Serine 9 and Tyrosine 216 Phosphorylation of GSK-3β Differentially Regulates Autophagy in Acquired Cadmium Resistance

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Glycogen synthase kinase-3β (GSK-3β) plays an important role in the regulation of apoptosis. To investigate its involvement in acquired cadmium (Cd) resistance, Cd-resistant cells (RH460) were established from H460 lung carcinoma cells. Cd resistance led to interruption of apoptosis and autophagy, as determined by an apoptotic sub-G1 population, procaspase-3 cleavage, and LC3-II induction. Cd-induced autophagy preceded apoptosis as determined by 3-methyladenine or zVAD and time-course experiments after Cd treatment. Despite β-catenin accumulation, phospho(p)-Ser/Tyr GSK-3α/β increased in the nucleus until 12 h after treatment and then p-Ser partly translocated to the cytoplasm. The GSK-3 inhibitor lithium augmented p-Ser/GSK-3α/β, which accumulated in the nucleus and cytoplasm, and increased autophagy. SB216763 inhibited p-Ser/p-Tyr GSK-3α/β and subsequent autophagy. GSK-3β knockdown decreased Cd-induced autophagy. Cd exposure to RH460 cells overexpressed with pcDNA-GSK-3β-HA strongly phosphorylated Serα/Tyr216 residues and decreased LC3-II. Constitutively active pcDNA-GSK-3b(S9A)-HA overexpression phosphorylated Tyr216 and decreased LC3-II, suggesting that p-Tyr inhibits autophagy. PI3K inhibitors decreased Cd-induced p-Ser GSK-3α/β and LC3-II, whereas a Ser/Thr phosphatase inhibitor, okadaic acid, hyperphosphorylated Ser residues, which accumulated in the nucleus and cytosol, and enhanced LC3-II. The general tyrosine kinase inhibitor genistein suppressed Cd-induced p-Tyr/p-Ser GSK-3α/β and LC3-II. Mouse lung tissues respond to long-term Cd exposure increased p-Tyr, downregulated LC3-II, and accumulated full-length Bax and procaspase-3. Taken together, this study shows that acquired Cd resistance is regulated by GSK-3β phosphorylation state, but not activation state, and intracellular localization of p-Ser GSK-3 regulates Cd-induced autophagy and apoptosis.

Key Words: glycogen synthase kinase-3; autophagy; cadmium; resistance; lung.

Cadmium (Cd), a widespread environmental and industrial pollutant, is a known human carcinogen (IARC, 1993). Its long biological half-life of over 20 years can lead to Cd accumulation and more serious problems, even at low levels (Goyer, 1991; Waalkes, 2003). In fact, epidemiological and experimental studies have shown that chronic exposure to Cd could be a cause of carcinogenesis in the lung, prostate, kidney, and liver (Goyer, 1991; Goyer et al., 2004). Cd carcinogenesis was shown to occur through the expression of proto-oncogenes, including c-jun, c-fos, c-myc, and p53 tumor suppressor, as well as by resistance to apoptosis (Huang et al., 2001; Jin and Ringertz, 1990; Wang and Templeton, 1998, Xu et al., 1999). Acquired Cd resistance could occur by suppression of apoptosis through interruption of JNK1/2 activity and metallothionein overexpression or expression of cytokeratin 8 (Lau and Chiu, 2007; Lau et al., 2006; Qu et al., 2006). Although the various harmful effects of Cd and its role in carcinogenesis are well documented, the precise mechanisms remain incompletely understood.

Glycogen synthase kinase-3 (GSK-3), a ubiquitously expressed serine/threonine kinase, has a wide range of cellular functions, including cell death, cell cycle, and carcinogenesis, and is an important regulator of various signal-transduction pathways, including the Wnt pathway (Forde and Dale, 2007). GSK-3 activity is regulated by phosphorylation of tyrosine (Tyr)216/276 or serine (Ser)216, as well as by its subcellular distribution (Bijur and Jope, 2003; Diehl et al., 1998; Meijer et al., 2004). Although GSK-3 is involved in survival signaling pathways (Crowder and Freeman, 2000; Hoeflich et al., 2000; Pap and Cooper, 1998), it is associated with apoptosis (Bhat et al., 2000; Hetman et al., 2000; Song et al., 2002). Moreover, GSK-3 was shown to modulate autophagy (Wang et al., 2009; Yang et al., 2010). These reports indicate that GSK-3 has multifunctional roles.
Autophagy is a process of lysosomal-dependent degradation of proteins and organelles in which portions of the cytoplasm are encompassed by double-membrane vesicles called autophagosomes and then fused with lysosomes. Their contents are subsequently degraded by lysosomal hydrolases and recycled, indicating that autophagy protects cells. However, accumulating data provide evidences that autophagy also plays a role in cell death (Codogno, 2005; Ouyang et al., 2012). Blocking apoptosis may lead to chemotherapeutic resistance (Kim et al., 2012), which may also involve autophagy (Huang et al., 2012; Sirichanchuen et al., 2012; Yang et al., 2012). However, the role of autophagy and the underlying molecular mechanisms for heavy metal–induced resistance remain to be elucidated.

Cd toxicity is related to multiple signaling pathways, caspase-dependent and caspase-independent apoptosis, and necrosis, and reactive oxygen species seem to be signaling molecules in these signaling pathways (Gobe and Crane, 2010; Oh et al., 2004). Recently, a role for GSK-3β-mediated autophagy was reported in Cd toxicity (Wang et al., 2009), suggesting that GSK-3 could play an important role in Cd-induced resistance. However, little is known about the role of GSK-3 in the molecular mechanisms that confer cellular resistance to Cd.

In this study, we investigated the involvement of GSK-3α/β and the underlying molecular mechanism of acquired Cd resistance. We found that acquired Cd resistance is caused by interruption of GSK-3α/β phosphorylation at serine residue and that its intracellular localization regulates Cd-induced autophagy and apoptosis.

**MATERIALS AND METHODS**

**Cell culture and culture conditions.** Human non–small cell lung carcinoma cells H460 and Cd-resistant RH460 cells were maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37°C in a 5% CO₂, 95% air-humidified incubator. 3-Methyladenine, bafilomycin A1 (BaF1), lithium chloride, genistein, sodium orthovanadate, chloroquine (CQ), and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO). The other chemicals used were of the purest grade available from Sigma (St Louis, MO).

**Cytotoxicity assay, flow cytometric analysis, and subcellular fractionation.** The MTT assay, cell cycle analysis, and nuclear, mitochondrial, and cytosol fractionation were performed as previously described (Choi et al., 2010; Oh et al., 2004).

**RNA interference and overexpression.** Cells were transfected with control siRNA, Atg5 siRNA or GSK-3β siRNA, and 1.5 μl lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in 6-well plates according to the manufacturer’s protocol. The plasmids pCDNA3.1-V5-GSK-3β WT and V5-GSK-3β S9A were kind gifts from Dr Yoon (Aju University, School of Medicine, Suwon, Korea), and GFP-IC5B plasmid DNA was kindly provided by Dr Xiao-Ming Yin (Indiana University School of Medicine, Indianapolis, IN). After cells were washed with OPTI-MEM medium (Gibco), DNA was transfected to cells using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the supplier’s protocol. After 4 h of incubation, the medium was exchanged to a complete medium containing 10% serum and antibiotics. The cells were incubated for an additional 24 h and treated as indicated in the figure 3A, 5A, and 5B.

**Immunofluorescence and immunoblot analysis.** Immunofluorescence staining and immunoblot analysis were performed as described previously (Choi et al., 2010; Yoon et al., 2010). Antibodies were as follows: anti-LC3B, anti-β-actin, and p-Ser17/19 GSK-3α/β (Cell Signaling Technology, Beverly, MA); GSK-3α/β (Millipore, Temecula, CA); anti-p-Tyr705/720 GSK-3α/β (Epitomics, Burlingame, CA; BD Pharmingen, San Diego, CA); anti-p-caspase3, β-catenin, and β-actin (Santa Cruz Biotechnology).

**Immunohistochemistry.** Preparation for sections from paraffin blocks were performed as previously described (Yoon et al., 2010). To detect p-Ser GSK-3, antigen retrieval was performed using microwave in 10mM sodium citrate buffer (pH 6) for 20 min, and the sections were incubated with p-Ser GSK-3α/β (1:50) for overnight at 4°C. A negative control with no primary antibody was performed for each specimen. Endogenous peroxidase activity was abolished by incubating the sections for 15 min in 0.3% H₂O₂. Next, procedures were performed using Polink-2 AP broad detection kit according to the supplier’s protocol (Life Science Division).

**Animals and Cd exposure.** Six weeks of age male C57BL/6 (C57) mice were purchased from the Orientbio (Suwon, Kyung-gi, Korea) and housed in controlled conditions of room temperature, a 12 h light/dark cycle and humidity of 50–60%. Animals received normal diet and drinking water ad libitum. Mice randomly divided into two groups of four to six mice and were injected ip daily with cadmium acetate (1 mg/kg body weight) until 8 weeks, then after injected alternately, or the same volume of saline for 20 weeks. Animals were then anesthetized with xylasol/ketamine and weighed. Animal care and all experiments were conducted in agreement with the Institutional Animal Care Committee of Chosun University.

**Statistical analysis.** All experiments were repeated at least three times, and the significance of the differences between treatments and respective controls was analyzed using Student’s t-test. Values are expressed as the mean ± SD.

**RESULTS**

**Cellular Sensitivity of H460 and RH460 Cells to Cd**

A Cd-resistant cell line (RH460) was established after several rounds of selection via exposure of H460 cells to a stepwise series of increasing Cd concentrations. Cytotoxicity to Cd in parental H460 and RH460 cells was analyzed by MTT assay after treatment with Cd for 24 h, and the IC₅₀ values for Cd were determined to be 8 and 70μM, respectively (Fig. 1A). RH460 cells were approximately 8.7-fold more resistant to Cd than H460 cells and exhibited resistance to Cd-induced apoptosis, as demonstrated by flow cytometry (Fig. 1B).

**Ser/Tyr Phosphorylation of GSK-3α/β in Response to Cd in H460 and RH460 Cells**

Involvement of GSK-3α/β in Cd resistance was assessed by its phosphorylation state. RH460 cells in response to Cd increased p-Ser in a dose- and time-dependent manner, and its induction delayed in RH460 compared with H460 cells. However, p-Tyr showed an increase within IC₅₀ concentrations of Cd, and β-catenin, a downstream target of GSK-3α/β (Miller and Moon, 1996), increased in both cells (Figs. 2A and 2B). To examine whether dual phosphorylation of GSK-3α/β was unique in H460 and RH460 cells, p-GSK-3α/β was evaluated...
in H1299 and Cd-resistant RH1299 cells and similar results were obtained (Supplementary fig. 1). These results suggest that RH460 cells did not sensitive to GSK-3 phosphorylation in response to Cd, which could be involved in Cd resistance.

Localization of p-GSK-3α/β was examined in cytosolic and nuclear fractions prepared from RH460 cells. In untreated control cells, GSK-3α/β is mainly localized to the nucleus and to a lesser extent the cytosol. After Cd treatment, p-Ser/p-Tyr accumulated in the nucleus, peaked at 12 h, and decreased. The p-Ser, but not p-Tyr, markedly accumulated in the cytoplasm at 24 h. Accumulation of β-catenin in the nucleus began at 1 h and remained elevated through 24 h. Immunoblots of β-actin and histone deacetylase 1 (HDAC 1) confirmed the complete separation of the cytosolic and nuclear fractions (Fig. 2C). The subcellular localization of p-Ser was confirmed using immuno fluorescence staining. Green fluorescence localized to the nuclear membrane of control cells. After 12 h of Cd treatment, it was aggregated in the cytoplasm or dispersed in the nucleus in some cells, which had a condensed or fragmented nucleus (Fig. 2D), and the number of such cells increased at 24 h. However, immunofluorescence staining for p-Tyr was observed in the nucleus of control cells, and the fluorescent intensity increased at 12 h and decreased at 24 h (Supplementary fig. 2A). The shuttling of p-GSK-3α/β between the nucleus and cytoplasm was further investigated in cells treated with increasing concentrations of Cd for 12 and 21 h, respectively (Supplementary figs. 2B and 2C). These data clearly point to a specific function of p-GSK-3α/β in the nucleus and cytoplasm.

**H460 and RH460 Cells Induce Autophagy and Caspase-3 Activation in Response to Cd**

To investigate whether autophagy is involved in Cd resistance, RH460 cells were transfected with green fluorescence protein (GFP)-LC3B plasmid DNA. In nontreated control cells, GFP fluorescence was evenly diffused in the cytoplasm; however, Cd treatment induced GFP puncta, indicating recruitment of LC3-II to the autophagosome (Fig. 3A). The autophagy induction by Cd treatment was confirmed by autophagic flux following treatment with BaF1 and CQ, and the membrane-bound LC3-II form was markedly accumulated (Fig. 3B). LC3-II began to be induced with decreasing of p62/SQSTM1 at 64 µM Cd treatment after 12 h in RH460 cells (Figs. 3D and 3F). In H460 cells, LC3-II began to be induced at 3 µM and at 6 h in dose- and time-dependent experiments, respectively (Figs. 3C and 3E). In addition, Cd treatment induced cleavage of procaspase-3, which appeared at 6 and 64 µM Cd treatments in H460 and RH460 cells, respectively. In a time-course experiment, active caspase-3 observed at 12 and 24 h in each cell line. These data indicate that autophagy proceeded to Cd-induced apoptosis. To examine whether blocking autophagy could reduce Cd-induced cell death, RH460 cells were treated with Cd in the presence of the autophagy inhibitor 3-methyladenine (MA) or pan-caspase inhibitor zVAD. 3-MA and zVAD alone did not induce obvious cell death compared with the control, but cotreatment with Cd resulted in a significant increase in cell viability compared with Cd alone and the effect was greater in 3-MA-treated cells than in zVAD-treated cells (Fig. 3G). Phase-contrast microscopy showed that the formation of vacuoles in the cytosol completely blocked by pretreatment with 3-MA, but still remained in zVAD-treated cells (Supplementary fig. 5) The Cd-induced cleavage of procaspase-3 was inhibited by 3-MA and zVAD, and the LC3-II level was attenuated by 3-MA but not by zVAD (Fig. 3H). These results were further confirmed by transfection with Atg5 siRNA (Fig. 3I), and knockdown of Atg5 gene significantly reduced cell death (Supplementary fig. 6). Therefore, Cd-induced cell death could result from cooperation between autophagy and apoptosis.

**Effects of Ser and Tyr Phosphorylation of GSK-3α/β on Cd-Induced Autophagy**

As shown in Figure 2A, Cd treatment led to dual phosphorylation of GSK-3α/β at Ser/Tyr residues. Thus, we investigated the site-specific effects of p-GSK-3α/β on Cd-induced autophagy. First, to determine the effects of p-Tyr, RH460 cells were treated with GSK-3 inhibitors SB216763 or lithium (Coghlan et al., 2000; Klein and Melton, 1996). Treatment with lithium alone, but not SB216763, slightly
decreased cell viability compared with control. The inhibitors had opposite effects on Cd-induced cell viability: lithium significantly decreased it, whereas SB216763 markedly increased it (Fig. 4A). Although both inhibitors accumulated β-catenin, they had different effects on the phosphorylation state of GSK-3α/β. Lithium treatment strongly accumulated the level of Cd-induced p-Ser GSK-3α/β in the nucleus and cytosol (Figs. 4B and 4C), indicating that lithium seems to inhibit GSK-3 activity by increasing the p-Ser level, whereas SB216763 only increased p-Ser21 and completely blocked Cd-induced p-Tyr279/216. Consistent with cell death, lithium enhanced the cleavage of procaspase-3 induced by Cd, which was reversed by SB216763. Interestingly, Cd-induced LC3-II was enhanced by lithium, but markedly decreased by SB216763 (Fig. 4B), suggesting that the phosphorylation state of GSK-3α/β, but not its activation state, seems to play a decisive role in Cd-induced cytotoxicity through autophagy. Thus, we examined the involvement of p-Tyr level on Cd-induced autophagy. RH460 cells were treated with the general tyrosine kinase inhibitor genistein, which decreased p-Ser as well as p-Tyr levels and led to a decrease in LC3-II and cleavage of procaspase-3, consequently increasing Cd-induced cell viability (Figs. 4D and 4E). Although a protein tyrosine phosphatase inhibitor ortho-vanadate had no effect on Cd-induced p-Tyr/p-Ser levels, further decreased Cd-induced cell viability (Figs. 4D and 4E).

Next, to examine the effect of p-Ser on Cd-induced autophagy regulation, RH460 cells were treated with PI3K inhibitors including wortmannin and Ly294002 (Vlahos et al., 1994), which block phosphorylation at serine residue of GSK-3α/β, a downstream of Akt (Pap and Cooper, 1998). PI3K inhibitors decreased the levels of Cd-induced p-Ser/p-Tyr GSK-3α/β and β-catenin, which caused a decrease of LC3-II level and procaspase-3 cleavage; consequently, cell viability increased compared with Cd-treated cells (Figs. 4F and 4G). However, the dephosphorylation of p-Ser by PI3K inhibitors
did not lead phosphorylation at Tyr residue of GSK-3α/β. In contrast, to increase p-Ser level of GSK-3α/β, RH460 cells were pretreated with OA, a Ser/Thr phosphatase inhibitor, and subsequently treated with Cd for 18h, which accumulated high amount of p-Ser in the nucleus and cytosol with dephosphorylation of Tyr residue, and increased LC3-II and procaspase-3 cleavage, significantly decreasing cell viability (Figs. 4H–J). Similar results were obtained when OA-pretreated cells were exposed to Cd for 10h (Supplementary fig. 3A–C), suggesting that strong phosphorylation in Ser residue led to Tyr dephosphorylation. Therefore, the subcellular localization of p-Ser GSK-3α/β may be critical for its functional role in the cell; cytoplasmic localization was associated with apoptosis. Consistently, in Cd-sensitive wild-type (wt) H460 cells, p-Ser localized to both compartments, even with low concentrations of Cd (Supplementary fig. 4). These data indicate that the phosphorylation state of GSK-3α/β at serine or tyrosine residue plays an important role in autophagy regulation in response to Cd.

Effects of Knockdown or Overexpression of GSK-3β on Cd-Induced Autophagy

Regulation of autophagy by p-Ser/p-Tyr GSK-3α/β was further confirmed by genetic knockdown and overexpression of GSK-3β. Transfection with GSK-3β siRNA markedly blocked total GSK-3β protein levels, which downregulated Cd-induced p-Ser/p-Tyr and LC3-II levels (Fig. 5A). Next, RH460 cells were transduced with hemagglutinin (HA)-tagged wt GSK-3β, a constitutively
active GSK-3β mutant (S9A), and pcDNA3.1 plasmids. Transduction of plasmid DNA was confirmed by immunoblotting for total GSK-3 protein and HA. Strong phosphorylation of Ser9 and Tyr216 in ectopically expressed GSK-3β was found in cells transduced with wt-GSK-3β after Cd exposure. Cells transduced with S9A showed Tyr216 phosphorylation, and the p-Ser-specific antibody did not recognize S9A-GSK-3β. Interestingly, LC3-II greatly decreased in Cd-exposed wt- and S9A-transduced cells, indicating that p-Tyr and p-Ser had opposite effects on Cd-induced autophagy, and strong phosphorylation in Tyr residue seemed to prevent the p-Ser GSK-3β (Fig. 5B). These data clearly showed the site-specific role of GSK-3β in Cd-induced autophagy, and they suggested the possibility that autophagy in Cd-resistant cells could be regulated by the check between p-Ser and p-Tyr of GSK-3β.

**Chronic Cd Exposure in Mice Blocks Ser Phosphorylation of GSK-3α/β and Autophagy in Lung Tissue**

To determine whether the in vitro results for Cd resistance could apply in vivo, mice were exposed to Cd through ip
injection for 20 weeks, and lysed lung tissues were used for immunoblot analysis (Fig. 6A). In saline-injected control mice, p-Ser GSK-3α/β remained at a basal level through 16 weeks, but the amount decreased at 20 weeks. The amount of p-Ser GSK-3α/β in Cd-injected mice at 2 weeks compared with control mice, and completely decreased through 16 weeks, increasing at 20 weeks. Interestingly, the amount of p-Tyr showed high level in Cd-injected mice compared with each control and further increased at 16–20 weeks. In control mice, LC3-I/II remained at basal levels throughout the 20 weeks. In Cd-injected mice, LC3-I/II increased at 2 weeks and continuously decreased through 16 weeks, increasing at 20 weeks. Interestingly, the amount of p-Tyr showed high level in Cd-injected mice compared with each control and further increased at 16–20 weeks. In control mice, LC3-I/II remained at basal levels throughout the 20 weeks. In Cd-injected mice, LC3-I/II increased at 2 weeks and continuously decreased through 16 weeks, slightly increasing at 20 weeks, which was confirmed by p62/SQSTM1 accumulation. Consistently, procaspase-3 and full-length Bax began markedly increasing at 8 weeks, indicating that Cd resistance is associated with the blocking of autophagy and apoptosis. The expression of p-Ser GSK-3α/β was confirmed by immunohistochemistry. In the epithelial cells of alveoli, bronchioles, and bronchus of the lung tissues from saline- or Cd-injected mice for 2 weeks, the strong positive staining was shown. However, the staining intensity was very faint in the lung tissue from Cd-injected mice for 8 weeks (Fig. 6B). Hematoxylin and eosin staining of lung tissues showed that all saline control animals had histologically normal lungs throughout the 20 weeks (Fig. 6C, a). However, the lungs of Cd-injected mice began to show severe abnormalities at 16 weeks through thickened alveoli walls and pulmonary fibrosis (Fig. 6C, b), which was further progressed at 20 weeks with shrunken air sacs (Fig. 6C, c).

**DISCUSSION**

Here, we provide the first evidence of the involvement of GSK-3α/β/autophagy signaling in acquired Cd resistance. Cd treatment induced dual phosphorylation of GSK-3α/β at Ser and Tyr residues and the checks between them play an important role in Cd-resistant cells. Phosphorylated-Ser GSK-3α/β mainly localized to the nucleus and translocated to the cytoplasm after Cd treatment. Treated cells had apoptotic nuclei, indicating that p-Ser GSK-3α/β localized in the cytoplasm is involved in cell death. Furthermore, Cd-induced autophagy was regulated by p-Ser GSK-3α/β and its subcellular localization, whereas it was prevented by p-Tyr, indicating that the level between the two phospho forms plays an important role on Cd-induced autophagy. In lung tissues from chronic Cd-injected mice, we found dephosphorylation of GSK-3α/β at Ser residue, blocking autophagy, and subsequent blocking of the cleavage of procaspase-3 and Bax. These results indicate that p-Ser GSK-3α/β could play an important role in the mechanism of resistance to Cd-induced autophagy and subsequent apoptosis.
It was reported that Cd-induced nephrotoxicity is regulated by the p-Tyr GSK-3-mediated autophagy (Wang et al., 2009). Moreover, our previous study found that Cd resistance was regulated by autophagy (Oh et al., 2009). Thus, we demonstrated the involvement of GSK-3α/β-autophagy signaling in Cd resistance and the underlying molecular mechanisms of how GSK-3α/β regulates autophagy in Cd resistance.

Activation of GSK-3α/β is regulated by Ser and Tyr phosphorylation or its subcellular distribution (Bijur and Jope, 2003; Diehl et al., 1998; Meijer et al., 2004). Phosphorylation of Tyr216 in GSK-3β plays a critical role in GSK-3 activation and function (Hughes et al., 1993), which is accompanied by the dephosphorylation of Ser (Forde and Dale, 2007; Qu et al., 2006). We found similar results in Cd-treated HepG2 cells (data not shown). However, Cd-treated H460 and RH460 cells, as well as H1299 and RH1299 cells, induced dual phosphorylation at Ser and Tyr residues, indicating that there was no correlation between both residues and Cd treatment. Despite Tyr phosphorylation, Cd treatment resulted in the accumulation of β-catenin, and we could not exclude the possibility that GSK-3 activity was not strictly correlated with the level of Tyr phosphorylation (Simón et al., 2008) or regulated by the check between inactive Ser and active Tyr forms of GSK-3 (Noël et al., 2011). In addition, Tyr216 may be autophosphorylated (Cole et al., 2004) or phosphorylated by other upstream kinases (Hartigan et al., 2001; Kim et al., 1999; Lesort et al., 1999). In the present study, phosphorylation at Tyr residue induced by transfection with GSK-3β into RH460 cells and its phosphorylation and functions were affected by a Tyr kinase inhibitor, indicating that Tyr phosphorylation was not an autophosphorylation event, at least in the present study. Inhibition of GSK-3 via Ser phosphorylation was regulated by several kinases, including PI3K, protein kinase C, p90Rsk, p70S6 kinase, and protein kinase A (Eldar-Finkelman, 2002). Treatment of PI3K inhibitors to RH460 cells decreased the level of Cd-induced p-Ser GSK-3α/β, indicating that GSK-3α/β is an Akt substrate. Interestingly, despite the degradation of β-catenin, Ser dephosphorylation with PI3K inhibitors did not lead to Tyr phosphorylation, rather decreased it. OA allowed accumulation of high amount of p-Ser, which caused dephosphorylation at Tyr residue, indicating that strong phosphorylation in Ser residue can prevent Tyr phosphorylation. Genistein is an inhibitor for Tyr kinase, as well as it has been reported to downregulate PI3K/Akt signaling pathway (Liang et al., 2012; Sahin et al., 2012). Thus, genistein treatment to RH460 cells caused dephosphorylation at both Ser/Tyr residues of GSK-3α/β. However, treatment of Tyr phosphatase inhibitor did not affect the Cd-treated p-Tyr level. These results support that the level of p-Ser GSK-3 could play an important role in Cd-induced toxicity.

The role of autophagy in cell survival and cell death is still controversial (Codogno, 2005). In the present study, Cd-induced autophagy delayed in RH460 cells compared with H460 cells. Similar results were found in H1299 and RH1299 cells. Genetic silencing or pharmacological inhibitors of autophagy attenuated the Cd-induced apoptosis through inhibition of caspase-3 activation, indicating that Cd-induced autophagy is involved in cell death through apoptosis. Moreover, the broad-caspase inhibitor zVAD did not affect Cd-induced autophagy and there were lower levels of cell viability in these cells than in 3-MA-treated cells. The time-course experiment showed that autophagy was a preceding event of apoptotic change at the biochemical level, indicating that Cd-induced apoptosis requires autophagy as a trigger of apoptosis.

We found the dual phosphorylation of GSK-3α/β in response to Cd in both wt and Cd-resistant cells within IC50 concentrations. In a previous report, Cd toxicity was related with p-Tyr GSK-3 (Wang et al., 2009). Thus, we examined the relationship between GSK-3α/β and autophagy in Cd resistance, as well as the effects of site-specific phosphorylation of Ser/Tyr residues on Cd-induced autophagy using GSK-3 inhibitors, genetic knockdown, GSK-3β overexpression, and kinase inhibitors. Despite the accumulation of β-catenin, pharmacological inhibitors of GSK-3 resulted in differences in the Cd-induced phosphorylation state of GSK-3α/β and autophagy; lithium strongly enhanced the Cd-induced p-Ser and increased the LC3-II level and procaspase-3 cleavage, consequently decreasing Cd-induced cell viability. SB216763 had completely opposite effects. RH460 cells overexpressed with constitutively active S9A mutant markedly decreased Cd-induced autophagy, indicating that Cd-induced autophagy is blocked by p-Tyr GSK-3. Furthermore, overexpression with wt-GSK-3β partially decreased Cd-induced autophagy, indicating that the Cd-induced autophagy is regulated by the check between two phospho forms of GSK-3, and strong phosphorylation in Tyr residue can prevent p-Ser-mediated autophagy. Genetic silencing of GSK-3β led to decreases in p-Tyr/p-Ser and LC3-II. In addition, Cd-induced LC3-II level was decreased by treatment with PI3K inhibitors or genistein. In contrast, OA treatment increased the level of LC3-II, indicating that Cd-induced autophagy was dependent on p-Ser GSK-3β. GSK-3 inhibitors did not show a correlation between p-Tyr and p-Ser (Bhat et al., 2000; Noël et al., 2011; Simón et al., 2008; Tan et al., 2005). Lithium induced p-Ser responses to nerve growth factor withdrawal without affecting the p-Tyr level in mouse neuronal cells (Bhat et al., 2000). Some stimuli, including Lysophosphatidic acid and staurosporine, increase p-Tyr even when GSK activity is inhibited (Noël et al., 2011; Simón et al., 2008), indicating that GSK-3 kinase activity is not necessarily correlated with the extent of p-Tyr or that GSK-3 inhibitors can act in a cell type–specific manner. Although the present study does not clearly discriminate the role of p-Tyr GSK-3β on Cd-induced autophagy, it does show the possibility that p-Tyr can block autophagy as demonstrated by the results obtained from S9A-mutant-transduced cells and in vivo study. In addition, the function of GSK-3 may be regulated by intracellular localization (Bijur and Jope, 2003; Diehl et al., 1998). In cells treated with high
concentrations of Cd or OA or lithium, p-Ser GSK-3α/β was localized in the cytoplasm, indicating that redistribution of GSK-3α/β could be caused by its distinctive function. In a previous study, Ser phosphorylation led to Bax-mediated mitochondrial apoptosis in colorectal cancer cells (Tan et al., 2005). Furthermore, in staurosporine-treated human neuroblastoma SH-SY5Y cells and nerve growth factor withdrawal from rat pheochromocytoma PC12 cells, apoptosis is associated with nuclear accumulation of p-Tyr216 GSK-3β (Bhat et al., 2000). Apoptosis by serum withdrawal and heat shock or staurosporine in SH-SY5Y cells depends on nuclear accumulation of GSK-3β (Bijur and Jope, 2003). In the present study, cytoplasmic redistribution of p-Ser during Cd treatment was matched by the occurrence of autophagy in RH460 cells. Immunofluorescence staining and immunoblotting for p-Ser revealed its presence in the nucleus; however, it was present in the cytoplasm of cells with apoptotic nuclei at later times. Moreover, in Cd-treated H460 cells, p-Ser is distributed in both the cytosol and nuclear compartments, even at low concentrations of Cd. Thus, the function of p-GSK-3α/β appears to be dynamically regulated. These results are supported by OA treatment. OA pretreatment to RH460 cells before Cd exposure caused accumulation of p-Ser in the cytosol, which led to autophagy. Thus, these results suggest that the effect of GSK-3α/β on Cd-induced autophagy could be dependent on the cellular distribution of p-Ser GSK-3α/β. Moreover, our results did not consistent with a previous report, which demonstrated p-Tyr-mediated autophagy.

To determine whether the results observed in cell culture models could be applied in vivo, we used chronic Cd-exposed mice. An indicator of Cd resistance was confirmed by expression of a multidrug resistance–associated protein (data not shown). p-Ser GSK-3α/β was induced early, leading to autophagy; however, it was almost dephosphorylated after 4 weeks, leading to an interruption of autophagy, which was confirmed by accumulation of p62/SQSTM1, an autophagy adaptor molecule (Mizushima et al., 2010). However, p-Tyr GSK-3α/β remained higher level compared with each control through 20 weeks. Moreover, autophagy inhibition resulted in the accumulation of procaspase-3 and full-length Bax, indicating that blocking autophagy led to apoptosis inhibition and fibrosis in mice that received chronic administration of Cd. Therefore, our in vivo data suggest that Cd resistance occurs through downregulation of p-Ser GSK-3-dependent autophagy, and p-Tyr seems to be involved in protection against Cd toxicity.

This is the first study to show that Cd resistance is regulated by the phosphorylation state of GSK-3 at Ser and Tyr residues and cytoplasmic localization of p-Ser, leading to autophagy and apoptosis. Thus, the mitagation of p-Ser GSK-3-mediated autophagy signaling plays a critical role in the development of Cd resistance, which could drive carcinogenesis. Therefore, our study suggests that the increasing of p-Ser GSK-3α/β level may be a useful target against Cd resistance. These findings also highlight the possibility that novel therapeutic approaches to resistance could be possible in chemo- or radiation therapy during cancer treatment.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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