$CoCl_2$-induced HIF-1α expression correlates with proliferation and apoptosis in MKN-1 cells: A possible role for the PI3K/Akt pathway

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Abstract. The exact mechanism behind the effect of hypoxia-inducible factor-1α (HIF-1α) on the proliferation and/or apoptosis of carcinoma cells is still a matter of debate. We treated a human gastric carcinoma cell line, MKN-1 (mutant P53), with 500 μM $CoCl_2$. A dual-phase pattern of HIF-1α expression with an increase until 4 h followed by a decrease until 36 h was observed. Immunocytochemistry showed that nuclear translocation was maximal at 4 h of treatment, while trypan blue staining showed a dual-phase pattern. Instead of G_1/S arrest, FACS showed an increase in the pre-G_1 fraction and G_2/M arrest that correlated with Cyclin-B1, SKP-2 and P27 expression. Starting at 6 h, the apoptotic index increased in a time-dependent manner, in correlation with the expression of HIF-1α, Bcl-2, Bcl-xL, Bax and cleaved-Caspase-9. Phosphorylation of Akt was inhibited by $CoCl_2$ treatment and LY294002 treatment inhibited HIF-1α expression in a dose-dependent manner. These results suggested that the alteration of $CoCl_2$-induced HIF-1α expression correlated with proliferation and apoptosis in MKN-1 cells. A possible role for the PI3K/Akt pathway was indicated in this model of hypoxia.

Introduction

Hypoxia-inducible factor-1 (HIF-1), a transcription factor, is critical to cell survival under hypoxic conditions. HIF-1 is composed of the O_2- and growth factor-regulated HIF-1α subunit, and the constitutively expressed HIF-1ß subunit (aryl hydrocarbon receptor nuclear translocator, ARNT), both of which belong to the basic-helix-loop-helix (bHLH)-PAS (PER, ARNT, SIM) protein family (1,2).

Under normoxic conditions, HIF-1α is rapidly degraded through ubiquitinylation by pVHL and proteosomes. This is triggered through post-translational hydroxylation at specific proline residues (P402, P564 and N803 in human HIF-1α protein) within the oxygen-dependent degradation domain (ODDD) (3,4) by a specific HIF-prolyl hydroxylase (HPH_{1-3} also referred to as PHD_{1-3}). In turn, in the presence of iron, oxygen and 2-oxoglutarate, the hydroxylated HIF-1α protein is recognized and bound by pVHL, which functions as an E3 ubiquitin ligase (5,6). However, under hypoxic conditions, HIF-1α evades hydroxylation and is translocated to the nucleus where it heterodimerizes with HIF-1α. The heterodimerized HIF-1α binds to HRE (hypoxia response element) and drives the transcription of over 60 target genes important for adaptation and survival in a hypoxic environment. These genes include those for glycolytic enzymes, the glucose transporters Glut-1 and Glut-3, endothelin-1 (ET-1), VEGF (vascular endothelial growth factor), tyrosine hydroxylase, transferrin and erythropoietin (7,8) which mainly play roles in metabolism, angiogenesis and red blood cell production. Furthermore, activation of HIF-1α is closely associated with cancer cell growth and survival, tumor development, tumor angiogenesis, and poor clinical prognosis (9).

Some metals are known as hypoxic mimicking agents including cobalt chloride, nickel chloride and desferrioxamine (10). Epstein *et al* (3) reported that specific prolyl hydroxylases have an iron-binding centre and that iron is critical for their enzymatic activities. It is proposed that iron chelators can remove iron from the iron-binding center of the enzyme and that the iron can be replaced with cobalt at this site, which will inactivate the hydroxylation activity. Recently it was reported that $CoCl_2$ treatment induced HIF-1α expression by binding to the PAS domain resulting in blockage of HIF-1α-pVHL binding and thereby HIF-1α stability (11,12).

HIF-1α expression was detected immunohistochemically in >70% of specimens of human cancer and their metastases

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compared to the adjacent normal regions, including breast, oro-pharyngeal, nasopharyngeal, prostate, brain, lung, stomach, head and neck cancer (13). Its expression is suggested to promote an aggressive tumor phenotype by mediating angiogenesis, oxygen transport, glycolysis, glucose uptake, growth factor signalling, invasion and metastasis (2).

HIF-1α expression was reported to correlate with apoptotic and pro-apoptotic factors but not with the cell proliferation in lung cancer (14). Also, hypoxia and $CoCl_2$ treatment were reported to protect $HepG_2$ cells from apoptosis induced by serum deprivation and *tert*-butyl hydroperoxide (t-BHP) (15,16). Despite some reports on an apoptotic effect of HIF-1α on several cell lines (17-19), contradictory information on its role and mechanism of effect in the regulation of cell proliferation and apoptosis has been put forward in recent years (20-23).

Previously, Zhong *et al* detected HIF-1α expression immunohistochemically in some common human cancer including gastric carcinoma (24). Western blot analysis revealed the HIF-1α expression to occur in a radical oxygen species-dependent manner (25) in gastric cancer cells, and to be induced by Cysteamine (26) or by non-steroidal anti-inflammatory drugs (27) in the gastric mucosa. Studies are still necessary to elucidate precisely how the expression of HIF-1α is regulated and the role of this subunit in human gastric carcinoma.

Many of the components of the PI3K/Akt pathway have been reported to be involved in the regulation of HIF-1α. However, the exact role of PI3K/Akt signalling in the activation of HIF-1 is still a matter of debate (28). Mottet *et al* suggested that a PI3K/Akt-glycogen synthase kinase-3ß pathway regulated HIF-1α under hypoxic conditions (29), while another report argued that PI3K/Akt signalling is required for neither the hypoxic nor normoxic stabilization of HIF-1α (30). Alvarez-Tejado *et al* reported that the activation of Akt during hypoxia might depend on cell type (31).

In the present study, we examined the expression of hypoxia inducible factor-1α induced by $CoCl_2$ dependent on the PI3K/Akt pathway and elucidated its correlation with the cell proliferation and apoptosis in a human gastric carcinoma cell line, MKN-1.

Materials and methods

Cell lines and cell cultures. A human gastric carcinoma cell line, MKN-1, was cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml L-glutamine at 37ºC in 5% CO_2.

Hypoxic treatment. For the $CoCl_2$ (Wako, Osaka, Japan) experiments, $1x10^6$ cells of MKN-1 were seeded in 10-cm diameter dishes 24 h before being treated with 0, 100, 240, 400 and 500 μM $CoCl_2$ for 6 h. For the other experiments, MKN-1 cells were treated with or without 500 μM $CoCl_2$ and 25, 50 and 100 μM LY294002 (Cayman Chemical, Ann Arbor, MI) under 20% O_2 at 37˚C for 0, 2, 4, 6, 8, 10, 12, 24 and 36 h.

Immunocytochemistry. Using Tissue Culture Chamber/slides (Miles Laboratories Inc., Naperville, IL), $1x10^4$ cells were seeded 24 h before the experiments. After 0, 4 and 36 h of treatment with 500 μM $CoCl_2$, the cells were washed with PBS and fixed in 4% PFA (paraformaldehyde) at 4˚C for 20 min. After microwave-based antigent retrieval with citrate-buffer, the blocking of endogenous peroxide with H_2O_2, and a rinse with PBS, fixed-cells were labelled with blocking-antibody followed by HIF-1α antibody overnight. Immunoreactions were then visualized with diaminobenzidines and counterstaining with hematoxylin. The percentage of positive immunoreactions was calculated per 200 cells as the average of at least five microscopic-fields.

SDS-PAGE and Western blot analysis. The cells were washed in PBS and solubilized in lysis buffer (50 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.1% NP-40, 5 mM NaF, 1 mM PMSF, 1 ng/mg leupeptin, 10 ng/ml soybean trypsin inhibitor, 1 ng/ml aprotinin, and 10 ng/ml N-tosyl-L-phenylalanyl chloromethyl ketone) for 60 min on ice. Lysates were centrifuged at 2,500 g for 5 min. The protein concentration was determined using the Bradford protein assay (Bio-Rad Lab., Richmond, CA) with bovine serum albumin (BSA; Wako, Osaka, Japan) as the standard. Fifty micrograms of protein was resolved by electrophoresis on 12% or 15% polyacrylamide gels and electro-transferred to a polyvinylidene difluoride filter (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in PBS(-) containing 0.01% Tween-20 (T-PBS), the membrane was blotted with anti-HIF-1α monoclonal antibody (1:500, NB-100-131, Novus Biologicals, Littleton, CO), anti-Akt polyclonal antibody (1:1000, Cell Signalling Tech. Beverly, MA), anti-phospho-Akt Ser 473 polyclonal antibody (1:1000, Cell Signalling Tech.), anti-P27/Kip1 monoclonal antibody (1:500, EA10; Oncogene, Cambridge, MA), anti-SKP2 monoclonal antibody (1:500, Zymed Laboratories Inc., San Francisco, CA), anti-Cyclin-B1 monoclonal antibody (1:500, Santa Cruz Biotechnology), anti Bcl-2 monoclonal antibody (1:500, Dako A/S Glostrup, Denmark), anti-Bcl-xL polyclonal antibody (1:500, Cell Signaling Tech.), anti-Bax monoclonal antibody (1:500, Clone 4F11, MBL Co. Ltd., Nagoya Japan), anti-Caspase-9 polyclonal antibody (1:500, Santa Cruz Biotechnology), anti-cleaved-Caspase-9 polyclonal antibody (1:500, Cell Signalling Tec.) and anti-ß-actin monoclonal antibody (1:500, AC15; Sigma, St. Louis, MO). The blots were washed in T-PBS 3 times for 5 min each time. Then they were developed with peroxidase-labeled anti-mouse IgG or anti-rabbit IgG (1:1000, MBL Co., Ltd.) for 30 min at room temperature. After a wash in T-PBS, signals were detected with an enhanced chemiluminescence system (ECL Western blotting analysis system, Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cell viability and detection of apoptotic cells. After specific periods of time, the MKN-1 cells were washed in PBS. Then the cell viability was determined by trypan blue staining and the results were normalized to those for untreated cells. Apoptotic cells were assessed morphologically by Hoechst 33258 staining after fixing with Clarke's fixative (ethanol:acetic acid = 3:1) using a ultraviolet laser microscope (Optiphoto-2, Nikon,

Tokyo, Japan). The apoptotic index (AI, %) was defined as follows: AI = 100 x (apoptotic cells/400 cells). The experiments were performed in triplicate.

FACS analysis. The cell cycle distribution and pre-G_1 (apoptosis peak) fraction were determined by FACS analysis of DNA content (Becton Dickinson, San Jose, CA) after 36 h of treatment. Cell suspensions were prepared by trypsinization and $1x10^6$ cells/ml were washed twice with PBS(-). The cells were fixed overnight in 10 ml of 70% ethanol at 40˚C and incubated with RNase at a concentration of 0.25 mg/ml at 37˚C for 1 h. Next, the cells were treated with propidium iodide (50 μg/ml in PBS) and incubated for 30 min at 4˚C in the dark. Before cell injection, the samples were passed through a 25-gauge needle to prevent cell clumping. DNA histograms were analyzed using lysis-II software (Becton Dickinson) to evaluate the cell cycle components.

Results

$CoCl_2$-induced HIF-1α expression. The LD_{50} of $CoCl_2$ was reported to be 1.1 mM (32). $CoCl_2$ treatment for 6 h induced the expression of HIF-1α in MKN-1 cells in a dose-dependent manner until 500 μM (Fig. 1A). The lower concentrations induced a relatively similar level of expression of HIF-1α but with a longer exposure (data not shown). The treatment duration was deduced from several pre-experiments (data not shown) to maximize the effect of HIF-1α on the gastric carcinoma cell line and minimize the possible confounding effects of $CoCl_2$ itself on the cell culture.

A higher cell density was suggested to induce higher expression of HIF-1α. Unfortunately, a higher density would hypothetically induce micro-environmental hypoxia which might confuse the results especially in the untreated cells after long periods when the density will be relatively high (33).

Treatment with 500 μM $CoCl_2$ induced HIF-1α expression in MKN-1 cells in a time-dependent manner. The pattern of expression had two phases, a sharp increase until 4 h of treatment followed by a gradual tapering until 36 h (Fig. 1B).

Immunocytochemistry. Based on the protein expression of HIF-1α, immunocytochemistry was performed at 0, 4 and 36 h of treatment (Fig. 2A). Cytoplasmic immunoreactivity was detected at all time-points in the untreated cells, with a relatively weak positivity (5.7%, 6.3% and 9.3%, respectively). On the other hand, significant nuclear immunoreactions were found in the treated cells after 4 h and in a relatively low number after 36 h (76.6% and 10.3%, respectively). This pattern correlated with the HIF-1α protein expression and viability of $CoCl_2$-treated MKN-1 cells (Fig. 2B). Some treated cells also showed cytoplasmic immunoreactions without a nuclear immunoreaction after 4 and 36 h of treament (15.6% and 12.9%, respectively).

Cell viability, FACS analysis and cell cycle-related protein expression. Accordingly, the viability index of the treated cells also showed a sharp increase until 4 h (128.1%) followed by a gradual tapering until 36 h (14.9%) (Fig. 2B). It is reasonable to assume that a correlation exist between the cell viability index and HIF-1α expression.

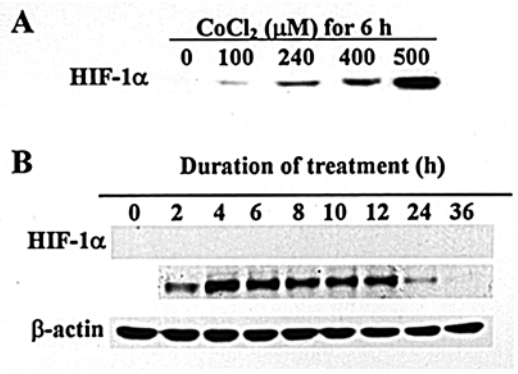

Figure 1. Expression of HIF-1α in MKN-1. A, HIF-1α expression under increasing doses of $CoCl_2$ for 6 h in MKN-1 cells. B, After incubation with 500 μM $CoCl_2$, HIF-1α expression shows a dual-phase pattern in a time-dependent manner, peaking after 4 h and then decreasing until 36 h. (Upper bar, untreated cells; lower bar, treated cells).

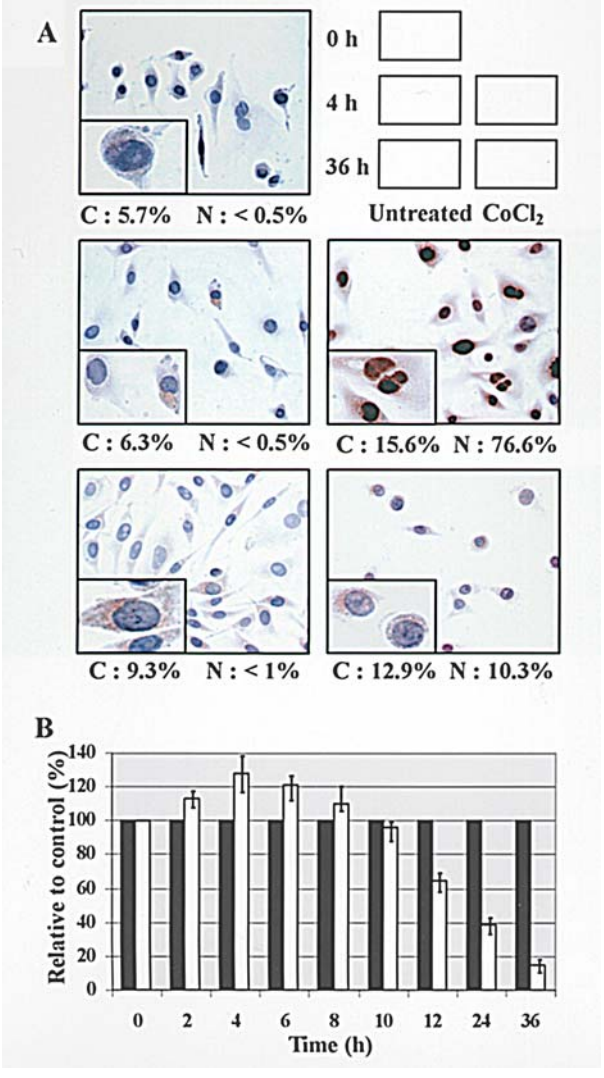

Figure 2. Immunocytochemistry and cell viability index. A, Immunocytochemistry shows cytoplasmic immunoreactions in the untreated cells at all the indicated time-points with relatively weak positivity. Significant immunoreactivity is found in the treated cells, especially after 4 h. [C, percentage (%) of cytoplasmic immunoreactions; N, percentage (%) of nuclear immunoreactions]. B, Cell viability index shows an increase until 4 h, followed by a decrease until 36 h. The graph shows averages from triplicate data of three separate experiments. (■, untreated cells; □, treated cells).

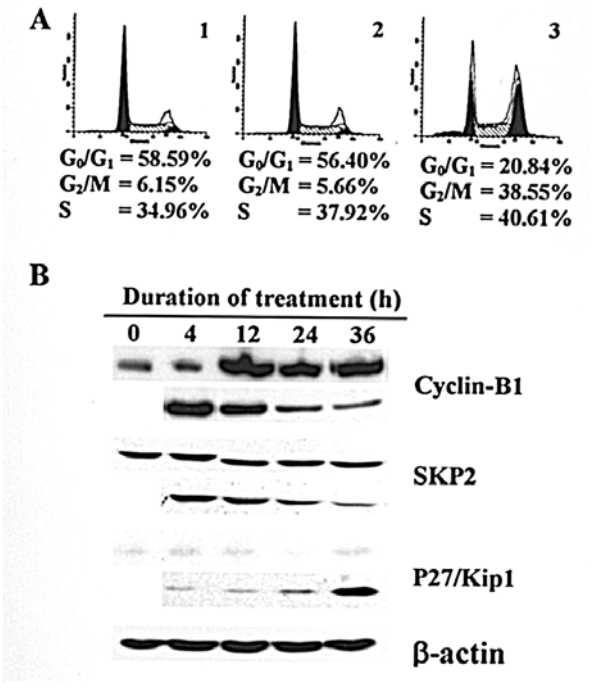

Figure 3. FACS and expression of cell cycle-related proteins. A, After 36 h of treatment, FACS shows an increase in the pre-G_1 (apoptotic fraction) and marked increase in the G_2/M (cycle arrest) fraction (1, untreated cells at 0 h; 2, untreated cells after 36 h; 3, treated cells after 36 h). B, Western blot shows that HIF-1α expression correlates with Cyclin-B1, SKP-2, and P27/Kip1 expression, under $CoCl_2$ treatment (for each, upper bar, untreated cells; lower bar, treated cells).

After 36 h, FACS analysis showed an increase in the pre-G_1 fraction and a marked increase in the G_2/M fraction in the $CoCl_2$-treated cells while no fraction showed a significant increase in the untreated cells (Fig. 3A). These results suggested that the decrease in expression of HIF-1α was correlated with G_2/M arrest and the induction of apoptosis in the $CoCl_2$-treated cells.

As shown in Fig. 3B, Western blot analysis revealed that the expression of Cyclin-B1 and SKP-2 protein expression had a similar pattern to that of HIF-1α. On the other hand, P27/Kip1 expression increased significantly after 36 h in the treated cells, which showed an inverse correlation with the alteration of HIF-1α expression. The expression of these proteins was also considered to correlate with the results of FACS analysis after 36 h of treatment.

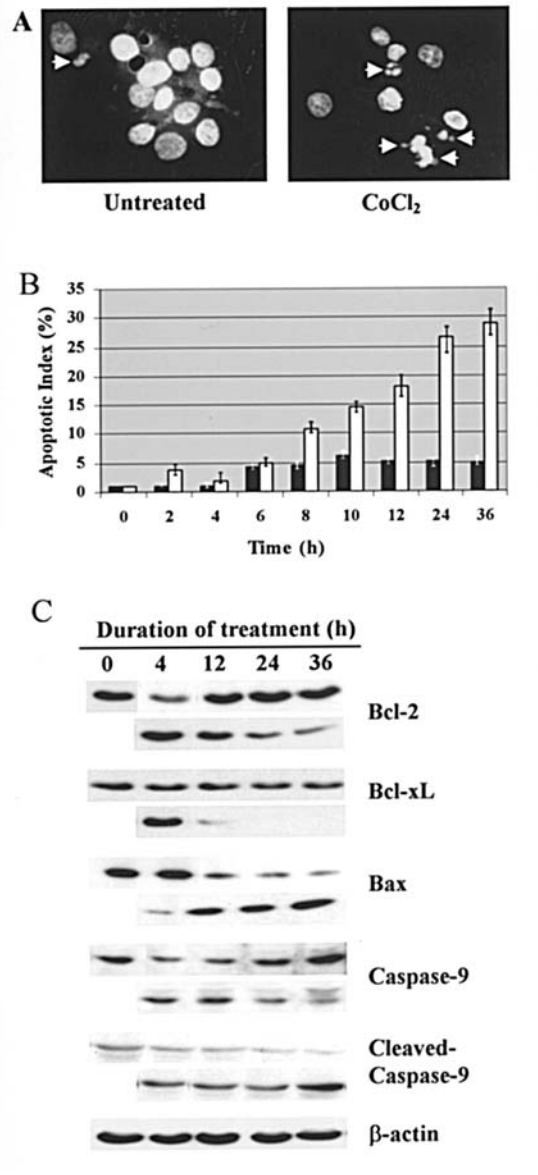

Figure 4. Correlation between HIF-1α expression and apoptosis. A, Hoechst staining shows the apoptotic cells (arrows) after 36 h in the $CoCl_2$-treated and untreated cells. B, Apoptotic index shows the correlation with HIF-1α expression; the increase starting after 6 h of treatment and coinciding with the decrease in HIF-1α expression. The graph shows averages from triplicate data of three separate experiments. (■, untreated cells; □, treated cells). C, Western blot analysis shows that the expression of HIF-1α correlates with that of Bcl-2 and Bcl-xL (anti-apoptotic) as well as Bax and cleaved-Caspase-9 (pro-apoptotic) (for each, upper bar, untreated cells; lower bar, treated cells).

Apoptotic index and expression of apoptosis-related proteins. The Hoechst 33258 staining revealed a significant number of apoptotic cells among the $CoCl_2$-treated cells at 36 h compared the untreated cells (Fig. 4A). The apoptotic index also reflected this increase, which started at 6 h and was time-dependent until 36 h, at which point values were 29.0% (treated-cells) vs. 5.0% (untreated cells) (Fig. 4B). This pattern was relevant to that of the cell viability index. It is reasonable to assume that the increase in the apoptotic index correlated with the decrease phase of HIF-1α expression.

Western blot analysis showed the expression of the anti-apoptotic Bcl-2 and Bcl-xL, in a pattern similar to that of HIF-1α, while the pro-apoptotic Bax and cleaved-Caspase-9 showed an increase in a time-dependent manner, coinciding with the decrease in HIF-1α expression (Fig. 4C). Overall, the patterns of Bcl-2, Bcl-xL, Bax and cleaved-Caspase-9 expression suggested a possible role for apoptosis in the correlation of HIF-1α expression with MKN-1 cell proliferation.

Expression of HIF-1α and phosphorylation of Akt. The expression of phosphorylated-Akt was inhibited after 36 h of $CoCl_2$ treatment (Fig. 5A). Furthermore, the pattern of expression of pAkt Ser473 expression correlated with that of cell-cycle related proteins (Cyclin-B1, SKP-2 and P27/Kip1 in Fig. 3B) and apoptosis-related proteins (Bcl-2, Bcl-xL,

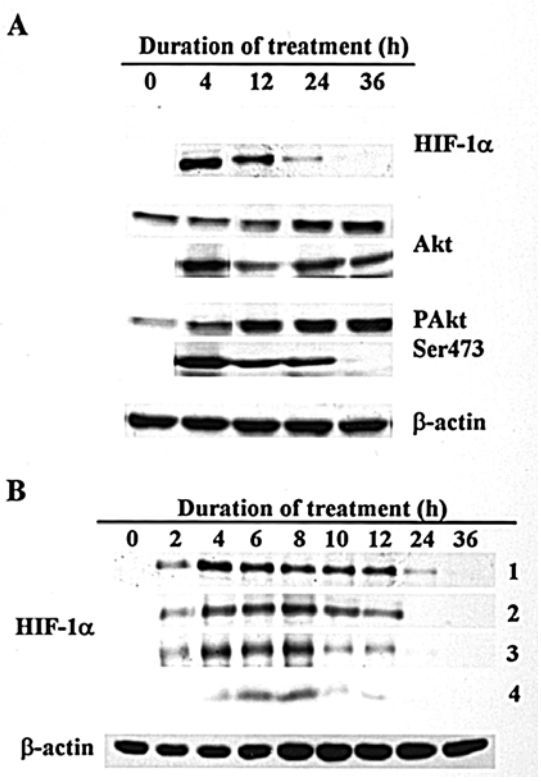

Figure 5. $CoCl_2$-induced HIF-1α expression and Akt phosphorylation. A, $CoCl_2$-treatment inhibits Akt phosphorylation after 36 h (for each, upper bar, untreated cells; lower bar, treated cells). B, Addition of LY294002 inhibits HIF-1α expression in a dose-dependent manner (treatment 1, 500 μM $CoCl_2$; co-treatment with LY294002 2, $CoCl_2$ + 25 μM; 3, $CoCl_2$ + 50 μM; 4, $CoCl_2$ + 100 μM).

Bax and cleaved-Caspase-9 in Fig. 4C). On the other hand, addition of LY294002, an Akt phosphorylation inhibitor, inhibited the expression HIF-1α in a dose-dependent manner (Fig. 5B).

Discussion

The present study demonstrated $CoCl_2$-induced HIF-1α expression by Western blot analysis as well as immunocytochemistry in a human gastric carcinoma cell line, MKN-1; the expression occuring in two phases in a time-dependent manner. The decrease that followed the initial phase of increase suggested a negative feed-back mechanism on prolonged exposure to hypoxia (34,35).

The immunocytochemistry suggested that the HIF-1α protein was constitutively expressed in the cytoplasm but at a level undetectable by Western blotting, pointing to a short half-life. The significant nuclear immunoreaction in the treated cells at 4 h was similar to the peak expression in the Western blot analysis, suggesting the translocation of HIF-1α to the nucleus before its sequential activities being initiated thereafter. After 36 h of treatment, relatively weak nuclear immunoreactions were still found even though the protein expression was undetectable by Western blotting.

The increase in cell viability, in correlation with HIF-1α expression, was suggested to be induced by an upregulation of the transcription of some target genes that support the cell growth (7-9), and was also attributed to resistance to apoptosis through a down-regulation of Bid and Bax expression (21). The present study showed that the down-regulation of Bax expression coincided with the increase of HIF-1α expression up until 4 h of treatment.

The precise role of HIF-1α in cell cycle arrest during hypoxia is still unclear, that is, whether it is essential for the up-regulation of P27/Kip1 and P21/WAF1 expression (36), whether the up-regulation is independent on HIF-1α (37), or whether the G_1/S arrest in response to severe hypoxia has a cell type-specific pattern (38). Also, P21/Waf1 and P27/Kip1 did not affect hypoxic growth arrest in immortalized cells although they were up-regulated in response to hypoxia (39). Interestingly, the FACS analysis in the present study showed an increase in the apoptotic fraction and marked G_2/M arrest instead of G_1/S arrest. The expression of Cyclin-B1, SKP2 and P27/Kip1 protein also altered in parallel, resulting in a decrease in the cell viability index. Previously, on exposure to hypoxia, HCT116 cells (a human colorectal carcinoma cell line) were reported to exhibit a transient arrest in G_2/M, but escape from this checkpoint and enter a long-term G_0/G_1 arrest (40). There is also a possibility that the hypoxia-mimicking conditions of $CoCl_2$-treatment in the present study were not strong enough and/or applied long enough to induce cells to enter long-term G_0/G_1 arrest compared to real hypoxia (41).

Recently, Stoeltzing *et al* reported that the injection of TMK-1 cells transfected with a dominant-negative mutant HIF-1α resulted in smaller xenograft tumors as well as smaller, thinner and less branching tumor vessels. However, no significant difference was found in the mean number of apoptotic cells compared to the control tumor model (42). Another report suggested that hypoxia-induced apoptosis through a mitochondrial pathway (22). Furthermore, Lee *et al* reported that HIF-1α-responsive HGTD-P/E2IG5 (estradiol-induced gene 5) facilitated cell death via a mitochondrial apoptotic cascade. Bcl-2/Bcl-xL inhibited this pro-apoptotic activity by forming a heterodimer with HGTD-P (23). In the present study, the increase of HIF-1α expression correlated with the up-regulation of Bcl-2 and Bcl-xL expression as well as higher cell survival as shown by the viability index. During the decrease phase, the expression of both decreased coinciding with the up-regulation of Bax and cleaved-Caspase-9 as well as an increase in the apoptotic index.

Regarding the PI3K/Akt pathway, $CoCl_2$ treatment in the present study inhibited Akt phosphorylation after 36 h. The addition of a PI3K/Akt inhibitor partially inhibited HIF-1α expression, suggesting a possible dose-dependent effect. The fact that the expression of both the HIF-1α and phosphorylated-Akt were inhibited on the longer treatment with $CoCl_2$ supported a previous report on the role of the PI3K/Akt-GSK3ß-HIF pathway (29). It was proposed that attenuation of phosphorylated-Akt may allow the GSK-3ß to be activated and depress the HIF-1α expression, though there is still the possibility that this is a cell-type specific effect (31). Also, that even the addition of 100 μM LY294002 did not fully inhibit HIF-1α expression, suggested some other mechanism(s) of regulation as reported previously (2,9,28).

Further studies are necessary to more precisely elucidate the correlation of HIF-1α expresssion with the proliferation

and apoptosis in carcinoma cells, especially MKN-1 cells and other human gastric carcinoma cell lines, as well as to clarify the role of the PI3K/Akt pathway in the expression and/or regulation of HIF-1α. Understanding the mechanism(s) more clearly, will facilitate manipulations for therapeutic purposes.

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