

## Green Tea Polyphenol Epigallocatechin-3-gallate Protects Cells against Peroxynitrite-Induced Cytotoxicity: Modulatory Effect of Cellular G6PD Status

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Glucose-6-phosphate dehydrogenase (G6PD) plays important roles in the maintenance of cellular redox balance. It was not until recently that the importance of G6PD in regulation of cellular growth and apoptosis emerged. In the present study, we found that G6PD-deficient fibroblasts were more susceptible to peroxynitrite-induced cytotoxicity. Treatment with peroxynitrite generator 3-morpholinosydnonimine (SIN-1) hydrochloride caused apoptosis in human fibroblast in a dose-dependent manner. This was preceded by a decrease in the intracellular level of glutathione (GSH) as well as accumulation of p53. The extent of apoptosis and glutathione depletion were greater in G6PD-deficient fibroblasts than in the normal counterpart. Pretreatment with green tea polyphenol epigallocatechin-3-gallate (EGCG) effectively blocked peroxynitrite-induced glutathione depletion, p53 accumulation, and apoptosis in both normal and G6PD-deficient cells. EGCG, administered to cells alone or as pretreatment, caused activation of Akt. The protective effect was abolished by phosphatidylinositol 3-kinase (PI3K) inhibitors, wortmannin, and LY294002. Our findings suggest that G6PD deficiency enhances the toxicity of peroxynitrite and that EGCG initiates cell survival signaling via the PI3K/akt pathway.

**KEYWORDS:** SIN-1; G6PD; peroxynitrite; apoptosis; glutathione; EGCG

### INTRODUCTION

The principal role of glucose-6-phosphate dehydrogenase (G6PD) is to generate reduced nicotinamide adenine dinucleotide phosphate (NADPH), which meets the cellular needs for reductive biosynthesis and maintenance of cellular redox homeostasis. The importance of G6PD is underscored by clinical manifestation of its deficiency. G6PD deficiency, a common enzymopathy affecting over 200 million individuals worldwide, can cause neonatal jaundice, drug- or infection-induced hemolytic crisis, favism, and, less commonly, nonspherocytic hemolytic anemia (1–3). Not until recently did novel findings on the role of G6PD in nucleated cells emerge. We have shown that G6PD-deficient fibroblasts grow at a reduced rate and have

an increased propensity for oxidant-induced senescence, compared with that of a normal control (4, 5). It is plausible that the G6PD status modulates cellular responses to growth factor or signaling molecules (6).

Nitric oxide (NO), as a signaling molecule, has multifarious physiological and pathogenic roles in different biological systems. The complexity of its biological effects lies in the formation of various derivatives as well as their numerous potential interactions with other molecules, such as metal ions and proteins (7). Reactive nitrogen species formed from NO can mediate oxidative or nitrosative stress. Peroxynitrite, the product from the reaction of nitric oxide with superoxide, is capable of oxidizing a wide variety of biomolecules (8–11). For instance, it causes the scission of DNA strand, particularly during replication and transcription (12). It oxidizes the tyrosine residue to 3-nitrotyrosine (13), possibly leading to alteration in tyrosine phosphorylation-dependent signaling, changes in protein conformation and subsequent inactivation, and/or enhancement of protein proteolysis. These molecular damages can be translated into cellular demise. Depending on condition, peroxynitrite can induce apoptosis or necrosis (14). Peroxynitrite-

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induced apoptosis has been reported in thymocytes (15), HL-60 cells (16), lymphoblastoid cells (17), dopaminergic SH-SY5Y cells (18), aortic endothelial cells (19), osteoblasts (20), HaCaT keratinocytes (21), myocytes (22), and islet cells (23). It is not completely understood how peroxynitrite triggers the apoptotic machinery. Being considered as a mediator of tissue injury in many biological systems, peroxynitrite is implicated in the pathophysiology of such diseases as acute endotoxemia, neurological disorders, atherosclerosis, and ischemia/reperfusion injury (24).

Tea, a beverage derived from the plant *Camellia sinensis*, is consumed widely around the world. Over 3 billion kilograms of tea are produced and consumed each year. Seventy-eight percent, 20%, and 2% of tea is black, green, and oolong tea, respectively (25). Green tea contains a group of polyphenolic compounds collectively known as catechins. These polyphenols include (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)-epicatechin (EC). These compounds, especially EGCG, possess profound pharmacological activities, such as antioxidant activity, inhibition of cell proliferation, modulation of cell cycle regulation, anticholesterolemic activity, suppression of angiogenesis, and anticarcinogenic effect (25–27).

We previously reported that G6PD-deficient fibroblasts and their normal counterparts exhibit completely different biological responses to NO (6). It is likely that the G6PD status, and hence intracellular redox milieu, determines how cells react to NO and its derivatives. In light of the fact that different reactive nitrogen species can elicit different biological effects, we examine whether G6PD status affects the cellular response to peroxynitrite. In this article, we report that SIN-1, a peroxynitrite-generating compound, exerted differential apoptogenic effect on G6PD-deficient and normal fibroblasts: the former were more susceptible to peroxynitrite-induced cytotoxicity than the latter. The cell death was preceded by glutathione depletion and accumulation of p53. Pretreatment with EGCG effectively reversed these molecular changes and protected the cells from apoptosis. The cytoprotective effect was associated with Akt activation and could be attenuated by wortmannin and LY294002, both of which are PI3K inhibitors. The present studies suggest that G6PD deficiency, and thus redox imbalance, predisposes cells to increased toxicity of peroxynitrite. EGCG preserves the intracellular glutathione reserve and spares the cells from peroxynitrite-induced apoptosis.

## MATERIALS AND METHODS

**Chemicals.** Unless stated otherwise, all chemicals were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, amphotericin, and trypan blue were purchased from Invitrogen (Carlsbad, CA). Antibodies to phosphorylated Akt (p-Ser 473), Akt, p53, and actin were available from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture and Treatments.** HFF1 cells carrying the Taiwan-Hakka (G6PD<sup>1376T</sup>) variant of the *G6PD* gene and the normal counterpart HFF3 cells were isolated as previously described (4–6). These cells were cultured in DMEM supplemented with 10% FCS, 100 units/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.25  $\mu$ g/mL of amphotericin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells at a population-doubling level (PDL) of 15–20 were used in experiments throughout the study.

For SIN-1 treatment, 2  $\times$  10<sup>5</sup> cells (in a 10 cm culture dish) or 2.5  $\times$  10<sup>3</sup> cells (in a well of a 96-well culture plate) were incubated with 2 mM or the indicated concentrations of SIN-1 in serum-free DMEM for 1 h. The medium was changed to complete medium, and cells were cultured for the indicated intervals. In experiments with EGCG and PI3K inhibitor, cells were preincubated with indicated concentrations

of EGCG, or EGCG in combination with 100 nM wortmannin or 10  $\mu$ M LY294002 for 30 min prior to 1 h of SIN-1 treatment.

**Determination of Cell Viability.** Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay (28). At the end of SIN-1 treatment, cells cultured in 96-well plates were incubated with 0.5 mg/mL of MTT at 37 °C for 2 h. The culture medium was removed, and 100  $\mu$ L of DMSO was added to solubilize the formazan formed. The absorbance of each well was measured using a microplate reader with a 570 nm test wavelength and a 690 nm reference wavelength.

**Determination of Intracellular Glutathione.** Intracellular glutathione level was assayed as described previously (29), with minor modification. Briefly stated, cells were washed twice with phosphate-buffered saline (PBS), incubated with 50  $\mu$ M monochlorobimane at 37 °C for 30 min, then washed and lysed in 0.2% Triton X-100 in PBS. Any debris was removed by centrifugation at 13 000g for 5 min. The concentration of protein in the supernatant was determined using Bio-Rad protein assay (Bio-Rad Laboratories, CA). The fluorescence in the supernatant was measured in a fluorescence microplate reader with excitation and emission wavelengths set at 400 and 480 nm, respectively. The concentration of GSH was calculated from standard curves and normalized with respect to protein concentration.

**Fluorescence Microscopy.** The nuclear morphology of the nuclei of the fibroblasts was observed by fluorescence microscopy. Cells cultured in slide chambers were fixed with 4% paraformaldehyde in PBS, for 30 min, and then stained with 10  $\mu$ M Hoechst 33342 at room temperature for 30 min. After a brief wash, the samples were observed under a fluorescence microscope with excitation and emission wavelengths of 355 and 465 nm, respectively.

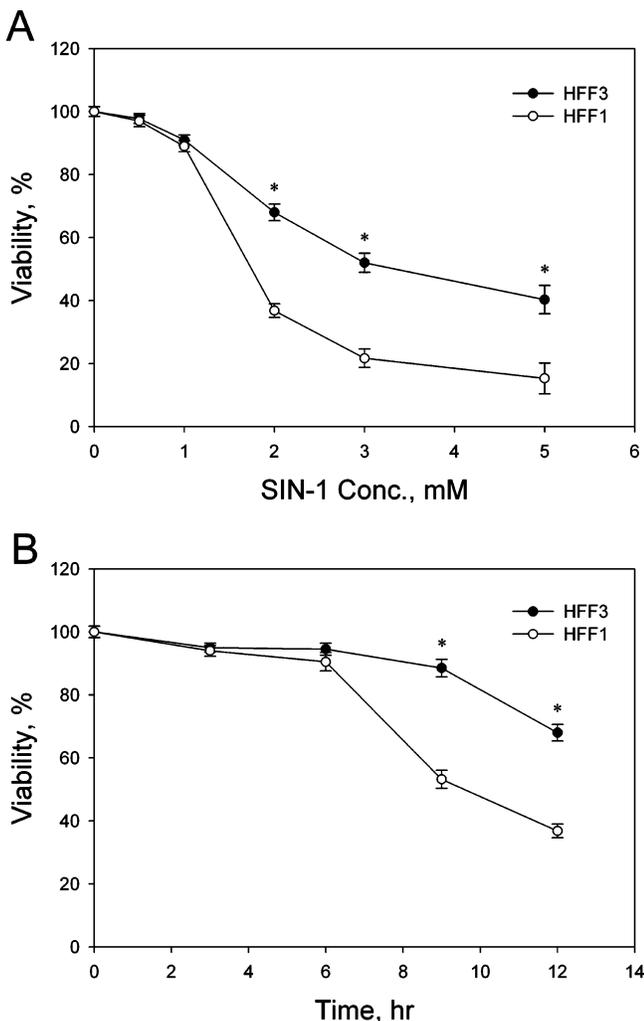
**Flow Cytometric Analysis of Apoptosis.** Cells were rinsed with ice-cold phosphate-buffered saline (PBS), trypsinized, and resuspended in 0.3 mL of PBS. They were then fixed and permeabilized by addition of 0.7 mL of ethanol. After a brief wash, the cells were gently resuspended in 1 mL of propidium iodide (PI) stain solution (40  $\mu$ g/mL propidium iodide (Sigma, St. Louis, MO), 100  $\mu$ g/mL RNase A, and 0.5% Triton X-100 in PBS) and incubated at room temperature for 30 min before analysis on a FACSCAN flow cytometer (Becton Dickinson, CA). The sub-G1 fraction is considered as the apoptotic cells and taken as a measure of the extent of apoptosis (30). The sub-G1 phase was quantified using Modfit software (Becton Dickinson, CA).

**Western Blotting.** The cells were rinsed with cold PBS, scraped, and collected by centrifugation. They were immediately lysed in lysis buffer (20 mM Tris-HCl (pH 8), 1% Triton X-100, 137 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM EGTA, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM  $\beta$ -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL aprotinin). The protein concentration of the lysate was determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA). The sample was analyzed by SDS-PAGE and immunoblotting with antibodies to phosphorylated Akt, Akt, p53, and actin according to manufacturers' instructions.

**Statistical Analysis.** Results are presented as mean  $\pm$  SD. Data were analyzed by *t*-test or two-way analysis of variance (ANOVA); *p* values of less than 0.05 were considered significant.

## RESULTS

**G6PD Deficiency Increases Susceptibility of Fibroblasts to Cytotoxicity of Peroxynitrite.** HFF1 cells were derived from the foreskin of a G6PD-deficient neonate, who had been identified to carry the Taiwan-Hakka variant (G6PD<sup>1376T</sup>) (4). The control HFF3 cells were isolated from the foreskin of a normal, sex- and age-matched infant. The G to T transversion caused a significant reduction in G6PD activity in HFF1 cells (0.11  $\pm$  0.005 U/mg of cell lysate) compared to that of HFF3 cells (0.3  $\pm$  0.01 U/mg of cell lysate). Treatment with peroxynitrite donor SIN-1 caused dose-dependent cell death in fibroblasts (Figure 1A). At low concentration of SIN-1 (<1 mM), the viability of HFF3 cells remained high; however, the viability dropped readily from 97.8  $\pm$  1.6% (*n* = 6) to 40.3  $\pm$

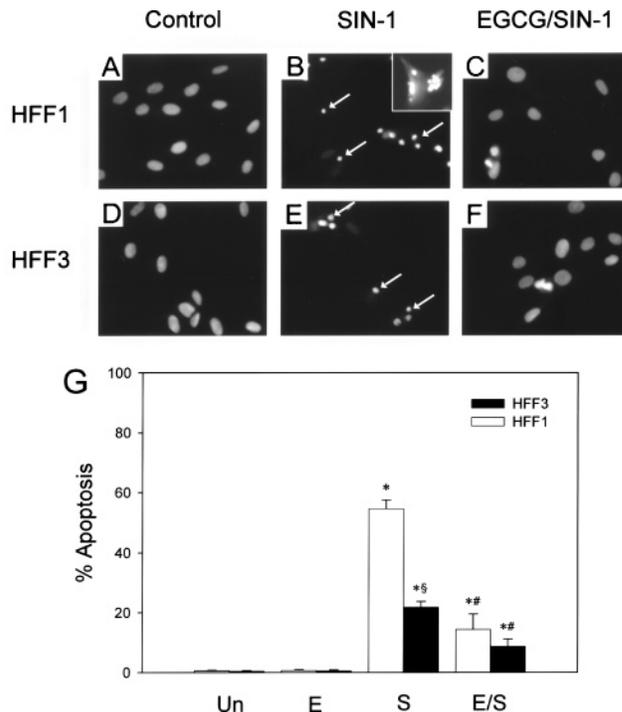


**Figure 1.** Effect of SIN-1 on the viability of HFF1 and HFF3 cells. (A) HFF1 or HFF3 cells were treated with the indicated concentrations of SIN-1 for 1 h. Cell viability was determined by MTT assay 12 h posttreatment and is expressed as the percentage of the untreated control. (B) HFF1 and HFF3 cells were treated with 2 mM SIN-1 for 1 h, and cell viability was determined at the indicated intervals after treatment. Data are presented as means  $\pm$  SD of six experiments. \*,  $p < 0.05$ , HFF3 vs HFF1.

4.5% ( $n = 6$ ) at SIN-1 concentrations ranging from 0.5 mM to 5 mM. Strikingly, G6PD deficiency enhances the cytotoxicity of peroxynitrite. Over 60% and 80% of HFF1 cells lost their viability at SIN-1 concentrations of 2 and 5 mM, respectively. When the temporal change in cell viability was followed after 1 h treatment with SIN-1, a significant decrease in this parameter was observed for both HFF1 and HFF3 from 9 h onward (Figure 1B). Apparently, HFF1 cells lost their viability much more rapidly than HFF3 cells. Nearly 50% of HFF1 cells underwent cell death 9 h posttreatment, whereas less than 15% of HFF3 cells did so at this time point. These data suggest that G6PD deficiency increases the cytotoxic effect of peroxynitrite.

#### Peroxyntirite-Induced Cell Death is Typical of Apoptosis.

Peroxyntirite-induced cell death was accompanied by nuclear fragmentation and formation of apoptotic bodies, which are small membrane-bound cell fragments containing highly condensed mass of DNA (Compare Figure 2, parts B and E with Figure 2, parts A and D). Microscopic examination revealed that a greater number of apoptotic bodies were present in HFF1 than in HFF3 cells. Such discrepancy was also revealed by the appearance of cells with a low DNA stainability (sub-G1

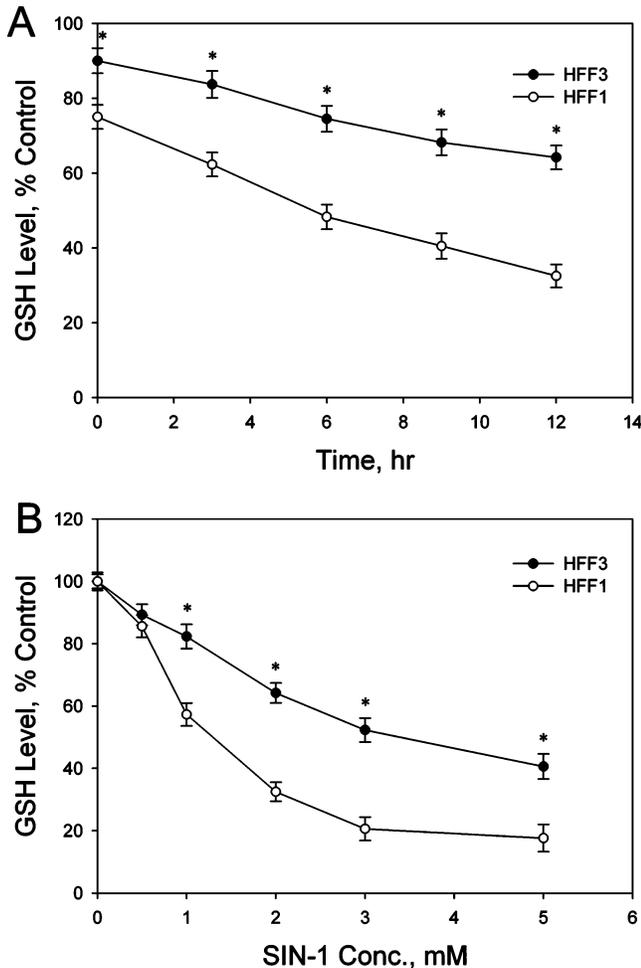


**Figure 2.** SIN-1 induced apoptosis in HFF1 and HFF3 cells. HFF1 (A–C) and HFF3 (D–F) cells were incubated with (C, F) or without (A, B, D, E) 50  $\mu$ M EGCG for 30 min and were subsequently treated with no (A, D) or 2 mM SIN-1 (B, C, E, F) for 1 h. Twelve hours later, the cells were stained with Hoechst 33342 and observed under a fluorescence microscope. White arrows indicate some typical apoptotic bodies. The inset of B shows a cell (on the right) undergoing cell blebbing and nuclear fragmentation (*karyorrhexis*) in that sample. The photographs shown here are representative of three experiments (original magnification:  $\times 200$ ). (G) HFF1 and HFF3 cells were untreated (Un) or treated with 50  $\mu$ M EGCG alone for 30 min (E), 2 mM SIN-1 for 1 h (S), or sequentially with 50  $\mu$ M EGCG for 30 min and 2 mM SIN-1 for 1 h (E/S). The level of apoptosis was analyzed by flow cytometry as described in the Materials and Methods. Data are presented as means  $\pm$  SD of six experiments. \*,  $p < 0.05$ , vs untreated control; §,  $p < 0.05$ , HFF3 vs HFF1; #,  $p < 0.05$ , cells with E/S treatment vs cells with S treatment.

fraction) when the SIN-1-treated samples were subject to flow cytometric analysis. As shown in Figure 2G, the extent of apoptosis in the untreated cells was negligible. Treatment with 2 mM SIN-1 resulted in  $54.21 \pm 3.01\%$  ( $n = 6$ ) and  $21.78 \pm 1.99\%$  ( $n = 6$ ) of apoptosis in HFF1 cells and HFF3 cells, respectively. It is clear that fibroblasts undergo apoptosis in response to peroxynitrite.

#### Peroxyntirite Causes Depletion of Intracellular GSH.

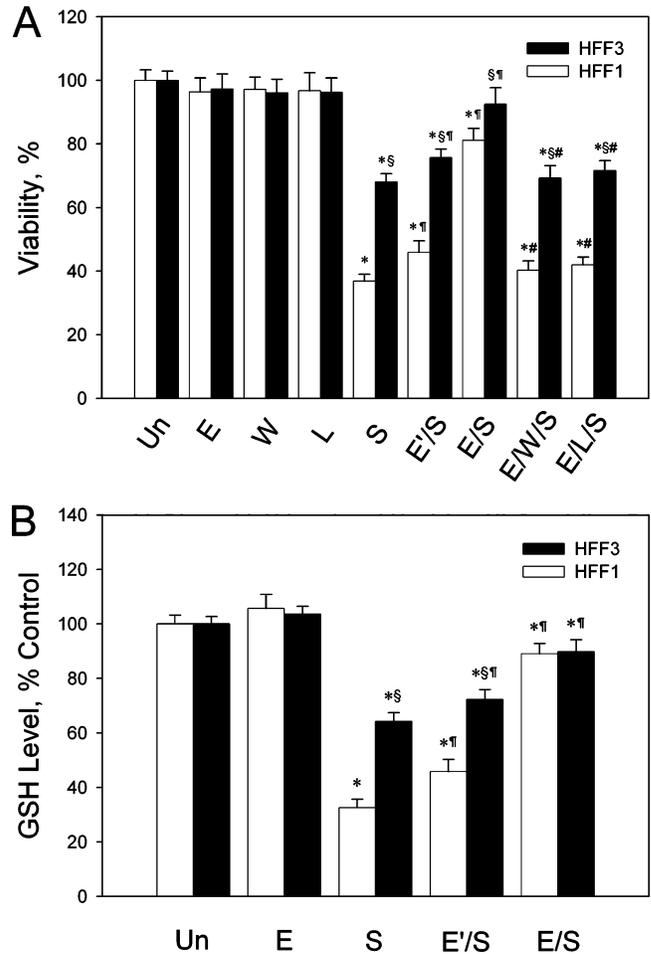
It can be hypothesized that G6PD deficiency debilitates the ability of cells to maintain a proper redox balance and sensitizes them to cytotoxic effect of peroxynitrite. To test such possibility, we examined the level of GSH, a putative target of peroxynitrite, in cells receiving SIN-1 treatment. Incubation with 2 mM SIN-1 caused small but significant changes in GSH content in both HFF1 and HFF3 cells just after treatment (Figure 3A). Such depletion became even more pronounced at later time points. The extent of GSH depletion was greater in HFF1 cells than in HFF3 cells at all the time points tested: the GSH levels decreased to  $32.5 \pm 3.1\%$  ( $n = 6$ ) in HFF1 cells versus  $64.2 \pm 3.2\%$  ( $n = 6$ ) in HFF3 cells at 12 h after treatment. Diminution of the glutathione level showed dose dependence (Figure 3B). Such effect was more prominent in HFF1 cells as compared



**Figure 3.** SIN-1 caused GSH depletion in HFF1 and HFF3 cells. (A) HFF1 and HFF3 cells were treated with 2 mM SIN-1 for 1 h. The intracellular GSH level was determined at the indicated intervals after treatment as described in the Materials and Methods and is expressed as the percentage of the untreated control. Data are presented as means  $\pm$  SD of six experiments. (B) HFF1 or HFF3 cells were treated with the indicated concentrations of SIN-1 for 1 h. The intracellular level of GSH was measured 12 h later. Data are presented as means  $\pm$  SD of six experiments. \*,  $p < 0.05$ , HFF3 vs HFF1.

with HFF3 cells. At SIN-1 concentrations ranging from 0.5 to 5 mM, the intracellular GSH level in cells was reduced from  $85.6 \pm 3.6\%$  ( $n = 6$ ) to  $17.7 \pm 4.4\%$  ( $n = 6$ ) in HFF1 cells, and from  $89.3 \pm 3.4\%$  ( $n = 6$ ) to  $40.6 \pm 4.1\%$  ( $n = 6$ ) in HFF3 cells. It is likely that GSH depletion represents one of the early events in peroxynitrite-induced apoptosis. In this manner, G6PD deficiency may weaken the capacity to maintain a proper GSH pool and exacerbate the situation.

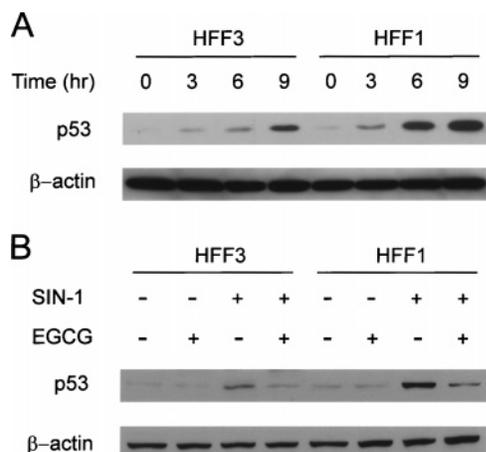
**EGCG Protects Cells against Peroxynitrite-Induced Apoptosis.** We investigated whether EGCG might protect fibroblasts from peroxynitrite-induced cell death. Thirty minutes' preincubation with 10  $\mu$ M EGCG significantly inhibited peroxynitrite-induced cell death (Figure 4A). Such protective effect displayed dose dependence. The viability of HFF1 and HFF3 cells, receiving pretreatment with 50  $\mu$ M EGCG and subsequent SIN-1 treatment, was elevated to  $81.1 \pm 3.8\%$  ( $n = 6$ ) and  $92.5 \pm 5.2\%$  ( $n = 6$ ), respectively. Consistent with this, preincubation with 50  $\mu$ M EGCG inhibited apoptosis, as judged by morphologic and flow cytometric criteria (Figure 2, parts C, F, and G). These EGCG-treated cells had a lower degree of nuclear fragmentation and formation of apoptotic bodies. The sub-G1



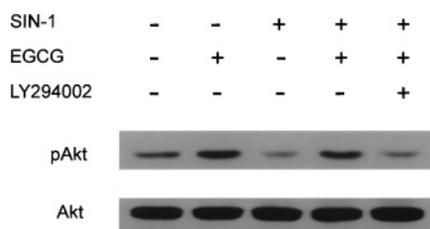
**Figure 4.** EGCG protected cells from glutathione depletion and peroxynitrite-induced loss of viability. (A) HFF1 and HFF3 cells were subject to various treatments: no treatment (Un); treatment with 50  $\mu$ M EGCG alone (E); treatment with 100 nM wortmannin alone (W); treatment with 10  $\mu$ M LY294002 alone (L); treatment with 2 mM SIN-1 (S); sequential treatment with 10  $\mu$ M EGCG and 2 mM SIN-1 (E/S); sequential treatment with 50  $\mu$ M EGCG and 2 mM SIN-1 (E/S); coinubation with 100 nM wortmannin and 50  $\mu$ M EGCG followed by SIN-1 treatment (E/W/S); coinubation with 10  $\mu$ M LY294002 and 50  $\mu$ M EGCG followed by SIN-1 treatment (E/L/S). Cell viability was determined as described in the legend to Figure 1. Data are presented as means  $\pm$  SD of six experiments. \*,  $p < 0.05$ , vs untreated control; §,  $p < 0.05$ , HFF3 vs HFF1; ¶,  $p < 0.05$ , cells with E/S or E/S treatment vs cells with S treatment; #,  $p < 0.05$ , cells with E/W/S or E/L/S treatment vs cells with E/S treatment. (B) HFF1 and HFF3 cells were subject to Un, E, S, E/S, or E/S treatment. The intracellular level of GSH was determined as described in the legend to Figure 3. Data are presented as means  $\pm$  SD of six experiments. \*,  $p < 0.05$ , vs untreated control; §,  $p < 0.05$ , HFF3 vs HFF1; ¶,  $p < 0.05$ , cells with E/S or E/S treatment vs cells with S treatment.

fraction of HFF1 cells with EGCG pretreatment decreased to  $14.29 \pm 5.21\%$  ( $n = 6$ ) and that of HFF3 cells to  $8.65 \pm 2.37\%$  ( $n = 6$ ) (Figure 2G).

It is wondered if EGCG could reverse the change in the GSH pool. We determined the GSH level in cells with SIN-1 treatment alone, or in combination with preincubation of EGCG. There was no significant change in the GSH content in cells treated with 50  $\mu$ M EGCG (Figure 4B). On the contrary, EGCG counteracted the SIN-1-induced exhaustion of GSH. After treatment with 50  $\mu$ M EGCG, the GSH pool increased to  $89.0 \pm 3.8\%$  ( $n = 6$ ) of control in HFF1 cells and  $89.8 \pm 4.4\%$  ( $n = 6$ ) in HFF3 cells.



**Figure 5.** Differential effect of EGCG and SIN-1 on p53 accumulation. (A) HFF1 and HFF3 cells were treated with 2 mM SIN-1 for 1 h. Cell lysates were prepared at the indicated intervals after treatment and analyzed by western blotting with antibody to p53. (B) HFF1 and HFF3 cells were incubated with or without 50  $\mu$ M EGCG for 30 min and then treated with 2 mM SIN-1 for an additional hour. Cell lysates were prepared at 9 h after treatment and subsequently analyzed by western blotting with antibody to p53. The blots shown here are representative of three experiments.



**Figure 6.** Activation of the PI3K/Akt pathway by EGCG. HFF1 cells were incubated with or without 50  $\mu$ M EGCG for 30 min in the presence or absence of 10  $\mu$ M LY294002. These cells were then treated with 2 mM SIN-1 for an additional hour. Cell lysates were prepared 30 min posttreatment and subsequently analyzed by western blotting with antibody to phosphorylated Akt (pAkt) and total Akt. The blots shown here are representative of three experiments.

**EGCG Reverses the Peroxynitrite-Induced p53 Accumulation.** The p53 tumor suppressor protein controls expression of genes involved in maintenance of genomic integrity, growth arrest, and apoptosis (31). To study if p53 is up-regulated during SIN-1-induced apoptosis, we performed western blotting with an extract from treated HFF1 and HFF3 cells. As shown in **Figure 5A**, there was an accumulation of p53 molecules in HFF1 cells at 3 h posttreatment. Likewise, a small but significant increase in p53 level was detected in HFF3 cells at the same time point. Apparently, p53 accumulates to a higher level in HFF1 cells at all the time points tested, reflecting a greater extent of apoptosis in these cells. In agreement with its antiapoptotic effect, pretreatment with 50  $\mu$ M EGCG suppressed the peroxynitrite-induced accumulation of p53 in both HFF1 and HFF3 cells (**Figure 5B**).

**PI3K Mediates the Protective Effect of EGCG.** PI3K/Akt is an important pathway signaling the antiapoptotic functions (32). It is speculative whether this pathway mediates the protective effect of EGCG. To test such possibility, we incubated HFF1 or HFF3 cells with EGCG in the presence of wortmannin or LY294002 before SIN-1 treatment and subsequently determined the cell viability. As shown in **Figure 4A**, the presence

of 10  $\mu$ M LY294002 or 100 nM wortmannin alone had little effect on viability. However, these compounds abolished the protective effect of EGCG on SIN-1-treated cells. LY294002 and wortmannin caused 88.3% and 92.1% inhibition of the cytoprotective effect of EGCG in HFF1. Similar levels of inhibition were found in HFF3 cells. Consistent with this, EGCG was found to affect phosphorylation and activation of Akt. As shown in **Figure 6**, treatment with EGCG alone led to robust activation of Akt, as shown by the phosphorylation at Ser<sup>473</sup>, which is essential to activation. In contrast, SIN-1 had an opposite effect. The level of Akt phosphorylation/activation remained unabated in cells subject to sequential treatments with EGCG and SIN-1. Akt activation was inhibited in the presence of LY294002. These findings suggest that the PI3K/Akt pathway may transmit the survival signal of EGCG.

## DISCUSSION

In the present study, we show that G6PD status is critical for modulation of cellular responses to peroxynitrite. Though SIN-1 treatment caused apoptosis in both G6PD-deficient and normal fibroblasts, the extent of apoptotic death was greater in the former than in the latter (**Figures 1 and 2**). Peroxynitrite-induced apoptosis was preceded by depletion of the cellular glutathione pool and p53 accumulation (**Figures 3 and 5**), implying that these events may be important in the initiation stage of apoptosis. EGCG inhibited these changes and conferred cytoprotection on SIN-1-treated cells (**Figures 4 and 5**). Such antiapoptotic effect may be attributed to activation of the PI3K/Akt pathway (**Figures 4 and 6**).

Reactive nitrogen species can exist in a number of chemical forms, such as NO<sup>+</sup>, NO<sup>•</sup>, NO<sup>-</sup>, NO<sub>2</sub>, N<sub>2</sub>O<sub>3</sub>, ONOO<sup>-</sup>, and ONOOCO<sub>2</sub>. These species show a multitude of chemical reactivities, for instance, oxidation, nitrosation, and nitration, toward various biological molecules (7). Being released from SIN-1, NO and O<sub>2</sub><sup>-•</sup> are present in equimolar quantities and react to form peroxynitrite at a constant rate. Peroxynitrite is highly reactive. The reported activities include nitrosation/nitration and hydroxylation of aromatic compounds, including tyrosine and tryptophan, as well as S-nitrosation/oxidation of sulfhydryl- and thioether-containing compounds (7, 13, 33). Reactions of peroxynitrite with biomolecules have been shown to influence cellular functions. Tyrosine nitration has been implicated in dysfunction of superoxide dismutase, actin, and cytochrome P450 (34); covalent modification of thiol groups in Ras, glyceraldehydes-3-phosphate dehydrogenase, and creatine kinase may affect their functions (35–37). Furthermore, peroxynitrite converts glutathione to disulfide, draining the cellular antioxidant capacity. These molecular and cellular changes may evoke the apoptotic events, such as p53 accumulation.

Peroxynitrite-induced GSH depletion was prolonged with respect to SIN-1 treatment. The initial fall in GSH level (i.e., during and/or just after SIN-1 treatment) may be, at least, in part accounted for by the direct nonenzymatic reaction between GSH and peroxynitrite. The decrement after the 1 h treatment is probably the consequence of alternative pathways of GSH depletion. Peroxynitrite inhibits superoxide dismutase and glutaredoxin (38–41), resulting in intracellular oxidant production and consumption of GSH. Moreover, repair processes themselves may use up GSH. Glutathione S-transferase and glutathione peroxidase catalyze the GSH-dependent repair, such as denitration/denitrosation (42, 43), of modified proteins. Excessive repair activities may further drain the SIN-1-treated cells of GSH reserve. In support of this, it was shown that cells isolated from glutathione peroxidase 1 (GPX1) knockout mice

are resistant to peroxynitrite-induced apoptosis (44). It is probable that these mechanisms operate in parallel in cells to effect the SIN-1-induced GSH depletion.

It is not unprecedented that glutathione depletion can trigger apoptosis (45–47). Here we show that depletion of intracellular GSH occurred immediately after SIN-1 treatment and preceded p53 accumulation and morphological changes characteristic of apoptosis. Moreover, EGCG that prevented GSH depletion also blocked apoptosis. Thus, GSH depletion may play an important role in peroxynitrite-induced apoptosis. This adequately explains why G6PD status modulates the cytotoxicity of peroxynitrite. As an NADPH-generating system, G6PD helps maintain a proper GSH pool and, hence, offers resistance to apoptosis. It follows that G6PD-deficient cells had considerably higher susceptibility to peroxynitrite. These findings underscore the essential role of G6PD in redox homeostasis and cell survival.

Several mechanisms may account for the inhibitory effect of EGCG on peroxynitrite-induced apoptosis. EGCG may work by efficiently scavenging the precursors of peroxynitrite, NO, and superoxide (48, 49) and by preventing its interaction with targets (50). Additionally, EGCG may function via an indirect manner. The present study shows that EGCG caused phosphorylation and activation of Akt, whose activity remained high even after SIN-1 treatment. Wortmannin and LY294002 that inhibited PI3K nullified the cytoprotective effect of EGCG. The PI3K/Akt pathway has antiapoptotic properties, as shown in a variety of experimental systems with overexpression of constitutively active or dominant negative mutants of Akt (51). Akt-mediated phosphorylation may alter the activity of proteins such as NF- $\kappa$ B, forkhead transcription factors, and Bcl-2 family members (32). It has been recently shown that LY294002 potentiates the arsenic oxide-induced apoptosis via GSH depletion, suggesting the involvement of PI3K/Akt pathway in regulation of cellular GSH reserve (52, 53). Whether EGCG modulates GSH metabolism through PI3K/Akt activation remains to be clarified. Whatever the mechanism, Akt appears to be a prime mediator of the cytoprotective effect of EGCG.

Our results may have clinical implications. For example, peroxynitrite has been implicated in pathological processes of such diseases as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (54). The susceptibility of different brain cell types to peroxynitrite may depend on factors such as the intracellular GSH content. Glutathione depletion is the earliest biochemical change in the substantia nigra of Parkinsonian patients and precedes the decrease in mitochondrial function and dopamine level (55). The loss of GSH results in tyrosine nitration of mitochondrial complex subunits in cultured dopaminergic cells (56). The converse is also true. Administration with  $\gamma$ -glutamylcysteine ethyl ester protects gerbils' cortices against peroxynitrite-induced oxidative stress (57). Though these findings suggest a neuroprotective role for GSH, the beneficial effect of GSH is not restricted to the nervous system. Glutathione supplementation enhances survival during peroxynitrite-mediated liver injury in mice (58). Taken together with these reports, our findings suggest that administration of EGCG could be used, as a means of GSH augmentation, for therapeutic intervention of degenerative diseases. This supports the notion that habitual green tea drinking might help to prevent or delay the onset of these diseases. Furthermore, the finding that the antiapoptotic effect of EGCG can be nullified by PI3K inhibitors has another implication: the protective effects and therapeutic usefulness of EGCG could be interfered by other compounds. More studies should be conducted to study interactions between EGCG and pharmacologic compounds.

In summary, the present study demonstrates that peroxynitrite-induced apoptosis is accompanied by GSH depletion, and as such, it explains how G6PD status affects the sensitivity of cells to peroxynitrite. Moreover, EGCG restores the cellular GSH reserve and protects cells from peroxynitrite-induced apoptosis.

## ABBREVIATIONS USED

G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; SIN-1, 3-morpholininosydnonimine; PI3K, phosphatidylinositol 3-kinase; EGCG, epigallocatechin gallate; Akt, the cellular homologue of the oncogene transduced by the acute transforming retrovirus AKT8 (Akt is also known as protein kinase B); HFF1, G6PD-deficient fibroblasts; HFF3, normal fibroblasts.

## LITERATURE CITED

- (1) Luzzatto, L.; Battistuzzi, G. Glucose-6-phosphate dehydrogenase. *Adv. Hum. Genet.* **1985**, *14*, 217–329.
- (2) Beutler, E. The genetics of glucose-6-phosphate dehydrogenase deficiency. *Semin. Hematol.* **1990**, *27*, 137–164.
- (3) Beutler, E. Glucose-6-phosphate dehydrogenase deficiency. *N. Engl. J. Med.* **1991**, *324*, 169–174.
- (4) Ho, H. Y.; Cheng, M. L.; Lu, F. J.; Chou, Y. H.; Stern, A.; Liang, C. M.; Chiu, D. T. Y. Enhanced oxidative stress and accelerated cellular senescence in glucose-6-phosphate dehydrogenase (G6PD)-deficient human fibroblasts. *Free Radical Biol. Med.* **2000**, *29*, 156–169.
- (5) Cheng, M. L.; Ho, H. Y.; Wu, Y. H.; Chiu, D. T. Y. Glucose-6-phosphate dehydrogenase-deficient cells show an increased propensity for oxidant-induced senescence. *Free Radical Biol. Med.* **2004**, *36*, 580–591.
- (6) Cheng, M. L.; Ho, H. Y.; Liang, C. M.; Chou, Y. H.; Stern, A.; Lu, F. J.; Chiu, D. T. Y. Cellular glucose-6-phosphate dehydrogenase (G6PD) status modulates the effects of nitric oxide (NO) on human foreskin fibroblasts. *FEBS Lett.* **2000**, *475*, 257–262.
- (7) Wink, D. A.; Mitchell, J. B. Chemical biology of nitric oxide: insight into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biol. Med.* **1998**, *25*, 434–456.
- (8) Pryor, W. A.; Jin, X.; Squadrito, G. L. One- and two-electron oxidations of methionine by peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11173–11177.
- (9) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. Peroxynitrite oxidation of sulfhydryls: the cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **1991**, *266*, 4244–4250.
- (10) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **1991**, *288*, 481–487.
- (11) Moreno, J. J.; Pryor, W. A. Inactivation of  $\alpha$ -1-proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol.* **1992**, *5*, 425–431.
- (12) Wink, D. A.; Hanbauer, I.; Grisham, M. B.; Laval, F.; Nims, R. W.; Laval, J.; Cook, J.; Pacelli, R.; Liebmann, J.; Krishna, M.; Ford, P. C.; Mitchell, J. B. Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. *Curr. Top. Cell. Regul.* **1996**, *34*, 159–187.
- (13) Radi, R.; Peluffo, G.; Alvarez, M. N.; Naviliat, M.; Cayota, A. Unraveling peroxynitrite formation in biological systems. *Free Radical Biol. Med.* **2001**, *30*, 463–488.
- (14) Kim, P. K. M.; Zamora, R.; Petrosko, P.; Billiar, T. R. The regulatory role of nitric oxide in apoptosis. *Int. Immunopharmacol.* **2001**, *1*, 1421–1441.
- (15) Virag, L.; Scott, G. S.; Cuzzocrea, S.; Marmer, D.; Salzman, A. L.; Szabo, C. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly(ADP-ribose) synthetase (PARS) activation. *Immunology* **1998**, *94*, 345–355.

- (16) Lin, K. Y.; Xue, J. Y.; Lin, M. C.; Spokas, E. G.; Sun, F. F.; Wong, P. Y. Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. *Am. J. Physiol.* **1998**, *274*, C855–C860.
- (17) Li, C. Q.; Trudel, L. J.; Wogan, G. N. Genotoxicity, mitochondrial damage, and apoptosis in human lymphoblastoid cells exposed to peroxynitrite generated from SIN-1. *Chem. Res. Toxicol.* **2002**, *15*, 527–535.
- (18) Saeki, M.; Kamisaki, Y.; Maeda, S. Involvement of mitogen-activated protein kinase in peroxynitrite-induced cell death of human neuroblastoma SH-SY5Y cells. *Neurosci. Res.* **2000**, *38*, 213–216.
- (19) Foresti, R.; Sarathchandra, P.; Clark, J. E.; Green, C. J.; Motterlini, R. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem. J.* **1999**, *339*, 729–736.
- (20) Reiff, D. A.; Kelpke, S.; Rue, L.; Thompson, J. A. Acidic fibroblast growth factor attenuates the cytotoxic effects of peroxynitrite in primary human osteoblast precursors. *J. Trauma* **2001**, *50*, 433–438.
- (21) Szabo, E.; Virag, L.; Bakondi, E.; Gyure, L.; Hasko, G.; Bai, P.; Hunyadi, J.; Gergely, P.; Szabo, C. Peroxynitrite production, DNA breakage and poly(ADP-ribose) polymerase activation in a mouse model of oxazolone-induced contact hypersensitivity. *J. Invest. Dermatol.* **2001**, *117*, 74–80.
- (22) Arstall, M. A.; Sawyer, D. B.; Fukazawa, R.; Kelly, R. A. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ. Res.* **1999**, *85*, 829–840.
- (23) Hadjivassiliou, V.; Green, M. H.; James, R. F.; Swift, S. M.; Clayton, H. A.; Green, I. C. Insulin secretion, DNA damage, and apoptosis in human and rat islets of Langerhans following exposure to nitric oxide, peroxynitrite, and cytokines. *Nitric Oxide* **1998**, *2*, 429–441.
- (24) Beckman, J. S.; Koppenol, W. H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. *Am. J. Physiol.* **1996**, *271*, C1424–C1437.
- (25) Yang, C. S.; Landau, J. M. Effects of tea consumption on nutrition and health. *J. Nutr.* **2000**, *130*, 2409–2412.
- (26) Ahmad, N.; Mukhtar, H. Green tea polyphenols and cancer: biologic mechanisms and practical implications. *Nutr. Rev.* **1999**, *57*, 78–83.
- (27) Yang, C. S.; Maliakal, P.; Meng, X. Inhibition of carcinogenesis by tea. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 25–54.
- (28) Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **1986**, *89*, 271–277.
- (29) Papadopoulos, M. C.; Koumenis, I. L.; Dugan, L. L.; Giffard, R. G. Vulnerability to glucose deprivation injury correlates with glutathione levels in astrocytes. *Brain Res.* **1997**, *748*, 151–156.
- (30) Nicoletti, I.; Migliorati, G.; Pagliacci, M. C.; Grignani, F.; Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **1991**, *139*, 271–279.
- (31) Sionov, R. V.; Haupt, Y. The cellular response to p53: the decision between life and death. *Oncogene* **1999**, *18*, 6145–6157.
- (32) Osaki, M.; Oshimura, M.; Ito, H. PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis* **2004**, *9*, 667–676.
- (33) Szabo, C. Multiple pathways of peroxynitrite cytotoxicity. *Toxicol. Lett.* **2003**, *140–141*, 105–112.
- (34) Greenacre, S. A.; Ischiropoulos, H. Tyrosine nitration: localization, quantification, consequences for protein function and signal transduction. *Free Radical Res.* **2001**, *34*, 541–581.
- (35) Lander, H. M.; Hajjar, D. P.; Hempstead, B. L.; Mirza, U. A.; Chait, B. T.; Campbell, S.; Quilliam, L. A. A molecular redox switch on p21ras: Structural basis for the nitric oxide-p21<sup>ras</sup> interaction. *J. Biol. Chem.* **1997**, *272*, 4323–4326.
- (36) Mohr, S.; Stamler, J. S.; Brune, B. Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite and related nitrosating agents. *FEBS Lett.* **1994**, *348*, 223–227.
- (37) Konorev, E. A.; Hogg, N.; Kalyanaraman, B. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett.* **1998**, *427*, 171–174.
- (38) Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **1992**, *298*, 431–437.
- (39) MacMillan-Crow, L. A.; Crow, J. P.; Thompson, J. A. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry* **1998**, *37*, 1613–1622.
- (40) Mohr, S.; Stamler, J. S.; Brune, B. Mechanism of covalent modification of glyceraldehyde-3-phosphate dehydrogenase at its active site thiol by nitric oxide, peroxynitrite and related nitrosating agents. *FEBS Lett.* **1994**, *348*, 223–227.
- (41) Aykac-Toker, G.; Bulgurcuoglu, S.; Kocak-Toker, N. Effect of peroxynitrite on glutaredoxin. *Hum. Exp. Toxicol.* **2001**, *20*, 373–376.
- (42) Kuo, W. N.; Kocis, J. M.; Mewar, M. Protein denitration/modification by glutathione-S-transferase and glutathione peroxidase. *J. Biochem. Mol. Biol. Biophys.* **2002**, *6*, 143–146.
- (43) Kuo, W. N.; Kocis, J. M. Nitration/S-nitrosation of proteins by peroxynitrite-treatment and subsequent modification by glutathione S-transferase and glutathione peroxidase. *Mol. Cell. Biochem.* **2002**, *233*, 57–63.
- (44) Fu, Y.; Sies, H.; Lei, X. G. Opposite roles of selenium-dependent glutathione peroxidase-1 in superoxide generator diquat- and peroxynitrite-induced apoptosis and signaling. *J. Biol. Chem.* **2001**, *276*, 43004–43009.
- (45) Armstrong, J. S.; Steinauer, K. K.; Hornung, B.; Irish, J. M.; Lecane, P.; Birrell, G. W.; Peehl, D. M.; Knox, S. J. Role of glutathione depletion and reactive oxygen species generation in apoptotic signaling in a human B lymphoma cell line. *Cell Death Differ.* **2002**, *9*, 252–263.
- (46) Suzuki, S.; Higuchi, M.; Proske, R. J.; Oridate, N.; Hong, W. K.; Lotan, R. Implication of mitochondria-derived reactive oxygen species, cytochrome C and caspase-3 in N-(4-hydroxyphenyl)retinamide-induced apoptosis in cervical carcinoma cells. *Oncogene* **1999**, *18*, 6380–6387.
- (47) Sanchez, A.; Alvarez, A. M.; Benito, M.; Fabregat, I. Apoptosis induced by transforming growth factor-beta in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates. *J. Biol. Chem.* **1996**, *271*, 7416–7422.
- (48) Haenen, G. R.; Paquay, J. B.; Korthouwer, R. E.; Bast, A. Peroxynitrite scavenging by flavonoids. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 591–593.
- (49) Robak, J.; Gryglewski, R. J. Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **1988**, *37*, 837–841.
- (50) Pannala, A. S.; Rice-Evans, C. A.; Halliwell, B.; Singh, S. Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem. Biophys. Res. Commun.* **1997**, *232*, 164–168.
- (51) Datta, S. R.; Brunet, A.; Greenberg, M. E. Cellular survival: a play in three Acts. *Genes Dev.* **1999**, *13*, 2905–2927.
- (52) Ramos, A. M.; Fernandez, C.; Amran, D.; Sancho, P.; de Blas, E.; Aller, P. Pharmacologic inhibitors of PI3K/Akt potentiate the apoptotic action of the antileukemic drug arsenic trioxide via glutathione depletion and increased peroxide accumulation in myeloid leukemia cells. *Blood* **2005**, *105*, 4013–4020.
- (53) Koh, S. H.; Kim, S. H.; Kwon, H.; Kim, J. G.; Kim, J. H.; Yang, K. H.; Kim, J.; Kim, S. U.; Yu, H. J.; Do, B. R.; Kim, K. S.; Jung, H. K. Phosphatidylinositol-3 kinase/Akt and GSK-3 mediated cytoprotective effect of epigallocatechin gallate on oxidative stress-injured neuronal-differentiated N18D3 cells. *Neurotoxicology* **2004**, *25*, 793–802.
- (54) Torreilles, F.; Salman-Tabcheh, S.; Guerin, M. C.; Torreilles, J. Neurodegenerative disorders: the role of peroxynitrite. *Brain Res. Rev.* **1999**, *30*, 153–163.

- (55) Sriram, K.; Shankar, S. K.; Boyd, M. R.; Ravindranath, V. Thiol oxidation and loss of mitochondrial complex I precede excitatory amino acid-mediated neurodegeneration. *J. Neurosci.* **1998**, *18*, 10287–10296.
- (56) Bharath, S.; Andersen, J. K. Glutathione depletion in a midbrain-derived immortalized dopaminergic cell line results in limited tyrosine nitration of mitochondrial complex I subunits: implications for Parkinson's disease. *Antioxid. Redox Signaling* **2005**, *7*, 900–910.
- (57) Drake, J.; Kanski, J.; Varadarajan, S.; Tsoras, M.; Butterfield, D. A. Elevation of brain glutathione by  $\gamma$ -glutamylcysteine ethyl ester protects against peroxynitrite-induced oxidative stress. *J. Neurosci. Res.* **2002**, *68*, 776–784.
- (58) Bajt, M. L.; Knight, T. R.; Farhood, A.; Jaeschke, H. Scavenging peroxynitrite with glutathione promotes regeneration and enhances survival during acetaminophen-induced liver injury in mice. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 67–73.

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