0026-895X/04/6506-1475–1484$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:1475–1484, 2004

Vol. 65, No. 6
3167/1152167
Printed in U.S.A.

Ceramide Negatively Regulates Glutathione *S*-transferase Gene Transactivation via Repression of Hepatic Nuclear Factor-1 That Is Degraded by the Ubiquitin Proteasome System

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Received December 19, 2003; accepted March 2, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The level of cellular ceramide, an apoptotic rheostat, is increased by sphingomyelinase or de novo synthesis. The expression of the glutathione *S*-transferase (GST) gene, whose induction accounts for cell viability, is regulated by activation of CCAAT/enhancer binding protein-β (C/EBPβ) and NF-E2-related factor-2 (Nrf2). Hepatic nuclear factor-1 (HNF1) is a transcription factor necessary for cell survival. This study investigated the role of HNF1 in *GSTA2* gene transactivation, the ubiquitin proteasomal degradation of HNF1, and the inhibition of activating HNF1 by ceramide for GSTA2 repression. C2-ceramide (C2), a cell-permeable analog, repressed the GSTA2 expression in H4IIE cells, whereas dihydro-C2, an inactive analog, had no effect. Immunoblot, immunocytochemical, and gel shift analyses revealed that C2 decreased the level of nuclear HNF1 and protein binding to the HNF response element (HRE). Deletion of the HRE or the *GSTA2* gene promoter region containing the HRE reduced luciferase reporter expression. Immunoprecipitation and immunoblot analyses showed that C2 decreased the level of ubiquitinated HNF1, which was reversed by treatment with MG132, a proteasome inhibitor. C2 suppressed GSTA2 induction by oltipraz via inhibition of inducible HNF1 DNA binding. The functional role of HRE for C2 repression of GSTA2 gene transactivation by oltipraz was verified by both the luciferase reporter gene expression and the transfection experiment with ΔHNF-pGL-1651 lacking the HRE. C2 similarly repressed the induction of GSTA2 promoter-luciferase by *tert*-butylhydroquinone via HNF1 suppression, suggesting that constitutive HNF1 activation is required for GSTA2 induction. C2 also inhibited GSTA3/5 expression. In conclusion, the HRE in the *GSTA2* promoter region is functionally active for the constitutive and inducible gene expression, and ceramide inhibits GST gene transactivation through decrease in nuclear HNF1, which is degraded by the ubiquitin proteasome system.

Ceramide serves as a key regulator of apoptotic cell death. Ceramide specifically induces cytotoxicity, which involves the process of apoptosis. Hydrolysis of sphingomyelin generates ceramide in response to extracellular stimuli including a variety of cytokines and stress inducers (Strum et al., 1994; Bose et al., 1995; Zhang et al., 1996). The levels of cellular ceramide increase at early times in response to oxidative stress, UV and ionizing radiation, cytotoxic agents, and cytokines through the activities of the neutral membrane-bound Mg^{+2}-independent sphingomyelinase (*N*-Smase) and acidic Smase (Kolesnick and Golde, 1994; Kolesnick and Krönke, 1998). Ligand activation of the tumor necrosis factor receptor causes activation of *N*-Smase through depletion of cellular glutathione and activation of cPLA2 (Luberto et al., 2002). Accumulation of ceramide leads to activation of the cellular effectors that are involved in the apoptotic process (e.g., proteases). Phosphatidylinositol 3-kinase (PI3-kinase) is down-regulated by ceramide, leading to inhibition of Akt and decreased phosphorylation of the death effector Bad (Basu et al., 1998; Scheid and Duronio, 1998; Salinas et al., 2000; Schubert et al., 2000). Thus, the cellular ceramide level may act as a general apoptotic rheostat controlling cell survival by regulating PI3-kinase-mediated antiapoptotic effector mechanisms.

Hepatic nuclear factor-1 (HNF1), a dimeric transcriptional

This study was supported by the National Research Laboratory Program (2001), Korea Institute of Science and Engineering Evaluation and Planning, Ministry of Science and Technology, The Republic of Korea.

ABBREVIATIONS: Smase, sphingomyelinase; PI3-kinase, phosphatidylinositol 3-kinase; HNF1, hepatic nuclear factor-1; GST, glutathione *S*-transferase; C/EBP, CCAAT/enhancer binding protein; ARE, antioxidant response element; Nrf2, NF-E2-related factor-2; C2, C2-ceramide; *t*-BHQ, *tert*-butylhydroquinone; PBS, phosphate-buffered saline; HRE, hepatic nuclear factor response element; SP-1, specific protein-1; ODN, oligodeoxynucleotide; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; bp, base pair(s).

regulator containing homeodomain, is one of the most important transactivators of liver-specific gene transcription. HNF1α, but not HNF1β, is expressed in hepatocytes (Schrem et al., 2002). HNF1α is also expressed in the extrahepatic tissues such as kidney, intestine, and pancreatic islets (Parrizas et al., 2001; Schrem et al., 2002). Homodimer of HNF1α is found in the liver, whereas heterodimers are detected in other organs. A number of genes are positively regulated by HNF1α interacting with the respective *cis*-acting HNF1 binding element in the promoters of the target genes, which include albumin, aldolase B, fibrinogen, α-lipoprotein AII and B, glucose-6-phosphatase, ferrochelatase, and cytochrome P450 2E1 (Borlak and Thum, 2001; Schrem et al., 2002). On the contrary, induction of dominant negative mutant of HNF1α induces mitochondrial hyperpolarization, cytochrome *c* release, and activation of caspase-3 and caspase-9 (Wobser et al., 2002). In addition, inhibition of HNF1 increases sensitivity to ceramide, but not to the other cytotoxic stimuli (e.g., high glucose-induced apoptosis) (Wobser et al., 2002). Yet, the molecular mechanism by which ceramide controls HNF1α for repression of the target genes has not been studied.

Glutathione *S*-transferases (GSTs) are an important family of detoxifying and cytoprotective enzymes. Glutathione conjugates of xenobiotics formed in the liver can be excreted intact in bile, or they can be converted to mercapturic acids in the kidney and excreted in urine. The elucidation of the mechanisms responsible for the transcriptional regulation of GSTs is one of the major challenges in understanding detoxification and the cell survival process. The *GSTA2* gene is a representative member of class α GSTs that contains xenobiotic response element, phenobarbital-responsive element, and glucocorticoid-responsive element. Analyses of the 5′-flanking region of the *GSTA2* gene from our and other research laboratories revealed the specific sequences defining the location of regulatory regions, namely the CCAAT/enhancer binding protein (C/EBP) response element and the antioxidant response element (ARE) (Kang et al., 2001, 2003). Our previous studies have shown that the PI3-kinase pathway regulates nuclear translocation of activating C/EBPβ and Nrf2 and controls activation of the C/EBP response element and the ARE for the induction of GSTs.

The regulatory region localized to nucleotides −867 to −857 bp in the promoter region of the *GSTA2* gene had been identified as an HNF recognition site (Rushmore et al., 1990). Although the HNF binding site was found to contribute to the maximum basal expression of the *GSTA2* gene (Rushmore et al., 1990), little information is available on the role of HNF1 transcription factor and the HNF response element for the expression of the GST genes. In the present study, we sought to determine 1) whether HNF1 activates the HNF binding element in the promoter region of the *GSTA2* gene and 2) whether ceramide inhibits activation of HNF1 and HNF-dependent GSTA2 gene transactivation. We used C2-ceramide (C2) as a cell-permeable analog of ceramide to assess the effects of ceramide on HNF1 and the *GSTA2* gene expression in H4IIE cells. In addition, we studied the role of the ubiquitin proteasome system in HNF1 degradation. Finally, we determined the effects of ceramide on the expression of other GST forms, GSTA3/5.

Materials and Methods

Materials. [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Anti-HNF1 and anti-ubiquitin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These antibodies specifically recognized their respective transcription factors without any cross-reactivity. C2 and dihydro-C2-ceramide (dihydro-C2) were provided by Calbiochem (San Diego, CA). MG132 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-dithiol-3-thione) was kindly gifted from CJ Co. (Seoul, Korea). *tert*-Butylhydroquinone (*t*-BHQ) (97%) was obtained from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase- or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was purchased from Zymed Laboratories (South San Francisco, CA). The plasmid pGTB-1.65 construct containing the rat GSTA2-promoter region (−1651 to +66) was kindly provided by Dr. C. B. Pickett (Schering Plough, Kenilworth, NJ).

Cell Culture. H4IIE, a rat hepatocyte-derived cell line, was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were treated with C2 (1–25 μM) or dihydro-C2 (20 μM) and dissolved in dimethyl sulfoxide for the indicated time period. For some experiments, H4IIE cells were incubated with 10 μM oltipraz or 10 μM *t*-BHQ for 6 to 24 h in the presence or absence of C2 or dihydro-C2 at 37°C. MG132 (10 μM) was used to inhibit proteasomal degradation. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) before sample preparation.

Preparation of Cytosolic Fractions. H4IIE cells were washed twice with PBS, scraped from their dishes, and sonicated to disrupt the membranes. Cytosolic fractions were prepared by differential centrifugation at 15,000*g* for 15 min and stored at −70°C until use. Protein content was determined by the Bradford assay (Bio-Rad protein assay kit; Bio-Rad, Hercules, CA).

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to previously published methods (Kang et al., 2003). In brief, H4IIE cells in dishes were washed twice with ice-cold PBS and then scraped from the dishes with PBS and transferred to microtubes. Cells were then centrifuged at 3000*g* for 3 min and allowed to swell after the addition of 100 μl of hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. The lysates were incubated for 10 min on ice and then centrifuged at 7200*g* for 6 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. The samples were then centrifuged at 15,000*g* for 10 min to obtain supernatants containing nuclear fractions. Nuclear fractions were stored at −70°C until use.

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to the previously published procedure (Kim et al., 1997). In brief, the cytosolic and nuclear fractions were separated by 7.5% and 12% gel electrophoresis, respectively, and were electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with each of the antibodies directed against GSTα (Detroit R&D, Detroit, MI) and HNF1, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Specificity of the anti-GSTα antibody to the GSTA2 subunit has been confirmed previously (Kim et al., 1997; Kang et al., 2003). Anti-GSTα antibody recognized GSTA3/5 subunits, the migration of which differed from that of GSTA2 subunit. Immunoreactive protein was visualized by an ECL chemiluminescence detection kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) (Kang et al., 2002, 2003). Equal loading of proteins was verified by actin immunoblotting with goat anti-actin

antibody (Santa Cruz Biotechnology, Inc.). Changes in the levels of proteins were determined by scanning densitometry of the blots. At least three separate experiments were performed with different samples to confirm changes in the protein levels.

Scanning Densitometry. Scanning densitometry of the immunoblots was performed with the Image Scan and Analysis System (Alpha Innotech, San Leandro, CA). The area of each lane was integrated using the software AlphaEase version 5.5, followed by background subtraction.

Gel Shift Assay. A double-stranded DNA probe containing the *GSTA2* gene HNF response element (HRE) was end-labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase and used for gel shift analysis. The sequence of the HNF binding oligonucleotide was 5′-TAGGTAATGATTAATAACCAAGACCCATGAA-3′. The reaction mixture contained 4 μl of 5× binding buffer (containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC, and 50 mM Tris-Cl, pH 7.5), 10 μg of nuclear extracts, and sterile water up to a total volume of 20 μl. The reaction mixture was preincubated without probe at room temperature for 10 min. The probe (1 μl, containing 10^6 cpm) was then added, and DNA-binding reactions were carried out for 30 min at room temperature. In some analyses, specificity of binding was determined by competition experiments, which were carried out by adding a 20-fold molar excess of an unlabeled HNF binding oligonucleotide to the reaction mixture before the labeled probe was added. SP-1 oligonucleotide (5′-ATTCGATCGGGGCGGGGCGAGC-3′) was used as a negative control for competition experiments. In other analyses, known as immuno-inhibition assays, antibody to HNF1 or HNF3β (2 μg each) was added to the reaction mixture 20 min after the labeled probe was added, and the reaction was then continued for another hour at 25°C. Samples were separated on 4% polyacrylamide gels at 100 V. The gels were fixed with 40% methanol/10% acetic acid, dried, and subject to autoradiography.

Immunocytochemistry. H4IIE cells were grown on Lab-TEK chamber slides (Nalge Nunc International, Naperville, IL) and incubated in serum-free Dulbecco's modified Eagle's medium for 6 h at 37°C. Standard immunocytochemical methods were used for immunostaining of HNF1 as described previously (Kang et al., 2003). In brief, cells were fixed in 100% methanol for 30 min, washed three times with PBS, and blocked in PBS containing 5% bovine serum albumin for 1 h at 37°C. The cells were then incubated for 1 h with a polyclonal rabbit anti-HNF1 antibody (1:100) in PBS containing 0.5% bovine serum albumin at 37°C, washed several times with PBS, and incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:100) at 37°C. Counterstaining with propidium iodide (2 μg/ml) was used to verify the location and integrity of nuclei. Negative control experiments were carried out with goat anti-rabbit IgG antibody as a primary antibody or with only fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody. Stained cells were washed five times with PBS and examined using a laser-scanning confocal microscope (Leica TCS NT; Leica, Wetzlar, Germany).

Construction of GSTA2 Promoter-Luciferase Constructs and Luciferase Assay. The pGL-1651 reporter gene construct was generated by ligating the 1.65-kb upstream region from the transcription start site of the *GSTA2* gene to the firefly luciferase reporter gene coding sequence. The pGL-797 reporter plasmid, in which the DNA region comprising the HRE was deleted from the promoter, was previously constructed (Kang et al., 2003). To specifically delete the HNF binding site from pGL-1651, the DNA fragments containing the nucleotides from −1651 to −873 bp and from −844 to +66 bp were PCR amplified using specific primers. The DNA fragments were ligated and cloned into the *Kpn*I/*Xho*I and *Xho*I/*Bgl*II sites of pGL3 (Promega, Madison, WI) luciferase reporter plasmid, which was indicated as ΔHNF-pGL-1651. The DNA sequences of the constructs were verified by sequence analysis using an ABI7700 DNA cycle sequencer and by restriction enzyme digestion.

To determine the promoter activity, we used the dual-luciferase reporter assay system (Promega). In brief, H4IIE cells (7×10^5 cells/well) were replated in six-well plates overnight, serum starved for 12 h, and transiently transfected with each GSTA2 promoter-luciferase construct and pRL-SV plasmid (a plasmid that encodes for *Renilla* luciferase and is used to normalize transfection efficacy) in the presence of LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA) for 3 h. Transfected cells were incubated in Dulbecco's modified Eagle's medium containing 1% fetal calf serum for 3 h. The cells were treated with 20 μM C2 for 1 h at 37°C and then exposed to 10 μM oltipraz or *t*-BHQ for 18 h in the continuing presence of C2. Firefly and *Renilla* luciferase activities in cell lysates were measured using a Luminoskan luminometer (Thermo Labsystems, Helsinki, Finland). The activity of firefly luciferase was measured by adding Luciferase Assay Reagent II (Promega) according to the manufacturer's instruction, and the *Renilla* luciferase reaction was initiated by adding Stop and Glo reagent (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of *Renilla* luciferase.

Decoy Oligodeoxynucleotide Technique. Double-stranded oligodeoxynucleotide (ODN) was prepared from complementary single-stranded phosphorothioate-bonded ODN (Bioneer, Chungbuk, Korea) by melting at 95°C for 5 min followed by a cool-down phase at ambient temperature (Cho et al., 2002). The sequences of the single-stranded ODN were as follows. Underlined letters denote phosphorothioate-bonded bases: HNF1, 5′-CACTGGTTAATGATTAACCACCA-3′; and mutant HNF1, 5′-CACTACCTAATAACAAAGCACCA-3′ (Locker et al., 2002; Hakkola et al., 2003). Cells were transfected with pGL-1651 and incubated with 2 μM decoy ODN or mutant decoy ODN for 24 h. Luciferase activity was measured as described above.

Immunoprecipitation. To determine the ubiquitinated HNF1, total cell lysate (250 μg in 1 ml) was incubated with anti-HNF1 or anti-ubiquitin antibody overnight at 4°C. The antigen-antibody complex was immunoprecipitated after incubation for 2 h at 4°C with protein G-agarose. Immune complexes were solubilized in 2× Laemmli buffer and boiled for 5 min. Samples were separated and analyzed using 7.5% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The samples were then immunoblotted with anti-ubiquitin or anti-HNF1 antibody. Blots were developed using an ECL chemiluminescence detection kit to visualize immunoblots.

Statistical Analysis. One-way analysis of variance was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons among multiple group means. The data were expressed as means ± S.E.

Results

The Constitutive Expression of GSTA2. H4IIE cells were treated with various concentrations of C2 to assess changes in the constitutive expression of GSTA2. The levels of GSTA2 protein were 52% and 80% suppressed in the cells treated with 20 and 25 μM C2 for 24 h, respectively. In contrast, dihydro-C2 did not change the level of constitutive GSTA2 (Fig. 1, A and B). The MTT assay showed that H4IIE cells exposed up to 25 μM C2 for 24 h were >90% viable. The viability of H4IIE cells incubated with 30 μM C2 for 24 h decreased to 10% of untreated control cells, presumably because of apoptosis. These data suggested that C2 might repress the cellular factor(s) required not only for GSTA2 expression but also for cell viability.

The Constitutive Nuclear HNF1. HNFs serve as the essential transcription factors for cell survival, which has been shown in hepatocytes and other types of cells (Schrem et al., 2002; Ryffel, 2001). The regulatory region localized to

nucleotides −857 to −867 bp, identified as an HNF recognition site, contributed to the maximal basal expression of the *GSTA2* gene (Rushmore et al., 1990). To determine whether ceramide altered the level of activating HNF1, immunoblot experiments were first conducted with the nuclear fractions. The degradation kinetics of HNF1 was studied in the cells treated with C2 for 3 to 12 h (Fig. 2A). The level of nuclear HNF1 decreased 3 h after treatment of H4IIE cells with C2. HNF1 was weakly detectable in the nuclear fraction at 6 h or a later time point. Treatment of the cells with 20 μM dihydro-C2 caused no change in the level of nuclear HNF1 (Fig. 2A). The level of nuclear GATA, which was determined as a comparative purpose, was not changed 3 to 6 h after C2 treatment compared with control, but rather increased 12 h after treatment (data not shown).

To verify whether decreases in band intensities obtained in the immunoblot analyses occurred as a result of alterations in the nuclear HNF1, the subcellular localization of HNF1 was determined by immunocytochemical analysis (Fig. 2B). Although HNF1 was located in both the cytoplasm and the nuclei of untreated H4IIE cells, HNF1 began to show only cytoplasmic localization 3 h after 20 μM C2 treatment. HNF1 disappeared in the nuclei of the cells treated with C2 for 6 to 12 h.

We determined whether C2 decreased HNF1 DNA binding. Gel shift analysis of protein binding to the HNF binding site was performed with the nuclear extracts of H4IIE cells using a radiolabeled HNF binding oligonucleotide. Treatment of H4IIE cells with 20 μM C2 gradually decreased the band intensity of HNF1 binding to the DNA as a function of time (i.e., 3–12 h), compared with control (Fig. 2C, left). Competition experiments with the antibody directed against HNF1 or

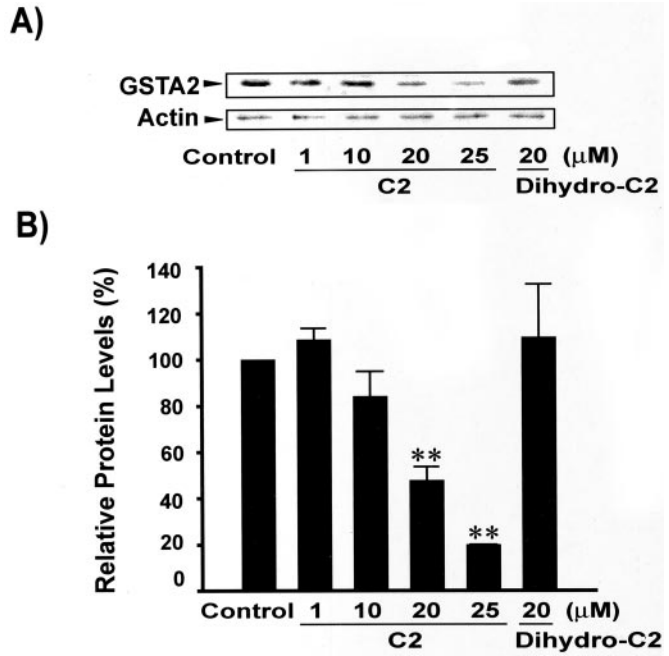

Fig. 1. Suppression of the constitutive expression of GSTA2 by C2. A, representative immunoblot analysis of the GSTA2 protein in the H4IIE cells incubated with C2 or dihydro-C2 for 24 h. Each lane was loaded with 10 μg of cytosolic proteins. Immunoreactive protein was visualized through incubation with horseradish peroxidase-conjugated secondary antibody and an ECL chemiluminescence detection kit. B, relative levels of GSTA2 in the cells treated with 1 to 25 μM C2 or 20 μM dihydro-C2 for 24 h. Equal loading of proteins was verified by probing the replicate blot for actin. Changes in GSTA2 protein levels relative to control were assessed by scanning densitometry. Data represent the mean ± S.E. of three separate experiments (significant compared with control, **, $p <$ 0.01; the level of GSTA2 in control cells = 100%).

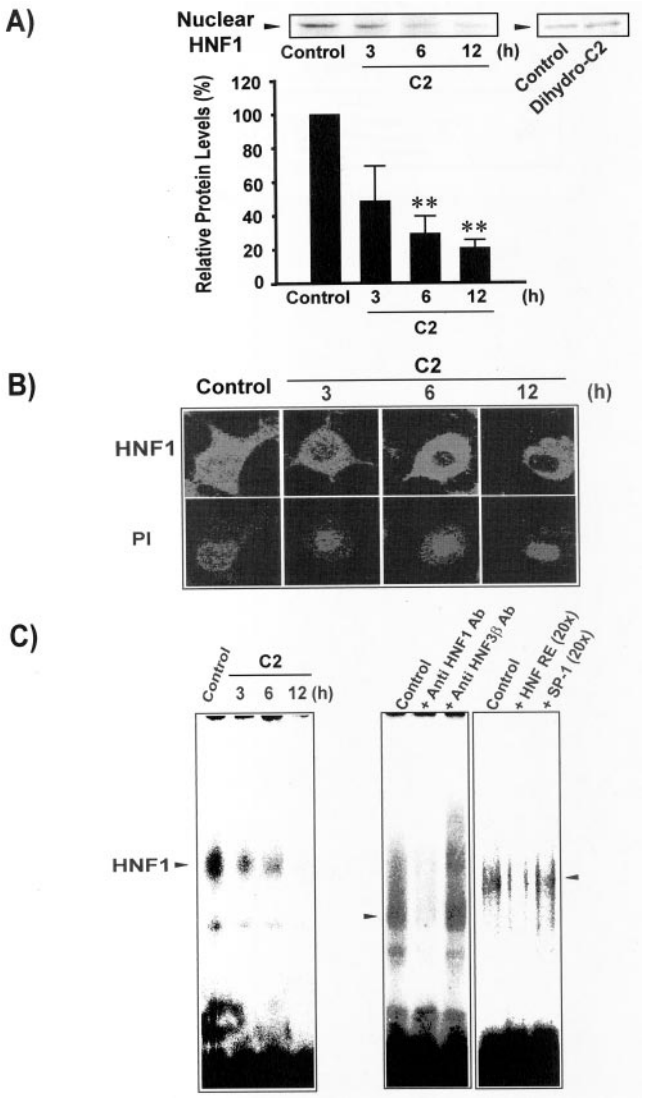

Fig. 2. Inhibition of constitutive HNF1 activation by C2. A, immunoblot analysis of nuclear HNF1 in H4IIE cells. The levels of HNF1 were determined in the nuclear fractions of the cells treated with 20 μM C2 for 3 to 12 h. Cells were also treated with 20 μM dihydro-C2. The relative HNF1 levels were measured by scanning densitometry of the immunoblots. Data represent the mean ± S.E. of at least three separate experiments (significant compared with control, **, $p < 0.01$) (Control = 100%). B, immunocytochemical analysis of HNF1. HNF1 was immunochemically localized using anti-HNF1 antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody in the H4IIE cells treated with C2 for 3 to 12 h. The same fields were counterstained with propidium iodide (PI) to verify the location and integrity of nuclei. Results were confirmed by separate experiments. C, gel shift analyses of protein binding to the HRE. Nuclear extracts were prepared from control cells or the cells incubated with 20 μM C2 for 3 to 12 h. Five micrograms of nuclear extracts was then incubated for 30 min with 5 ng of radiolabeled HNF binding oligonucleotide. Arrowhead indicates the DNA bound with HNF (left). Immunocompetition assay of HNF binding to the HNF binding oligonucleotide. Nuclear extracts prepared from the H4IIE cells were incubated with polyclonal antibodies against HNF1 or HNF3β (2 μg each) for 1 h. The nuclear extracts immunodepleted with anti-HNF antibody were then mixed with labeled probe for the HNF binding oligonucleotide (middle). For competition assays, a 20-fold molar excess of unlabeled HNF oligonucleotide or SP-1 oligonucleotide was added to the nuclear extracts from control cells (right).

HNF3β indicated that HNF DNA binding activity was specifically dependent on HNF1 (Fig. 2C, middle). Addition of a 20-fold excess of an unlabeled HNF binding oligonucleotide to the nuclear extract completely abolished the binding activity, whereas excess unlabeled SP-1 oligonucleotide failed to inhibit binding, suggesting that the binding protein was HNF (Fig. 2C, right). These data provided evidence that C2 decreased the level of activating nuclear HNF1, which was accompanied by GSTA2 repression.

The Role of the HRE in the *GSTA2* Gene Transactivation. Next, we examined the functional role of the HNF binding site in the GSTA2 promoter to assess whether C2 repressed transcription of the *GSTA2* gene via down-regulation of activating HNF1 that binds to the HRE. When the DNA sequence containing the core HNF binding site located between the nucleotide −857 bp and the nucleotide −867 bp in pGL-1651 was deleted (Fig. 3A) (i.e., ΔHNF-pGL-1651), the constitutive expression of luciferase activity was reduced to 45% of control (Fig. 3B). To further determine the functional role of the HRE in the *GSTA2* gene expression, we used GSTA2 promoter deletion analysis. Reporter gene assays were also performed using the H4IIE cells transfected with the mammalian cell expression vector pGL-797, which contained the ARE but lacked the DNA region comprising the HRE (Fig. 3A). Transfection of H4IIE cells with pGL-797 resulted in a 50% decrease in luciferase activity compared with that obtained with pGL-1651 (Fig. 3B). To further verify the role of the HNF1 consensus site in the *GSTA2* gene, pGL-1651-transfected cells were incubated with the HNF1-specific decoy ODN for 24 h, and the expression of luciferase was determined. HNF1, but not mutant HNF1, decoy ODN significantly reduced luciferase expression from pGL-1651 (Fig. 3C). These results indicated that the HRE was functionally active for the constitutive transactivation of the *GSTA2* gene.

Decrease in Nuclear HNF1 That Is Degraded by the Ubiquitin Proteasome System. Certain transcription factors are degraded in the proteasomes after multi-ubiquitination (Shim and Smart, 2003; Stewart et al., 2003). To determine whether ubiquitins are conjugated to HNF1, we subjected the lysates obtained from H4IIE cells treated with C2 or MG132, a proteasome inhibitor, to immunoprecipitate with an anti-ubiquitin antibody and then analyzed the immunoprecipitates by immunoblotting using an anti-HNF1 antibody. Multi-ubiquitination showing a ladder pattern was observed in untreated control cells, whereas the intensity of one of the major ubiquitinated HNF1 bands was notably decreased in the cells treated with C2 (Fig. 4A, left). In contrast, the protein species corresponding to that decreased by C2 treatment was accumulated in the cells exposed to MG132. We obtained similar results by anti-HNF1 immunoprecipitation and anti-ubiquitin immunoblotting analyses (Fig. 4A, right).

To assess whether the level of activating HNF1 decreased in parallel with a decrease in ubiquitinated HNF1, the nuclear proteins prepared from the cells treated with C2 and/or MG132 were immunoblotted with an anti-HNF1 antibody (Fig. 4B). Treatment of H4IIE cells with 20 μM C2 markedly decreased the HNF1 level in the nuclear fraction. HNF1 was notably accumulated in the cells treated with C2 plus MG132. Treatment of cells with MG132 alone (6 h) also resulted in accumulation of HNF1 in the nuclei, as evidenced by enhancement of the immunoblotted band intensity of HNF1. These results indicate that ceramide decreases the level of nuclear HNF1 that is subjected to ubiquitin proteasomal degradation.

The Effect of C2 on GSTA2 Induction by Oltipraz. A previous study from this laboratory has shown that oltipraz induces GSTA2 through activation of C/EBPβ and its binding to the C/EBP response element (Kang et al., 2003). We were interested in whether oltipraz-inducible GSTA2 expression was affected by C2 treatment. C2 at the concentration of 10 μM partially, but significantly, inhibited the expression of GSTA2 induced by 10 μM oltipraz (Fig. 5A). GSTA2 induction by oltipraz was completely suppressed 24 h after 20 μM C2 treatment. Dihydro-C2 (20 μM) failed to significantly decrease oltipraz-inducible GSTA2 expression (Fig. 5A).

Our preliminary study revealed that oltipraz activated HNF1, as supported by nuclear translocation of HNF1 and an increase in protein binding to the HNF binding site (M. K. Cho and S. G. Kim, unpublished data). We assessed whether C2 decreased HNF1 activation inducible by oltipraz. Immunoblot and immunocytochemical analyses revealed that C2 at the concentration of 20 μM blocked the increase in the level of nuclear HNF1 in the cells treated with oltipraz (Fig. 5B, left). This was confirmed by immunocytochemical analysis (Fig. 5B, right). We then examined whether C2 changed the extent of protein binding to the HNF binding oligonucleotide in the cells exposed to oltipraz. Gel shift analysis of protein binding to the HNF binding site was performed with nuclear extracts of H4IIE cells using a radiolabeled HNF binding oligonucleotide. Treatment of H4IIE cells with oltipraz + C2 for 6 h resulted in a decrease in HNF binding to the DNA compared with oltipraz alone (Fig. 5C). Antibody competition experiment showed that the complex of protein binding to the DNA comprised HNF1 but not HNF3β.

To correlate the functional role of the HRE with C2-mediated suppression of the *GSTA2* gene transactivation by oltipraz, we used the ΔHNF-pGL1651 plasmid. Reporter gene assays showed that C2 was capable of repressing oltipraz-inducible gene transactivation (Fig. 5D). Inhibition of oltipraz-inducible pGL-1651 luciferase expression by C2 was comparable with that observed in the cells transfected with ΔHNF-pGL-1651. These data provided evidence that C2 inhibited HNF1 activation by oltipraz, which resulted in suppression of the *GSTA2* gene transactivation.

The Effect of C2 on GSTA2 Induction by *t*-BHQ. *t*-BHQ induces GSTA2 primarily through activation of Nrf2 and binding of activated Nrf2 to the ARE (Kang et al., 2000, 2001). We determined the effect of C2 on *t*-BHQ-inducible GSTA2 expression to assess whether the constitutive HNF1 activation was required for the gene induction by Nrf2 activation. C2 (10 or 20 μM) significantly inhibited GSTA2 induction by 10 μM *t*-BHQ, whereas dihydro-C2 did not change *t*-BHQ-inducible GSTA2 expression (Fig. 6A).

We then determined whether C2 inhibited the constitutive HNF1 activation in the cells treated with *t*-BHQ. Nuclear HNF1 was not affected by treatment with *t*-BHQ alone (Fig. 6B, left). The level of HNF1 in the nuclear fraction was decreased by 20 μM ceramide treatment (Fig, 6B, left). Immunocytochemistry of the cells treated with *t*-BHQ in the presence or absence of C2 verified HNF1 suppression in *t*-BHQ-treated cells (Fig. 6B, right). Gel shift analysis

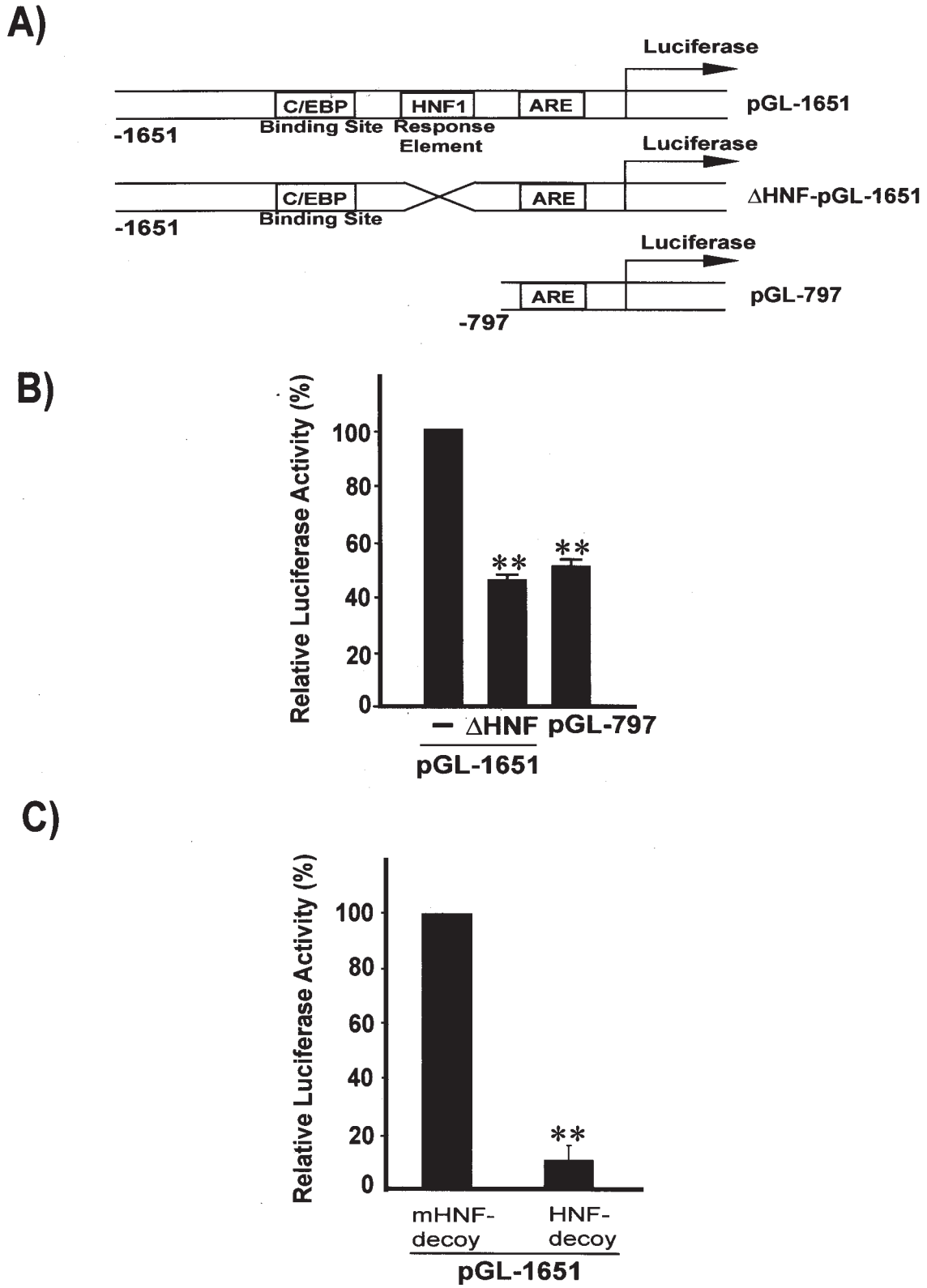

Fig. 3. The role of HNF binding element in the constitutive transcription of GSTA2 promoter-luciferase gene. A, GSTA2-luciferase reporter constructs. B, decrease in the luciferase activity by deletion of the HNF binding element. Dual luciferase reporter assays were performed in H4IIE cells transiently transfected with ΔHNF-pGL-1651 (ΔHNF), in which the HRE was specifically deleted, or with pGL-797, in which the DNA region from −797 to −1651 bp comprising the HRE was deleted. The relative luciferase inducibility was obtained from the ratio of activity in ΔHNF-pGL-1651- or pGL-797-transfected cells to that in pGL-1651-transfected cells (100%). The experimental value for luciferase activity represented the mean ± S.E. of three separate experiments (significant compared with pGL-1651-transfected cells, **, $p < 0.01$). C, effect of HNF1 consensus decoy ODN on the pGL-1651 luciferase expression. Cells were transfected with pGL-1651 and incubated with 2 μM HNF1 decoy ODN or mutant HNF1 (mHNF1) decoy ODN for 24 h. Luciferase activity was monitored as described above. The experimental value for luciferase activity represented the mean ± S.E. of three separate experiments (significant compared with mHNF decoy, **, $p < 0.01$).

showed that C2 decreased HNF1 DNA binding (data not shown).

The luciferase reporter gene analysis demonstrated that the expression of GSTA2 promoter-luciferase inducible by *t*-BHQ was inhibited by C2 treatment or by transfection with ΔHNF-pGL1651 (Fig. 6C). These results suggested that the constitutive HNF1 activation is necessary for Nrf2-mediated GSTA2 induction by *t*-BHQ.

The Effects of C2 on the Expression of GSTA3/5. To explore whether C2 also suppressed the expression of other GSTα forms that are controlled by HNF1, we immunoblotted for GSTA3/5 in the lysates of control cells or the cells treated with oltipraz or *t*-BHQ in the presence or absence of C2. The constitutive expression of GSTA3/5 was repressed by treatment of the cells with 20 or 25 μM C2 but not with dihydro-C2 (20 μM) (Fig. 7A). GSTA3/5 induction by oltipraz or *t*-BHQ was repressed by concomitant treatment of the cells with 20 μM C2 (Fig. 7, B and C). Dihydro-C2 very weakly inhibited oltipraz-inducible GSTA3/5, which was not statistically significant. Our results demonstrated that C2 was capable of repressing GSTA3/5.

Discussion

Cellular responses to ceramide culminate in increased apoptosis and decreased proliferation and differentiation of cells (Zundel and Giaccia, 1998; Pettus et al., 2002; Kolesnick and Fuks, 2003). Suppression of HNF1α activates the apoptotic cell death machinery via alterations in gene expression and mitochondrial dysfunction (Wobser et al., 2002). Knockout experiments revealed that mice lacking HNF1α die around weaning after a progressive wasting syndrome with a marked liver enlargement (Wobser et al., 2002), supporting the notion that HNF1 serves as an essential transcription factor necessary for cell survival. In the present study, we demonstrated for the first time, that ceramide as a signaling molecule for apoptosis decreased the level of nuclear HNF1

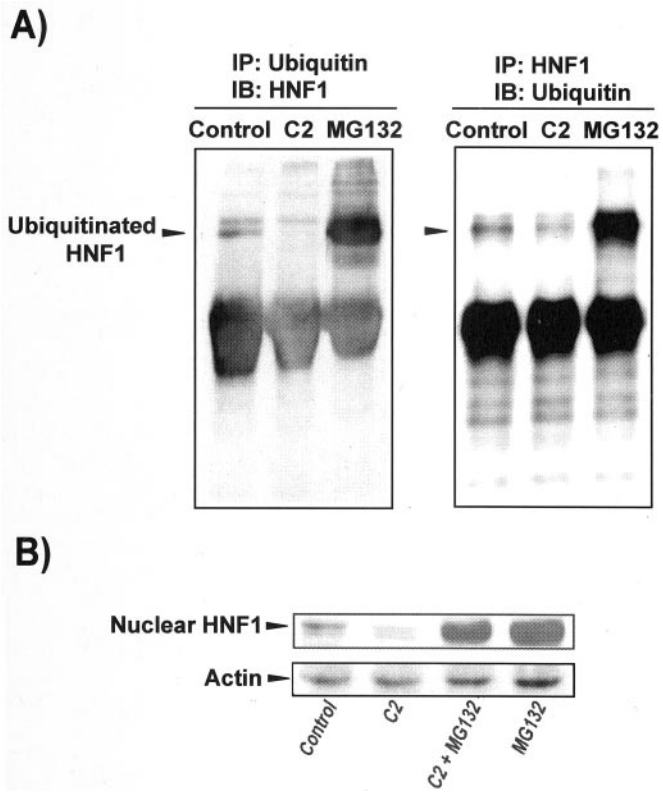

Fig. 4. The effects of C2 on the level of ubiquitinated HNF1 in H4IIE cells. A, immunoblot analyses of ubiquitinated HNF1. The lysates prepared from the H4IIE cells treated with 20 μM C2 for 6 h were immunoprecipitated with an anti-ubiquitin antibody followed by immunoblotting with an anti-HNF1 antibody. Immunoblot analysis for ubiquitin was also carried out with anti-HNF1 immunoprecipitates. B, immunoblot analysis of nuclear HNF1. The levels of HNF1 were determined in the nuclear fractions obtained from the H4IIE cells treated with 20 μM C2 and/or 10 μM MG132. Each lane was loaded with 15 μg of nuclear proteins. Equal loading of proteins was verified by probing the replicate blot for actin. Results were confirmed by repeated experiments.

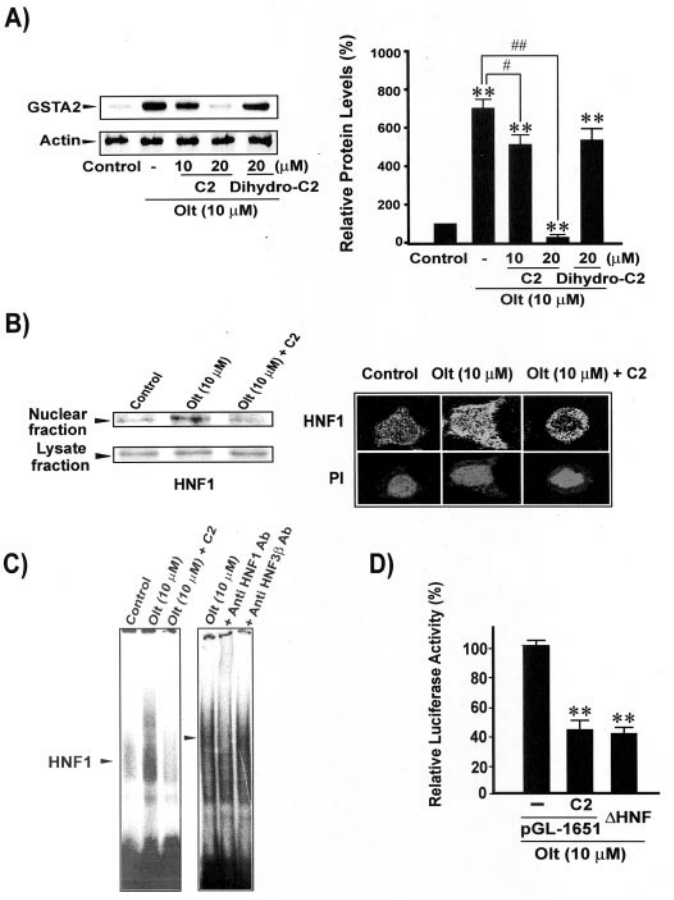

Fig. 5. The effect of C2 on HNF1-mediated GSTA2 gene transactivation by oltipraz. A, immunoblot analysis of GSTA2 protein in the H4IIE cells incubated with oltipraz (10 μM), oltipraz + C2 (20 μM), or oltipraz + dihydro-C2 (20 μM) for 24 h. Each lane was loaded with 10 μg of cytosolic proteins. Equal loading of proteins was verified by probing the replicate blot for actin. Data represent the mean ± S.E. of at least three separate experiments (significant compared with control, *, $p < 0.05$; **, $p < 0.01$; significant compared with oltipraz alone, #, $p < 0.05$; ##, $p < 0.01$) (the level of GSTA2 in control cells = 100%). B, effect of C2 on the level of HNF1 in the nucleus. HNF1 was immunoblotted in the nuclear and lysate fractions of the cells treated with 10 μM oltipraz in the presence or absence of 20 μM C2 for 6 h. HNF1 was also immunochemically localized using anti-HNF1 antibody, as described in Fig. 2B. C, gel shift analysis of HNF DNA binding. Nuclear extracts were prepared from control cells or the cells incubated with oltipraz or oltipraz + C2 for 6 h. Arrowhead indicates the DNA bound with HNF1. Immunocompetition assay of HNF DNA binding was carried out as described in Fig. 2C. D, GSTA2-luciferase activities in the pGL-1651-transfected cells treated with oltipraz or oltipraz + C2 or in the ΔHNF-pGL-1651-transfected cells (ΔHNF) treated with oltipraz for 18 h. Dual luciferase reporter assays were performed as described in the legend to Fig. 3. The experimental value for luciferase activity represented the mean ± S.E. of three separate experiments [significant compared with pGL-1651-transfected cells treated with oltipraz alone (100%), **, $p < 0.01$]. Olt, oltipraz.

and thus reduced its binding to the HNF binding site. We also found that the ubiquitin proteasome system degraded HNF1. This was directly evidenced by ubiquitination of HNF1 and also by decrease in ubiquitinated HNF1 in parallel with that in nuclear HNF1 by C2. The role of the ubiquitin proteasome system for HNF1 degradation was further supported by accumulation of ubiquitinated HNF1 in the cells treated with MG132, a proteasome inhibitor. The decrease in nuclear HNF1 accompanied a decrease in the amount of nuclear ubiquitinated HNF1. Ceramide may cause cells to decrease the rate of HNF1 synthesis, thereby reducing the supply of HNF1 (the half-lives of proteins decrease in general in the cells under stress). Alternately, the reduction in activating HNF1 in the nucleus may have resulted from a consequence of a more rapid mode of proteasomal degradation of HNF1 that requires ubiquitination. Hence, we speculate that the reduction in activating HNF1 by ceramide may result from the disturbed homeostatic balance between HNF1 synthesis and its degradation. The exact mechanism of degradation process of HNF1 remains to be further identified.

Previous studies have shown that the HRE located in the *GSTA2* gene promoter is functional and that HNF1α, but not HNF1β, binds to the HNF binding site (Voss et al., 2002). Low levels of GST expression in kidney cells correlated with low levels of HNF1 (Clairmont et al., 1994). On the contrary, decrease in the expression of GST by interleukin-6 and dexamethasone was not accompanied by any decrease in binding of HNF1 to the HRE (Voss et al., 2002). HNF1 is required as an essential transcription factor for the transcription of the genes responsible for the hepatocyte viability. Nevertheless, the activation of HNF1 and the role of activating HNF1 for the HNF binding site present in the promoter region of the target gene have not been studied in association with apoptotic cell death signal. In the present study, we found the role of activating HNF1 in the constitutive and inducible *GSTA2* gene transactivation. We also established the inhibitory effect of ceramide on HNF1-mediated GST gene expression.

The expression of GST and many other genes (e.g., albumin) requires HNF1 for the maximal transcription activity

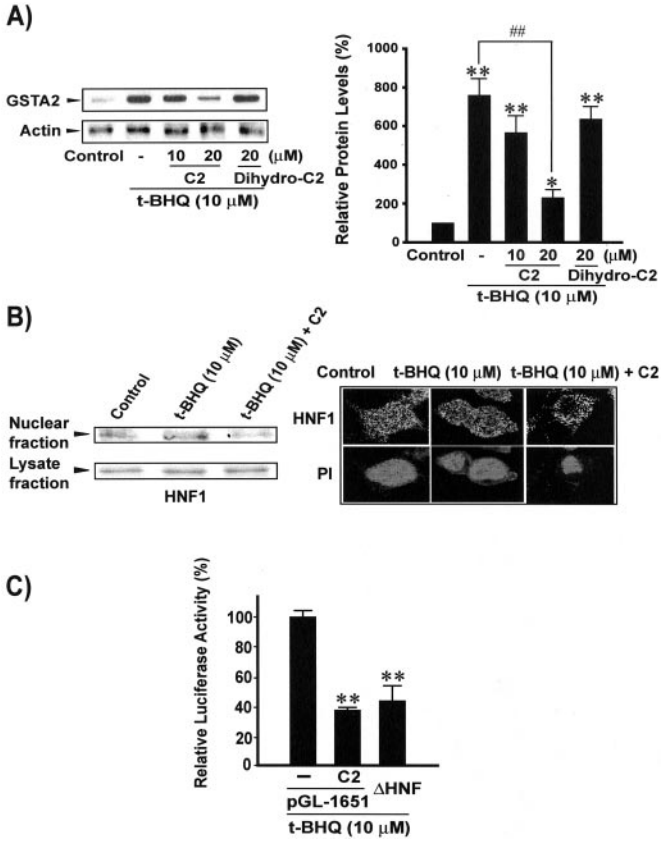

Fig. 6. The effects of C2 on the *GSTA2* gene transactivation by *t*-BHQ. A, immunoblot analysis of GSTA2 protein in the H4IIE cells incubated with *t*-BHQ (10 μM), *t*-BHQ + C2 (20 μM), or *t*-BHQ + dihydro-C2 (20 μM) for 24 h. Equal loading of proteins was verified by probing the replicate blot for actin. Data represent the mean ± S.E. of at least three separate experiments (significant compared with control, *, $p < 0.05$; **, $p < 0.01$; significant compared with *t*-BHQ alone, ##, $p < 0.01$) (the level of GSTA2 in control cells = 100%). B, effect of C2 on the level of HNF1 in the nucleus. HNF1 was immunochemically monitored in the cells treated with 10 μM *t*-BHQ in the presence or absence of 20 μM C2 for 6 h, as described in the legends to Fig. 2, A and B. HNF1 was also immunochemically localized using anti-HNF1 antibody, as described in Fig. 2B. C, GSTA2-luciferase activities in the pGL-1651-transfected cells treated with *t*-BHQ or *t*-BHQ + C2 or in the ΔHNF-pGL-1651-transfected cells (ΔHNF) treated with *t*-BHQ for 18 h. Dual luciferase reporter assays were performed as described in the legend to Fig. 3. The experimental value for luciferase activity represented the mean ± S.E. of three separate experiments [significant compared with pGL-1651-transfected cells treated with *t*-BHQ alone (100%), **, $p < 0.01$].

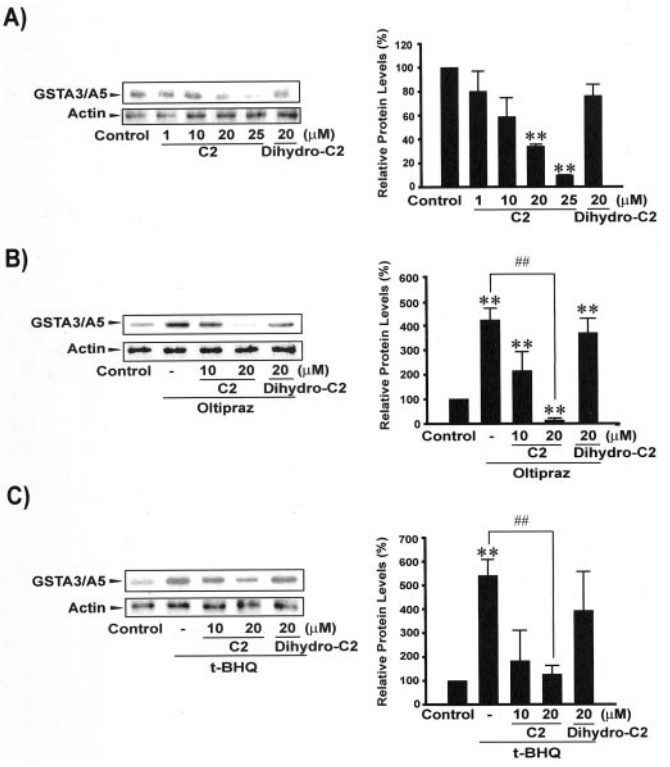

Fig. 7. The effects of C2 on the expression of GSTA3/5 in H4IIE cells. A, immunoblot analysis of GSTA3/5. The constitutive expression of GSTA3/5 protein was assessed in the cells treated with 1 to 25 μM C2 or 20 μM dihydro-C2 for 24 h. B, immunoblot analysis of oltipraz-inducible GSTA3/5. The expression of GSTA3/5 protein was assessed in the cells treated with 10 μM oltipraz in the presence or absence of 10 to 20 μM C2 or 20 μM dihydro-C2 for 24 h. C, immunoblot analysis of *t*-BHQ-inducible GSTA3/5. The expression of GSTA3/5 protein was assessed in the cells treated with 10 μM *t*-BHQ in the presence or absence of 10 to 20 μM C2 or 20 μM dihydro-C2 for 24 h. Each lane was loaded with 10 μg of cytosolic proteins. Equal loading of proteins was verified by probing the replicate blot for actin. Data represent the mean ± S.E. of at least three separate experiments (significant compared with control, *, $p < 0.05$; **, $p < 0.01$; significant compared with oltipraz or *t*-BHQ alone, ##, $p < 0.01$; the level of GSTA2 in control cells = 100%).

(Schrem et al., 2002). In the present study, the functional role of the HRE for C2 repression of the *GSTA2* gene transactivation was verified by the luciferase reporter gene expression and the promoter deletion analysis. Also, the crucial role of activating HNF1 binding to the HRE was demonstrated by the luciferase reporter experiment using an HRE-deleted mutant of the *GSTA2* gene promoter (i.e., ΔHNF-pGL1651). Multiple coactivators of HNF1 and other HNFs have been previously identified, including CBP, p300, and p/CAF (Schrem et al., 2002). The transcription factors are members of the p160 protein family and have been shown to exhibit an intrinsic histone acetyltransferase activity (Ma et al., 1999). Physical interaction of HNF1 with CBP, p/CAF, SRC-1, and SRC-3 leads to increased HNF1-dependent transcription of a genome-integrated promoter (Ma et al., 1999; Jung and Kullak-Ublick, 2003). Sphingolipid is involved in the down-regulation of genes such as cytochrome P450s. For example, accumulation of ceramide is responsible for the repression of CYP2C11 by interleukin-1 (Chen et al., 1995). Degradation by ceramide of HNF1 and other transcription factors may also be responsible for the transcriptional repression of cytochrome P450s and other gene products.

The C/EBP genes play a role in normal tissue development and cellular function, cell proliferation, and functional development (Matsuno et al., 1996; Shim and Smart, 2003). C/EBP family members and bZIP transcription factors possess a leucine zipper and a basic DNA-binding domain in their C-terminal region and N-terminal transcription factors (Yeh et al., 1995). Previously, we have shown that nuclear translocation of C/EBPβ and its binding to the C/EBP binding site in the *GSTA2* gene, which is activated by oltipraz, leads to strong and persistent induction of the gene (Kang et al., 2003). The pathway of PI3-kinase plays an essential role in oltipraz-inducible nuclear translocation of C/EBPβ (Kang et al., 2003). Oltipraz induces GSTA2 through enhanced nuclear translocation of HNF1 and HNF1 binding to the HRE (M. K. Cho and S. G. Kim, unpublished data) and through activating C/EBPβ binding to the C/EBP response element (Kang et al., 2003). Ceramide decreased the transcriptional activity of C/EBPβ with a strong decrease in its phosphorylation (Sprott et al., 2002; Kostova and Wolf, 2003). We also found that ceramide stimulates degradation of C/EBPβ in H4IIE cells (I. N. Park, I. J. Cho, and S. G. Kim, unpublished data). Thus, the apoptotic effect of ceramide, which counterbalances the cell surviving effect of oltipraz, may be mediated with repression of HNF1 and with other transcription factors, such as C/EBP proteins.

The compounds including *t*-BHQ and β-naphthoflavone induce Nrf2 phosphorylation, which leads to an increase in its stability and subsequent transactivation activity (Nguyen et al., 2003). Ceramide was capable of inhibiting Nrf2 (I. N. Park, I. J. Cho, and S. G. Kim, unpublished data), indicating that ceramide represses the induction of GSTA2 at least in part through suppression of Nrf2. Taken together, the current study provides the first evidence that ceramide mediates degradation of the activating transcription factors that are required for gene expression and that the ability of ceramide in decreasing the essential transcription factors is accompanied by repression of the GST gene. Repression of GST in response to toxic stimuli probably parallels decrease in cell viability through alterations in the levels of activating nuclear HNF1 and other transcription factors such as C/EBPβ and Nrf2. It has been shown that both C/EBPβ and Nrf2 are degraded via the ubiquitin proteasome system (Hattori et al., 2003; Itoh et al., 2003).

In addition, we found that C2 inhibited the constitutive expression of GSTA3/5 and blocked GSTA3/5 induction by oltipraz or *t*-BHQ. A GenBank database search revealed that the promoter regions of the *GSTA3* (GenBank accession no. AF067442) and *GSTA5* (GenBank accession no. AH005038) genes contain the consensus DNA binding site for HNF (Fotouhi-Nidakani and Baist, 1999). Therefore, repression of GSTA3/5 by C2 is highly likely to result from suppression of the transcription factor commonly required for their gene expression. In this study, we have shown the several important findings that help in understanding the correlation of GST expression with cell viability in conjunction with the fate of transcription factors.

In conclusion, the present study demonstrated that 1) the HNF binding element present in the promoter region of the *GSTA2* gene is essentially required for the constitutive and inducible gene expression; 2) ceramide, a general apoptotic rheostat controlling cell survival, decreases the level of nuclear HNF1 that is subjected to degradation by the ubiquitin proteasomes, and 3) the degradation of HNF1 by ceramide is responsible for GST repression.

Acknowledgment

The kind donation of pGTB-1.65 containing the GSTA2-promoter region from Dr. Cecil B. Pickett is gratefully acknowledged.

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