Induction of Glutathione Transferase in Insulin-Like Growth Factor Type I Receptor-Overexpressed Hepatoma Cells

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ABSTRACT
Insulin-like growth factor type I receptor (IGF-IR) is frequently overexpressed in human hepatocellular carcinoma cells (HCC), and this overexpression has been correlated with increased tumor growth. The protective response of HCC to reactive oxygen species (ROS) produced by chemotherapeutic agents is mediated with the induction of phase II detoxifying genes including glutathione transferase (GST). To understand the roles of IGF-IR overexpression in HCC in terms of its detoxifying effect on ROS and conferred resistance to chemotherapy, we analyzed whether IGF-IR overexpressions affect IGF-1-inducible GST expression. GSTα was induced by exposure to IGF-1 in IGF-IR cells but not in cells expressing normal levels of IGF-IR. Furthermore, IGF-IR-overexpressed HCCs (IR-HCC) are more resistant to doxorubicin than control HCC cells, which was associated with the increased GST induction by IGF-1. Molecular analyses using GSTA2 promoter supported the involvement of xenobiotic response element (XRE) in GSTα induction. IGF-1 caused the nuclear translocation of CCAAT/enhancer-binding protein α (C/EBPα), which might be responsible for XRE activation. In addition, IGF-1 increased the activities of phosphatidylinositol 3-kinase (PI3-kinase) and extracellular signal-regulated kinase in IR-HCCs. Moreover, the inhibition of PI3-kinase completely abolished the nuclear translocation of C/EBPα and the up-regulation of GSTα protein in IR-HCC treated with IGF-1. However, specific inhibitors against extracellular signal-regulated kinase, c-Jun N-terminal kinase, or p38 kinase did not alter IGF-1-inducible GSTα expression. These results provide evidence that one of the pathological consequences of IGF-IR overexpression in HCCs is the potentiation of GSTα induction by IGF-1. Moreover, this potentiation of GST may be associated with decreased susceptibility to chemotherapeutic agents such as doxorubicin.

Hepatocellular carcinoma is the fifth most common malignant tumor worldwide. Hepatomas are likely to rise from normal cells through the accumulation of multiple genetic changes, which include the functional loss of tumor suppressor genes and the presence of activating oncoprotein mutations. In addition to genetic changes in these genes, the expressions of growth factor receptors are up-regulated in hepatocellular carcinoma cells (HCC) (Yamaguchi et al., 1995; Scharf and Braulke, 2003). One of the growth factors predominantly implicated in hepatocarcinogenesis is insulin-like growth factor type I (IGF-1). IGFs are synthesized and secreted by hepatocytes, and the interaction of IGF-1 with its receptor, IGF type I receptor (IGF-IR), plays a central role in the proliferation of a variety of cell types (Pietrzkowski et al., 1993; Scharf et al., 2001). Differentiated hepatocytes are not believed to be a major target for the actions of IGF-1, because only a few
IGF-I binding sites have been demonstrated on adult hepatocytes (Caro et al., 1988; McElduff et al., 1988). Whereas increased expression of IGF-IR in malignant hepatoma cells has been reported (Tsai et al., 1988), the physiological role of IGF-IR overexpression in HCC has not been completely clarified.

Reactive oxygen species (ROS) are persistently produced as a result of mitochondrial respiration during the uncontrolled growth of cancer cells. The capacity of cancer cells to maintain cellular functions during oxidative stress resides in the rapid induction of protective enzymes, which decrease oxidative stress. Protective adaptive response to electrophiles or ROS is mediated by enhancing the expression of phase II detoxifying genes. Glutathione transferases (GSTs) are a major group of phase II detoxification enzymes that display broad substrate specificity and provide protection against oxidative stress (Hayes and Pulford, 1995).

Based on the hypothesis that IGF-IR overexpression increases the inducibility of GST and that this induction is required for the efficient removal of ROS during uncontrolled cell growth or exposure to chemotherapeutic agents such as doxorubicin, we assessed the effect of IGF-1 on GSTα expression in both H4IIE hepatoma cells and hepatoma cells overexpressing IGF-IR (IGF-IR-H4IIE cells). Here, we demonstrate for the first time that low concentrations of IGF-1 cause the induction of GSTα in IGF-IR-overexpressing hepatoma cells and that the susceptibility of cells to ROS-generating doxorubicin is diminished by IGF-IR overexpression.

Our previous studies showed that Nrf2/antioxidant response element (ARE) and CCAAT/enhancer-binding protein β (C/EBPβ)/xenobiotic response element (XRE) pathways play essential roles in GSTα induction and that this induction is mediated through a phosphatidylinositol 3-kinase (PI3-kinase) pathway (Kang et al., 2000, 2001, 2003a). Because the signaling pathways required for GST induction by growth factors have not been studied, our second objective was to determine whether the activation of PI3-kinase by IGF-1 is required for the C/EBPβ or Nr2-mediated induction of GSTα. Herein, we demonstrate that the induction of GSTα by IGF-1 is controlled by PI3-kinase-dependent C/EBPβ activation in IGF-IR-overexpressing H4IIE cells.

Materials and Methods

Materials. Anti-GSTα antibody was supplied by Detroit R&D (Detroit, MI). Horseradish peroxidase-conjugated donkey anti-rabbit IgG, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). 5-Bromo-4-chloro-3-indoly1-phosphate (BCIP)/nitroblue tetrazolium (NBT) was from Invitrogen (Carlsbad, CA). Anti-Nrf2, anti-IGF-IRβ, anti-C/EBPβ, anti-proliferating cell nuclear antigen, and anti-lamin B1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phosphorylated Akt, Akt, phosphorylated extracellular signal-regulated kinase (ERK), ERK, phosphorylated c-Jun N-terminal kinase (JNK), JNK, phosphorylated p38 kinase, and p38 kinase antibodies were purchased from Cell Signaling Technology (Danvers, MA). Neutralizing monoclonal antibody against IGF-IR was supplied from Chemicon (Temecula, CA). Most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). Myc/s5 overexpression vector was provided by Dr. A. Toker (The Boston Biomedical Research Institute, Boston, MA). The mitogen-activated protein (MAP) kinase 1 (MKK1) dominant-negative mutant was a gift from Dr. S. J. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder, CO). The JNK1 dominant-negative mutant (KmJNK1) and dominant-negative mutant of p38 kinase (dp38K) were provided by Dr. N. Dharsekaran (Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia, PA) and Dr. H. S. Choi (College of Pharmacy, Chosun University, Gwangju, Korea), respectively.

Cell Culture. H4IIE and HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA) and Korea Cell Line Bank (Seoul, Korea), respectively. SK-Hep1 cells were kindly provided from Dr. K. Y. Lee (Chonnam National University, Gwangju, Korea). These three cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere.

Construction of IGF-IR Retroviral Plasmid and the Infection of H4IIE Cells. IGF-IR was stably expressed in H4IIE cells using an MSCV-GFP retrovirus system provided by Dr. Scot W. Lowe at Cold Spring Harbor Laboratories (Cold Spring Harbor, NY). IGF-IR cDNA was subcloned into MSCV-GFP retroviral vector, and Phoenix cells (a packaging cell line) were transfected with MSCV-GFP (control) or MSCV-IGF-IR/GFP (IGF-IR-overexpressed) plasmid. Supernatants containing ecotropic replication-independent retroviruses were collected and then stored at −80°C until required. H4IIE cells that were 30% confluent were multiply infected (eight times) with retrovirus particles. Intensities of infection were monitored by GFP-fluorescence, and Western blot analysis using a specific antibody.

Preparation of Nuclear Fractions. Nuclear extracts were prepared essentially as described by Schreiber et al. (1990). In brief, cells in dishes were washed with ice-cold phosphate-buffered saline (PBS), scraped, transferred to microtubes, and allowed to swell for 15 min on ice. The samples were then centrifuged at 7200 g for 5 min. Pellets containing crude nuclei were resuspended in 60 mM of extraction buffer containing 20 mM HEPES, pH 7.9, 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiobitol, and 0.5 mM phenylmethylsulfonyl fluoride. Cell membranes were disrupted by vortexing, and lysates were incubated for 10 min on ice and centrifuged at 7200 g for 5 min. Pellets containing crude nuclei were resuspended in 60 μl of extraction buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiobitol, and 1 mM phenylmethylsulfonyl fluoride and then incubated for 30 min on ice. The samples were then centrifuged at 15,800 g for 10 min to obtain supernatants containing nuclear extracts, which were stored at −80°C until required.

Immunoblot Analysis. After washing H4IIE or IGF-IR-H4IIE cells with sterile PBS, they were lysed in buffer containing 20 mM Tris-Cl, pH 7.5, 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethysulfonyl fluoride, and 1 μg/ml leupeptin. Cell lysates were centrifuged at 10,000 g for 10 min to remove debris, and the supernatant proteins were fractionated using a 10% separating gel. Fractionated proteins were then electrophoretically transferred to nitrocellulose paper, and proteins were immunoblotted with specific antibodies. The secondary antibodies used were horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibody. Nitrocellulose papers were developed using BCIP/NBT or an enhanced chemiluminescence system.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide Cell Viability Assay. To determine cell viabilities, cells were plated at 104 cells/well in 96-well plates. Viable adherent cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (2 mg/ml) for 4 h. Media were then removed, and the formazan crystals produced were dissolved by adding 200 μl of dimethyl sulfoxide per well, and absorbance was assayed at 540 nm. Cell viabilities are expressed as relative ratios to untreated control cells.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays were performed using an in situ cell death detection kit according to manufacturer's instructions.
Colonies were maintained in 1 ml of 0.3% basal medium Eagle's agar containing 10% fetal bovine serum and IGF-1 (10 ng/ml) with or without doxorubicin (0.1 μM) at 37°C in a humidified incubator for 14 days. Cell colonies were counted using three different plates under microscope.

**Determination of Caspase-3 Activity.** Caspase-3 activity was assessed using a commercially available kit (CaspACE assay system; Promega, Madison, WI). In brief, caspase-3 activity was assayed with cell lysates (50 μg) in the buffer solution, pH 7.5, containing 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 100 mM HEPES, 10% sucrose, and 10 mM dithiothreitol. Reaction was initiated by the addition of 100 μM acetyl-Asp-Glu-Val-Asp-Asp-5-nitroanilide as a colorimetric substrate and continued for 4 h at 37°C. Absorbance was measured at 405 nm.

**Construction of GSTA2 Promoter-Luciferase Constructs and Luciferase Assay.** Firefly luciferase reporter gene construct pGL-1651 was generated by ligating pGL3-Basic vector (Promega) with the -1.65 promoter region of the GSTA2 gene, and the chimeric gene constructs, pGL-197, pGL-197 and pGL-AC/EPP (~905 to ~699 lacking reporter) were prepared using polymerase chain reaction-amplified-deleted GSTA2 promoter regions (Kang et al., 2003a,b). To determine promoter activity, we used a dual-luciferase reporter assay system (Promega). In brief, cells (3 × 10^5 cells/well) were plated in 12-well plates overnight and transiently transfected with GSTA2 promoter-luciferase construct and pRL-SV plasmid (Renilla reniformis luciferase expression for normalization) (Promega) using Genejuice reagent (Novagen, Madison, WI). The cells were then exposed to IGF-1 for 18 h, and firefly and R. reniformis luciferase activities in cell lysates were measured using a luminesimeter (TD-20; Turner Designs, Sunnyvale, CA). Relative luciferase activities were calculated by normalizing GSTA2 promoter-driven firefly luciferase activity versus R. reniformis luciferase.

**Gel-Shift Analysis.** A double-stranded C/EBP consensus oligonucleotide was used for gel-shift analysis after end-labeling the probe with [γ-32P]ATP using T7 polymerase kinase. The sequence of the C/EBP consensus oligonucleotide was 5’-TGGAGATTGG-CATCTGCA-3’. Reaction mixtures contained 4 μl of 5× binding buffer containing 20% glycerol, 5 mM MgCl2, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly(dI-dC), 50 mM Tris-Cl, pH 7.5, 10 or 15 μg of nuclear extracts, and sterile water to a total volume of 20 μl. The reaction mixtures were preincubated for 10 min. DNA binding reactions were carried out at room temperature for 20 min after adding 1 μl of probe (106 cpm). Binding specificity was determined by using competition experiments, which were carried out by adding a 20-fold excess of an unlabeled C/EBP, specific protein-1, or activator protein-1 oligonucleotide to reaction mixtures before the DNA binding reaction. For immunohibination assays, antibodies (2 μg of each) were added to reaction mixtures after an initial 20-min incubation and then incubated for a further 1 h at 25°C. Samples were loaded onto 4% polyacrylamide gels at 100 V, and run gels were dried and autoradiographed.

**Quantification of C/EBPβ Bound to XRE Region of GSTA2 Promoter.** Biotin-labeled double-stranded oligonucleotide of XRE region of GSTA2 promoter was used for the quantification of C/EBPβ bound to XRE region. Reaction mixtures contained 10 μl of 5× binding buffer containing 20% glycerol, 5 mM MgCl2, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly(dI-dC), 50 mM Tris-Cl, pH 7.5, 30 μg of nuclear extracts, and sterile water to a total volume of 50 μl. DNA binding reactions were carried out at room temperature for 40 min. The samples were additionally incubated with 100 μl of streptavidin-conjugated magnetic beads (MagnaBind-Streptavidin; Pierce, Rockford, IL) for 30 min, and the DNA-bound beads were separated. After washing with PBS, the XRE-bound proteins were eluted with the buffer containing 0.1 M glycine-HCl, pH 2.8 and fractionated using a 10% separating gel. The fractionated proteins were then electrophoretically transferred to nitrocellulose paper and immunoblotted with C/EBPβ antibody.

**Immunocytochemistry Analysis.** IGF-IR-H4IIE cells were grown on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) and incubated in serum-deprived medium for 48 h. For immunostaining, the cells were fixed in 4% paraformaldehyde for 1 h and washed three times with PBS. After blocking in 5% bovine serum in PBS for 1 h at room temperature, they were incubated for 1 h with anti-IGF-IR, anti-C/EBPβ, or anti-Nrf2 antibody (1:100) in PBS containing 0.5% bovine serum albumin, serially washed with PBS, and incubated with tetramethylrhodamine B isothiocyanate-conjugated secondary antibody (1:100; Zymed Laboratories Inc., San Francisco, CA). Counterstaining with DAPI verified the locations and integrities of nuclei. Stained cells were washed and examined under a fluorescence microscope (Axiovert 200M; Carl Zeiss, Hamburg, Germany; supported from Chosun University, Gwangju, Korea).

**Data Analysis.** Scanning densitometry was performed using an Image Scan and Analysis System (Alpha-Innotech, San Leandro, CA) or FLA-7000 (Fujifilm, Japan). One-way analysis of variance procedures were used to assess significant differences between treatment groups. When treatment was found to have a significant effect, the Newman-Keuls test was used to compare multiple group means. Statistical significance was accepted at either p < 0.05 or p < 0.01.

**Results**

**GSTα Induction by IGF-1 in IGF-IR-Overexpressed Hepatoma Cells.** Although differentiated hepatocytes express limited amounts of IGF-IR (McElduff et al., 1988), its expression level is up-regulated during hepatocarcinogenesis (Tsai et al., 1988). To mimic malignant hepatoma, we established H4IIE cells stably expressing IGF-1 (IGF-IR-H4IIE cells) by retroviral infection. As shown in Fig. 1A, immunocytochemistry using IGF-IRβ-specific antibody showed that IGF-IRβ was mainly located in the transmembrane and cytoplasmic region and that the level of fluorescence was higher in IGF-IR-H4IIE cells than in GFP-overexpressed control (GFP-H4IIE) cells. We also determined IGF-IR expression levels by Western blot analysis in both cell types. Basal IGF-IR expression levels in IGF-IR-H4IIE cells were higher than those in GFP-H4IIE cells, and exposure of these cells to IGF-1 for 24 h caused the down-regulation of IGF-IR expression (Fig. 1B).

The hepatic expression of GST is affected by oxidative stress or chemotherapeutic agents (Kensler et al., 1987; Pinkus et al., 1996; Kim et al., 1997; Kang et al., 2001), and many types of cells produce small quantities of ROS in response to a variety of extracellular growth signals (Sundaresan et al., 1995; Bae et al., 1997); however, the role of growth factor(s) in GST expression has not been studied. Hence, we further examined the IGF-1-inducible expression of GST subtypes in both GFP-H4IIE and IGF-IR-H4IIE cells. GSTα in

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Fig. 1. GSTα induction by IGF-1 in IGF-IR-overexpressing hepatoma cells. A, immunocytochemistry of IGF-IR. GFP-overexpressing H4IIE cells (GFP-H4IIE) and IGF-IR overexpressing H4IIE cells (IGF-IR-H4IIE) were stained with antibody highly specific for IGF-IR. B, the expression of IGF-IR in GFP-H4IIE and IGF-IR-H4IIE cells. Western blot analysis was performed using cell lysates (10 μg/lane) and anti-IGF-IR antibody. C, selective induction of GSTα by IGF-1 in IGF-IR-overexpressing hepatoma cells. A representative immunoblot shows GSTα protein in both GFP-H4IIE and IGF-IR-H4IIE cells incubated with IGF-1 (1–100 ng/ml) for 18 h. Each lane was loaded with 10 μg of protein. Equal protein loading was verified using actin as an internal standard. Relative changes in the GSTα subunit were assessed by scanning densitometry. Data represent the means ± S.D. of three separate experiments (significant versus the control: *, p < 0.05; **, p < 0.01; control level = 1). D, IGF-1-stimulated induction of GSTα in SK-Hep1 and HepG2 cells. SK-Hep1 cells were incubated with IGF-1 (10–100 ng/ml) for 18 h. The immunoblots show IGF-IRβ (left, duplicated samples) and GSTα proteins (right) in SK-Hep1 and HepG2 cells. Each lane was loaded with 10 μg of protein.
GFP-H4IIE cells was unaffected by IGF-1 at 3 to 100 ng/ml (Fig. 1C, left). In contrast, GSTα was significantly induced by 10 ng/ml IGF-1 in IGF-IR-H4IIE cells, and the extent of GSTα induction was further enhanced by higher concentrations of IGF-1 (30 or 100 ng/ml) (Fig. 1C, right). Other GST subunits such as GSTμ and GSTπ were unaffected by IGF-1 in both cell types (Fig. 1C). Moreover, supraphysiological concentration of IGF-1 (1 μg/ml) did not induce GSTα in GFP-H4IIE cells (data not shown). These results suggest that IGF-1 signaling is coupled with the process of GSTα induction and imply that physiological concentrations of IGF-1 induce GSTα in IGF-IR-overexpressed hepatoma cells but not in GFP-overexpressed hepatoma cells.

Next, we also determined whether IGF-1 increased the expression of GSTα in human hepatoma cell lines, SK-Hep1 and HepG2 cells. Basal IGF-IR levels in SK-Hep1 and HepG2 cells were higher than those in H4IIE cells, and 100 ng/ml IGF-1 induced GSTα protein in both of the hepatoma cell lines (Fig. 1D).

The Induction of GSTα in IGF-IR-Overexpressed Hepatoma Cells Was Associated with Chemoresistance against Doxorubicin. The cytotoxic effect of doxorubicin can be modulated by GST expression (Liu et al., 2001), and transfection with GSTα cDNA protected HL-60 cells from doxorubicin-induced cytotoxicity (Cheng et al., 2001). Hence, we compared the doxorubicin-induced cytoxicities in IGF-IR-overexpressing H4IIE cells and GFP-overexpressing H4IIE cells (control cells). MTT assays showed that doxorubicin (30 or 100 μM, 24-h incubation) reduced cell viability in GFP-H4IIE control cells pretreated with IGF (30 ng/ml) for 18 h (Fig. 2A). In comparison to the control group, doxorubicin-induced cytotoxicity was significantly reduced in IGF-IR-H4IIE cells pretreated with IGF-1 (Fig. 2A).

We also assessed doxorubicin-induced apoptosis in both cell types to establish the mechanistic basis of doxorubicin cytotoxicity. Representative TUNEL assay photographs showed that exposure of cells to doxorubicin for 24 h caused control cell apoptosis (Fig. 2B). TUNEL-positive indices were lower in IGF-IR-H4IIE cells than in GFP-H4IIE cells (Fig. 2B), which was consistent with MTT assay results. MTT assay represents the indices of chemical-induced cell death, whereas colony formation assay represents transformation or proliferation potential of cancer cells. We additionally

Fig. 2. A, cell viability after treating with doxorubicin. After treating GFP-H4IIE and IGF-IR-H4IIE cells with doxorubicin (3–100 μM) for 24 h, cell viabilities were determined using MTT assays. Data represent means ± S.D. of eight separate samples (significant versus the control: **, p < 0.01; significant versus cell viability in same concentration group of GFP-H4IIE cells, ##, p < 0.01). B, representative photographs of TUNEL assays on cells cultured with or without 30 μM doxorubicin for 18 h. 30 ng/ml IGF-1 was pretreated 18 h before doxorubicin exposure. Results were confirmed by multiple experiments. Apoptosis (42%) was observed at 30 μM doxorubicin-treated GFP-H4IIE cells. C, colony formation assay. GFP-H4IIE and IGF-IR-H4IIE cells were incubated for 14 days with or without 0.1 μM doxorubicin and 10 ng/ml IGF-1 in soft agar plates. The numbers below each representative picture show average number ± S.D. of colonies. D, caspase-3 activity. Caspase-3 activity was determined in cells incubated with or without 30 μM doxorubicin for 24 h. Data represent means ± S.D. of four separate experiments (significant versus the control: **, p < 0.01; significant versus caspase-3 activity in GFP-H4IIE cells; ##, p < 0.01). E, left, effect of N-acetylcysteine (1 or 10 mM) on the cytotoxicity induced by 30 μM doxorubicin in GFP-H4IIE cells. Right, effect of ethacrynic acid (20 or 50 μM) on the cytotoxicity induced by doxorubicin in IGF-IR-H4IIE cells. Data represent means ± S.D. of eight separate samples (significant versus the control: **, p < 0.01; significant versus doxorubicin-treated group: ##, p < 0.01).
performed colony formation assays using GFP-H4IIE and IGF-IR-H4IIE cells. IGF-treated IGF-IR-H4IIE cells are more resistant to the 0.1 μM doxorubicin-mediated reduction of colony formation (Fig. 2C).

Caspase-3 plays an important role in the development of the morphological changes associated with apoptosis, such as DNA fragmentation and chromatin condensation. The apoptotic cell death caused by doxorubicin is also mediated by caspase-3 activation (Tsang et al., 2003). Thus, we investigated whether caspase-3 activation is inhibited in IGF-IR-overexpressing hepatoma cells. Exposure of GFP-H4IIE cells to 30 μM doxorubicin for 24 h markedly activated caspase-3 (3.3-fold increase) (Fig. 2D), which is in contrast to that in IGF-IR-H4IIE control cells, in which doxorubicin-inducible caspase-3 activity was 51% reduced (Fig. 2D).

Enhanced expression of GSTα in IGF-IR-overexpressing hepatoma cells may be associated with reduced chemosensitivity. One plausible mechanism of doxorubicin-chemoresistance in cancer cells concerns the reduced formation of ROS by doxorubicin (Müller et al., 1998). Thus, we examined whether N-acetylcysteine, an antioxidant, blocked doxorubicin-induced cytotoxicity in GFP-H4IIE cells. MTT assay showed that apoptosis induced by doxorubicin was not reversed by 1 mM N-acetylcysteine but was completely reversed by 10 mM N-acetylcysteine (Fig. 2E). In addition, a minimal cytotoxicity by doxorubicin in IGF-IR-H4IIE cells was potentiated by a chemical inhibitor of GST, ethacrynic acid (Fig. 2E). The data suggest that increased GSTα expression reduces cellular ROS formation by doxorubicin and that this might be related to chemoresistance to doxorubicin.

C/EBP/XRE-Dependent Transactivation of the GSTA2 Gene. The role of AREs in the inducible expression of phase II enzymes by antioxidants or radicals has been studied extensively (Kang et al., 2001, 2002a). Recently, we also showed that C/EBPβ activation is involved in chemopreventive agent-induced GSTα gene expression via XRE element (Kang et al., 2003a,b). Given the roles of ARE or XRE in GSTα expression, we performed reporter gene analyses using

![Fig. 3. Analysis of C/EBP-responsive element in the GSTA2 promoter. A, induction of luciferase activity by IGF-1 in IGF-IR-H4IIE cells transiently transfected with GSTA2 chimeric gene constructs pGL-1651 (containing both XRE and ARE elements), pGL-797 (containing ARE), pGL-AC/EBP (lacking C/EBP/XRE), or pGL-197 (minimal promoter). Dual luciferase reporter assays were performed on lysed cells cotransfected with GSTA2-luciferase gene constructs (firefly luciferase) and pRL-SV (R. reniformis luciferase) (ratio of 100:1) after exposure to IGF-1 (30 ng/ml) for 18 h. Reporter gene activation was calculated as the relative ratio of firefly luciferase to R. reniformis luciferase activity. Data represent the means ± S.D. of 4 to 10 separate samples (significant versus the control: **, p < 0.01; control level = 1). B, left, immunocytochemistry and immunoblotting of Nrf2. IGF-IR-H4IIE cells were treated with 30 ng/ml IGF-1 for 6 h. Nrf2 was localized by immunostaining with anti-Nrf2 antibody. The same fields were counterstained with DAPI (1 μg/ml). B, right, levels of Nrf2 were assessed immunochemically in nuclear fractions of IGF-IR-H4IIE cells incubated with IGF-1 (30 ng/ml) for 1 to 24 h. C, role of C/EBP in IGF-1-inducible pGL-1651 reporter gene activation. Cells were cotransfected with pGL-1651/pRL-SV40 (100:1) and AC/EBP (dominant-negative C/EBP) or C/EBPβ-overexpressing vector in 1:1 ratio, and luciferase activities were measured 18 h after transfection. Luciferase activities are expressed as ratios of those of control cells transfected with pCMV500 (significant versus the control: **, p < 0.01; significant versus IGF-1 treated IGF-IR-H4IIE cells transfected with pCMV500: ##, p < 0.01; control level = 1).
from IGF-IR-H4IIE cells treated with or without 30 ng/ml IGF-1 for 12 h.

and streptavidin-coated magnetic beads. Nuclear extracts were obtained

determination of XRE-bound C/EBP

DNA binding reactions were performed by gel-shift analysis. B, determination

right, competition assays with unlabeled C/EBP oligonucleotide or spe-

Figs. 4. Activation of C/EBPβ by IGF-1 in IGF-IR-overexpressing hepa-

toma cells. A, left, gel-shift analysis of the C/EBP transcription complex.

Nuclear extracts were prepared from IGF-IR-H4IIE cells cultured in the

presence or absence of 30 ng/ml IGF-1 for 1 to 24 h. All lanes contained 10 µg of nuclear extracts and 5 ng of the labeled C/EBP consensus sequence. Right, competition assays with unlabeled C/EBP, specific protein-1 (SP-1), activator protein-1 (AP-1) oligonucleotide, C/EBPβ antibody, or C/EBPα antibody to the nuclear extracts of cells treated with IGF-1 for 6 h. All lanes contained 15 µg of nuclear extracts and 5 ng of the labeled C/EBP consensus sequence. DNA binding reactions were performed by gel-shift analysis. B, determination of XRE-bound C/EBPβ using biotin-labeled XRE oligonucleotide and streptavidin-coated magnetic beads. Nuclear extracts were obtained from IGF-IR-H4IIE cells treated with or without 30 ng/ml IGF-1 for 6 h. C, immunocytochemistry and immunoblotting of C/EBPβ. IGF-IR-H4IIE cells were treated with 30 ng/ml IGF-1 for 3 or 24 h. C/EBPβ localization was detected by immunostaining with anti-C/EBPβ antibody. The same

pGL-1651, which contained the luciferase structural gene and a −1.65 kilobase GSTA2 promoter, and tried to identify the transcription factor(s) involved in GSTA2 gene transactivation by IGF-1. IGF-1 (30 ng/ml) caused a 3-fold increase in the luciferase activity in IGF-IR-H4IIE cells transfected with pGL-1651 plasmid (Fig. 3A), which suggests that the induction of GSTα by IGF-1 is mediated through the transcriptional activation of GSTA2 gene. Promoter deletion analyses were performed to clarify the binding site(s) required for this inducing activity of IGF-1. IGF-1-inducible luciferase activities were not observed in IGF-IR-H4IIE cells transfected with pGL-797 or pGL-AC/EBP, which contained ARE but not the XRE/CEBP binding region (Fig. 3A). Moreover, pGL-197, which lacked nucleotides −1651 to −198 of the GSTA2 gene flanking region, was not induced by IGF-1 (Fig. 3A). Nrf2 is believed to be an essential transcription factor for ARE activation (Huang et al., 2000; Kang et al., 2002b). Immunocytochemistry and Western blot analyses confirmed that IGF-1 (30 ng/ml) did not cause the nuclear translocation of Nrf2 or affect the nuclear level of Nrf2 (Fig. 3B). These results indicate that the XRE/CEBP binding region, but not the ARE/Nrf2 binding region, in GSTA2 promoter is essentially required for the induction of GSTα by IGF-1.

To further determine whether C/EBP is associated with XRE activation by IGF-1, C/EBP-specific dominant-negative mutant (AC/EBPβ) (Kang et al., 2003a) was expressed in combination with pGL-1651 luciferase reporter in IGF-IR-H4IIE cells. pGL-1651 reporter activity was almost completely inhibited by dominant-negative mutant C/EBPβ expression, whereas transfection with pCMV500 (control vector) did not affect an increase in pGL-1651 reporter activity by IGF-1 (Fig. 3C). Moreover, overexpression of rat C/EBPβ (Lee and Kim, 2006) significantly potentiated pGL-1651 reporter activity by IGF-1 (Fig. 3C). These findings indicate that the C/EBP-binding sequence in the XRE region of the GSTA2 gene is responsible for GSTα induction by IGF-1 in IGF-IR-overexpressed hepatoma cells.

Activation of C/EBPβ by IGF-1. To test whether IGF-1 activates C/EBP, electrophoretic mobility shift assays were performed to determine C/EBP DNA binding activity using nuclear extracts prepared from IGF-IR-H4IIE cells treated with IGF-1. IGF-1 (30 ng/ml) resulted in a time-dependent increase in C/EBP binding compared with untreated controls. C/EBP binding activity began to increase 1 h after treatment and was sustained for up to 24 h (Fig. 4A, left). Moreover, C/EBP binding activity was completely reversed by a 10-fold excess of unlabeled C/EBP in nuclear extracts, which confirmed the specificity of protein binding to the C/EBP binding site (Fig. 4A, right). Competitive experiments using specific antibodies against C/EBPα or C/EBPβ showed that IGF-1-induced C/EBP DNA binding activity specifically depends on the C/EBPβ form. Moreover, anti-C/EBPβ antibody almost completely eliminated the band intensity and supershifted the retarded band (Fig. 4A, right). To quantify the XRE-bound C/EBPβ, we also used biotin-labeled XRE oligonucleotide and streptavidin-coated magnetic beads. All fields were counterstained with 1 µl/ml DAPI. For Western blot analysis of C/EBPβ in nuclear fractions, nuclear extracts were obtained from IGF-IR-H4IIE cells treated with IGF-1 for 1 to 24 h, and C/EBPβ was detected immunochemically with specific antibody.
though C/EBPβ bound to XRE oligonucleotide was minimal in control sample, IGF-1 treatment caused an increase in XRE-bound C/EBPβ (Fig. 4B). Nuclear lamin B1 levels were determined as loading controls.

Because C/EBPβ is mainly located in the cytoplasmic fraction and is translocated to the nucleus upon activation, we determined the nuclear levels of C/EBPβ by subcellular fractionation and immunoblotting. Nuclear levels of C/EBPβ were increased from 1 to 24 h after exposing cells to 30 ng/ml IGF-1, which was consistent with the gel-shift assay results (Fig. 4C). To verify this result, we also carried out immunocytochemical analysis using C/EBPβ-specific antibody. C/EBPβ was located predominantly in the cytoplasm of control cells (Fig. 4C), whereas it had a nuclear localization in cells treated with 30 ng/ml IGF-1 (3 h), which suggested that C/EBPβ might move into the nucleus. At later time points (i.e., 24 h after IGF-1 exposure), C/EBPβ was observed to be predominantly distributed in the nucleus (Fig. 4C).

Role of PI3-Kinase in GSTα Induction by IGF-1. Previous studies have shown that PI3-kinase and its downstream kinases (e.g., Akt and p70S6 kinase) are involved in cellular responses to growth factor(s) (Dupont and Le Roith, 2001; Cho and Kim, 2003). Moreover, a series of our studies also demonstrated that PI3-kinase plays a key role in the induction of phase II detoxifying genes (Kang et al., 2000, 2001, 2003a). To estimate whether IGF-1 activates PI3-kinase in IGF-IR-H4IIE cells, we determined the extent of the phosphorylation of Akt, a downstream kinase of PI3-kinase. IGF-1 (30 ng/ml) transiently (5–10 min) increased phosphorylated Akt levels in IGF-IR-H4IIE cells (Fig. 5A). However, IGF-1 failed to activate PI3-kinase in GFP-H4IIE cells (Fig. 5A). We then assessed the role of PI3-kinase in C/EBPβ-mediated GSTα induction in IGF-IR-overexpressing cells. Pretreatment of these cells with LY294002 (20 μM), a specific inhibitor of PI3-kinase, completely blocked the induction of GSTα and the nuclear accumulation of C/EBPβ in IGF-IR-H4IIE cells by IGF-1 (Fig. 5B). To confirm the role of PI3-kinase, IGF-1-inducible pGL-1651 reporter activity was monitored in IGF-IR-H4IIE cells overexpressing the myc85 functions as a kind of dominant-negative. Myc85 overexpression also inhibited an increase in pGL-1651 reporter activity by IGF-1 (Fig. 5C). These results raised the possibility that the PI3-kinase pathway controls C/EBPβ and C/EBPβ-dependent GSTα gene expression.

Previously, it was proposed that ERK1/2 and p38 kinase might be associated with the induction of quinone reductase and γ-glutamylcysteine synthetase, respectively (Yu et al., 1999; Zipper and Mulcahy, 2000). To study whether MAP kinase pathways are also stimulated by IGF-1 in IGF-IR-H4IIE cells, we measured the activity of each MAP kinase as

![Fig. 5. PI3-kinase-dependent GSTα induction by IGF-1 in IGF-IR-overexpressing hepatoma cells. A, IGF-1-inducible PI3-kinase activation. PI3-kinase activity was assessed using phosphospecific Akt antibody. B, effects of PI3-kinase inhibitor on C/EBPβ activation and GSTα protein induction by IGF-1. The levels of GSTα (18 h) and C/EBPβ (6 h) were determined by Western blot analysis in IGF-IR-H4IIE cells cultured with 30 ng/ml IGF-1 in the presence or absence of 20 μM LY294002 (LY). C, effect of myc85 overexpression on pGL-1651 reporter activity. pGL-1651 reporter activity change by IGF-1 (30 ng/ml) was measured in IGF-IR-H4IIE cells transiently transfected with 1 μg of myc85 or 1 μg of pCMV5.](molpharm.aspetjournals.org)
a function of time after IGF-1 treatment. The level of active phosphorylated ERK1/2 was increased at 5 to 30 min after IGF-1 treatment and peaked at 10 min (Fig. 6A). However, the phosphorylation intensities of JNK or p38 kinase were only slightly affected in IGF-IR-H4IIE cells stimulated by 30 ng/ml IGF-1 from 5 min to 3 h (Fig. 6A). To investigate whether blockade of the MAP kinase cascade led to a change in the expression of GSTα, we determined pGL-1651 reporter activity in IGF-IR-H4IIE cells transfected with dominant-negative mutants of MKK1 (ERK inhibition), JNK, and p38 kinase. Transfection of each dominant-negative mutant did not alter the IGF-1-mediated increase in pGL-1651 reporter activity (Fig. 6B). We also assessed whether the GSTα expression is changed by MAP kinase inhibitors. IGF-IR IGF-IR-H4IIE cells were preincubated with specific MAP kinase inhibitors for 30 min and then exposed to 30 ng/ml IGF-1 for an additional 18 h. Neither U0126 (an ERK inhibitor, 10 μM) nor SB203580 (a p38 kinase inhibitor, 10 μM) suppressed GSTα protein induction (Fig. 6C). Moreover, SP600125, a selective JNK inhibitor, failed to block GSTα induction (Fig. 6C). These data show that none of the MAP kinase pathways is responsible for the induction of GSTα by IGF-1 in IGF-IR-overexpressed hepatoma cells.

Increase in Doxorubicin Sensitivity by Blocking of IGF-IR and PI3 Kinase. To finally test whether the activities of IGF-IR and PI3-kinase pathways are associated with the acquisition of doxorubicin resistance in IGF-IR-H4IIE cells, we determined the doxorubicin cytotoxicity after exposure of cells to IGF-IR-neutralizing antibody and LY294002. Blocking of either IGF-IR or PI3-kinase significantly enhanced doxorubicin-induced cell death in IGF-IR-H4IIE cells (Fig. 7, A and B).

Discussion

The parenchymal cells of the liver synthesize and secrete IGF-1 and IGF-2, and these secreted IGFs are believed to be participated in postnatal growth. The expression of IGF-IR is very low in adult hepatocytes, whereas its expression is obvious in Kupffer and stellate cells, which suggests that hepatocytes respond poorly to IGFs (Caro et al., 1988). However, a growing body of evidence indicates that IGF-IR expression is increased in the human cirrhotic liver, liver cancer, and HBs-infected hepatoma cells compared with normal adult liver cells (Caro et al., 1988; Kim et al., 1996; Scharf et al., 1998). Moreover, antisense IGF-1 gene therapy may be useful for preventing hepatoma progression (Lafarge-Frayssinet et al., 2002). Recently, it was shown that blocking IGF-IR enhances chemo- and radiotherapy responses and inhibits tumor growth (Min et al., 2005). Hence, IGF signaling seems to be involved both in the pathological progress of hepatoma and in the acquisition of chemotherapy resistance.

In the present study, we show for the first time that IGF-1-mediated GSTα expression is potentiated in hepatoma cells overexpressing IGF-IR and that enhanced GSTα expression in IGF-IR-overexpressing hepatoma cells coincides with a reduced cytotoxic response to doxorubicin. Anthracycline anticancer agents such as doxorubicin and daunorubicin increase reactive oxygen species in cells, and oxidative stress is known to be correlated with the cytotoxicity of these chemotherapeutic agents (Muller et al., 1998). We also found that a representative antioxidant, N-acetylcysteine, efficiently blocked doxorubicin-induced cytotoxicity in GFP-H4IIE cells. The induction of GST family protein is a protective adaptive response to oxidative stress (Bergelson et al., 1994). GST functions as an efficient ROS-removing enzyme in cancer cells, and increases in ROS and subsequent apoptosis by doxorubicin were reduced to control levels by GST overexpression expressing H9C2 cells (L’Ecuyer et al., 2004). In this study, resistance to doxorubicin cytotoxicity in IGF-IR-H4IIE cells was completely reversed by a chemical inhibitor of GST, ethacrynic acid. These results provide evidence that enhanced GSTα expression in IR-HCCs by IGF-1 is associated with hepatoma resistance to chemotherapy.

Fig. 6. Effects of MAP kinase inhibition on GSTα up-regulation by IGF-1 in IGF-IR-overexpressing hepatoma cells. A, the effects of IGF-1 (30 ng/ml) on MAP kinase phosphorylation. The activations of ERK, p38 kinase, and JNK were assessed by immunoblotting the phosphorylated forms of these MAP kinases. B, the effects of dominant-negative mutants of MKK1 (MKK1DN), JNK (JNKNK1), and p38 kinase (p38KDN) on pGL-1651 reporter activity. pGL-1651 reporter activity changed by IGF-1 (30 ng/ml) was measured in IGF-IR-H4IIE cells transiently transfected with 1 μg of each dominant-negative mutant or 1 μg of pCMV5. C, the effects of ERK, p38 kinase, and JNK inhibitors on GSTα expression. IGF-IR-H4IIE cells were treated with 30 ng/ml IGF-1 for 18 h with or without specific inhibitors (10 μM U0126, U; 10 μM SB203580, SB; 10 μM SP600125, SP).
A number of studies have been conducted on the mechanistic basis of the phase II enzyme induction by monofunctional and bifunctional inducers. Monofunctional inducers transcriptionally activate the expression of GST\(\alpha\) genes through ARE, whereas bifunctional inducers act through both XRE and ARE (Prochaska et al., 1985; Wattenberg, 1985). Moreover, Nrf2 binding to ARE plays a critical role in the induction of phase II detoxifying enzymes, and a lack of Nrf2/ARE activation increases sensitivity to xenobiotics (Enomoto et al., 2001; McMahon et al., 2001). In the present study, we found that IGF-1 failed to stimulate the nuclear translocation of Nrf2 in IR-HCCs. This implies that different mechanisms are involved in the induction of GST\(\alpha\) by IGF-1.

We demonstrated previously that the chemopreventive agent, oltipraz, activates C/EBP\(\beta\) and that its binding to the C/EBP/XRE sequence in GSTA2 promoter is required for the induction of GST\(\alpha\) by oltipraz (Kang et al., 2003a). It has also been reported that exposure to growth factors, including IGF-1 and serum, cause C/EBP\(\alpha\) activation (Harris et al., 2001; LaVoie et al., 2004). Thus, we examined whether IGF-1 activates C/EBP\(\beta\) and whether this activation is involved in IGF-1-inducible GST\(\alpha\) expression. Here, we show that the activation of C/EBP\(\beta\) by IGF-1 precedes GST\(\alpha\) protein up-regulation, which, in combination with gel shift, luciferase-reporter gene, and promoter deletion assays, strongly supports the essential role of C/EBP\(\beta\)/XRE in GST\(\alpha\) induction by IGF-1 in IR-HCCs. The N-terminal transactivation domains of C/EBP\(\beta\) are known to interact with CBP/p300 coactivator, and these interactions are critical for C/EBP\(\beta\) transactivation (Mink et al., 1997). Thus, C/EBP\(\beta\) binding to the C/EBP/XRE site would result in its cooperative interaction with CBP/p300 coactivator to transactivate the GSTA2 gene. A recent study has revealed that C/EBP\(\alpha\) acts as a negative regulator that binds to GST-P enhancer 1 sequence and suppresses GST\(\tau\) expression in normal rat liver (Ikeda et al., 2006). In the promoter region of GST-\(P\) gene, Nrf2 also binds to GPE1 region and activates the transcription of GST-\(P\) gene in preneoplastic region and hepatoma (Ikeda et al., 2004). In the present study, we showed that C/EBP\(\beta\) but not C/EBP\(\alpha\) is essentially required for the IGF-1-induced GST\(\alpha\) expression. Moreover, GST\(\tau\) is not inducible by IGF-1 in IGF-IR-H4IIE cells. Hence, it seems that distinct C/EBP isoforms differentially contribute to the activation of ARE (C/EBP\(\alpha\)/- or XRE (C/EBP\(\beta\)/-mediated induction of GST enzymes.

A number of transcription factors are phosphorylated by distinct members of the kinase family, and these phosphorylations are triggered by a variety of growth factors (Pessin and Okada, 1999; Vincent and Feldman, 2002). And, in a series of studies, we demonstrated that PI3-kinase is essentially required for the induction of phase II detoxifying enzymes (Kang et al., 2000, 2001, 2003a). Signaling pathways downstream of PI3-kinase affect cell growth, survival, and motility, and IR-HCCs treated with IGF-1 showed transient PI3-kinase activation, which is consistent with previous studies (Cao et al., 2004). Either chemical inhibition or p85 subunit transfection suppressed C/EBP\(\beta\) expression by IGF-1 in IR-HCCs. Moreover, the inhibition of C/EBP/XRE activation and GST\(\alpha\) expression by IGF-1 in IR-HCCs. Hence, the inhibition of C/EBP/XRE activation and GST\(\alpha\) up-regulation by blocking PI3-kinase demonstrates the essential role of PI3-kinase in the regulation of IGF-mediated phase II detoxifying-enzyme expression.

We showed that IGF-1 increased the phosphorylation of ERK but not those of p38 MAP kinase or JNK. Extensive studies support the role of ERK activation as a cell proliferation or cancer progression signal (Fang and Richardson, 2005). It also has been reported that ERK is required for the activation of Nrf2/ARE during the induction of phase II detoxifying genes (Yu et al., 1999; Owuor and Kong, 2002). In the present study, we found that U0126, an ERK-specific inhibitor, failed to inhibit GST\(\alpha\) expression in IR-HCCs. As evidenced by either chemical inhibitors or by dominant-negative mutants, neither p38 kinase nor JNK was responsible for the induction of GST\(\alpha\) by IGF-1 in IR-HCCs. Hence, the
MAP kinase pathways, including ERK, are unlikely to be involved in IGF-1-inducible C/EBP/XRE activation in IR-HCCs. Taken together, the present study shows that C/EBPβ is activated by IGF-1 in IRG-IR-overexpressing hepatoma cells and that the PI3-kinase pathway plays a critical role in the activation of C/EBP/XRE. Our observation that GSTAs induced by IGF-1 is enhanced in IRG-IR-overexpressing hepatoma cells versus normal hepatoma cells has significant implications for the pathological role of IRG-IR up-regulation during liver carcinogenesis. Recently, evidence has been accumulated that IGF-IR is a promising target for cancer therapy. Increased expression of IGF-IR is found in a variety of tumors, which is correlated with reduced survival, metastasis development and tumor dedifferentiation (Scharf and Braulke, 2003). Although fully differentiated hepatocytes contain few IGF-IRs (McElduff et al., 1988), IGF-IR is frequently overexpressed in HCC (Tsai et al., 1988; Scharf et al., 2001). This may be associated with the aggressive characteristics of HCC, because binding of IGFRs to the IGF-IR results in the uncontrolled proliferation of cancer cells through either stimulating cell cycle progression or preventing apoptosis. Hence, IGF-IR is believed to be an attractive therapeutic target for several cancers, including HCC. In fact, IGF-IR-blocking antibodies or selective inhibitors to block IGF-IR kinase activity have been introduced as potential chemotherapeutic agents (Scotlandi et al., 1998; Höpfner et al., 2006). In this study, we suggest that odoxorubicin in IGF-IR-overexpressed hepatoma is in part related with the induction of GSTAs. Thus, IGF-IR-targeting therapy would be effective to enhance chemotherapeutic activities of antineoplastic agents.

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