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5-Hydroxytrytophan Inhibits *tert*-Butylhydroperoxide (*t*-BHP)-Induced Oxidative Damage via the Suppression of Reactive Species (RS) and Nuclear Factor-κB (NF-κB) Activation on Human Fibroblast

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5-Hydroxytryptophan (5HTP), an analogue of tryptophan, is a precursor of serotonin that also has effective antioxidative and anti-apoptotic properties (*1*). However, the cellular mechanisms underlying these properties of 5HTP have not been explored. In this study, we tested the hypothesis that 5HTP exerts its antioxidative action against oxidative stress and inflammation by suppressing the activation of the key pro-inflammatory transcriptional pathways, p38 mitogen-activated protein kinase (p38MAPK) and nuclear factor-κB (NF-κB). The study was carried out using human fibroblast cells that were challenged by *tert*-butylhydroperoxide (*t*-BHP)-induced oxidative damage. Results show that 5HTP significantly reduced *t*-BHP-induced oxidative damage in human fibroblast cells, as determined by cell cytotoxicity, intracellular reactive species (RS) and peroxynitrite ($ONOO^-$) generation, and inducible nitric oxide synthase expression. Moreover, 5HTP protected human fibroblast cells against *t*-BHP-induced oxidative DNA damage, as determined by 4,6-diamidino-2-phenylindole (DAPI) staining. Pretreatment of human fibroblast cells with 5HTP also dose-dependently inhibited glutathione (GSH) depletion, indicating that it protects cells against *t*-BHP-induced oxidative damage. Western blot analysis revealed that 5HTP also markedly increased Bcl-2 expression and suppressed both p38MAPK and NF-κB activation in the *t*-BHP-treated human fibroblast cells. When these results are taken together, they strongly indicate that 5HTP has beneficial and protective effects against *t*-BHP-induced cell death *in vitro*, as demonstrated by its antioxidative and anti-inflammatory actions. Data further showed that the protective mechanisms underlying the actions of 5HTP against oxidative stress-induced damage are associated with RS/$ONOO^-$ scavenging and the inhibition of lipid peroxidation and GSH depletion.

KEYWORDS: 5-Hydroxytryptophan; *tert*-butylhydroperoxide; oxidative stress; human fibroblast; apoptosis

1. INTRODUCTION

Cellular oxidative stress can be induced by various endogenous processes, whereby the balance between oxidants and oxidative reductants slants toward an oxidative state. Major sources of oxidants are derived from oxygen- and nitrogen-based reactive species (RS), including reactive oxygen species (ROS) and reactive nitrogen species (RNS) (*2*), and lipid peroxidation byproducts.

RS are generated under normal physiologic conditions, including various cellular metabolic processes, and some species (e.g., hydrogen peroxide) may function as an important physiologic mediator. However, when RS overproduction is not well-regulated, increased oxidative stress may result that could have far-reaching consequences (*3*). Oxidative stress caused by RS has also been linked to apoptosis (*4*−*6*). One of the most diverse consequences of oxidative stress is a disrupted redox balance, which is known to activate multiple cellular signaling pathways, among them the activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) (*7*).

A number of pro-oxidants, such as *tert*-butylhydroperoxide (*t*-BHP), are implicated in cell damage from oxidative stress. As a

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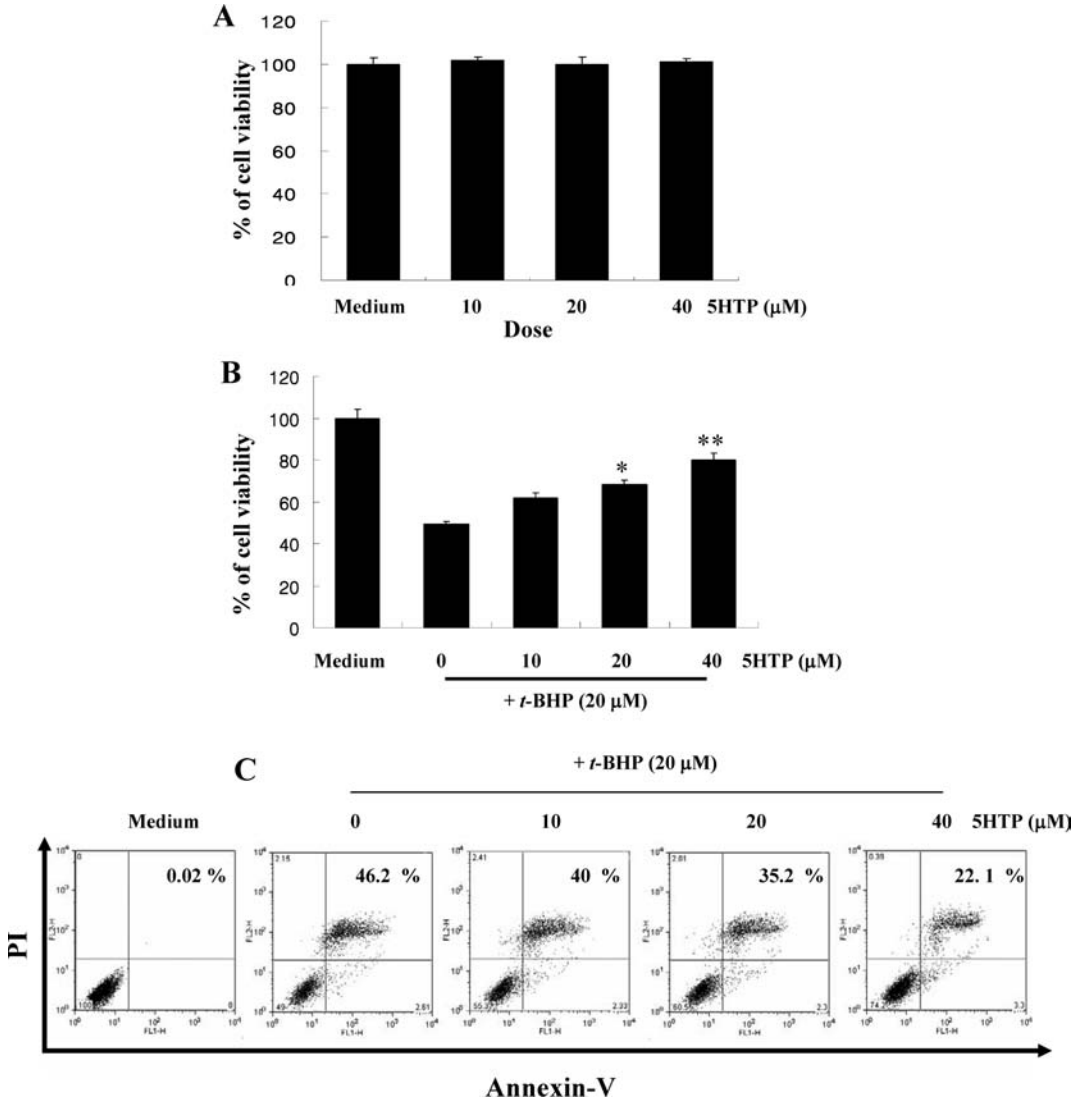

Figure 1. Effect of 5HTP on cell viability and *t*-BHP-induced cytotoxicity in human fibroblast cells. Human fibroblast cells at the concentration of 1×10^5 cells/well were preincubated using various concentrations of 5HTP for 2 h and then incubated with 20 μM *t*-BHP for another 24 h. (A and B) Cell viability was assessed by the MTT reduction assay and expressed as the percentage of absorbance values of the control group. (C) Cells analyzed by two-color flow cytometry using annexin-V/PI staining kit are shown. The cells in the control group were cultured in the medium alone, and the cells in 20 μM *t*-BHP-treated alone were used as the positive group. Values represent means $\pm$ SD of triplicate, independent experiments. A difference was considered statistically significant when (*) $p < 0.05$ and (**) $p < 0.01$ against only the *t*-BHP-treated group.

short-chain analogue of lipid hydroperoxides, *t*-BHP has been used as a model compound to investigate the mechanisms of cell damage initiated by oxidative stress (*8*) and can be metabolized to initiate lipid peroxidation and damage cell integrity (*9*).

One of the most physiologically important RS derived from nitrogen is nitric oxide (NO), and its essential role in vascular function is well-recognized. However, unregulated NO could have far-reaching, adverse effects as a cytotoxic mediator by reacting with hydrogen peroxide or superoxide to produce the potent oxidant, peroxynitrite ($ONOO^-$) (*10*). $ONOO^-$ may act to modify lipid peroxidation and cytosolic proteins, inactivate key enzymes (*11*), and mediate apoptosis in various kinds of cells (*12*).

5-Hydroxytryptophan (5HTP) is a well-known precursor of serotonin, having psychoactive properties (*13*). However, 5HTP also has antioxidative and anti-inflammatory properties that have not been fully explored, particularly with regard to the molecular basis of its actions, which are almost unknown to date.

In this study, we investigated the protective effects of 5HTP against *t*-BHP-induced oxidative stress and apoptosis in human fibroblast cells by examining several markers of oxidative damage and apoptosis-related proteins, such as intracellular glutathione (GSH), lipid peroxidation, RS and $ONOO^-$ generation, DNA damage, inducible nitric oxide synthase (iNOS), as well as Bax and Bcl-2 levels.

In this report, we present data showing that 5HTP inhibits *t*-BHP-induced oxidative damage and apoptosis through the downregulation of RS and iNOS expression in human fibroblast cells.

2. MATERIALS AND METHODS

2.1. Materials. Human fibroblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA). 5HTP and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich Chemical (St. Louis, MO), as were all reagents, unless specifically stated elsewhere. Fetal bovine serum (FBS), penicillin−streptomycin, and trypsin−ethylenediaminetetraacetic acid (EDTA) were purchased from Gibico BRL (Ground Island, NY). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was provided by Duchefa Biochemie (Haarlem, The Netherlands). Antibodies of human Bax and Bcl-2 were purchased

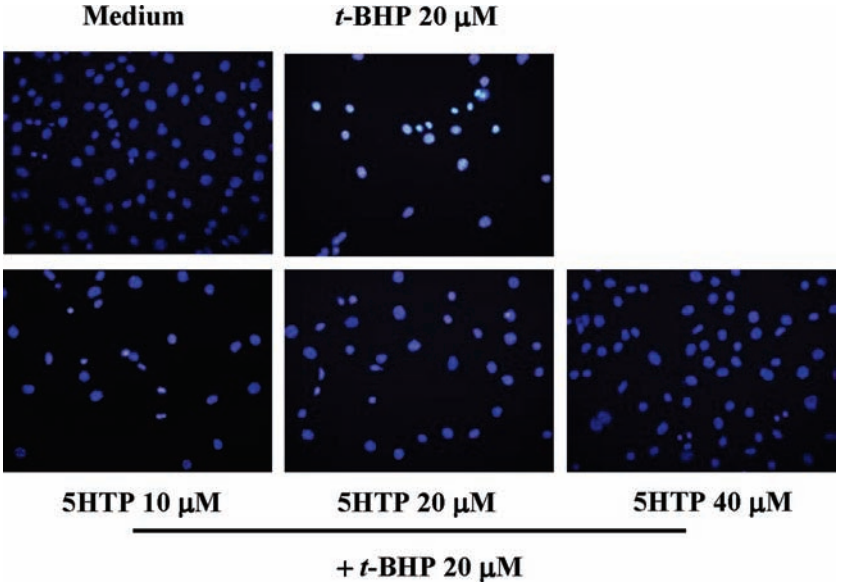

Figure 2. Effect of 5HTP on *t*-BHP-induced oxidative DNA damage in human fibroblast cells. Cells were treated with 20 μM *t*-BHP after pretreatment with 5HTP (10, 20, or 40 μM) for 2 h, and oxidative DNA damage was evaluated using DAPI staining, as described in the Materials and Methods. The DAPI-stained cells were evaluated using fluorescence microscopy (400×). One representative result is presented from triplicate experiments that yielded similar results.

form Santa Cruz Biotechnology (Santa Cruz, CA). Human α-tubulin antibody and 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Horseradish-peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were provide by Santa Cruz Biotechnology (Santa Cruz, CA). 5HTP was dissolved in dimethyl sulfoxide (DMSO) for culturing with cells, and the final culture concentration of DMSO was $<0.1\%$.

2.2. Cell Culture and MTT Assay. Human fibroblast cells were cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Fibroblast cells were plated at 90−95% confluence in all experiments. Nontoxic concentrations of 5HTP used in 24 h culture experiments were ≤40 μM, as determined by the MTT assay. At the end of the incubation period, the MTT assay was performed to quantify cellular viability. Human fibroblast cells were incubated in a fresh medium containing 0.5 mg/mL MTT for 4 h at 37 °C. After removal of unconverted MTT, the purple formazan product was dissolved in 0.5 mL of DMSO through shaking. The absorbance of formazan dye was measured colorimetrically at $\lambda = 570$ nm.

2.3. Flow Cytometry Assay. Flow cytometric analysis of human fibroblasts was performed using annexin-V and propidium iodide (PI) staining kit. After incubation of cells for 24 h with either 0.1% DMEM (as a medium control) and 20 μM *t*-BHP only or pretreatment of 5HTP (10, 20, and 40 μM), the cells were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and then incubated with fluorescein isothiocyanate (FITC)-conjugated annexin-V and PI (BD Biosciences Pharmingen, San Diego, CA). The fluorescein-stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.4. 4,6-Diamidino-2-phenylindole (DAPI) Staining. Cell death was studied morphologically using a fluorescent nuclear dye, DAPI. Apoptotic cells with condensed chromatin show nuclear fragmentation that produce characteristic, irregular unclear staining. Briefly, human fibroblast cells treated with various concentrations of 5HTP and 20 μM *t*-BHP were washed with PBS. Cells were fixed with 4% paraformaldehyde and permeabilized by incubation in 0.1% sodium citrate containing 0.1% Triton X-100 for 5 min at 4 °C. They were then stained with DAPI (1 μg/mL) solution for 30 min at 25 °C and examined by a fluorescence microscopy under blue light (BX50-FLA, Olympus, Tokyo, Japan).

2.5. Lipid Peroxidation and RS Production Assays. The malondialdehyde (MDA) level was measured by the method described by Draper and Hadley (*14*). The color produced by the reaction of thiobarbituric acid with MDA was measured at 533 nm. The result was expressed as picomoles of MDA per milligram of protein.

The intracellular generation of RS was measured using carboxy-H_2DCF-DA, a cell-permeable dye. Inside the cells, this compound is oxidized by ROS to form a fluorescent carboxydichlorofluorescein (DCF). Briefly, cells seeded in 6-well plates at 1×10^5 cells/well were treated with or without 5HTP and *t*-BHP and then incubated with 10 μM carboxy-H_2DCF-DA at 37 °C for 10 min. The cells were then washed twice with PBS. The rate of oxidation of the dye in the cells was monitored by flow cytometry.

2.6. Glutathione Assay. After 24 h of passage of cells into 60 mm culture dishes, the cells were incubated with or without 5HTP and *t*-BHP for 24 h. The medium was aspirated, and the cells were washed twice with PBS. A total of 1 mL of 10% trichloroacetic acid (TCA) was added to each dish, and the sample was left in ice for 30 min. The mixture was sonicated and centrifuged at 12 000 rpm for 10 min. Reduced GSH was measured fluorometrically using the fluorochrome *ortho*-phthaladehyde (OPT) by the method by Hissin and Hilf (*15*).

2.7. Measurement of Peroxynitrite. $ONOO^-$ generation was assessed by $ONOO^-$-dependent oxidation of dihydrorhodamine 123 (DHR123) to rhodamine 123 (RH123) as described previously (*16*). Cell were incubated with DHR123 (20 μM, Molecular Probe, Eugene, OR) for 30 min, and the fluorescence of RH123 was measured. Briefly, cells seeded in 6-well plates at 1×10^5 cells/well and treated with or without 5HTP and *t*-BHP were incubated with 20 μM DHR123 at 37 °C for 30 min. The cells were then washed twice with PBS. The rate of oxidation of the dye in the cells was monitored by flow cytometry.

2.8. Measurements of iNOS, p38 Mitogen-Activated Protein Kinase (p38MAPK), NF-κB, Bax, and Bcl-2 Expression. Human fibroblast cells were lysed in an ice-cold, whole-cell lysate buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride for 30 min at 4 °C. Cell debris was remove by microcentrifugation, and supernatants were quickly frozen. Protein concentrations were measured using Bio-Rad colorimetric protein assay kits (Bio-Rad, Hercules, CA). Proteins (30 μg) were separated on 10 or 12% sodium dodecyl sulfate (SDS)−polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell GmbH, Dassel, Germany). Anti-α-tubulin, anti-iNOS antibodies, anti-phospho-p38MAK, anti-p38MAPK, anti-phospho-p65, anti-Bax, and anti-Bcl-2 antibodies (Cell Signaling, Danvers, MA) were used as primary antibodies, and horseradish-peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Amersham Pharmacia Biotech GmbH, Freiburg, Germany) were used as the secondary antibodies. Bands were detected by enhanced chemiliminescence (ECL) kits.

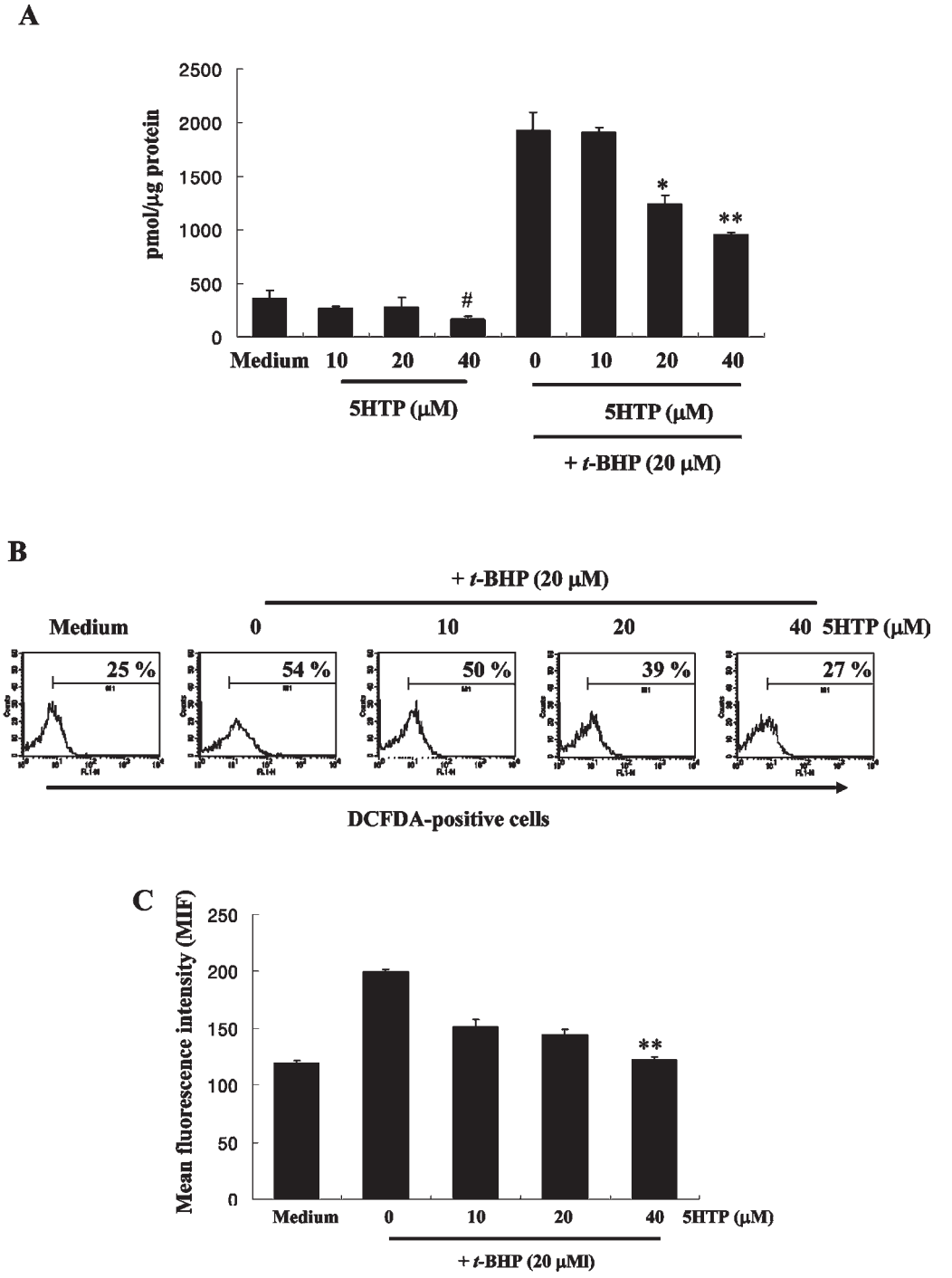

Figure 3. Effects of 5HTP on lipid peroxidation and RS generation by *t*-BHP in human fibroblast cells. (A) Human fibroblast cells were pretreated with 5HTP (10, 20, or 40 μM) for 2 h; then 20 μM *t*-BHP was added for a period of 24 h; and then lipid peroxidation was evaluated by the MDA assay. The formation of ROS after 12 h of *t*-BHP exposure was detected using $DCFH_2$-DA oxidation and fluorescence in the pretreatment of 5HTP. Human fibroblast cells were cultured for 12 h in standard DMEM containing 10% FBS. The rate of DCF formation was then evaluated by (B) flow cytometry and (C) mean fluorescence intensity. The results are representative of triplicate, independent experiments. (#) $p < 0.05$ versus the control and (∗) $p < 0.05$ and (∗∗) $p < 0.01$ versus the group treated by only 20 μM *t*-BHP.

2.9. Statistical Analysis. Results are presented as the means ± standard deviation (SD). Data were analyzed by one-way analysis of variation (ANOVA) followed by Duncan's post hoc test using SPSS version 11.0 (SPSS, Inc., Chicago, IL). The differences were considered statistically significant when p values were < 0.05.

3. RESULTS

3.1. Protection against *t*-BHP-Induced Cytotoxicity by 5HTP in Human Fibroblast Cells. To test the extent of the protective action of 5HTP against *t*-BHP-induced cytotoxicity, human fibroblast cells were incubated with different concentrations (10, 20, and 40 μM) of 5HTP for 24 h and then cell viability was evaluated by determining the percentage of cell viability.

As shown in **Figure 1A**, there were no marked differences in the percentage of dead cells, as evidenced by the MTT assay. To test a protective effect of 5HTP against *t*-BHP was evaluated by measuring the extent of inhibition of *t*-BHP-induced reduction

in cell percentages using human fibroblast cells. The total cell percentage by the MTT assay was indicative of the proliferative activity of the human fibroblast cells, and reductions of cell percentages were associated with *t*-BHP-induced cell death. Treatment with *t*-BHP induced significant decreases in total cell numbers, while pretreatment with 5HTP inhibited *t*-BHP-induced cell death in a dose-dependent manner (**Figure 1B**). We also confirmed this finding by annexin-V/PI staining, in which pretreatment with 5HTP reduced *t*-BHP-induced cell death, while *t*-BHP treatment in the absence of 5HTP induced significant increases in annexin-V/PI double-positive cells.

3.2. Attenuation of *t*-BHP-Induced DNA Damage by 5HTP in Human Fibroblast Cells. Condensation and degradation of chromosomal DNA are cardinal features of apoptosis. The nuclear staining assay was used to assess the morphological changes of apoptosis in human fibroblast cells. The effect of 5HTP at a concentration range of 10−40 μM on the chromatin condensation of *t*-BHP-treated human fibroblast cells was determined by DAPI staining.

As shown in **Figure 2**, the normal cells exhibited uniformly dispersed chromatin and intact cell membranes. On the other hand, treatment with *t*-BHP (20 μM) for 24 h caused approximately 52% of the cells to undergo DNA condensation. We also found that *t*-BHP-induced DNA condensation was decreased by around 80% by pretreatment with 5HTP (40 μM). These results indicated that 5HTP impairs *t*-BHP-induced DNA condensation in human fibroblast cells.

3.3. Suppression of *t*-BHP-Induced Lipid Peroxidation and Intracellular ROS Production by 5HTP. Lipid peroxidation and ROS production are well-established mechanisms of cellular injury. In the case of lipid peroxidation, it leads to the production of aldehydes and, finally, to the destruction of membrane lipids. RS are reported to induce cell death, including apoptosis and lipid peroxidation. To explore whether 5HTP modulates the induction of lipid peroxidation and ROS generation by *t*-BHP treatment, we followed the formation of MDA and intracellular ROS generation. Treatment with 5HTP alone did not change the amount of MDA produced in comparison to the medium group (chemically untreated). However, exposure to 20 μM *t*-BHP for 12 h increased the amount of MDA in the human fibroblast cells, and 5HTP treatment significantly reduced *t*-BHP-induced MDA production in a dose-dependent manner (**Figure 3A**). Next, to confirm that 5HTP suppressed *t*-BHP-induced oxidative stress in human fibroblast cells, we assessed intracellular ROS generation using the fluorescent probe carboxy-H_2DCF-DA assay. When cells treated with *t*-BHP were compared to those pretreated with 5HTP, we found that increased ROS was clearly suppressed in a dose-dependent manner (panels **B** and **C** of **Figure 3**).

3.4. Suppression of *t*-BHP-Induced GSH Depletion and iNOS Expression by 5HTP. To further examine the inhibitory effects of 5HTP on *t*-BHP-induced oxidative damage in human fibroblast cells, GSH levels and iNOS expression were determined. GSH is a well-known non-protein antioxidant that provides electrons for enzymes, such as GSH peroxidase, which reduces RS. GSH has been shown to be crucial for the regulation of apoptosis and cell proliferation (*17*). Therefore, we analyzed the effects of 5HTP on changes in GSH levels and *t*-BHP-induced cell death in human fibroblast cells (**Figure 4A**).

5HTP was found to markedly and dose-dependently inhibit GSH depletion, indicating that it protects cells against *t*-BHP-induced oxidative damage. Another factor that might participate in ROS-related apoptosis is iNOS expression because it synthesizes a large amount of NO production over long periods of time. Western blot analysis was used to measure the expression of iNOS. As shown in **Figure 4B**, when human fibroblast cells were

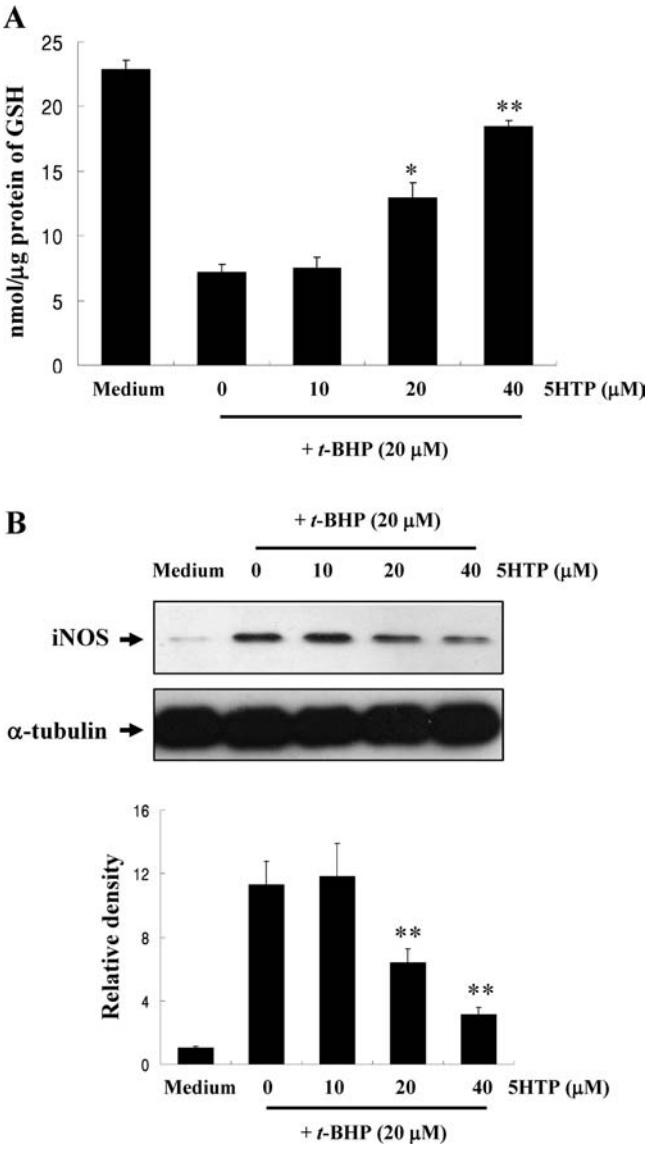

Figure 4. Effects of 5HTP on the GSH level and iNOS expression in *t*-BHP-treated human fibroblast cells. (A) Human fibroblast cells were pretreated with 5HTP (10, 20, or 40 μM) for 2 h; then 20 μM *t*-BHP was added for 24 h; and then GSH levels were assessed by the GSH assay. (B) Equal amounts of proteins were separated by SDS−polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The membranes were then probed with the indicated antibodies (anti-iNOS and α-tubulin) and detected by ECL. α-Tubulin was used as the internal control. The results are representative of triplicate, independent experiments. (∗) $p < 0.05$ and (∗∗) $p < 0.01$ versus the group treated by only 20 μM *t*-BHP.

exposed to 20 μM *t*-BHP for 24 h, the expression rate of iNOS was markedly increased from 1 ± 0.12-fold (medium) to 11.3 ± 1.5-fold. In contrast, when the human fibroblast cells were pretreated with various concentrations of 5HTP for 2 h, the expression rate of iNOS decreased at 20 and 40 μM but not 10 μM 5HTP treatment. These data indicate that 5HTP not only inhibited the depletion of GSH levels but also prevented the upregulation of iNOS expression induced by *t*-BHP.

3.5. Inhibition of *t*-BHP-Induced $ONOO^-$ Generation and p38MAPK and NF-κB Activation by 5HTP. $ONOO^-$, a potent oxidizing nitrating species, initiates lipid peroxidation, causes DNA damage, and reacts with thiols (*18*). $ONOO^-$ generation and iNOS synthesis are regulated by the transcription

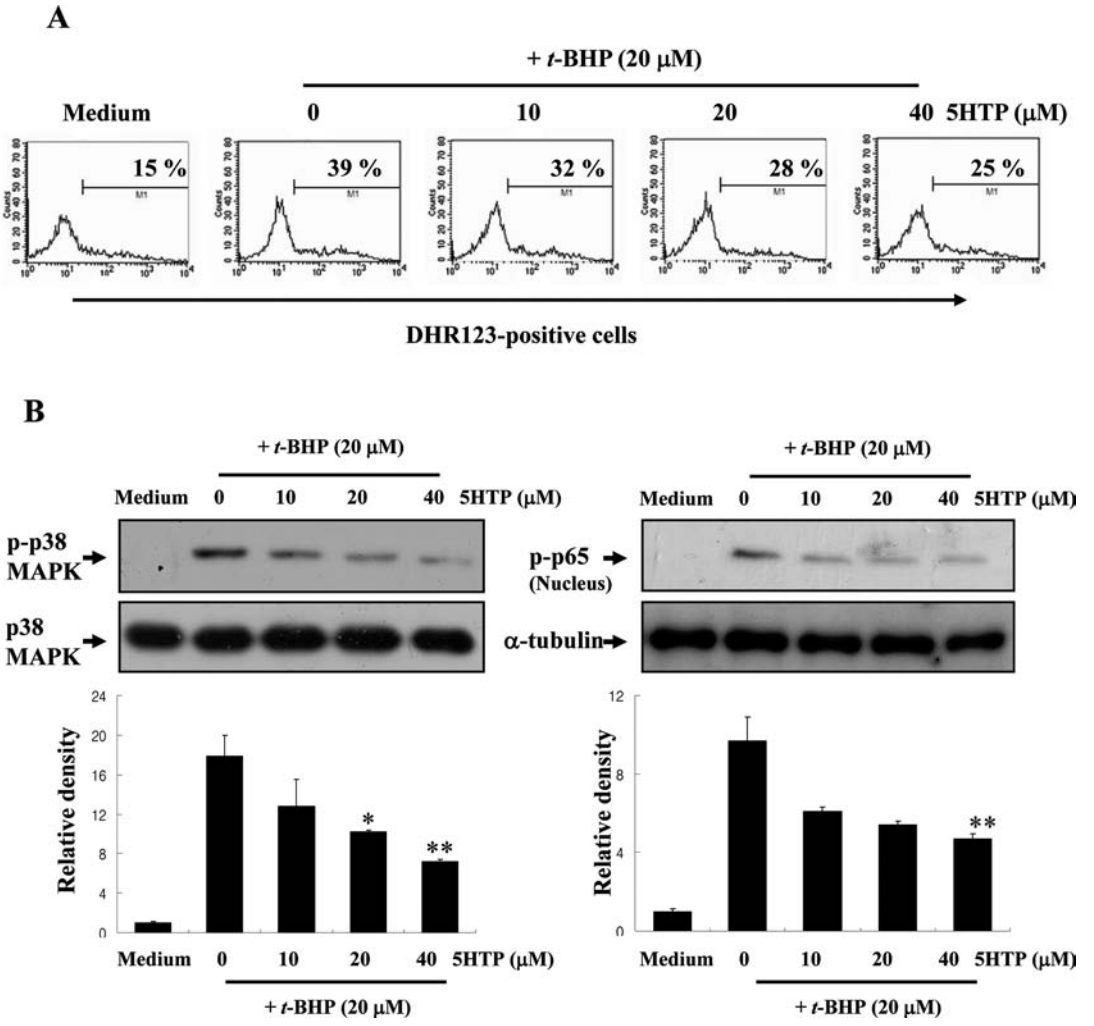

Figure 5. Effect of 5HTP on $ONOO^-$ generation in *t*-BHP-treated human fibroblast cells. (A) Human fibroblast cells were pretreated with 5HTP (10, 20, or 40 μM) for 2 h; then 20 μM *t*-BHP was added for 24 h; and then the rate of DHR123 oxidation was evaluated by flow cytometry. (B) Formation of $ONOO^-$ after 12 h of *t*-BHP exposure was detected using DHR123 oxidation and fluorescence in the pretreatment with 5HTP. The cells were pretreated with various amounts of 5HTP for 1 h before *t*-BHP stimulation (20 μM *t*-BHP). The cell lysates were prepared and blotted with anti-phosho-p38MAPK, anti-p38MAPK, anti-phosho-p65, and anti-α-tubulin antibodies. The bound antibodies were visualized using biotinylated goat anti-human IgG. The results shown represent triplicate, independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ versus the group treated by only 20 μM *t*-BHP.

factor pathways, NF-κB and p38MAPK (*19*). In this experiment, $ONOO^-$ levels were determined using a fluorescent probe, DHR123. Cells treated with *t*-BHP had increased fluorescence intensity (DHR oxidation) by 39% [364 ± 17.5 median fluorescence intensity (MFI)] compared to unstimulated cells.

As shown in **Figure 5A**, when human fibroblast cells were exposed to 20 μM *t*-BHP for 12 h, $ONOO^-$ generation markedly increased from 15% [263 ± 21.2 MFI (medium)] to 39% (364 ± 17.5 MFI). In contrast, when human fibroblast cells were pretreated with various concentrations of 5HTP for 2 h, the expression rate of $ONOO^-$ decreased.

To determine whether the p38MAPK and NF-κB signal pathways are involved in the *t*-BHP-induced $ONOO^-$ generation, we investigated the effect of 5HTP on the generation of $ONOO^-$ (**Figure 5B**). The *t*-BHP-induced activation of p38MAPK and NF-κB translocation were reduced by 5HTP treatment in a dose-dependent manner. 5HTP dose-dependently both inhibited $ONOO^-$ generation and suppressed p38MAPK and NF-κB activation at 40 μM 5HTP.

3.6. Modulation of the Bcl-2 Protein by 5HTP in *t*-BHP-Treated Human Fibroblast Cells. Several apoptosis mechanisms have been identified in oxidative-damaged cells that include the downregulation of anti-apoptotic proteins, such as Bcl-2, and the upregulation of pro-apoptotic proteins, such as Bax (*20*, *21*). To explore whether 5HTP modulated the expression of Bcl-2 in *t*-BHP-treated human fibroblast cells and its responses to oxidative damage, Bcl-2 expression levels were determined by western blot analysis. As shown in **Figure 6**, when human fibroblast cells were exposed to 20 μM *t*-BHP for 24 h, the expression rate of Bcl-2 decreased from 1 ± 0.1-fold (medium) to 0.57 ± 0.07-fold. In contrast, when human fibroblast cells were pretreated with various concentrations of 5HTP for 2 h, the expression rate of Bcl-2 increased from 0.57 ± 0.07-fold (*t*-BHP) to 0.98 ± 0.47-fold. Moreover, the expression rate of Bax decreased from 4 ± 0.12-fold (*t*-BHP) to 1 ± 0.2-fold. The results reported above show that 5HTP impaired the expression of Bcl-2 in the *t*-BHP-treated human fibroblast cells.

4. DISCUSSION

In this study, we document that 5HTP possesses protective effects against *t*-BHP-induced oxidative cell damage and apoptosis in human fibroblast cells. Furthermore, we obtained evidence from a molecular basis of the anti-inflammatory properties

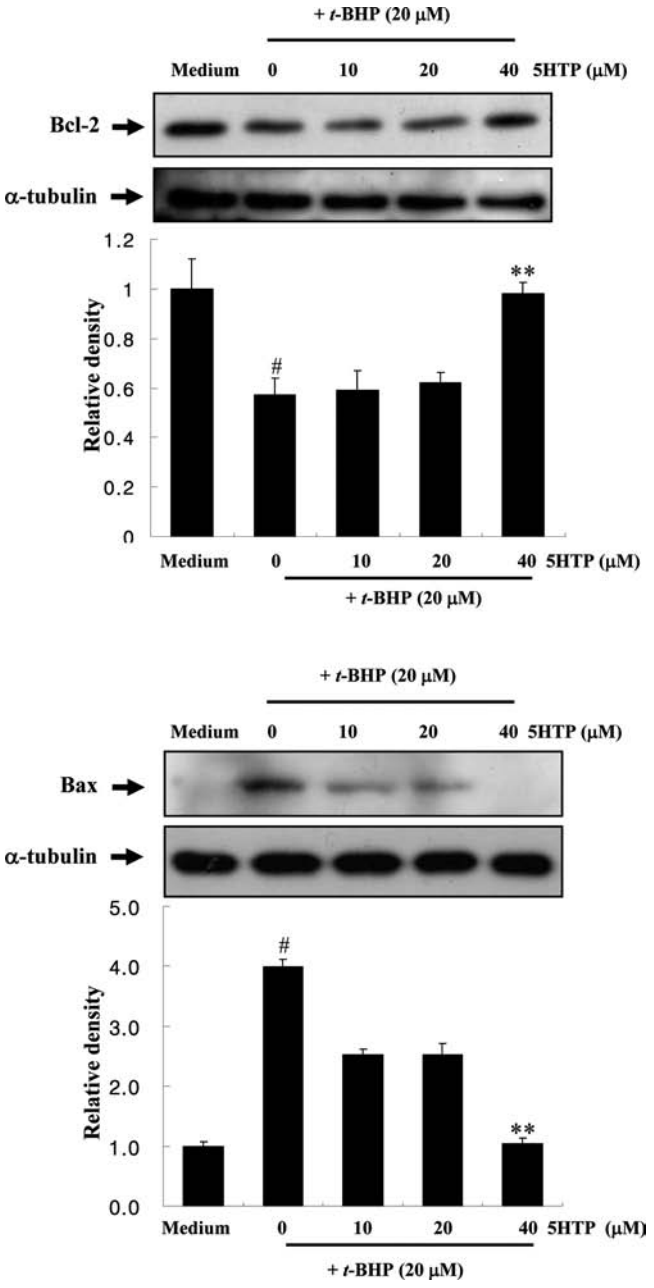

Figure 6. Effects of 5HTP on Bcl-2 and Bax expressions in *t*-BHP-treated human fibroblast cells. Human fibroblast cells were incubated with various concentrations of 5HTP in the presence of 20 μM *t*-BHP for 24 h and lysed, and then equal amounts of proteins were separated by SDS−PAGE and transferred to nitrocellulose membranes. The membranes were then probed with the indicated antibodies (anti-Bcl-2, anti-Bax, and α-tubulin) and detected by ECL. α-Tubulin was used as the internal control. (#) $p < 0.05$ versus the control and (∗) $p < 0.05$ and (∗∗) $p < 0.01$ versus the group treated by only 20 μM *t*-BHP.

of 5HTP in its ability to modulate key pro-inflammatory transcriptional pathways.

We found that the lipid peroxidation induced by *t*-BHP in human fibroblast cells was attenuated in cells pretreated with 5HTP in a dose-dependent manner (**Figure 3A**). The involvement of lipid peroxidation in cell death induction was studied as a consequence of oxidative damage inducing apoptosis (*22*, *23*). Oxidative damage of DNA leads to DNA fragmentation and mutation and then to further cell death. Although a model for *t*-BHP-induced DNA damage in normal cells has been reported (*24*, *25*), we have found the ability of 5HTP to protect against *t*-BHP-induced DNA damage in human fibroblast cells. As shown in **Figure 2**, the incubation of fibroblast cells with *t*-BHP induced DNA damage, as illustrated by the DAPI staining (**Figure 2**). Although exposure to 20 μM *t*-BHP significantly induced DNA damage, pretreatment with 5HTP inhibited *t*-BHP-induced DNA damage in a dose-dependent manner. Oxidative damage is often reflected in the GSH/glutathione disulfide (GSSG) ratio, and GSH depletion is causatively related to mitochondrial dysfunction and apoptosis induction (*26*). Our results show that pretreatment with protective 5HTP led to a reduction in GSH depletion that may well be related to its protective action against *t*-BHP-induced cell death. Interestingly, 5HTP has a 5-hydroxy group (5′-OH) in its B ring. This group is reported to be the principal site of antioxidant reaction (*32*).

Another physiological mediator that might participate in *t*-BHP-related cell death is NO (*27*, *28*). NO is a radical product of arginine in cells stemming from a reaction catalyzed by NOS. We found that the *t*-BHP-induced upregulation of iNOS in human fibroblast cells was attenuated by the pretreatment of these cells with 5HTP (**Figure 4B**). NO, as a source of a nitro-oxidative stressor, can induce apoptosis by interacting with superoxide to produce the more potent $ONOO^-$, leading to the activation of MAPK and NF-κB(*29*). In a previous study using confocal laser microscopy, $ONOO^-$ was confirmed to mediate apoptosis in endothelial cells (*30*). Also, several apoptosis inducers are shown to cause intracellular oxidation that can be blocked by anti-apoptotic Bcl-2. In addition, Bcl-2 has been shown to suppress RS generation, thereby inhibiting apoptotic induction (*31*). Our current data on the protective actions of 5HTP against cellular damage and apoptosis are consistent with those reported findings. The modulation of pro-apoptotic Bax and anti-apoptotic Bcl-2 expressions by pretreatment with 5HTP may well provide molecular protection against *t*-BHP-induced cell death.

In conclusion, our data clearly show the antioxidative and anti-inflammatory actions of 5HTP at cellular and molecular levels. At the cellular level, the protective effects of 5HTP against *t*-BHP-induced oxidative stress in human fibroblast cells are, namely, preventing cell death, lipid peroxidation, ROS and $ONOO^-$ generation, and GSH depletion. Furthermore, the modulation of major pro-inflammatory pathways by 5HTP provides new insights into the properties of 5HTP. Taken together, 5HTP has, therefore, been shown to be an efficacious agent against oxidative stress when cells are exposed to increased intracellular RS generation, lipid peroxidation, GSH depletion, and increased iNOS expression.

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