfusion (control). Again, broccoli significantly reduced the number of apoptotic cardiomyocytes to 18 ± 1.5%.

To further confirm broccoli-mediated prevention of cardiomyocyte apoptosis, we determined the activity of cytochrome *c*. **Figure 4** shows the results. Cytochrome *c* was significantly increased after ischemia/reperfusion, correlating with the results of **Figure 3**. Broccoli prevented the increase in cytochrome *c*. The activity of pro-caspase 3 was significantly reduced in the ischemic reperfused myocardium, and broccoli prevented the loss of pro-caspase 3.

Effects of Broccoli on mRNA Transcripts of the Trx Superfamily. Having confirmed that broccoli reduced myocardial ischemic reperfusion injury, we determined if broccoli provided cardioprotection by inducing the genes of the thioredoxin superfamily. The mRNA transcripts of Trx, Trx-reductase, and Grx were determined by RT-PCR. As shown in **Figure 5**, ischemia/reperfusion reduced the transcripts of mRNAs of all members of the Trx superfamily and not only did broccoli preserve these transcripts, it actually up-regulated them over the control values.

Effects of Broccoli on the Induction of Proteins of the Trx Superfamily. We then determined if broccoli also induced the expression of Trx proteins. Similar to mRNA transcripts, ischemia reperfusion significantly reduced the expression of Trx, Grx, and Prdx proteins (**Figure 6**). We failed to identify any Trx1 protein using the Trx1 antibody and were also unable to detect Grx2 protein because the proper antibody was not commercially available. In contrast to mRNA transcripts, Trx did not overexpress the proteins over the control values, but simply preserved them from ischemia/reperfusion-mediated loss.

Effects of Broccoli on the Expression Profiles of Nrf2. Because the induction of Trx from sulforaphane present in broccoli is likely to occur via the induction of ARE and because Keap1, a nuclear suppressor of Nrf2, is likely to be involved in this process, we examined the expression profiles of Nrf2 in response to ischemia/reperfusion and broccoli. **Figure 7** shows the results. Nrf2 was significantly down-regulated after ischemia and reperfusion, but broccoli prevented the Nrf2 protein from undergoing down-regulation.

Effects of Broccoli on the Induction of the HO-1 Protein. It has been known that during the induction of Trx from sulforaphane present in broccoli, HO-1 can be transactivated. To determine if this is true in our experimental setting, we studied the expression of the HO-1 protein. As shown in **Figure 8**, HO-1 was significantly reduced in the ischemic reperfused myocardium, and broccoli prevented the loss of HO-1.

Effects of Broccoli in the Generation of Survival Signal. We examined the members of the survival proteins triggered by broccoli. **Figure 9** shows the results. The phosphorylation of Akt was reduced after ischemia/reperfusion, but significantly up-regulated with broccoli. The phosphorylation of cSrc followed a similar pattern. Bcl2 was down-regulated after ischemia/reperfusion, but unlike Akt and cSrc, it was not activated over the control value. Broccoli prevented the loss of Bcl2 protein due to ischemia/reperfusion. The phosphorylation of IkB was almost abolished in the ischemic reperfused myocardium, but it was restored with broccoli.

DISCUSSION

There are several salient features of the study in which broccoli was fed for 1 month: (i) improved ventricular function compared to control; (ii) reduced myocardial infarct size and cardiomyocyte apoptosis; (iii) enhanced induction of the expression of both at mRNA and at protein levels of Trx1, Trx2, Grx1, Trx-reductase, Prdx, and Nrf2; and (iv) induction of antideath proteins and transcription factors in the survival pathway including phospho-Akt, Bcl-2, and NFκB.

A growing body of evidence supports the role of mitochondria in predicting the life and death of cardiomyocytes. For example, mitochondria are critically involved in apoptotic cell death triggered by ischemia and reperfusion (*14*). For the maintenance of mitochondrial integrity, membrane potential is likely to have influence on myocardial energy production and ultimate survival

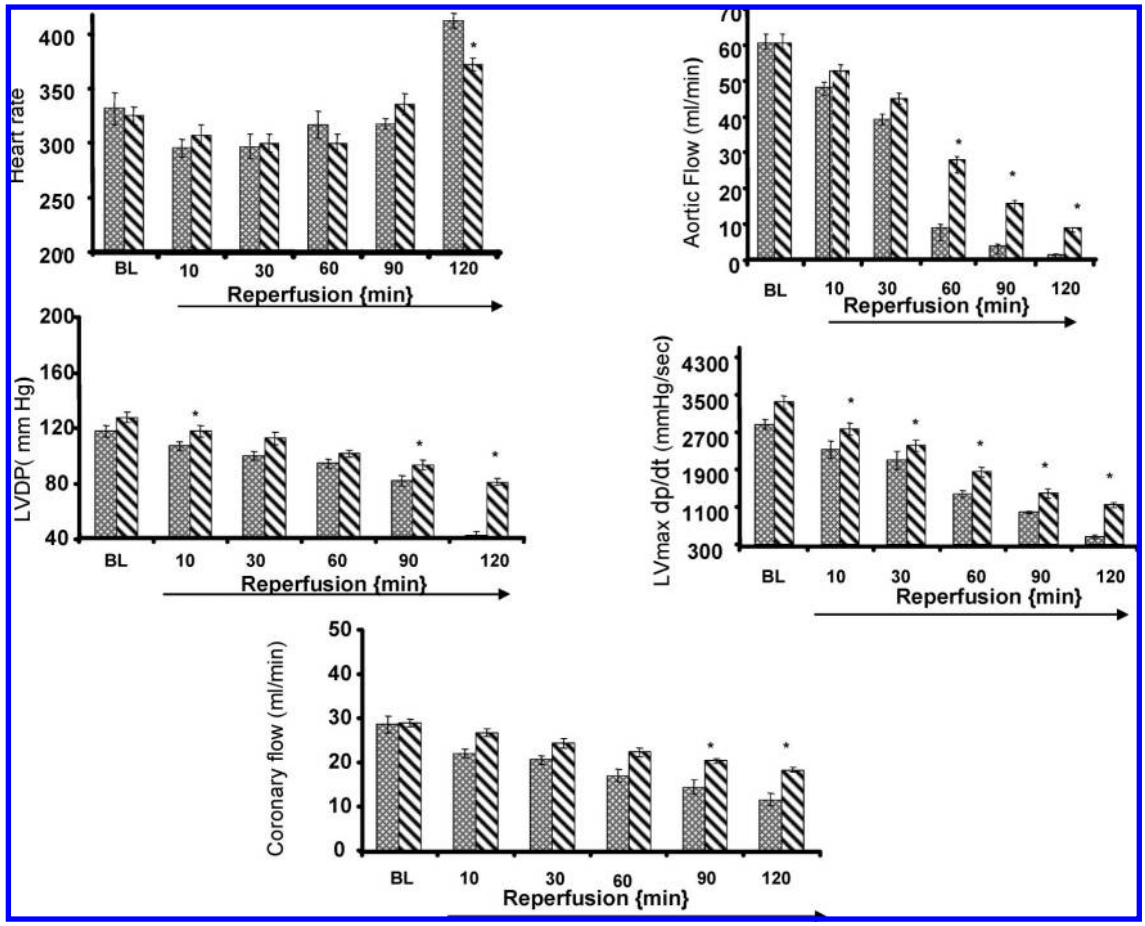

Figure 1. Effects of ischemia/reperfusion on heart rate (top left) aortic flow (top right), left ventricular developed pressure [LVDP] (middle left), maximum first derivative of developed pressure [LV_{max} dp/dt] (middle right), and coronary flow (bottom) of control rat heart (cross-hatched bars) and broccoli-treated rat heart (slashed bars). Results are shown as means ± SEM for six hearts in each group: ∗, $p < 0.05$ vs control.

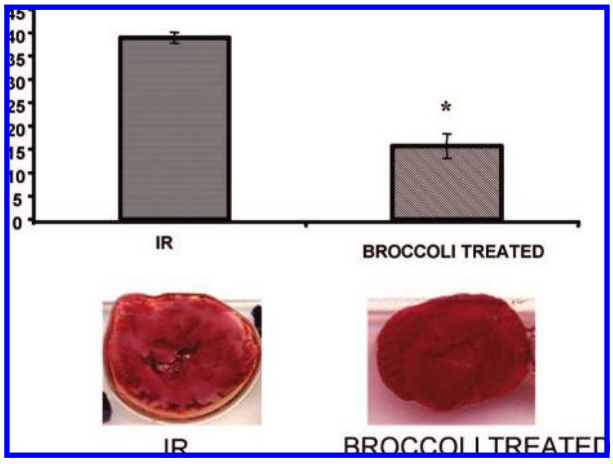

Figure 2. Effects of broccoli on myocardial infarct size. Isolated hearts from control rat ($n = 6$) and broccoli-treated groups ($n = 6$) were subjected to 30 min of global ischemia followed by 2 h of reperfusion in the working mode. Infarct size was measured by triphenyltetrazolium chloride (TTC) dye method. Values are shown as mean ± SEM: ∗, $p < 0.05$ vs control. Below the bar graph are shown representative photos for the myocardial infarct (white to yellow represents myocardial infarct).

of the cells. Cellular injury is directly related to changes of mitochondrial architecture including an irreversible loss of the matrix contents and integral membrane protein constituents such as cytochrome *c* (*14*). Once released, cytochrome *c* triggers the formation of an apoptotic complex, which readily activates caspase cascade initiated by caspase 9 leading to the activation of pro-caspase 3, the main executioner of apoptosis. The results of the present study showed an increased amount of cytochrome *c* and a reduced amount of pro-caspase 3 in the ischemic reperfused myocardium, which were completely reversed by broccoli, indicating its ability to generate antiapoptotic signal. Consistent with these findings, the number of apoptotic cardiomyocytes was significantly reduced by broccoli.

As mentioned earlier, broccoli contains significant amounts of glucosinolates, which can be hydrolyzed to produce isothiocyanates. Among isothiocyanates, sulforaphane has been studied extensively and found to possess abilities to induce phase II detoxification enzymes (*15*). Sulforaphane can also induce Trx through the antioxidant-responsive element (*16*). In ref *16* it was shown that ARE was involved in the mechanism of Trx induction by sulforaphane in RPE cells. The results of the present study confirmed this earlier study and further demonstrated that broccoli could prevent the loss of Nrf2 during ischemia/reperfusion.

Several recent studies from our laboratory have implicated a cardioprotective role of thioredoxins (*17–19*). The cellular changes associated with ischemic heart diseases are redox-regulated. Ischemia and reperfusion render the heart in the oxidized environment maintained by the stabilizing disulfides present in the extracellular surface, whereas the intracellular environment is maintained in the reduced state with the help of free sulfhydryl groups. The principal disulfide reductase responsible for maintaining the inside of the cell in the reduced state is thioredoxin (*20*). Thioredoxins and other members of the thioredoxin superfamily such as glutaredoxins and perox-

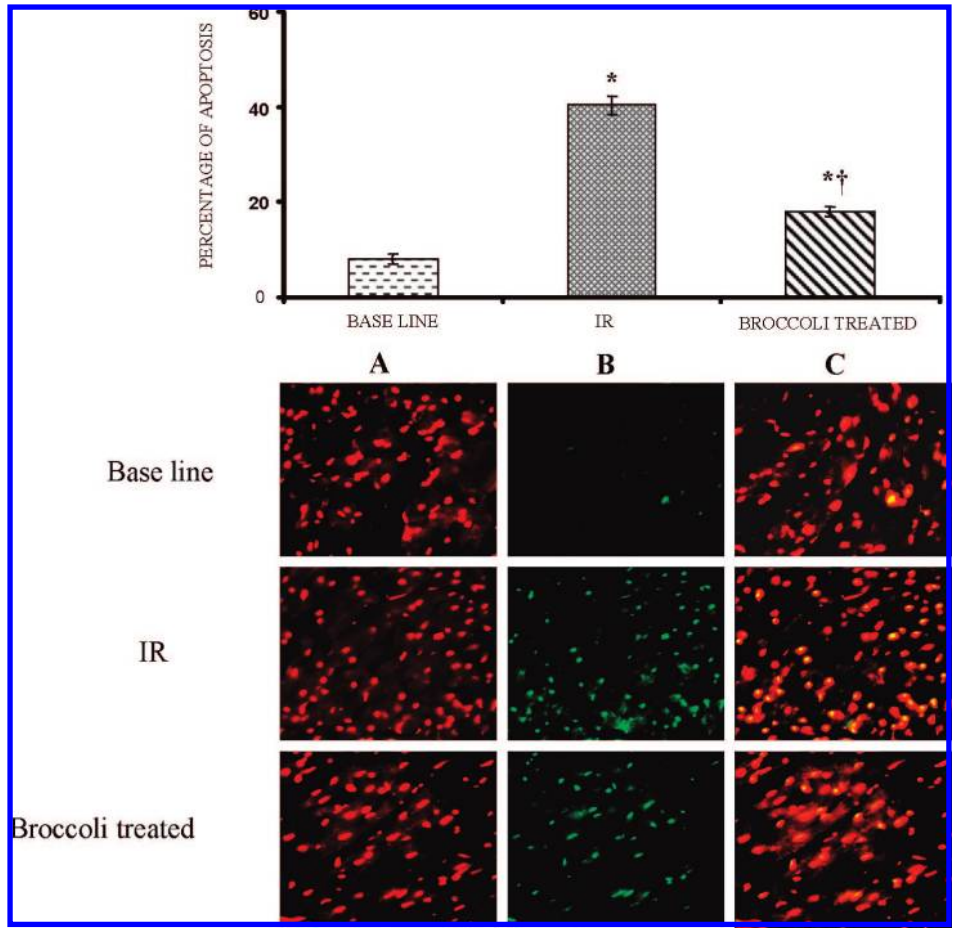

Figure 3. Effects of broccoli on cardiomyocyte apoptosis. Isolated heart from baseline hearts were perfused with the KHB buffer without subjection to ischemia and reperfusion: (**A**) total number of cells (red channel); (**B**) total number of apoptotic cells (green channels); (**C**) merged imaged of panels **A** and **B** (yellow dots in merged channel are the apoptotic cells). Values are mean ± SEM: ∗, $p < 0.05$ vs control; †, $p < 0.05$ vs ischemia reperfusion. Representative photomicrographs are shown below the bar graph.Isolated hearts from control rats ($n = 6$) and broccoli-treated rats ($n = 6$) were subjected to 30 min of global ischemia followed by 2 h of reperfusion in the working mode. Cardiomyocyte apoptosis was measured by Tdt-mediated dUTP nick end labeling with a Promega kit.

iredoxins are ubiquitously present in mammalian cells including hearts (*21*) and play an important role in maintaining the redox environment of the cell. Trx1 appears to play an important role in a variety of degenerative diseases including cardiovascular diseases. For example, Trx1 can reduce myocardial ischemia/reperfusion injury and decrease the incidence of ventricular fibrillation (*17–19, 22*). Measurements of plasma thioredoxin in healthy volunteers and during open-heart surgery demonstrated elevated levels of Trx in arterial plasma during reperfusion of the postcardioplegic heart of the patients (*23*). Trx increased during surgical preparation for cardiopulmonary bypass, but decreased during the bypass due to the release of oxidized Trx into the plasma. In an isolated perfused rat heart model, Trx protected the hearts from reperfusion-induced arrhythmia (*24*). In the early reperfusion period, h-thioredoxin reduced the incidence of ventricular fibrillation from 75% (untreated) to 42 and 25% at 0.01 and 0.1 μM concentrations, respectively. Interestingly, SOD was unable to protect these hearts from reperfusion-induced arrhythmias in this model. A recent study has indicated a possible association between plasma Trx concentrations and the severity of heart failure (*25*). Trx immunoreactivity was none to trivial in control specimens. Positive Trx staining was found in the hearts of the patients with active myocarditis and dilated cardiomyopathy. The positive staining was located in infiltrating cells and damaged myocytes in the perinecrotic lesions, suggesting that myocardial Trx was up-regulated in myocarditis and cardiomyopathy with active necrotic stage associated with DNA damage. In another related study, increased Trx expression was found in the skin biopsies (obtained during cardiac catheterization) of 29 of 35 patients with congestive heart failure, but in none of the 8 control subjects (*25*). Overexpression of Trx1 in the heart attenuated adriamycin-induced cardiotoxicity (*26*). Treatment with recombinant human Trx1 suppressed cardiomyocyte injury in adrenomycin-treated cardiomyocytes. In the present study, broccoli prevented ischemia/reperfusion mediated loss of total Trx protein as well as Trx2. In contrast, the mRNAs of both Trx1 and Trx2 were significantly increased as compared to those present in the nontreated hearts.

Thioredoxin reductase catalyzes the NADPH-dependent reduction of thioredoxin and plays an important role in the redox cycling of Trx (*27*). Selenium (Se) increases most selenoproteins involved in redox reactions including glutathione peroxidase and thioredoxin reductase, which contain a selenocysteine residue in the conserved C-terminal sequence Cys-SeCys- (*28*). In fact, thioredoxin reductase can reduce selenite into selenide (*29*). Thioredoxin reductase along with Trx forms a redox system that regulates a number of several other redox-regulated systems, thereby modulating the redox-sensitive transcription factor NFκB and oxidative stress (*30*). Cells with lower thioredoxin reductase activity are more susceptible to oxidative damage (*31*). Our results indicate significant loss of both mRNA

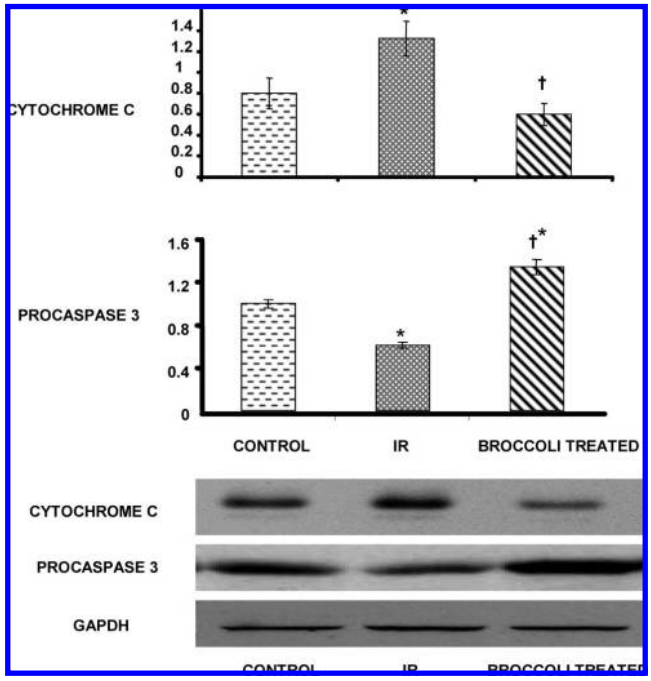

Figure 4. Western blot analysis of cytochrome *c* and pro-caspase 3 from cytosolic fraction of control (dashed bars), ischemic (cross-hatched bars), and broccoli-treated heart samples (slashed bars) (left ventricular tissue). Overexpression of cytochrome *c* was significantly blocked in broccoli-treated heart after ischemia/reperfusion compared to that of the ischemic heart. Expression of pro-caspase 3 was significantly reduced in ischemic heart, but in broccoli-treated heart the expression of pro-caspase 3 was significantly increased. GAPDH was used as loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times. Values are mean ± SEM: ∗, $p < 0.05$ vs control; †, $p < 0.05$ vs ischemia/reperfusion.

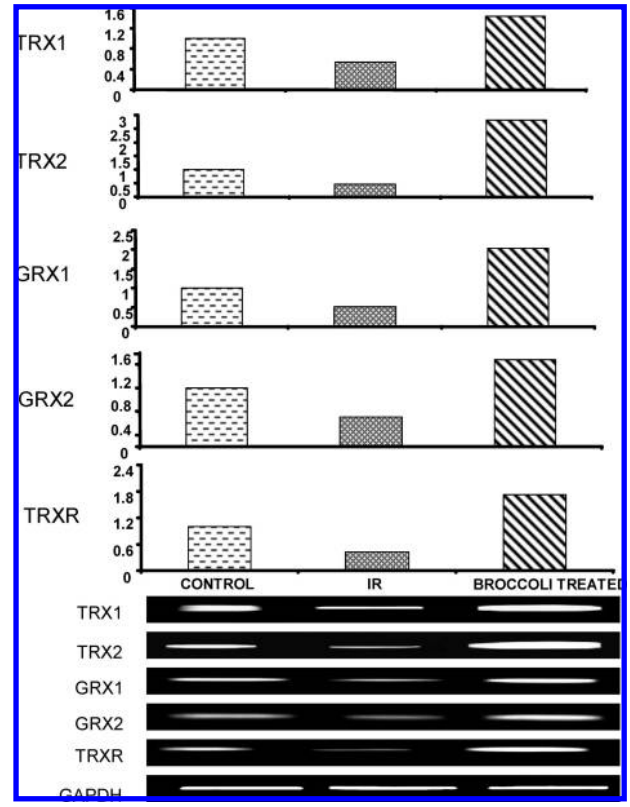

Figure 5. Total RNA was extracted from left ventricular tissue, and mRNA transcripts of Trx, Trx-reductase, and Grx were determined by RT-PCR from the isolated RNA of the control (dashed bars), ischemic reperfused (cross-hatched bars), and broccoli-treated heart samples (slashed bars). Ischemia/reperfusion reduced the transcripts of mRNAs of all Trx1, Trx2, Grx1, Grx2, and Trx-R, but broccoli up-regulated the transcripts of these mRNAs over control. RT-PCR of GAPDH was used as loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times.

and protein of thioredoxin reductase in the ischemic reperfused myocardium. Broccoli restored the thioredoxin reductase protein, but its mRNA level was significantly up-regulated as revealed by RT-PCR.

Glutaredoxin (Grx), also known as thiol transferase, is also a ubiquitous protein found in most of the organs including the heart. Two Grx genes have been found in mammals. Grx1 is initially considered to be a cytosolic enzyme. However, recent results of immunolabeling experiments have shown that it is also present in the nucleus (*32*). The second Grx (*Grx2*) gene, which was reported recently, encodes two proteins as a result of alteration of RNA splicing. One of the Grx2 isoforms is located in the mitochondria and the other in the nucleus (*33*). Similar to Trx, Grx also functions in catalyzing thiol/disulfide exchange (*34*). The active site of Grx [Cys-Pro-Tyr(Phe)-Cys] is oxidized during this process and is regenerated by using the reducing equivalents of GSH. The oxidized GSH is then recycled back to the reduced form of GSH by glutathione reductase. Because GSH is the major intracellular nonprotein thiol, it forms the majority of mixed disulfides inside the cells under oxidative stress. Grx1 is, therefore, expected to play a predominant role in reversing this process. In addition to its function in thiol/disulfide exchange, Grx1 also serves as an alternative electron donor to ribonucleotide reductase (*35*), participates in deiodination of thyroxin to triiodothyronine (*36*), and exhibits activity of dehydroascorbate reductase for regeneration of ascorbic acid (*37*). However, despite the potential role of Grx in the antioxidant defense mechanism, studies on its antioxidant function have been exceptionally limited. Our recent studies demonstrated that transgenic mice overexpressing Grx1 reduced the number of apoptotic cardiomyocytes in the ischemic reperfused heart (*38*). The results of the present study indicated down-regulation of both transcripts and proteins of Grx1 and Grx2 in the ischemic reperfused hearts. Broccoli increased the transcripts of both Grx1 and Grx2 compared to control, but the protein levels were restored only by broccoli.

Peroxiredoxins (Prdx), thioredoxin peroxidases, are the antioxidant components of the thioredoxin superfamily (*39*). Six Prdxs (Prdx1−Prdx6) have been identified, of which Prdx6 is found in the cytosolic fraction together with Prdx1, Prdx2, and Prdx4. Prdx4 is also found in mitochondria and peroxisome, whereas Prdx3 exists only in mitochondria. In the present study, we examined total Prdx activity and found that such activity was reduced in the ischemic reperfused myocardium and that broccoli could restore the activity.

Because sulforaphane is known to induce Trx through the antioxidant response element (ARE) (*16*), we determined the effects of broccoli on Nrf2. Redox-sensitive thiol groups of proteins such as Keap1, a cytosolic repressor of Nrf2, are involved in the regulation of redox responses, and isothiocyanates can activate a Keap1−Nrf2 redox-sensitive pathway that induces the expression of genes containing antioxidant responsive element (*40*). Sulforaphane appears to modify Keap1 from thionoacyl adducts, thereby releasing Nrf2 followed by its translocation into the nucleus and resulting in the activation of ARE-regulated genes (*41, 42*). The results of our study demonstrated down-regulation of Nrf2 in the ischemic reperfused myocardium and its restoration by broccoli.

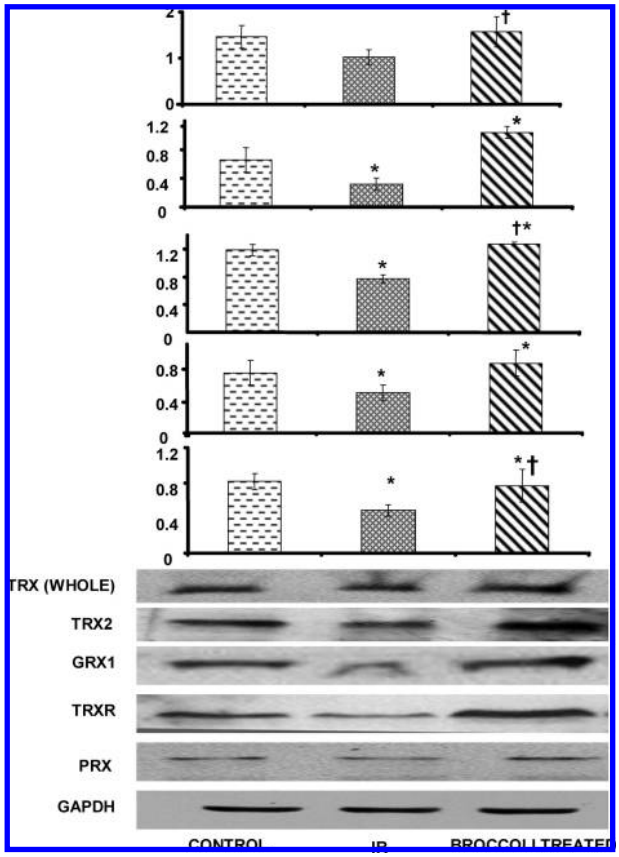

Figure 6. Western blot analysis of Trx (whole), Trx2, Grx1, Trx-R, and Prx from left ventricular tissue of time control (dashed bars), ischemic reperfused (cross-hatched bars), and broccoli-treated heart (slashed bars). Western blot analyses of Trx (whole), Grx1, Trx-R and Prx were performed with cytosolic fraction, and Western blot for Trx2 was performed with mitochondrial fraction. Expression of all of these Trx superfamily proteins was significantly reduced in ischemia reperfusion, but in broccoli-treated sample the reduction of expression of these proteins was blocked. GAPDH was used as loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times. Values are mean ± SEM: ∗, $p < 0.05$ vs control; †, $p < 0.05$ vs ischemia reperfusion.

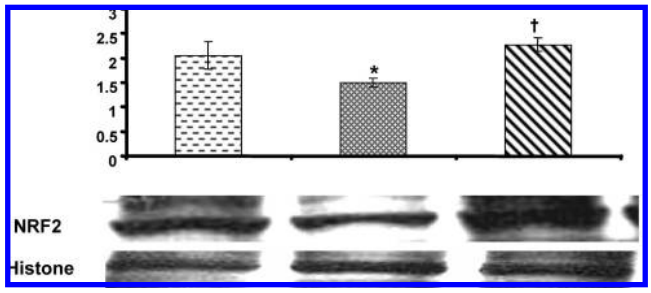

Figure 7. Western blot analysis of Nrf2 protein from nuclear fraction of control (dashed bar), ischemic reperfused (cross-hatched bar), and broccoli-treated heart sample (slahsed bar) (left ventricular tissue). Expression of Nrf2 was significantly down-regulated after ischemia and reperfusion, but in the case of broccoli-treated sample, down-regulation of Nrf2 expression after ischemia and reperfusion was prevented. Histone was used as loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times. Values are mean ± SEM: ∗, $p < 0.05$ vs control; †, $p < 0.05$ vs ischemia reperfusion.

Accumulating evidence supports the notion that Trx may facilitate the induction of HO-1 (*43*). Because HO-1 is a cardioprotective protein, we sought to determine if broccoli also

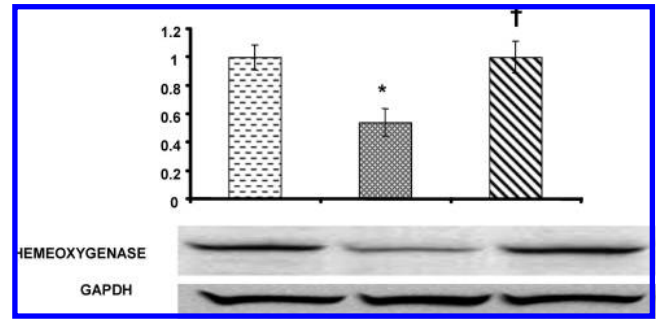

Figure 8. Western blot analysis of heme oxygenase protein from the cytosolic fraction of the heart: control (dashed bar), ischemic reperfused (cross-hatched bar), and broccoli-treated heart samples (slashed bar) (left ventricular tissue). In broccoli-treated samples the reduction of expression of heme oxygenase protein after ischemia reperfusion was prevented. GAPDH was used as loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times. Values are mean ± SEM: ∗, $p < 0.05$ vs control; †, $p < 0.05$ vs ischemia reperfusion.

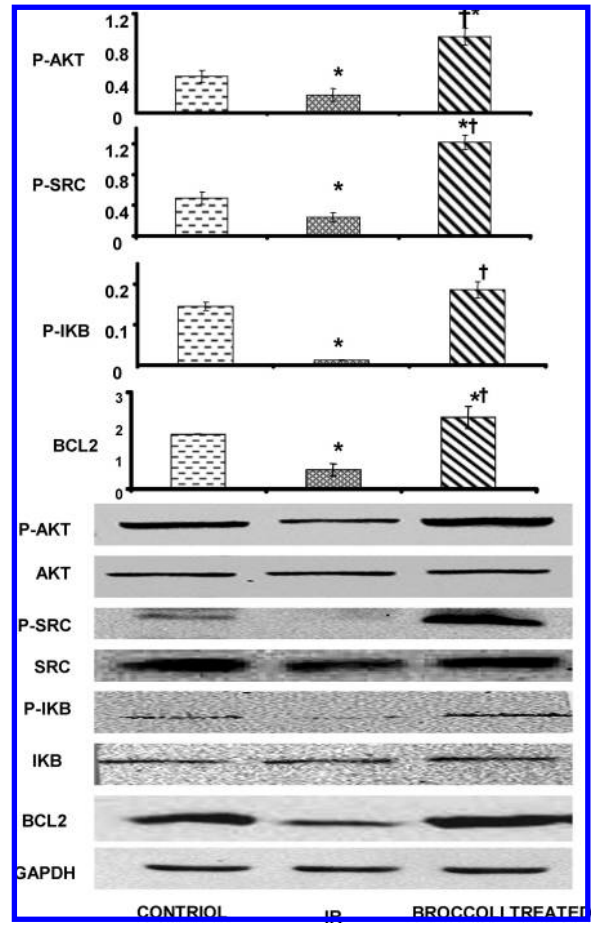

Figure 9. Western blot analysis of p-Akt, Akt, p-Src,Src, IkB, p-IkB, and Bcl2 proteins from cytosolic fractions of control (dashed bars), ischemic reperfused (cross-hatched bars), and broccoli-treated hearts (slashed bars). Expression of Akt, .cSrc, and Ikb proteins remained the same in all groups, but expression of p-Akt, p-Src,p-IkB was reduced after ischemia and reperfusion, and broccoli prevented such reduction. GAPDH was used as loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times. Values are mean ± SEM: ∗, $p < 0.05$ vs control; †, $p < 0.05$ vs ischemia control.

caused the transactivation of HO-1 through the induction of Trx. The results of our study showed down-regulation of HO-1 after

ischemia/reperfusion, which was prevented by broccoli. Although the mechanism of HO-1 induction was not determined, and whether Trx had a direct role in the induction of HO-1 was not confirmed, the prevention of ischemia/reperfusion-mediated loss of HO-1 protein by broccoli appears to play a role in cardioprotection.

Ischemia/reperfusion causes cardiomyocyte death by activating the death signaling pathway and/or inhibiting the survival signaling pathway (*44*). We examined several components of the survival pathway, which are known to be significantly affected by ischemia/reperfusion. Broccoli appears to rescue the cardiomyocytes in the ischemic reperfused heart through the PI-3-kinase-Akt survival pathway. Akt is a critical regulator of PI-3-kinase-mediated cell survival, and constitutive activation of Akt is sufficient to block cell death by a variety of apoptotic stimuli (*45*). We have also examined cSrc because Src tyrosine kinase acts as a membrane-attached molecular switch that links a variety of cues to crucial intracellular signaling pathways. cSrc has been implicated in mechanisms of cell survival and death, which are regulated by complex signal transduction processes (*46*). Our previous studies showed that activation of cSrc might represent a crucial step for cellular protection associated with ischemia and reperfusion (*47*). The present study showed that broccoli potentiated an increased phosphorylation of Akt and cSrc. Once activated, Akt can phosphorylate and inactivate proapoptotic proteins such as Bad and pro-caspase 9 and activate the antiapoptotic redox sensitive transcription factor NFκB, a finding consistent with our results that indicated increase in Bcl2 and activation of NFκB as evidenced by increased phosphorylation of IkB by broccoli.

NOTE ADDED AFTER ASAP PUBLICATION

In the version published ASAP on December 29, 2007, there was an error in the author affiliations. This was corrected in the version posted on January 16, 2008.

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