Stimulatory Heterotrimeric GTP-binding Protein Inhibits Hydrogen Peroxide-induced Apoptosis by Repressing BAK Induction in SH-SY5Y Human Neuroblastoma Cells*

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So-Young Kim‡, MiRan Seo§, Yeni Kim, Yun-Il Lee‡, Jung-Min Oh§, Eun-Ah Cho‡, Jae-Seung Kang‡, and Yong-Sung Juhnn

From the ‡Department of Biochemistry and Molecular Biology, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-799 and the §Department of Microbiology, College of Medicine, Inha University, Incheon 402-751, Korea

Heterotrimeric stimulatory GTP-binding protein (Gs) stimulates adenylyl cyclases to activate the cAMP signaling pathway. Although the cAMP pathway has been reported to be involved in apoptosis, the role of the Gs-cAMP signaling pathway during reactive oxygen species (ROS)-mediated apoptosis, which is involved in the resistance of cancer cells to chemotherapy and radiation, is not clearly understood. Thus, in this study we aimed to investigate the role of the α subunit of Gs (Gsα) in the ROS-induced apoptosis of cancer cells. The stable expression of constitutively active Gsα (GsαQL) inhibited the hydrogen peroxide-induced apoptosis of SH-SY5Y human neuroblastoma cells and reduced the hydrogen peroxide-induced increase in Bak and the decrease in Bcl-XL protein expression. Exogenous Bak expression abolished these inhibitory effects of GsαQL, but Bak small interfering RNA decreased hydrogen peroxide-induced apoptosis. Gsα repressed hydrogen peroxide-induced Bak expression by inhibiting the transcription of Bak mRNA, which resulted from the inhibition of the hydrogen peroxide-induced activation of transcription factors such as AP1, NF-κB, and NFAT. Moreover, Gsα also inhibited the hydrogen peroxide-induced binding of AP1, NF-κB, and NFAT to the Bak promoter. Furthermore, hydrogen peroxide-induced apoptosis was reduced by treating cells with prostaglandin E2, which activates Gsα, but this was augmented by CCPA, which activates Gsα causing a decrease in cAMP levels. From the results, we conclude that Gsα protects neuroblastoma cells from hydrogen peroxide-induced apoptosis by repressing Bak induction, which is mediated by the inhibition of the hydrogen peroxide-induced activations of AP1, NF-κB, and NFAT through cAMP-PKA-CREB signaling system.

The heterotrimeric GTP-binding proteins (G proteins) are composed of α, β, and γ subunits, and the α subunits of Gs protein (Gsα) are classified into four main families: Gαq, Gαi, Gαs, and Gα12. When a signaling molecule, such as a hormone or a neurotransmitter binds to a G protein-coupled receptor (GPCR), the receptor stimulates the replacement of GDP with GTP on the Gα. GTP-bound activated heterotrimer dissociates into an α subunit (Gs-GTP) and a βγ dimer (Gβγ), which can independently regulate effectors including adenylyl cyclases, phospholipases, phosphodiesterases, and ion channels. The hydrolysis of GTP to GDP by intrinsic GTPase, a process that is regulated by RGS (regulator of G-protein signaling) proteins, leads to the reassociation of the heterotrimer and the termination of the activation cycle (1–3). G protein signaling systems are involved in regulation of a variety of cellular responses, which include metabolism, neurotransmission, proliferation, differentiation, and apoptosis. Although signaling for cellular proliferation and apoptosis has generally been attributed to growth factor receptors that possess ligand-regulated protein-tyrosine kinase activity, growing evidence now indicates that GPCRs also regulate cellular growth and apoptosis (4–6).

Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic metabolism, and the most common ROS types are superoxide anions (O2•−), hydrogen peroxide (H2O2), and hydroxyl radicals (HO•) (7). ROS can react with DNA, proteins, carbohydrates, and lipids in a destructive manner due to their high levels of chemical reactivity. Thus, ROS are considered DNA-damaging agents that increase mutation rates and promote oncogenic transformation, and are implicated in the development of cancer and metastases (8, 9). Moreover, ROS participate in the modulation of apoptosis following treatment with various agents including Fas, ultraviolet, and chemotherapeutic drugs (10–12). Therefore, the ability of a cancer cell to defend itself against ROS is associated with resistance to chemo- and radiotherapy. Consequently, a more detailed understanding of the mechanisms that regulate ROS-induced apoptosis would contribute to our abilities to develop novel therapeutic strategies directed toward killing resistant cancer cells (13).
The α subunit of the stimulatory G protein (Gaα) activates adenylate cyclases to increase cellular cAMP, which regulates various cellular responses by activating cAMP-dependent protein kinase (PKA), guanine nucleotide exchange factor activated by cAMP, and cyclic nucleotide gated ion channels. Moreover, increased Gaα signaling was found to lead to the formation of tumors in endocrine cells (14), and thus, the activated mutant of Gaα is known as gsp oncogene. Increased Gaα signaling also leads to the apoptosis of cardiac myocytes (4) but protects neuronal apoptosis (15). However, the effect of Gaα signaling on ROS-mediated death of cancer cells is not understood. Thus, we undertook this study to examine whether Gaα can modulate hydrogen peroxide-induced cancer cell death, and if so, to elucidate the molecular mechanism responsible.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—SH-SY5Y human neuroblastoma cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (JBI, Korea) and 100 units/ml penicillin/streptomycin, in a 5% CO₂ incubator at 37 °C. SH-SY5Y cells stably expressing co’s modified Eagle’s medium containing 10% fetal bovine cells were purchased from the American Type Culture Collection. Moreover, increased Gaα signaling by cAMP, and cyclic nucleotide gated ion channels. Gaα activated mutant of Gaα is known as gsp oncogene. Increased Gaα signaling also leads to the apoptosis of cardiac myocytes (4) but protects neuronal apoptosis (15). However, the effect of Gaα signaling on ROS-mediated death of cancer cells is not understood. Thus, we undertook this study to examine whether Gaα can modulate hydrogen peroxide-induced cancer cell death, and if so, to elucidate the molecular mechanism responsible.

**Expression Constructs and Transient Transfection**—cDNA encoding constitutively active Gaα (GaαQL) was subcloned into pcDNA3 expression vector (Invitrogen). The BacpcDNA3 expression construct was kindly provided by Dr. Young-Jun Oh (Yonsei University, Korea). Reporter plasmids containing luciferase gene under the control of CRE, AP1, NFAT, and NF-κB, respectively, were from Stratagene (La Jolla, CA). Decay of nucleolus containing the acidic CRE response element (deoxy CRE) was constructed of phosphorothioate oligonucleotide (Genotech, Korea), with the following sequences 5’-TGACGTGACGTGACGTGCACTGCA-3’ (24 bp), and the sequences of control decoy were 5’-CTAGCTAGCTAGCTAGCTAGCTAGC-3’ (24 bp). pENTR/H1/TO-Gaα, and the Bac siRNA construct were generated by the BLOCK-it™ Inducible H1 RNAi Entry Vector Kit (Invitrogen) using the following primers: Gaα siRNA, a forward primer 5’-CCGCGGATGTTGACTGCGGATCAGTCCATG-3’, and a reverse primer 5’-AACGAACTGACGTGACGTGACGTGCACTGCA-3’. Bak siRNA, a forward primer 5’-CTACGCGACGTGACGTGACGTGCACTGCA-3’, and a reverse primer 5’-AAAACCGGACGTGACGTGACGTGCACTGCA-3’. Transfection was performed by electroporation using a Gene Pulser II (Bio-Rad) at 250 V/950 microfarads.

**Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—Total RNA was isolated from the cells using the acid guanidinium thiocyanate-phenol-chloroform extraction method (17). First-strand complementary DNA (cDNA) was synthesized using oligo(dT) primers and the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed with specific primers: Bak, forward primer 5’-TGAAGATGCGTGCAGGAGGC-3’, and reverse primer 5’-CATGAAGTAATCCTTATGTTAC-3’. Glyceraldehyde-3-phosphate dehydrogenase, forward primer 5’-ACCACGATCGGTGGGAT-3’, and reverse primer 5’-GCCACCACCTGTGGTCTGTA-3’. Real-time RT-PCR was performed in a 25-μl mixture composed of 200 nM forward and reverse primers, and qTMT SYBR Green Supermix (Roche, Basel, Switzerland) using iCyler and iQ software (Bio-Rad). After 40 cycles of PCR, average Bak threshold cycle (Ct) values from triplicate PCR were normalized against the average Ct values of glyceraldehyde-3-phosphate dehydrogenase.

**Immunoblot Analysis**—Immunoblotting was performed as previously described (18). Total cell lysates (50 μg of protein) were separated on 10 or 15% SDS-polyacrylamide gels, transferred to nitrocellulose paper, and analyzed with specific antibodies. Antibodies against Bcl2, Gaα, GSK-3β, IkBα, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against Bcl-xL, Bad, Bak, phosphorylated CREB (Ser133), cleaved caspase-3 (Asp175), c-Fos, phosphorylated GSK-3β (Ser9), poly(ADP-ribose) polymerase (PARP), JNK, phosphorylated JNK (Thr183/Tyr185), and phosphorylated c-Jun (Ser63, Ser73) were from Cell Signaling Technology (Beverly, MA). Antibody against NFA1T was purchased from Affinity Bioreagents (Golden, CO). Cytochrome c released into the cytoplasm was analyzed after subcellular fractionation by immunoblotting using an antibody obtained from BD Biosciences (19). The densities of visualized bands were quantified using NIH Image J software and relative band densities are expressed as percentages of corresponding control densities.

**Flow Cytometric Analysis of Annexin V and Propidium Iodide-stained Cells**—Apoptosis was quantified using Annexin V-FITC apoptosis kits (BD Biosciences) according to the manufacturer’s instructions. Stained cells were analyzed using a FACS Calibur flow cytometer and the CellQuest analysis program (BD Biosciences North Ryde, Australia).

**Generation of Bak Promoter Reporter Constructs**—Genomic DNA was isolated from SH-SY5Y cells by digestion with proteinase K and sequential phenol extraction (20), and used as a template for PCR to clone the Bak promoter. The forward primer designed for cloning the Bak promoter region contained an XhoI site at the 5’ terminus, with the sequence 5’-CCGCGGATGTTGACTGCGGATCAGTCCATG-3’. The reverse primer contained a HindIII site at the 5’ terminus, and had the sequence 5’-CCCAAGCTTGCATCATGTTATTGGAGATGG-3’. PCR was performed using LA Taq polymerase (Takara Shuzo Co., Japan), and the resulting 3500-bp PCR products were digested with XhoI and HindIII and then ligated upstream of the luciferase reporter gene in pG2 basic vector (Promega Corp., Madison, WI) to generate Bak-pLuc. A series of mutants with various deletions in the cloned Bak promoter region were prepared by PCR. The primers used were as follows: for mutant deleted between −3500 and −2500, (−2500) Bak-pLuc: a forward primer 5’-CCGCTCGAGGAGGTTGTGCTCCATCTCCAGA-3’, for mutant deleted between −3500 and −1500, (−1500) Bak-pLuc: a forward primer 5’-CCGCT-
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CGAGCTTCACCTGTCCCTGTCGG-3'; and for mutant deleted between -3500 and -522, (-522)Bak-pLuc: a forward primer 5'-CCGCTCGAGGGAACAGCTTAAAACCCCCCA-3'. Mutants deleted in API sites in Bak promoter of (-1500)Bak-pLuc were also constructed by PCR using the following primers: D1 (deletion upstream of -1216), 5'-CCGCTCAGGTGACTA-ACGATGTCACCTCG-3'; D2 (deletion between -912 and -919) products were constructed by ligation two PCR products into wild type plasmid digested with Xhol and HindIII (Xhol-SacII fragment: forward primer 5'-/H11002-AGCGATGTGACCTCGGGCACTCA-3'GCTGTGACCCCTCATC-3', reverse primer 5'-TCCCCGGAGAACCTT-GCTGTGACCCCTCATC-3', SacII-Xhol fragment: forward primer 5'-TCCCCGGAGAACCTTGGGACACTCAACTTTTT-3', reverse primer 5’-CCCAAGCTTGACCTCATGTTATGGGATGG-TGG-3'; D1 and D2 (deletion upstream of -912), 5'-CCGCTCGAGGGAATGTACCGTGGACACTCAACTTTTT-3', the same reverse primer (5’-CCCAAGCTTGACCTCATGTTATGGGATGG-TGG-3') was used all PCRs for mutagenesis.

Luciferase Activity Assays—SH-SY5Y cells were transfected with plasmids containing luciferase reporter gene by electroporation. Luciferase activities were assayed using the Bio luminescent Reporter Gene Assay System (Tropix, Bedford, MA) according to the manufacturer's instructions. At least three independent experiments in duplicate were performed, and promoter activities were normalized versus $\beta$-galactosidase activity.

**Electrophoretic Mobility Shift Assay by Chemiluminescence**—The cells were treated with hydrogen peroxide for 24 h, and then disrupted in lysis buffer (containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40). The cell lysates was centrifuged at 14,000 $\times$ g at 4°C for 6 min, and the resulting pellets were resuspended in extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM MgCl$_2$, 420 mM NaCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 25% glycerol, 0.2 mM EDTA, and 1% Nonidet P-40). After centrifugation, the supernatant was collected for study of DNA-protein interactions, which were analyzed by chemiluminescence using the LightShift Chemiluminescent EMSA Kit (Pierce). Oligonucleotides, which were analyzed by chemiluminescence using the supernatant was collected for study of DNA-protein interactions.

**Results**

$\text{Ga}_s$ Inhibited Hydrogen Peroxide-induced Apoptosis of SH-SY5Y Neuroblastoma Cells—To investigate the role of $\text{Ga}_s$ in hydrogen peroxide-induced apoptosis, a constitutively active mutant $\text{Ga}_s$, $\text{Ga}_s$Q227L ($\text{Ga}_s$QL), was stably expressed in SH-SY5Y human neuroblastoma cells; this was confirmed by Western blotting using antibodies against $\text{Ga}_s$ and hemagglutinin tag in $\text{Ga}_s$QL (Fig. 1A). The expression of $\text{Ga}_s$QL increased the basal cAMP level to 28.5 ± 0.5 pmol/mg protein ($p < 0.05$) from 3.4 ± 0.6 pmol/mg of protein in vector-transfected cells, and the CREB phosphorylation. Twenty-four hours after treatment with 100 $\mu$M hydrogen peroxide for 30 min, cells were stained with Annexin V to detect apoptosis. Stable expression of $\text{Ga}_s$QL reduced Annexin V positive and propidium iodide-negative cells to 9.6 ± 1.7 from 45.8 ± 4.5% ($p < 0.02$), and Annexin V-positive and propidium iodide-positive cells to 3.9 ± 1.1 from 15.0 ± 2.2% ($p < 0.02$) in vector-transfected control (Fig. 1B). $\text{Ga}_s$QL expression also decreased the cleavages of caspase-3 and PARP (Fig. 1C), and blocked cytochrome c release into cytosol (Fig. 2A) after 24 h of hydrogen peroxide treatment. To confirm the anti-apoptotic effect of $\text{Ga}_s$, the effects of $\text{Ga}_s$, siRNA were analyzed. The transient transfection of $\text{Ga}_s$ siRNA decreased the basal expression of $\text{Ga}_s$ in the $\text{Ga}_s$QL expressing cells (56.6 ± 2.9%, $p < 0.05$) and vector-transfected cells (46.0 ± 4.5%, $p < 0.05$) from siRNA-untransfected controls. Treatment with hydrogen peroxide augmented the siRNA effect resulting in a further decrease of $\text{Ga}_s$ expression in the $\text{Ga}_s$QL expressing cells (40.2 ± 1.4%, $p < 0.05$) and vector-transfected cells (26.2 ± 4.7%, $p < 0.05$) (Fig. 1D). Transfection of $\text{Ga}_s$ siRNA increased hydrogen peroxide-induced caspase-3 cleavage to 193.3 ± 2.3% ($p < 0.05$) and PARP to 146.3 ± 4.4% ($p < 0.05$) in vector-transfected cells, and it also increased hydrogen peroxide-induced caspase-3 cleavage from 36.6 ± 4.9 to 162.6 ± 4.6% ($p < 0.05$) and PARP from 24.0 ± 2.7 to 129.8 ± 4.7% ($p < 0.05$) in $\text{Ga}_s$QL expressing cells versus the hydrogen peroxide-treated vector-transfected control (Fig. 1D), indicating that $\text{Ga}_s$ protected SH-SY5Y neuroblastoma cells from hydrogen peroxide-induced apoptosis.

$\text{Ga}_s$ Inhibited Hydrogen Peroxide-induced Apoptosis by Repressing Increases in Bak Protein in SH-SY5Y Neuroblastoma Cells—To probe the mechanism whereby $\text{Ga}_s$ inhibits hydrogen peroxide-induced apoptosis of the neuroblastoma cells, we examined the effects of $\text{Ga}_s$ on the expression of Bcl2 family proteins. Treatment with hydrogen peroxide induced an increase in pro-apoptotic Bak protein, but decreases in Bax and Bad proteins. Moreover, the stable expression of $\text{Ga}_s$QL inhibited up-regulation of Bak and down-regulation of Bax and Bad induced by hydrogen peroxide (Fig. 2A). However, hydrogen peroxide treatment did not decrease anti-apoptotic Bcl-expression, and the overexpression of $\text{Ga}_s$QL did not change Bcl2 expression. The expression of Bcl-xL, another anti-apoptotic Bcl2 family protein, was reduced by hydrogen peroxide treatment, and the overexpression of $\text{Ga}_s$QL blocked the
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\( G_\alpha_s \) Repressed Hydrogen Peroxide-induced Bak Expression by Inhibiting the Transcription of Bak mRNA—To investigate the mechanism whereby \( G_\alpha_s \) represses the hydrogen peroxide-induced expression of Bak protein, we examined the degradation rate of Bak protein after blocking protein synthesis with cycloheximide. It was found that the degradation rate of Bak protein in \( G_\alpha_s \)-QL expressing cells was not significantly higher than that in vector-transfected cells (Fig. 3A). On the other hand, hydrogen peroxide treatment markedly increased Bak mRNA in vector-transfected cells, but only slightly in \( G_\alpha_s \)-QL-transfected cells (Fig. 3B). This finding was confirmed by real time RT-PCR, which showed a 29.0 ± 4.5-fold \((p < 0.02)\) increase in Bak mRNA in hydrogen peroxide-treated vector-transfected control cells, and a 6.5 ± 2.3-fold increase \((p < 0.02)\) in \( G_\alpha_s \)-QL expressing cells versus the untreated vector-transfected control cells. In addition, expression of \( G_\alpha_s \)-QL increased the basal Bak mRNA level to 3.2 ± 0.7-fold \((p < 0.02)\) versus the untreated vector-transfected control cells (Fig. 3C). Furthermore, hydrogen peroxide treatment increased luciferase activity under the control of the Bak promoter to 12.6 ± 0.3-fold \((p < 0.02)\) compared with untreated vector-transfected cells. The expression of \( G_\alpha_s \)-QL increased basal Bak luciferase activity to 1.8 ± 0.5-fold \((p < 0.02)\), but reduced hydrogen peroxide-stimulated luciferase activity to 3.1 ± 0.5-fold of the untreated vector-transfected cells \((p < 0.05)\).

\( G_\alpha_s \) Inhibited the Transcription of Bak mRNA via cAMP-PKA-CREB-dependent Pathways—In a study to probe the signaling pathway mediating the repression of Bak induction by \( G_\alpha_s \), we treated SH-SYSY neuroblastoma cells with forskolin. Forskolin treatment reduced the hydrogen peroxide-induced cleavages of PARP and caspase-3 and the release of cytochrome c to the cytosol (Fig. 4A). Similar protective effects were exhibited by treatment of the cells with dibutyryl cAMP (data not shown). Moreover, forskolin treatment blocked the increase in Bak and the decrease in Bad and Bak induced by hydrogen peroxide. Forskolin also reduced the observed down-regulation of Bcl-x\(_L\) by hydrogen peroxide, but did not change Bcl2 expression (Fig. 4A). Treatment of cells with H89, a cAMP dependent protein kinase (PKA) inhibitor, abolished the anti-apoptotic effects of \( G_\alpha_s \)-QL and the inhibitory effect of \( G_\alpha_s \)-QL on Bak induction, as evidenced by PARP and caspase-3 cleavage (Fig. 4B).
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![Image](https://example.com/image.png)

**FIGURE 2.** Gαs inhibited apoptosis by repressing Bak expression induced by hydrogen peroxide. A, effects of GαsQL on the hydrogen peroxide-induced expression of Bcl2 family proteins. B, effects of GαsQL on the hydrogen peroxide-induced expression of Bak. After incubation with 100 μM hydrogen peroxide for 24 h, cells were analyzed for Bcl2 family proteins and the mitochondrial distribution of Bak. Histograms represent mean ± S.E. and Bak densities expressed as -fold of the corresponding densities of control cells. Asterisks indicate significantly different versus vector-transfected cells (p < 0.05, Mann-Whitney U test). The effects of Bak overexpression (C) and Bak knockdown (D) on the anti-apoptotic effects of GαsQL. SH-SY5Y cells were transfected with 15 μg of Bak plasmids (pcDNA3-Bak or pENTR-Bak siRNA) or vector controls (pcDNA3 or pENTR-lacZ) by electroporation. After 48 h, cells were then treated with 100 μM hydrogen peroxide for 24 h and apoptotic levels were determined.

4B). Furthermore, transfection of decay CRE abolished the anti-apoptotic effects of GαsQL and its inhibition of Bak induction by hydrogen peroxide (Fig. 4C).

**Gαs Repressed Hydrogen Peroxide-induced Bak Expression by Inhibiting the Activation of Transcription Factors.—**To investigate the mechanism underlying the repression of hydrogen peroxide-induced Bak transcription by Gαs, the effects of GαsQL on the activation of transcription factors by hydrogen peroxide were analyzed. Treatment with hydrogen peroxide activates reporter luciferase under the control of transcription factors AP1, NFAT, and NF-κB, but not under the control of CREB. The expression of GαsQL activated CREB-luciferase activity to 3.5 ± 0.4-fold (p < 0.02) of the basal activity, but inhibited the hydrogen peroxide-induced activation of AP1-luciferase from 11.5 ± 1.2- to 3.8 ± 0.4-fold (p < 0.02), NFAT-luciferase from 4.3- to 1.6-fold (p < 0.02), and NF-κB-luciferase from 5.2 ± 0.6- to 1.6 ± 0.5-fold (p < 0.02) (Fig. 5A). Similarly, treatment with forskolin activated CREB-luciferase activity to 11.4 ± 0.7-fold (p < 0.02) of basal activity, but inhibited hydrogen peroxide-induced activation of AP1-luciferase from 9.7 ± 0.2- to 1.9 ± 0.2-fold (p < 0.02), NFAT-luciferase from 3.7 ± 0.3- to 1.2 ± 0.4-fold (p < 0.02), and NF-κB-luciferase from 4.2 ± 0.1- to 1.0 ± 0.3-fold (p < 0.02) (Fig. 5A). Furthermore, hydrogen peroxide-induced Bak-luciferase activities were inhibited by pre-treatment with SB203580 (an AP1 inhibitor), cyclosporine A (an NFAT inhibitor), or pyrrolidine dithiocarbamate (an NF-κB inhibitor) to 2.1 ± 1.1-, 4.3 ± 0.4-, and 3.3 ± 1.0-fold, respectively, from 13.6 ± 0.2-fold of the control (p < 0.02), but pre-treatment with CRE decoy enhanced hydrogen peroxide-induced Bak-luciferase activity to 29.8 ± 0.5-fold from the 13.6 ± 0.2-fold of the control (p < 0.02) (Fig. 5B).

Gαs Repressed Hydrogen Peroxide-induced Bak Expression by Inhibiting the Binding of Transcription Factors on Bak Promoter.—To localize the region of the Bak promoter responsible for hydrogen peroxide induction, the effects of serial deletion on Bak luciferase activity were analyzed in SH-SY5Y neuroblastoma cells. Treatment with hydrogen peroxide induced Bak luciferase activity to 12.9 ± 0.2-fold under the control of the 3500-bp untranslated region, to 9.5 ± 0.7-fold under the control of −2500-bp untranslated region, and 6.1 ± 0.1-fold under the control of −1500-bp untranslated region to p < 0.02 versus the vector-transfected control, indicating that induction-fold decreased in proportion to the 5′ deletion extent in the 5′-untranslated region of the Bak promoter (Fig. 6A). To study the mechanism of Gαs to inhibit hydrogen peroxide-induced Bak transcription mediated by transcription factors, the effects of Gαs on the binding of AP1, CREB, NFAT, and NF-κB to the Bak promoters were analyzed by electrophoretic mobility shift assay in SH-SY5Y cells. The expression of GαsQL inhibited the hydrogen peroxide-induced binding of AP1 probe from 3.2 ± 4.3- to 1.6 ± 2.3-fold (p < 0.05), NFAT probe from 2.2 ± 4.1- to 1.7 ± 1.8-fold (p < 0.05), and NF-κB probe from 2.6 ± 4.4- to 1.7 ± 2.9-fold (p < 0.05) to the Bak promoter. However, GαsQL
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FIGURE 3. Gαs inhibited the hydrogen peroxide-induced transcription of Bak in SH-SY5Y cells. A, effects of GαsQL on the degradation rate of Bak protein. SH-SY5Y cells were treated with 10 μg/ml cycloheximide (CHX), and Bak protein levels were quantified at various times by Western blotting analysis of total cell lysates. The graph shows the average densities of Bak bands. B and C, effects of GαsQL on Bak mRNA levels and Bak-luciferase activity. Bak mRNA expressions were measured by RT-PCR and quantitative real-time RT-PCR (C). Amounts of Bak mRNA were normalized versus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and presented as ratio versus vector-transfected controls. For luciferase assay, SH-SY5Y cells were transfected with 2.5 μg of (−3500 bp)Bak-pLuc and 2.5 μg of a β-galactosidase construct. After 24 h, cells were treated with 100 μm hydrogen peroxide for 24 h, and luciferase activities were measured and normalized versus β-galactosidase activity and are presented as ratio versus vector-transfected controls. Asterisks indicate significant differences (p < 0.05 versus vector-transfected cells, Mann-Whitney U test).

increased the basal binding of the CREB probe (2.2 ± 3.0-fold, p < 0.05) and inhibited the hydrogen peroxide-induced decrease in CREB binding from 0.17 ± 1.3- to 2.1 ± 2.9-fold (p < 0.05) (Fig. 6B). To assess the contribution of AP1 in Bak induction by hydrogen peroxide, the effect of two deletions in the AP1 site (−1221 to −1216 or −919 to −912) of (−1500)Bak-pLuc were analyzed. The deletions decreased hydrogen peroxide-induced luciferase activity from 6.1 ± 0.1-fold (p < 0.02) to 2.6 ± 0.5-fold, and to 1.8 ± 0.2-fold (p < 0.02), respectively, and deletion of both sites decreased to 1.1 ± 0.1-fold (p < 0.02) (Fig. 6C).

Gαs Inhibited Hydrogen Peroxide-induced Activation of NF-κB and NFAT in a PKA-dependent Pathway—To probe the mechanism for Gαs-CAMP signaling to inhibit hydrogen peroxide-induced AP1 activation, the effects of Gαs on the phosphorylation and expression of AP1 components: c-Jun and c-Fos. Treatment with hydrogen peroxide increased phosphorylation of c-Jun at Ser63 and Ser73, and expression of c-Jun in vector- and GαsQL-expressing cells. GαsQL expression blocked the hydrogen peroxide-induced phosphorylation of c-Jun, and inhibited the induction of c-Jun to 40.0 ± 7.9% (p < 0.02) and c-Fos to 60.0 ± 8.1% (p < 0.02) of that of the vector-transfected control (Fig. 6D). The phosphorylation of JNK, a major kinase that activates c-Jun was increased by hydrogen peroxide treatment. The expression of GαsQL also increased the basal phosphorylation of JNK as high as that of hydrogen peroxide-treated vector-transfected cells, without resulting in an increase in the phosphorylation and expression of c-Jun and c-Fos (Fig. 6D). Treatment of GαsQL-expressing cells with hydrogen peroxide did not cause a significant change in JNK phosphorylation. The expression of GαsQL also increased the phosphorylation of p38 and ERK mitogen-activated protein kinase as high as that of hydrogen peroxide-treated vector-transfected cells (data not shown), suggesting that Gαs inhibits hydrogen peroxide-induced AP1 activation by inhibiting other enzymes. Thus, the involvement of glycogen synthase kinase-3β (GSK-3β) was examined, because GSK-3β was reported to involve in c-Jun induction. Treatment of SH-SY5Y cells with hydrogen peroxide decreased the phosphorylation of GSK-3β at serine 9 to activate the enzyme (Fig. 6E). GαsQL expression increased the basal phosphorylation level of GSK-3β (Ser9) (2.3 ± 0.1-fold, p < 0.05) and decreased the basal c-Jun level (0.8 ± 0.1-fold, p < 0.05) versus vector-transfected cells. GαsQL expression repressed the decrease in GSK-3β (Ser9) phosphorylation induced by hydrogen peroxide (2.1 ± 0.1-fold from 0.5 ± 0.2-fold in vector-transfected cells, p < 0.05) and in expression of c-Jun (0.8 ± 0.1-fold from 1.9 ± 0.1-fold, p < 0.05) (Fig. 6E). Treatment with lithium chloride (selectively inhibit GSK-3) increased the phosphorylation of GSK-3β, and decreased hydrogen peroxide-induced phosphorylation and induction of c-Jun in both vector- and GαsQL-transfected cells. Moreover, treatment with H89 also blocked the increase in GSK-3β phosphorylation (Ser9) induced by GαsQL (data not shown). This result suggests that Gαs represses hydrogen peroxide-induced AP1 activation by inhibition of GSK-3β in a PKA-dependent pathway.

Next, the effects of Gαs on the degradation of IκBα following hydrogen peroxide treatment were examined to probe the mechanism for Gαs to inhibit hydrogen peroxide-induced activation of NF-κB. Treatment of SH-SY5Y cells with hydrogen peroxide decreased the IκBα level to activate NF-κB. GαsQL expression increased the basal expression...
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level of IκBα versus vector-transfected cells (1.9 ± 0.1-fold, p < 0.05), and repressed the hydrogen peroxide-induced decrease in IκBα levels from 0.4 ± 0.1- to 1.7 ± 0.1-fold (p < 0.05) (Fig. 6F). Treatment with H89 slightly decreased the basal level of IκBα, but augmented the hydrogen peroxide-induced decrease in the IκBα level, suggesting Gαs inhibit hydrogen peroxide-induced activation of NF-κB by PKA-dependent blocking of the IκBα degradation in SH-SY5Y cells. Finally, to probe the mechanism for Gαs-cAMP signaling to inhibit hydrogen peroxide-induced NFAT activation, the effects of Gαs on the nuclear translocation of NFAT following hydrogen peroxide treatment was analyzed, because NFATs are known to translocate into the nucleus after activation. Most of NFAT was localized in the cytosol fraction in SH-SY5Y cells before treatment, but hydrogen peroxide treatment induced translocation of most of NFAT from the cytosol to nuclear fraction (Fig. 6F). However, GαsQL expression decreased the hydrogen peroxide-induced translocation of NFAT.
FIGURE 6. 

\[ \text{G}_{\alpha_s} \] repressed hydrogen peroxide-induced Bak expression by inhibiting the binding of transcription factors on Bak promoter in a PKA-dependent manner.  

A, effects of serial deletions in the Bak promoter on Bak-luciferase activity induced by hydrogen peroxide.  

B, effects of \( \text{G}_{\alpha_s} \) on the binding of AP1, CREB, NFAT, and NF-\( \kappa B \) to Bak promoter. The bindings of transcription factors to Bak promoter were analyzed by electrophoretic mobility shift assay using a chemiluminescent biotin 5’ end-labeled DNA probe corresponding to the AP1, CREB, NFAT, or NF-\( \kappa B \) consensus sequences. For competition experiments, 100-fold excesses of unlabeled AP1, CREB, NFAT, or NF-\( \kappa B \) oligonucleotide were added.  

C, effects of AP1 binding site deletions on Bak-luciferase activity induced by hydrogen peroxide. SH-SYSY cells were co-transfected with Bak-luciferase plasmids containing various deletions or AP1 binding site deletions in Bak promoter and \( \beta \)-galactosidase plasmids, and then Bak luciferase activity was analyzed and normalized to \( \beta \)-galactosidase.  

D, effects of \( \text{G}_{\alpha_s} \) on the hydrogen peroxide-induced activation of JNK.  

E, effects of \( \text{G}_{\alpha_s} \) on the hydrogen peroxide-induced activation of GSK-3\( \beta \).  

F, effects of \( \text{G}_{\alpha_s} \) on the hydrogen peroxide-induced degradation of I\( \kappa B \) and nuclear translocation of NFAT. SH-SYSY cells expressing \( \text{G}_{\alpha_s} \) were treated with hydrogen peroxide (100 \( \mu M \)) for 12 h in the presence or absence of 10 \( \mu M \) LiCl or 10 \( \mu M \) H89. The expressions and phosphorylations of JNK, GSK-3\( \beta \), NFAT, c-Jun, and c-Fos were analyzed by Western blotting using a respective specific antibody. For analysis of nuclear translocation of NFAT, cytosolic and nuclear proteins were prepared. Histograms represent mean ± S.E. of band densities expressed as -fold of the corresponding densities of control cells.
NFAT to the nuclear fraction (0.2 ± 0.1- from 0.8 ± 0.2-fold, p < 0.05), without significant change in its distribution before the treatment. Treatment with H89 caused a decrease in cytosolic distribution and concomitant increase in nuclear distribution of NFAT in vector-transfected cells and Gαs-transfected cells, suggesting that Gαs inhibits hydrogen peroxide-induced NFAT activation by blocking its nuclear translocation in a PKA-dependent pathway.

Contrary to the cAMP level, CREB phosphorylation induced by PGE2 reached a peak (31.6 ± 4.2-fold) at 1 h, and then slowly decreased to maintain elevated levels of phosphorylation (10.8 ± 4.3-fold p < 0.05) until 24 h, showing a similar temporal pattern of CREB phosphorylation by forskolin. This result suggests that PGE2 can maintain CREB activation for several hours after cAMP concentration is elevated for a short period in SH-SY5Y cells (Fig. 7B).
Furthermore, the expression of constitutively active Go_{12} (Go_{12QL}), which antagonizes Go_{s} effects, augmented hydrogen peroxide-induced Bak expression and the cleavages of caspase-3 and PARP, and partially eliminated the inhibitory effect of Go_{sQL} on hydrogen peroxide-induced Bak expression and cleavages of caspase-3 and PARP (Fig. 7C). However, Go_{sQL} expression also increased the basal expression level of the Bak protein in vector-transfected cells and Go_{sQL}-transfected cells, implying that Go_{s} may increase basal Bak expression by a CREB-independent pathway because Go_{s} was shown to decrease CREB phosphorylation in SH-SY5Y cells (data not shown). Treatment with CCPA, which (binding of CCPA to adenosine A1 receptor activates Go_{12} to inhibit adenylate cyclases) augments hydrogen peroxide-induced Bak expression, CREB dephosphorylation, and cleavages of PARP and caspase-3. The hydrogen peroxide-induced cleavage of caspase-3 was increased by CCPA from 5.8 ± 0.3- to 7.9 ± 0.7-fold (p < 0.02), and of PARP from 6.8 ± 0.1- to 9.9 ± 0.1-fold (p < 0.05) (Fig. 7D).

**DISCUSSION**

This study was performed to determine whether the α subunit of stimulatory protein, Go_{s}, can modulate hydrogen peroxide-induced apoptosis and if so, to elucidate the molecular mechanism responsible. We found that Go_{s} inhibits hydrogen peroxide-induced apoptosis by repressing Bak induction in SH-SY5Y human neuroblastoma cells. This finding is supported by the following results. First, the overexpression of constitutively active Go_{sQL} was found to protect SH-SY5Y cells from hydrogen peroxide-induced apoptosis, and Go_{s} siRNA augmented apoptosis. Second, Go_{sQL} overexpression repressed the hydrogen peroxide-induced up-regulation of Bak protein by inhibiting transcription of Bak. Third, Bak overexpression abolished the protective effect of Go_{s} on hydrogen peroxide-induced apoptosis, and knockdown of Bak (using siRNA) inhibited the apoptosis. We also found that the repression of Bak induction by Go_{s} is mediated by cAMP, PKA, and CREB. This was evidenced by the observations that B_{cAMP} and forskolin repressed Bak induction and protected cells against hydrogen peroxide-induced apoptosis; moreover, treatment with the PKA inhibitor H89 or transfection with decoy CRE restored Bak promoter activity, augmented hydrogen peroxide-induced apoptosis, and abolished the protective effect of Go_{sQL}. In addition, the protective effect of Go_{s} was abolished by co-expressing constitutively active mutant Go_{12}, which antagonizes Go_{s} by inhibiting adenylate cyclase.

The Go_{s}-cAMP signaling pathway has been reported to regulate cell death in various ways. Treatment with cholera toxin (which activates Go_{s}) was found to protect neuronal cells from glutamate-induced ROS-mediated apoptosis by up-regulating the anti-apoptotic protein Bcl2 in a cAMP-dependent manner (15), and forskolin has been reported to prevent the hydrogen peroxide-induced apoptosis of PC12 pheochromocytoma cells by increasing the level of antioxidant glutathione (21). B_{cAMP} inhibited apoptosis by blocking the induction of Bax mRNA (22). Moreover, PKA was reported to protect cells from apoptosis by inducing Bad phosphorylation (23), whereas CREB induces the transcription of the anti-apoptotic gene Bcl2. However, it has not been previously reported that the Go_{s}-cAMP signaling pathway modulates apoptosis by regulating Bak expression. Thus, this study presents the first evidence, to the best of our knowledge, that the Go_{s}-cAMP signaling pathway modulates cancer cell apoptosis by repressing Bak induction.

Bak protein is a multidomain pro-apoptotic protein of the Bcl2 family, and acts as an essential gateway to intrinsic cell death pathways by facilitating the release of cytochrome c from mitochondria (24, 25). Moreover, Bak^{-/-} double knock-out cells have been reported to be resistant to anticancer drug-induced apoptosis in many human cancer cells (26). In addition, Bak has been reported to be up-regulated and to act as a downstream mediator of an oxidative stress pathway that leads to the apoptosis of SH-SY5Y neuroblastoma cells in response to fenretidine (27). These reports demonstrate that Bak mediates hydrogen peroxide-induced apoptosis, and support our finding that Go_{s} can exert a protective effect against hydrogen peroxide-induced apoptosis by repressing Bak expression in SH-SY5Y neuroblastoma cells.

In our study to investigate the mechanism underlying the repression of hydrogen peroxide-induced Bak expression by Go_{s}, we found that Go_{s} represses Bak expression by inhibition of activation and binding of several transcription factors, *i.e.* AP1, NF-κB, and NFAT, to Bak promoter following hydrogen peroxide treatment. This conclusion was reached because the transcription factors AP1, NFAT, and NF-κB, but not CREB, were found to be activated by hydrogen peroxide when assessed by luciferase assay; specific inhibitors of the transcription factors blocked Bak transcription induced by hydrogen peroxide; and Go_{s} or forskolin were found to inhibit the hydrogen peroxide-induced activations of AP1, NFAT, and NF-κB, and thus to repress Bak gene transcription. In addition, Go_{s} also inhibited the binding of AP1, NF-κB, and NFAT to the Bak promoter induced by hydrogen peroxide treatment. The binding sites for these transcription factors seems to be scattered widely in the Bak promoter region (~3500 bp), because Bak luciferase was found to be increased in proportion to the extent of deletion in this region. The nuclear transcription factor AP1 is composed of dimers of *fos* and *jun* proto-oncogenes, and has been linked to a variety of cellular events including proliferation, differentiation, transformation, and apoptosis. Moreover, oxidative stress is known to induce the expressions and phosphorylations of *Fos* and *Jun* (28, 29), and the DNA binding activity of AP1 in various cell types is known as a prelude to cell death (30, 31). This study indicates that AP1 is involved in the repression of hydrogen peroxide-induced Bak expression by Go_{s}. This is based on the results that the deletion of the two AP1 binding sites in the Bak promoter blocked Bak induction by hydrogen peroxide, and that Go_{s} repressed the expression and phosphorylation of both c-Fos and c-Jun. Recently, it was reported that nitric oxide activates an apoptotic cascade in human brain tumor cells, involving sustained INK activation to activate c-Jun and AP1 DNA binding activity and subsequent Bak induction (32). These reports show that AP1 mediates the apoptosis induced by oxidative stress, and support our finding that the repression of AP1 activation by the Go_{s}-cAMP signaling system protects cells from apoptosis. We found that Go_{s} represses...
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hydrogen peroxide-induced activation of GSK-3β by phosphorylation of GSK-3β at serine 9, which can result in inhibition of the phosphorylation and induction of c-Jun to repress AP1 activation. Furthermore, a GSK-3 inhibitor, lithium, was found to block the hydrogen peroxide-induced phosphorylation and induction of c-Jun. The phosphorylation of GSK-3β in GαQL-expressing cells was blocked by the PKA inhibitor, as suggested by the report that PKA can directly phosphorylate GSK-3β at serine 9 and inhibit its apoptotic activity in neurons (33). GSK-3 was reported to act in tandem with JNK to coordinate the full execution of the c-Jun stress response and AP-1-induced death (34). Together with these reports, our finding suggests that Gαs-cAMP signaling inhibits hydrogen peroxide-induced AP1 activation by PKA-dependent phosphorylation and inhibition of GSK-3β. However, GαQL expression increased the basal and hydrogen peroxide-stimulated phosphorylation of JNK, p38, and ERK without a significant increase in the phosphorylation and expression of c-Jun and c-Fos, suggesting that Gαs repressed hydrogen peroxide-induced AP1 activation in a mechanism that is independent of JNK, p38, and ERK. NF-κB-induced transcription has been reported to play a central role in host defense and inflammatory response, and to prevent apoptosis by increasing the expressions of anti-apoptotic genes including Bcl2, and by decreasing the expression of pro-apoptotic Bax protein in many human tumors. Thus, NF-κB may be a major factor that controls the ability of cells to resist apoptosis-based tumor surveillance systems (35). However, NF-κB has also been reported to stimulate apoptosis by inducing the expressions of death-promoting genes including p53, the death receptor Fas, and its ligand, TNF-α, and Bak (36–39) and by linking ferretine-induced ROS production to apoptosis. NF-κB dimers are held in an inactive cytoplasmic complex with a family of inhibitory proteins, the IκBs, including IκBa. Phosphorylation of IκBs at two serine residues in their N-terminal regulatory domain by the IκB kinase complex targets them for rapid ubiquitin-mediated proteasomal degradation, which induces the nuclear translocation of NF-κB to function (40). Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide were reported to inhibit the activation-induced cell death in T cells by repressing expression of Fas ligand through stabilization of IκBa via the cAMP signaling system (41). cAMP was found to decrease NF-κB activation in response to the superoxide anion (42, 43), and to increase the IκBa levels to inhibit lipopolysaccharide-induced NF-κB activation in THP-1 cells (44). Furthermore, activation of the PKA pathway is suggested to inhibit NF-κB transcription by phosphorylating CREB, which competes with p65 for limiting amounts of CBP, because both NF-κB and NFAT require the coactivator CBP for transcriptional activity (45). These reports support our finding that Gαs increases the IκBa level in a PKA-dependent pathway to inhibit hydrogen peroxide-induced activation of NF-κB in neuroblastoma cells. This finding indicates that the Gαs-cAMP signaling pathway protects cells from apoptosis by inhibiting the hydrogen peroxide-induced activation of NF-κB, and implies that NF-κB mediates apoptosis by stimulating Bak expression. NFAT's constitute a family of transcription factors that transduce calcium signals and coordinate the expressions of proteins involved in cell growth and apoptosis. NFAT is known to be activated by activation of various receptors inducing calcium mobilization, and the receptor activation and calcium mobilization result in activation of many intracellular enzymes including the calcium- and calmodulin-dependent phosphatase calcineurin. Calcineurin is a major upstream regulator of NFAT proteins, and causes the dephosphorylation and translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells (46, 47). NFAT has been reported to mediate oxidative stress-induced apoptosis by inducing Fas ligand expression. Moreover, Bcl2 and Bcl-xL were found to mediate anti-apoptotic effects by inhibiting NFAT activation and translocation to the nucleus through the sequestration of calcineurin (48). cAMP signaling has been reported to inhibit NFAT activity by direct phosphorylation of NFAT by PKA resulting in inhibition of nuclear entry and transcriptional activity (41, 49). Together with these reports, our finding that Gαs inhibits hydrogen peroxide-induced nuclear translocation of NFAT, which was inhibited by a PKA inhibitor, suggest that Gαs-cAMP signaling inhibits hydrogen peroxide-induced activation of NFAT by a PKA-dependent phosphorylation. This study shows that the Gαs-cAMP signaling system might act upstream of transcription factors such as AP1, NF-κB, and NFAT to block their activation induced by hydrogen peroxide, the resulting Bak expression, and then apoptosis of SH-SY5Y neuroblastoma cells. The present study also shows that GαQL expression increases the basal level of the Bak protein and CREB binding to the Bak promoter, but that GαQL expression inhibits Bak induction by hydrogen peroxide. Furthermore, it was also shown that hydrogen peroxide reduced CREB binding to the Bak promoter, and that the transfection of decoy CREB increased hydrogen peroxide-induced Bak luciferase activity. Thus, to interpret this seemingly paradoxical phenomenon, we speculate that the Gαs-cAMP signaling system stimulates basal CREB binding to the Bak promoter to increase the basal level of transcription, which is reduced by hydrogen peroxide, but that it represses hydrogen peroxide-induced Bak expression by inhibiting activation of transcription factors mediating Bak expression in a CREB-dependent manner. In addition to Bak expression, Gαs was found to repress the hydrogen peroxide-induced down-regulation of Bcl-xL in SH-SY5Y human neuroblastoma cells, and the resulting increase in Bcl-xL expression shifted the ratio of anti-apoptotic Bcl2 family proteins to favor cell survival (50). This finding suggests that the Gαs-cAMP signaling system maintains mitochondrial integrity and protects cells from apoptosis not only by repressing the induction of pro-apoptotic Bak but also by maintaining the levels of anti-apoptotic Bcl-xL proteins, which implies that Gαs-cAMP affects multiple pro- and anti-apoptotic Bcl2 family proteins.

Because Gαs was found to protect cells from hydrogen peroxide-induced apoptosis, we examined whether PGE2, an agonist of the receptors that couple with Gαs, protects SH-SY5Y cells from hydrogen peroxide-induced apoptosis, and found this to be the case. This finding is similar to reports that PGE2 protects human colon cancer cells from apoptosis (51, 52). In fact, EP receptors have been identified as potential targets for the treatment and prevention of colorectal cancer (53). PGE2 binds to four different receptors termed EP1, EP2, EP3,
EP4, and of these EP2 and EP4 couple to Gαs (54). PGE2 induced a transient increase in cAMP levels, but maintained CREB phosphorylation for several hours, which might be involved in the anti-apoptotic effect of the PGE2-Gαs signaling system. On the other hand, treatment with CCPA, an adenosine A1 receptor agonist, enhanced hydrogen peroxide-induced apoptosis in SH-SYSY neuroblastoma cells. Adenosine A1 receptor interacts with the pertussis toxin-sensitive G protein that inhibits adenylate cyclase, and is considered a promising therapeutic target in cancer (55). The anti-apoptotic effect of PGE2 and the pro-apoptotic effect of CCPA were accompanied by corresponding changes in Bak induction after hydrogen peroxide treatment, indicating that both PGE2 and CCPA influence apoptosis by modulating the Gαs/Gαi-cAMP signaling pathway that regulates Bak expression. This result suggests that apoptosis induced by various stimuli including γ-radiation and chemotherapeutic agents, thus their therapeutic efficiency, can be improved by co-treatment with specific GPCR ligands that modulate sensitivity or resistance to apoptosis.

Based on the findings of the present study, we concluded that Gαs can protect neuroblastoma cells from hydrogen peroxide-induced apoptosis by repressing Bak induction, and that this is mediated by the inhibition of hydrogen peroxide-induced transcription factor activation (e.g. AP1, NF-κB, and NFAT) by the cAMP-PKA-CREB signaling pathway. We also conclude that hydrogen peroxide-induced apoptosis can be modulated by the administration of receptor agonists that activate Gαs or Gαi. These findings provide a better understanding of how Gαs signaling pathways modulate ROS-induced apoptosis, and have important clinical implications, namely, that the apoptosis of cancer cells mediated by hydrogen peroxide following chemotherapy or radiation can be enhanced by administering agonists or antagonists of cancer cell-specific receptors to improve the therapeutic efficiency.

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