BIOCELL
2010, VOL. 34(1): 15-21

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Cobalt chloride stimulates phosphoinositide 3-kinase/Akt signaling through the epidermal growth factor receptor in oral squamous cell carcinoma

Mi Heon Ryu[1,a], Jeong Hee Park[1,a], Ji Eun Park[1], Jin Chung[2], Chang Hun Lee[3] and Hae Ryoun Park[1*]

1. Department of Oral Pathology, School of Dentistry, Pusan National University, Beomeo-ri, Mulgeum-Eup, Yangsan 626-870, South Korea
2. Department of Oral Microbiology, School of Dentistry, Pusan National University, Beomeo-ri, Mulgeum-Eup, Yangsan 626-870, South Korea
3. Department of Pathology, School of Medicine, Pusan National University, Beomo-ri, Mulgeum-Eup, Yangsan, South Korea

a. These authors contributed equally to this work.

Key words: hypoxia, DNA synthesis, S-phase

ABSTRACT: Tumor cells are often found under hypoxic conditions due to the rapid outgrowth of their vascular supply, and, in order to survive hypoxia, these cells induce numerous signaling factors. Akt is an important kinase in cell survival, and its activity is regulated by the upstream phosphoinositide 3-kinase (PI3K) and receptor tyrosine kinases (RTKs). In this study, we examined Akt activation and RTKs/PI3K/Akt signaling using the hypoxia-mimetic cobalt chloride in oral squamous carcinoma cells. Cobalt chloride increases Akt phosphorylation in both a dose- and time-dependent manner. Blocking the activation of the PI3K/Akt pathway using LY294002 abolished Akt activation in response to cobalt chloride, suggesting that Akt phosphorylation by cobalt chloride is dependent on PI3K. In addition, activation of the PI3K/Akt pathway seems to rely on the epidermal growth factor receptor (EGFR), since the inhibition of EGFR attenuated cobalt chloride-induced Akt activation. The results in this study also demonstrate that cobalt chloride increases EGFR protein levels and induces oral squamous cell carcinoma cells to enter S phase.

Introduction

Hypoxia, defined as a state where oxygen tension drops below normal limits, plays a pivotal role in pathological conditions, including tumors. Tumors, especially those that are cancerous, tend to be hypoxic because they grow too fast to contain enough blood vessels to maintain their oxygen supply. Hypoxia can limit tumor growth and tumors with poor vascularization fail to grow, leading to apoptosis. In contrast, certain types of cancer cells can survive and even proliferate under hypoxic conditions by triggering specific cellular and systemic adaptive responses (Hockel and Vaupel, 2001; Semenza, 2002).

When cells are exposed to hypoxia, a large number of factors respond to the altered environment, resulting in either cell death or survival. Of these factors, Akt is one of the most important signaling molecules for determining cell fate under hypoxic conditions. A few studies have reported that hypoxia or the hypoxia-mimicking condition produced by cobalt chloride ($CoCl_2$) treatment can activate Akt, whereas other investigations

*Address correspondence to: Hae Ryoun Park.
E-mail: *parkhr@pusan.ac.kr*
Received: August 28, 2009. Revised version received: January 28, 2010. Acepted: February 5, 2010.

have suggested that it inhibits, or has no effect on Akt activation (Barthel *et al.*, 2007; Chen *et al.*, 2001; Lee *et al.*, 2008a, b). Thus, Akt activity in response to hypoxia remains controversial.

Akt activity is mainly dependent on phosphoinositide 3-kinase (PI3K). Active PI3K phosphorylates membrane phosphatidylinositol phospholipids, and these lipid products act as second messengers that mediate the diverse cellular functions of PI3K. One of the targets of these lipid second messengers is the serine/threonine kinase Akt (Franke *et al.*, 1997; Toker and Cantley, 1997). The PI3K/Akt pathway is activated by receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), the insulin growth factor 1 receptor (IGF1R), and the platelet-derived growth factor receptor (PDGFR). The RTKs are stimulated by their respective growth factors, leading to cell metabolism and growth through downstream signaling systems (Alessi *et al.*, 1996; Blume-Jensen and Hunter, 2001; Chen *et al.*, 2001). In addition to growth factor ligands, it has been suggested that heat shock and ultraviolet light can activate RTKs, resulting in the activation of PI3K and its target Akt (Coffer *et al.*, 1995; Lin *et al.*, 1997). Though numerous RTK candidates and the consecutive PI3K/Akt signaling pathways have been studied, there are few reports on how hypoxia affects RTKs/PI3K/Akt signaling.

This study examined the effect of hypoxia on signaling pathways in oral squamous cell carcinoma cells using $CoCl_2$ to induce a hypoxia-mimicking condition. First, we investigated whether $CoCl_2$ can activate or inhibit the PI3K/Akt pathway in oral squamous carcinoma cells. Second, we studied which growth factor signaling pathway is used when the stimuli evoke cellular responses. Finally, we investigated how the hypoxia-mimetic $CoCl_2$ affects the cell cycle in oral squamous carcinoma cells.

Materials and Methods

Cell culture

An YD8 cell line was established from the cancer of an oral squamous cell carcinoma patient and was gifted from the Department of Oral Pathology, College of Dentistry, Yonsei University (Seoul, Korea). The cells were grown in RPMI 1640 medium (Gibco, Rockville, MD) with 10% fetal bovine serum (Gibco) and incubated in a humidified atmosphere containing 5% CO_2 at 37ºC.

Reagents

LY294002 (PI3K inhibitor), PD168393 (EGFR inhibitor), and picropodophyllin (IGF1R inhibitor) were obtained from Calbiochem (La Jolla, CA). DMPQ (PDGFR inhibitor) was purchased from TOCRIS (Ellisville, MO). Cobalt chloride was purchased from Sigma (St. Louis, MO). The antibodies against phospho-Akt (Ser473), phospho-Akt (Thr308), and Akt were purchased from Cell Signaling Technology (Beverly, MA). The goat anti-actin antibody, anti-rabbit IgG antibody and rabbit anti-mouse IgG antibody were purchased from Santa Cruz (Santa Cruz, CA).

Western blot analysis

YD8 cells were lysed in lysis buffer (150 mM NaCl, 0.2% Nonidet P-40, 50 mM NaF, 100 μM Na_3VO_4, pH 7.2) in the presence of protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) on ice for 30 min, followed by clarification by centrifugation at 12,000 g for 15 min. Protein concentrations were determined using the Bio-Rad Protein assay (Bio-Rad, Hercules, CA). Proteins (50 μg) were resolved on 10% (w/v) polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS, pH 7.6) containing 0.1% Tween20 for 1 h at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies at 4ºC. The membranes were washed in Tris-buffered saline/Tween20 and were incubated with the respective horseradish peroxidase-conjugated secondary antibody in Tris-buffered saline/Tween20. After washing in Tris-buffered saline/Tween20, bound secondary antibodies were detected using Supersignal West Femto substrate (Pierce, Madison, WI). The immunoreactive bands were captured and quantified using a ChemiDoc system (Bio-Rad). All the experiments were done at least three times independently.

Cell cycle analysis

Cells were washed with phosphate buffered saline containing 1% bovine serum albumin and centrifuged at 2,000 rpm for 10 min. Cells were then washed with phosphate buffered saline and resuspended in 3 ml of ice-cold 75% ethanol for 24-72 h. The cells were incubated with DNase-free RNase (0.1 mg/ml; Sigma) and propidium iodide (50 μg/ml; Sigma) at 4ºC for at least 1 h in a dark room prior to flow cytometric analysis.

The percentages of cells in different phases of the cell cycle were measured with a flow cytometer (Beckman Coulter, Fullerton, CA). A minimum of 10^5 cells were measured and analyzed for cell cycle analysis.

Statistical analysis

Results are presented as mean ± standard deviation of separate experiments, and were expressed as a ratio of treated cells to control cells. ANOVA or Student *t*-test was used for statistical analysis using a software package (SPSS 14.0).

Results

A hypoxic condition induced by $CoCl_2$ increases Akt phosphorylation in oral squamous carcinoma cells

Akt activation by hypoxia was studied using $CoCl_2$, a hypoxia-mimicking agent. YD8 cells were treated with 0, 10, 50, 100, 200, or 300 μM of $CoCl_2$ for 3 h. The activation of Akt was assessed via immunoblotting using specific antibodies raised against threonine (Thr) 308 and serine (Ser) 473 of phospho-Akt. These antibodies were chosen because full activation of Akt requires the phosphorylation of both residues (Alessi *et al.*, 1996). Treatment with $CoCl_2$ resulted in an appreciable increase in detectable Thr308 and Ser473 phosphorylation without affecting total Akt expression in YD8 cells (Fig. 1). The increases of Akt phosphorylation at both residues were statistically significant ($P<0.01$). Next, to determine the effect $CoCl_2$ has on Akt activation over a time course, cells were treated with 300 μM of $CoCl_2$ for 1-24 h. As shown in figure 2, a significant increase in the level of phosphorylated Akt at Ser473 was observed 3 h after $CoCl_2$ treatment. This increase peaked at 6 h and returned to a basal Akt phosphorylation level at 24 h. However, phosphorylation at Thr308 was significantly higher in $CoCl_2$-treated cells for 24 h, though the expression of the protein was weakened. These observations reveal that the hypoxia-mimetic $CoCl_2$ increases Akt phosphorylation at both residues in a dose- and time-dependent manner and that Thr308 is more sensitive to phosphorylation than Ser473 in the presence of $CoCl_2$.

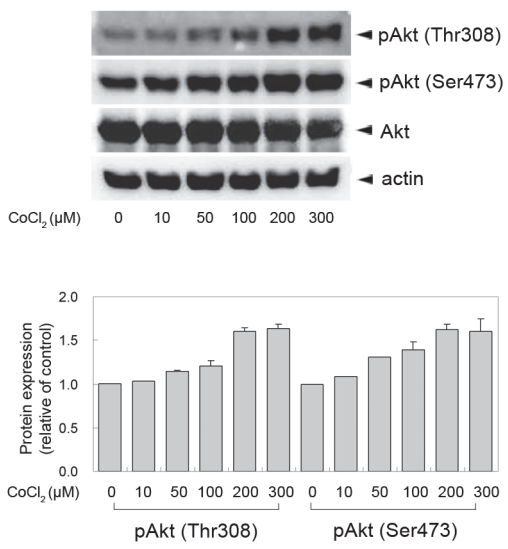

FIGURE 1. The effects of $CoCl_2$ treatment on Akt activation. $CoCl_2$ increases in a dose-dependent fashion. YD8 cells were treated with varying concentrations of $CoCl_2$ as indicated for 3 h, and the expression of phospho-Akt (Thr308) and phospho-Akt (Ser473) implying Akt activation were analyzed by western blot analysis. The density of the bands for phosphorylated Akt was quantitated by densitometric analysis and data are expressed as a ratio of the control, set at 1. The error bars indicate the standard deviation of the three separate experiments. The increase of phosphorylated Akt protein by $CoCl_2$ treatment was statistically significant (P<0.05; ANOVA analysis).

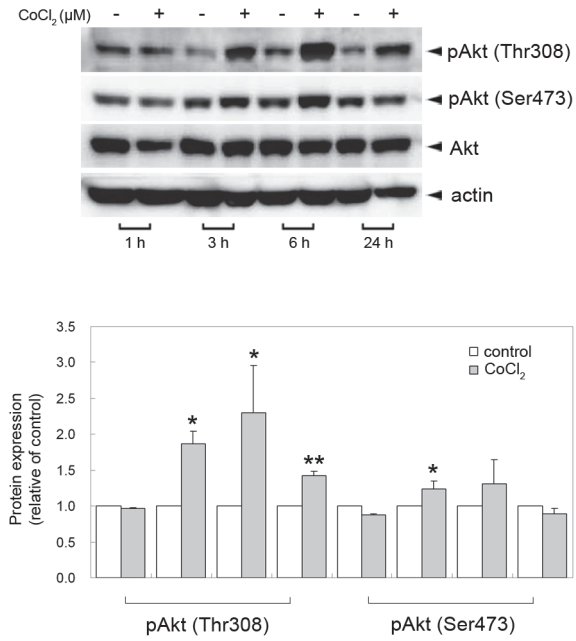

FIGURE 2. The effects of $CoCl_2$ treatment on Akt phosphorylation. YD8 cells were treated with 300 μM of $CoCl_2$ for the indicated times. Then, the expression of phospho-Akt (Thr308) and phospho-Akt (Ser473) was analyzed by western blot analysis. Quantitative data of densitometric analysis were presented in the histograms at the bottom of figures. The data are presented as a ratio of the control, set at 1. * and ** indicate a statistical significance of p <0.05 and P<0.01, respectively, as determined by paired Student *t*-test.

$CoCl_2$ activates Akt in a PI3K-dependent manner and through EGFR

We also attempted to investigate the molecular mechanism by which $CoCl_2$ induces Akt activation in oral squamous carcinoma cells. The level of Akt phosphorylation reflects PI3K activity, since Akt phosphorylation is invariably stimulated by active PI3K (Cantley, 2002; Toker and Cantley, 1997). To confirm the involvement of PI3K in $CoCl_2$-induced Akt phosphorylation, we pretreated the cells with 20 μM of LY294002, a PI3K inhibitor, for 1 h and then added 300 μM of $CoCl_2$ to the cells for 3 h. $CoCl_2$-stimulated Akt phosphorylation at Thr308 and Ser473 was significantly inhibited in oral squamous carcinoma cells in the presence of LY294002, suggesting that the phosphorylation of Akt is PI3K-dependent (Fig. 3).

Increased Akt phosphorylation by $CoCl_2$ in this study indicates that the hypoxic condition provides survival signals to cells, such as growth factors. To clarify the involvement of growth factor receptors and to define which growth factor signaling pathways contribute to the activation of the PI3K/Akt pathway by $CoCl_2$, we investigated how blocking individual RTKs affects Akt activation in cells exposed to $CoCl_2$. We used 1 μM PD168393, 2 nM picropodophyllin (PPP), and 200 nM DMPQ as specific inhibitors of EGFR, IGF1R, and PDGFR, respectively. Though picropodophyllin showed inhibitory activity of Akt phosphorylation, it had no inhibitory effect on Akt phosphorylation induced by $CoCl_2$. $CoCl_2$-induced Akt phosphorylation was suppressed only by PD168393, but not by picropodophyllin or DMPQ, implying that hypoxic signaling is mediated by EGFR (Fig. 4).

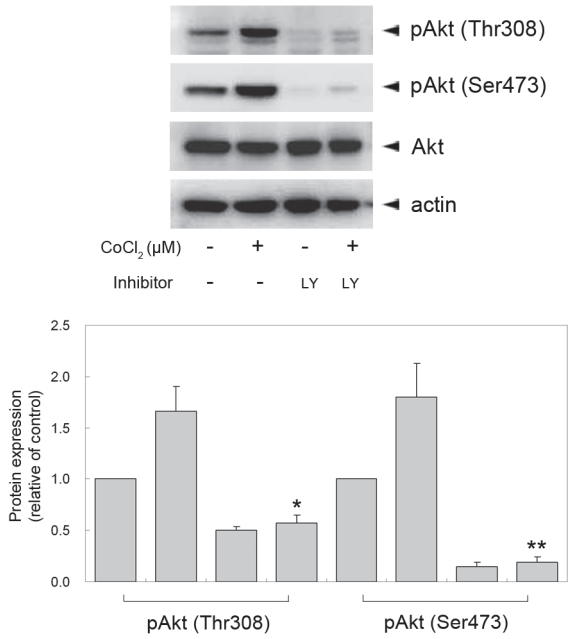

FIGURE 3. The effects of a PI3K inhibitor on the phosphorylation of Akt in cells exposed to $CoCl_2$. Cells were pretreated with the PI3K inhibitor for 1 h and then exposed to 300 μM of $CoCl_2$. Following 3 h incubation, lysates were subjected to western blot analysis for the detection of phosphorylated Akt proteins. The density of the bands was quantified by densitometric analysis as reported in the histograms at the bottom of the figure. Data are expressed as a ratio of the non-treated control, set at 1. The PI3K inhibitor (LY; 20 μM LY294002) significantly suppressed $CoCl_2$-induced Akt phosphorylation (*, p <0.01; **, P<0.05; paired Student *t*-test).

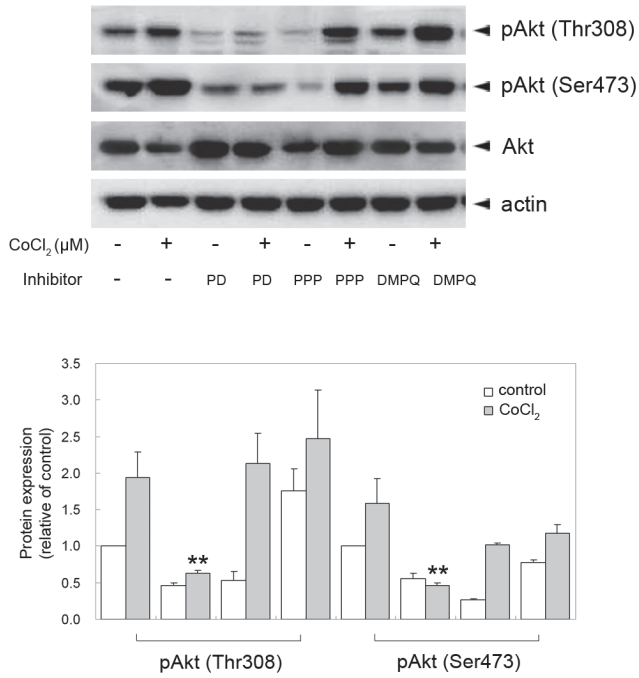

FIGURE 4. The effects of various kinase inhibitors on the phosphorylation of Akt in cells exposed to $CoCl_2$. Cells were treated with the kinase inhibitors for 1 h and then exposed to 300 μM of $CoCl_2$ for 3 h. The expression of phospho-Akt (Thr308) and phospho-Akt (Ser473) was analyzed by western blot analysis. The density of the bands was quantified by densitometric analysis. The effect of kinase inhibitors on Akt phosphorylation was expressed relative to that of untreated control cells, set at 1. In the histograms data are presented as mean ± standard deviation. PD, 1 μM PD168393 (EGFR inhibitor); PPP, 2 nM picropodophyllin (IGF1R inhibitor); DMPQ, 200 nM DMPQ (PDGFR inhibitor). The effects of inhibitors on $CoCl_2$-induced Akt phosphorylation was examined and analyzed (**, p <0.01; paired Student *t*-test).

$CoCl_2$ treatment results in the upregulation of EGFR and an increase in DNA synthesis

To investigate whether $CoCl_2$ directly affects EGFR itself, the level of EGFR expression was evaluated in YD8 cells in the presence of varying $CoCl_2$ concentrations. EGFR protein levels were increased by $CoCl_2$ in a dose-dependent manner. When cells were treated with 300 μM of $CoCl_2$ for 24 h, the increase in EGFR expression was comparably prominent (Fig. 5). In addition, the effect of $CoCl_2$ on the cell cycle was examined using a flow cytometer. $CoCl_2$ increased the percentage of the cell population in the S-phase (45.6%) compared to control-treated cells (21.9%) (Fig. 6).

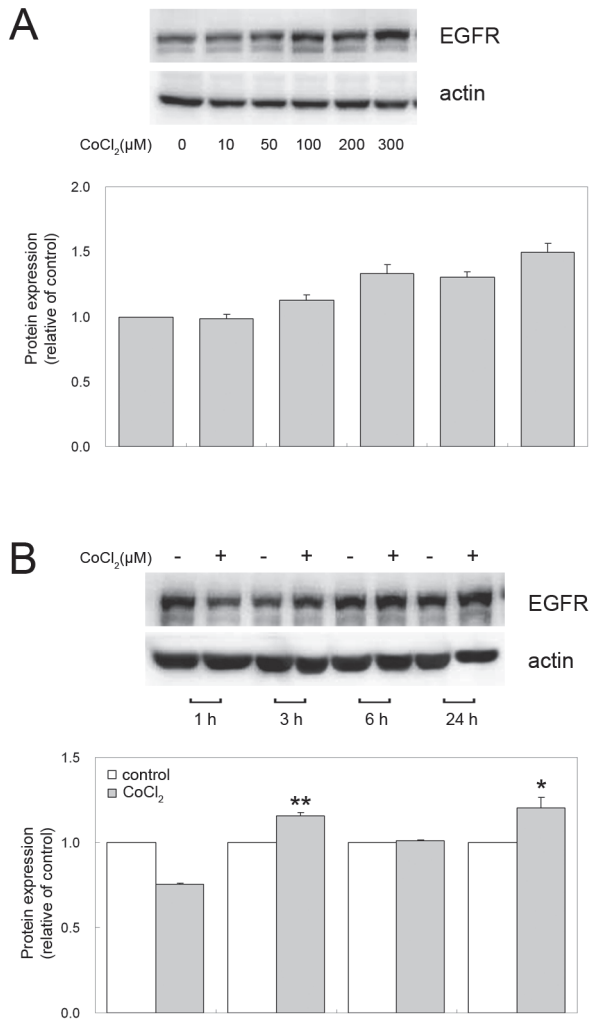

FIGURE 5. The effect of $CoCl_2$ treatment on EGFR expression. $CoCl_2$ treatment increases the expression of EGFR protein in a dose- and time-dependent manner. (A) YD8 cells were treated with varying concentrations of $CoCl_2$ as indicated. After 3 h, the cells were analyzed for EGFR. The expression of EGFR protein was significantly increased by $CoCl_2$ treatment ($P< 0.05$; ANOVA analysis). (B) YD8 cells were treated with 300 μM of $CoCl_2$ for 1-24 h. Cells were harvested and analyzed for Akt phosphorylation. The density of bands was quantitated and EGFR levels were expressed relative to that of untreated control cells, set at 1 (*, $p < 0.01$; **, $P<0.05$; paired Student *t*-test).

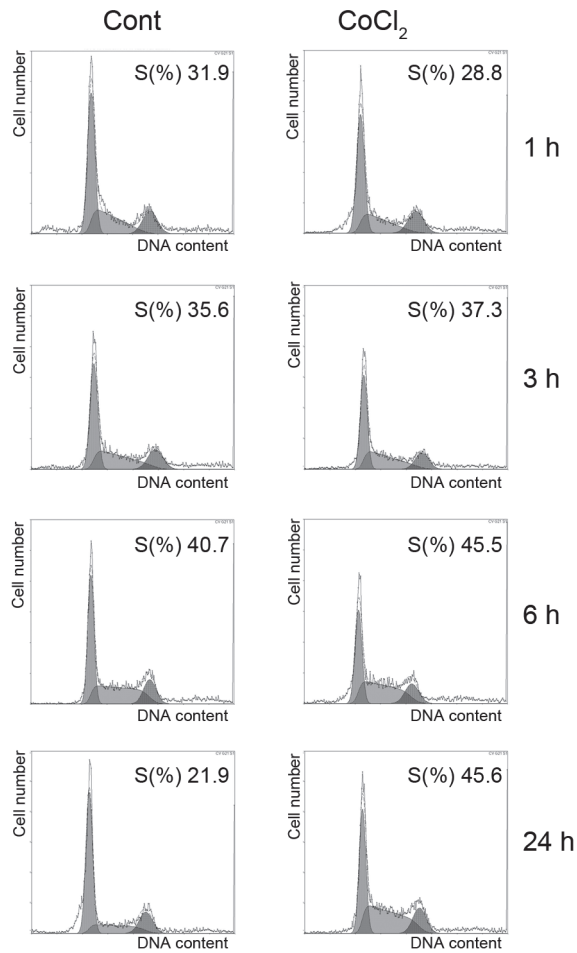

FIGURE 6. Signaling by the hypoxia-mimetic $CoCl_2$ results in S-phase entry. Cells were incubated with 300 μM of $CoCl_2$. After 1 h, 3 h, 6 h, and 24 h, cells were harvested, fixed in ethanol, and stained with propidium iodide. The cell cycle was analyzed by flow cytometry. The number of S (%) depicts the percentage of S-phase fraction of control or $CoCl_2$-treated cells.

Discussion

Activation of the PI3K/Akt pathway drives tumors towards a more malignant phenotype, which can survive under harsh conditions (Nicholson and Anderson, 2002). A study by Izuishi *et al*. (2000) investigated the role of Akt in cell tolerance to nutrient deprivation, and found that high cellular Akt expression was associated with a higher tolerance of liver and pancreatic cancer

cell lines to nutrient deprivation. The present study shows that $CoCl_2$, a well-known hypoxia-mimetic agent, activates Akt in oral squamous carcinoma cells, which concurs with previous reports using Hela and PC12 cells (Alvarez-Tejado *et al.*, 2001; Jin *et al.*, 2007). In contrast to our data and the data observed in Hela and PC12 cells, numerous studies have reported that hypoxia or cobalt ions have no effect on Akt phosphorylation and even inhibit Akt phosphorylation in certain cells, including smooth muscle cells, gastric carcinoma cells, and COS cells (Ardyanto *et al.*, 2006; Humar *et al.*, 2002; Loberg *et al.*, 2002). Finally, it has been proposed that the activation of Akt during hypoxia may be cell-type dependent and not a general effect of hypoxia (Alvarez-Tejado *et al.*, 2001). However, the differences in Akt activation by hypoxia or $CoCl_2$ cannot be explained solely by the cell type because Akt phosphorylation results differed in previous reports using the same cell line. For example, Jin *et al*. reported that Akt was activated in Hela cells in response to $CoCl_2$ exposure, whereas Barthel *et al*. observed that $CoCl_2$ had no effect on Akt activation in these cells, though other metal ions, such as copper and zinc, significantly induced Akt phosphorylation (Barthel *et al.*, 2007; Jin *et al.*, 2007). The main experimental difference between these two studies was the $CoCl_2$ incubation time. Barthel *et al*. (2007) investigated Akt phosphorylation after only a 30 min exposure to $CoCl_2$, while Jin *et al*. (2007) observed Akt activation after 6 h of $CoCl_2$ treatment, similar to the present study. In a report using PC12 cells, Alvarez-Tejado *et al*. (2001) suggested that Akt is activated several hours after exposure to a hypoxic environment, whereas growth factors stimulate Akt within minutes (Alvarez-Tejado *et al.*, 2001). The authors concluded that Akt activation by hypoxia or the hypoxia-mimetic $CoCl_2$ may depend not only on the cell type, but also on the exposure time to these conditions. Furthermore, the hypoxic condition requires several hours to induce its effects on the Akt pathway, though short-period hypoxia clearly activates Akt.

Although IGF1R and PDGFR are well-known PI3K/Akt stimulators, the presence of inhibitors specifically targeting IGF1R or PDGFR does not block $CoCl_2$–induced Akt activation in oral squamous carcinoma cells. The results of the present study showed that $CoCl_2$ induces the activation of PI3K/Akt and that this occurs through an EGFR-related pathway, but unlikely through IGF1R or PDGFR. In addition to the inhibition of $CoCl_2$-induced Akt activation by the EGFR inhibitor, the upregulation of EGFR by $CoCl_2$ was also observed in this study. A recent study using oligonucleotide array analyses showed that hypoxia induced EGFR as well as other well-known hypoxia-induced genes, such as VEGF. Using immunohistochemistry, this study observed the induction of EGFR in tumor cells in the vicinity of necrotic areas, which is a histological indicator of tumor hypoxia (Wang *et al.*, 2007). Also, it has been suggested that the hypoxic microenvironment induces the overexpression of EGFR via HIF-2α activation (Franovic *et al.*, 2007). Taken together, these results suggest that oral squamous carcinoma cells use the EGFR signaling pathway to adapt and survive in a hypoxic environment.

It has been reported that Akt plays an important role in the transition from the G1 phase to the DNA synthesis phase of the cell cycle. Lee *et al*. (2008a) have suggested that short periods of hypoxia increase the percentage of the cell population in the S phase by inducing cell cycle regulatory proteins through the PI3K/Akt/mTOR pathways in mouse embryonic cells. It was also previously reported that a short exposure time to hypoxia increases DNA synthesis in primary cultured chicken hepatocytes (Lee *et al.*, 2008b). While currently no such results have been reported for human cells, it seems that exposure to hypoxia increases DNA synthesis in oral squamous carcinoma cells. This transition to the S phase may allow cancer cells to survive and become resistant to radiotherapy and/or chemotherapy.

In conclusion, this study demonstrates that $CoCl_2$–induced hypoxia in oral squamous carcinoma cells increases Akt activation through EGFR signaling, causing the cells to enter S phase. We propose that oral squamous carcinoma cells are resistant to hypoxia and that under this condition, EGFR expression is induced, resulting in the activation of Akt. This Akt induction may promote cell cycle progression and be an important factor in resistance to chemotherapy and/or radiotherapy in hypoxic oral squamous carcinoma cells.

Acknowledgments

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (2009-0075867).

References

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal* **15**: 6541-6551.

Alvarez-Tejado M, Naranjo-Suarez S, Jimenez C, Carrera AC, Landazuri MO, del Peso L (2001). Hypoxia induces the activation of the phosphatidylinositol 3-kinase/Akt cell survival pathway in PC12 cells: protective role in apoptosis. *The Journal of Biological Chemistry* **276**: 22368-22374.

Ardyanto TD, Osaki M, Tokuyasu N, Nagahama Y, Ito H (2006). CoCl2-induced HIF-1alpha expression correlates with proliferation and apoptosis in MKN-1 cells: a possible role for the PI3K/Akt pathway. *International Jjournal of Oncology* **29**: 549-555.

Barthel A, Ostrakhovitch EA, Walter PL, Kampkotter A, Klotz LO (2007). Stimulation of phosphoinositide 3-kinase/Akt signaling by copper and zinc ions: mechanisms and consequences. *Archives of Biochemistry and Biophysics* **463**: 175-182.

Blume-Jensen P and Hunter T (2001). Oncogenic kinase signalling. *Nature* **411**: 355-365.

Cantley LC (2002). The phosphoinositide 3-kinase pathway. *Science (New York, N.Y.)* **296**: 1655-1657.

Chen EY, Mazure NM, Cooper JA, Giaccia AJ (2001). Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. *Cancer Research* **61**: 2429-2433.

Coffer PJ, Burgering BM, Peppelenbosch MP, Bos JL, Kruijer W (1995). UV activation of receptor tyrosine kinase activity. *Oncogene* **11**: 561-569.

Franke TF, Kaplan DR, Cantley LC, Toker A (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science (New York, N.Y.)* **275**: 665-668.

Franovic A, Gunaratnam L, Smith K, Robert I, Patten D, Lee S (2007). Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 13092-13097.

Hockel M, Vaupel P (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *Journal of the National Cancer Institute* **93**: 266-276.

Humar R, Kiefer FN, Berns H, Resink TJ, Battegay EJ (2002). Hypoxia enhances vascular cell proliferation and angiogenesis *in vitro* via rapamycin (mTOR)-dependent signaling. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **16**: 771-780.

Izuishi K, Kato K, Ogura T, Kinoshita T, Esumi H (2000). Remarkable Tolerance of Tumor Cells to Nutrient Deprivation: Possible New Biochemical Target for Cancer Therapy. *Cancer Research* **60**: 6201-6207.

Jin HO, An S, Lee HC, Woo SH, Seo SK, Choe TB, Yoo DH, Lee SB, Um HD, Lee SJ, Park MJ, Kim JI, Hong SI, Rhee CH, Park IC (2007). Hypoxic condition- and high cell density-induced expression of Redd1 is regulated by activation of hypoxia-inducible factor-1alpha and Sp1 through the phosphatidylinositol 3-kinase/Akt signaling pathway. *Cellular Signalling* **19**: 1393-1403.

Lee SH, Lee MY, Han HJ (2008a). Short-period hypoxia increases mouse embryonic stem cell proliferation through cooperation of arachidonic acid and PI3K/Akt signalling pathways. *Cell Proliferation* **41**: 230-247.

Lee SH, Lee MY, Lee JH, Han HJ (2008b). A potential mechanism for short time exposure to hypoxia-induced DNA synthesis in primary cultured chicken hepatocytes: Correlation between Ca(2+)/PKC/MAPKs and PI3K/Akt/mTOR. *Journal of Cellular Biochemistry* **104**: 1598-1611.

Lin RZ, Hu ZW, Chin JH, Hoffman BB (1997). Heat shock activates c-Src tyrosine kinases and phosphatidylinositol 3-kinase in NIH3T3 fibroblasts. *The Journal of Biological Chemistry* **272**: 31196-31202.

Loberg RD, Vesely E, Brosius FC (2002). Enhanced glycogen synthase kinase-3beta activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism. *The Journal of Biological Chemistry* **277**: 41667-41673.

Nicholson KM, Anderson NG (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cellular Signalling* **14**: 381-395.

Semenza GL (2002). HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends in Molecular Medicine* **8**: S62-7.

Toker A, Cantley LC (1997). Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387**: 673-676.

Wang T, Niki T, Goto A, Ota S, Morikawa T, Nakamura Y, Ohara E, Ishikawa S, Aburatani H, Nakajima J, Fukayama M (2007). Hypoxia increases the motility of lung adenocarcinoma cell line A549 via activation of the epidermal growth factor receptor pathway. *Cancer Science* **98**: 506-511.