Transcriptional repression of the human *p53* gene by cobalt chloride mimicking hypoxia

Seok Geun Lee, Hyeon Lee, Hyune Mo Rho*

School of Biological Sciences, Seoul National University, Seoul 151-742, South Korea

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Abstract The switch to an angiogenic phenotype is known to be a fundamental determinant of neoplastic growth and tumor progression. We herein report that the transcription of the human *p53* gene was repressed by treatment with a hypoxia-mimicking concentration of cobalt chloride and alone by hypoxia-inducible factor 1α. Analyses of serial deletions, site-directed mutageneses and heterologous promoter systems showed that the site responsible for the repression by both factors was the E-box element in the promoter of the *p53* gene. These results alongside previous data suggest that the loss of *p53* including the transcriptional repression may play an important role in the angiogenic switch during tumorigenesis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: *p53* tumor suppressor gene; Transcription; Hypoxia; Cobalt chloride

1. Introduction

Regions of vascular deficiency or defective microcirculation in growing tumors are deprived of O_2, glucose, and other nutrients. Apoptosis induced by nutrient deficiency counterbalances cell proliferation and limits tumor growth [1,2]. Clonal evolution of tumor cells in this hypoxic microenvironment results from selection of subpopulations that not only resist apoptosis, but also promote the formation of new blood vessels [3,4]. In addition to promoting further growth of the primary tumor, cellular adaptation to hypoxia and tumor neovascularization are strongly correlated with the risk of invasion and metastasis [4–6]. The switch to an angiogenic phenotype is considered to be a fundamental determinant of neoplastic progression [1,7].

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that regulates O_2 homeostasis and physiologic responses to O_2 deprivation [8,9]. HIF-1 consists of two subunits, HIF-1α and HIF-1β that belong to a subfamily of basic helix–loop–helix transcription factors containing a PAS (Per-ARNT-Sim) motif [10]. A decrease in cellular O_2 tension leads to elevation of HIF-1 activity via stabilization of the HIF-1α protein; conversely, ubiquitin-mediated proteolysis of HIF-1α on exposure to a normoxic environment results in rapid decay of HIF-1 activity [10–12]. HIF-1 is a key transcriptional mediator of metabolic adaptation and VEGF-mediated angiogenesis in response to hypoxia [8,9,13–17]. Although these responses serve to maintain O_2 homeostasis in normal tissues, they are also experienced by tumors to facilitate neovascularization and growth. Akin to their role in vascular development and remodeling in normal tissues, HIF-1α and VEGF facilitate tumor angiogenesis, and both HIF-1α and VEGF are overexpressed in a wide variety of human cancers [4,14,16,18–22].

The human *p53* tumor suppressor gene encodes a multifunctional transcription factor that mediates cellular responses to diverse stimuli, including DNA damage and hypoxia [23]. In addition to being an integral component of the surveillance mechanisms that arrest cell cycle progression under adverse conditions, p53 is also involved in mediating hypoxia-induced apoptosis and inducing inhibitors of angiogenesis such as thrombospondin-1 (TSP-1) [1,24,25]. Evidence also suggests that p53 negatively regulates *VEGF* expression [26–28]. Somatic mutations of the *p53* gene represent one of the most common genetic alterations in human cancers, and the acquisition of such defects is strongly associated with tumor progression and metastasis [29]. The p53 is known to promote Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α, and the loss of *p53* in tumor cells has also been known to enhance HIF-1α levels and augment HIF-1-dependent transcriptional activation in response to hypoxia [30]. Here, we investigated whether hypoxia-mimicking cobalt chloride ($CoCl_2$) [31] and HIF-1α would regulate the transcription of the *p53* gene.

2. Materials and methods

2.1. Plasmids

p53p1CAT reporter plasmid was kindly provided by David Reisman [32], and p53-Luc vector was purchased from Stratagene. The other reporter plasmids (p53-416, p53-220, p53-67, p53mCPE, p53mκB, p53mEbox, p53mE2F, p53Eboxtk, and p53mEboxtk) were constructed as described previously [33]. HIF-1α expression vector was presented as a gift by Dr. T.K. Kim (Harvard University).

2.2. Transient transfection assay

All transient transfections and CAT assays were performed as described previously [33]. The conversion of chloramphenicol to its acetylated form was quantified by using the BAS radio-analytic imaging system according to the manufacturer's instructions (Fuji). Luciferase assay was conducted as described previously [34]. Normalization of transfection efficiency was conducted using the Bradford assay (Bio-Rad). All experiments were repeated at least three times, and a representative autoradiograph is shown. Standard deviations were $<12\%$ in all cases.

*Corresponding author. Fax: (82)-2-888-7784.
E-mail address: hyunerho@plaza.snu.ac.kr (H.M. Rho).

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2.3. RT-PCR

Total RNA was extracted from HeLa cells using the acid guanidinium thiocyanate–phenol–chloroform method [35]. RT-PCR was conducted as described previously [34]. The *cyclophilin* gene was used as an internal control. Oligonucleotide sequences are as follows: p53 upper (5′-ATGGAGGAGCCGCAGTCAGATCCTA-3′), p53 lower (5′-CGCGTCGACTCAGTCTGAGTCAGGCCCTTCTGTC-3′), cyclophilin upper (5′-AGCACTGGAGAGAAAGGATT-3′) and cyclophilin lower (5′-AACCACTCAGTCTTGGCAGT-3′)

3. Results

In order to study the effect of $CoCl_2$, mimicking hypoxia, on the promoter activity of the human *p53* gene, the *p53* promoter-CAT construct (p53p1CAT) was transfected into HeLa cells. As shown in Fig. 1A, the CAT activity of the *p53* promoter was gradually repressed by up to about 3.5-fold as a result of increasing the concentration of $CoCl_2$. These results imply that $CoCl_2$ represses the activity of the p53 promoter. As the HIF-1α subunit is known to play major roles in response to hypoxia [8,15,17,36], we also observed that $CoCl_2$ induced the HIF-1α protein in HeLa cells as previously reported (data not shown) [10,37]. Therefore, we also investigated whether HIF-1α would regulate the activity of the *p53* promoter. To study the effect of HIF-1α on the promoter activity of the human *p53* gene, p53p1CAT reporter plasmid was cotransfected with HIF-1α expression vector. The CAT activity of the *p53* promoter was gradually repressed by up to about 4.5-fold through increasing the HIF-1α expression vector (Fig. 1B). These results imply that the expression of the *p53* gene was repressed by hypoxia-mimicking $CoCl_2$ and HIF-1α.

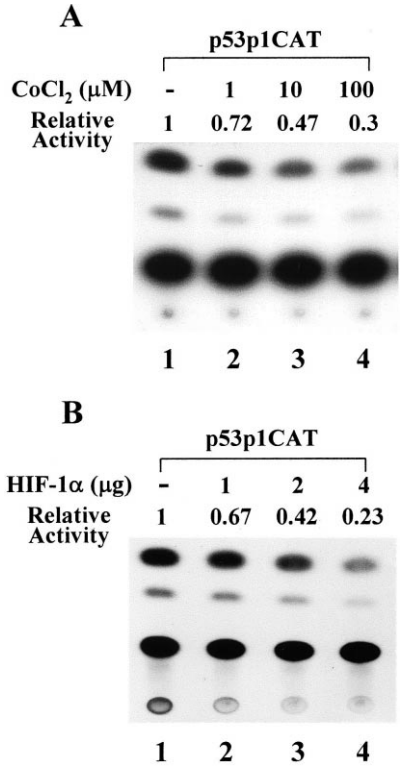

Fig. 1. The effect of $CoCl_2$ and HIF-1α on the promoter of the human *p53* gene. A: The p53-CAT reporter plasmid (p53p1CAT) was transfected into HeLa cells, and the transfected cells were treated with $CoCl_2$ for 16 h as indicated. The relative CAT activity is shown compared to that obtained for the untreated cells. B: Transcriptional activity of the *p53* promoter was measured by cotransfection with the HIF-1α expression vector as indicated. The basal CAT activity of p53p1CAT is designated as 1.0 and each CAT activity is shown as a value relative to this basal activity.

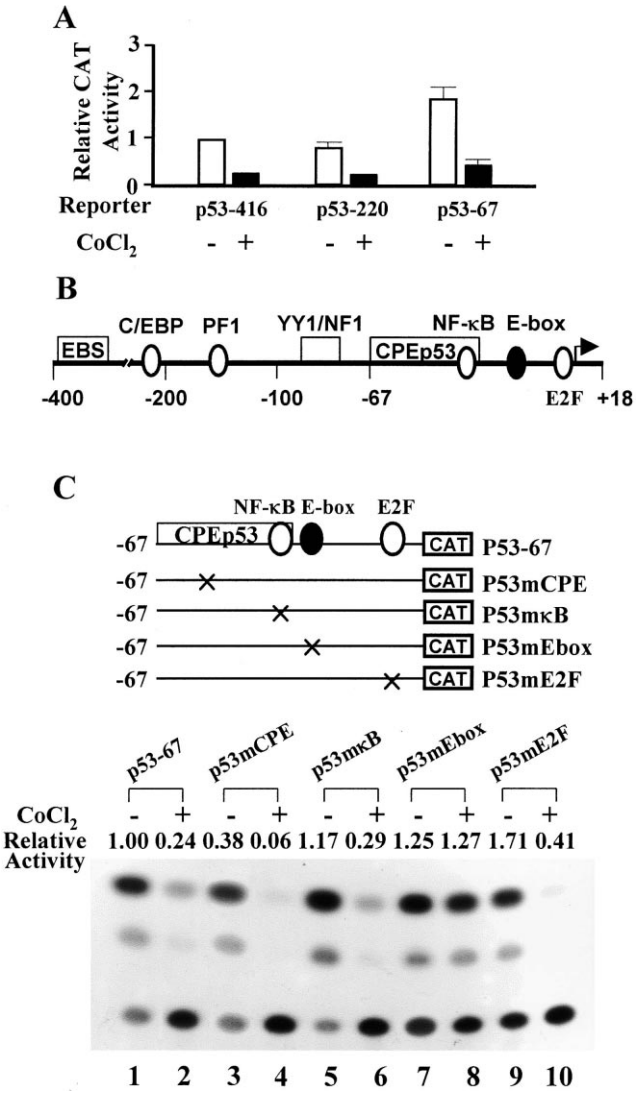

Fig. 2. Identification of the repression site by $CoCl_2$ in the *p53* promoter. A: Serial deletion mutant reporter plasmids of the *p53* promoter were transfected into HeLa cells, and the transfected cells were treated with $CoCl_2$ (100 μM) for 16 h as indicated. CAT activities without (−) or with (+) $CoCl_2$ are indicated by the blank bars and solid bars, respectively. The basal CAT activity of p53-416 is designated as 1.0 and each CAT activity is shown as a value relative to this basal activity. $CoCl_2$-mediated repression is observed in each construct to be about four-fold. Error bars indicate standard errors. B: The schematic representation of the transcriptional binding sites and approximate location in the *p53* promoter. EBS, ETS binding site; C/EBP, CCAAT/enhancer binding protein site; CPEp53, p53 core promoter element; arrow, major transcription start site. C: Transient transfection assays were performed using each of the mutant *p53* promoter-CAT reporter plasmids, and the transfected cells were treated with $CoCl_2$ (100 μM) for 16 h as indicated. The basal CAT activity of p53-67 is designated as 1.0 and each CAT activity is shown as a value relative to this basal activity.

In order to identify the target sequence of $CoCl_2$-mediated repression in the upstream region of the *p53* gene, deletion mutants of the *p53* promoter were prepared and transfected into HeLa cells. $CoCl_2$-mediated repression was observed in

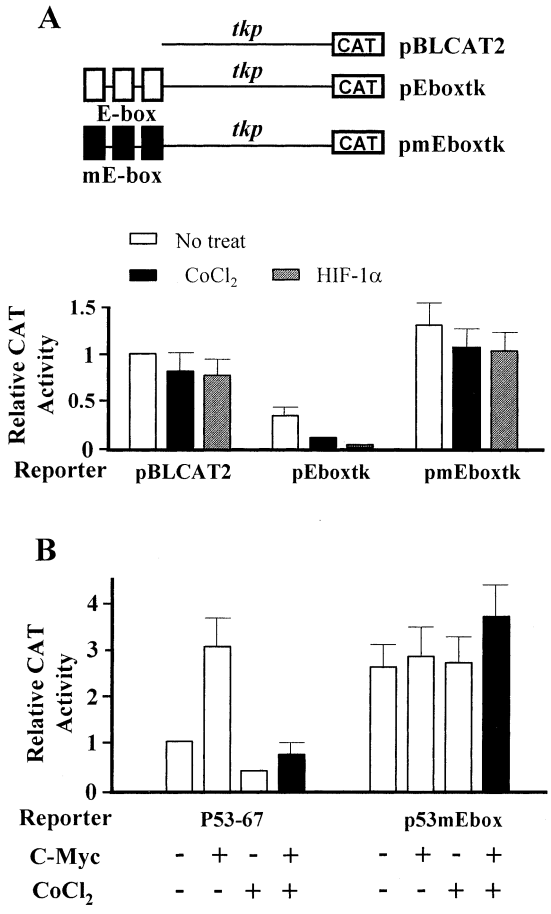

Fig. 3. A: The effect of $CoCl_2$ and HIF-1α on the synthetic E-box and mutated E-box in the heterologous promoter. The plasmid pBLCAT2 has a *tk* promoter (*tkp*). The plasmid pEboxtk and pmEboxtk have three copies of the E-box and mutated E-box element, respectively, in front of *tkp* in the pBLCAT2. Each heterologous reporter plasmid was transfected with HIF-1α expression vector, or the transfected cell with each reporter plasmid was treated with 100 μM $CoCl_2$ for 16 h. B: The inhibitory effect of $CoCl_2$ on the transcriptional activation of the *p53* promoter by c-Myc. Each plasmid of p53-67 and p53mEbox was transfected with c-Myc expression vector into HeLa cells, and the transfected cells were treated with 100 μM $CoCl_2$ for 16 h, as indicated. Error bars indicate standard errors.

each construct by three- to five-fold regardless of the promoter strength (Fig. 2A). These results suggested that the target sequence of $CoCl_2$-mediated repression is located in the region of nt −67 to +18, in which there are four transcriptional factor binding sites (CPEp53, NF-κB, E-box element and E2F) (Fig. 2B). As shown in Fig. 2A (lanes 1, 3, and 5), the basal CAT activity of each deletion reporter plasmid resulted in a similar pattern as in a previous report [33]. To determine which site is specifically involved in $CoCl_2$-mediated repression of the *p53* promoter activity, we constructed four plasmids bearing mutations on each p53 core promoter element (CPEp53), NF-κB binding site, E-box element and E2F binding site (Fig. 2C). When each mutant transfected into the HeLa cells, $CoCl_2$-mediated repression was maintained in the p53mCPE, p53mκB mutants and p53mE2F, but not in the plasmid p53mEbox (Fig. 2C). These results indicate that the transcriptional repression of the *p53* gene by $CoCl_2$ is mediated through the E-box element. The basal promoter activity of p53mCPE was lower than that of the other plasmids (Fig. 2C, lane 3) as previously reported [38].

The effect of $CoCl_2$ and HIF-1α was tested on the E-box elements attached to the thymidine kinase (*tk*) promoter of herpes simplex virus. The $CoCl_2$ and HIF-1α did not change the activity of the *tk* promoter (Fig. 3A, lanes 1–3). The $CoCl_2$ and HIF-1α repressed the CAT activity of pEboxtk, which suggests the repression by $CoCl_2$ and HIF-1α through the E-box element (Fig. 3A, lanes 4–6). In contrast, $CoCl_2$ and HIF-1α did not repress the pmEboxtk (Fig. 3A, lanes 7–9). These results showed that the transcriptional repression of the *p53* promoter by $CoCl_2$ and HIF-1α was also mediated through the E-box element.

The promoter of the human *p53* tumor suppressor gene is also transactivated by the c-Myc/Max heterodimer, which binds to the E-box element [32]. We therefore investigated whether $CoCl_2$ affects the transactivation of the human *p53* gene by c-Myc. The c-Myc expression vector was cotransfected with the plasmid containing the E-box element. The c-Myc activated the *p53* promoter about three-fold, presumably through the heterodimerization with endogenous Max protein (Fig. 3B, lane 2). However, when treated with 100 μM $CoCl_2$, the c-Myc did not activate *p53* promoter activity (Fig. 3B, lane 4). Rather, it was more strongly repressed in the

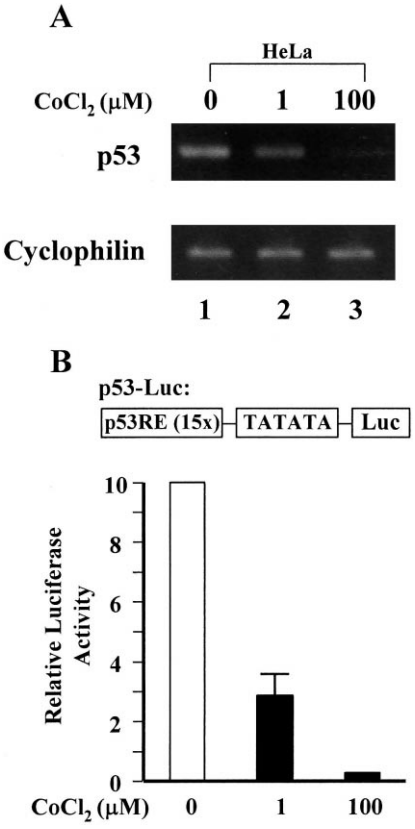

Fig. 4. Repression of the expression of the *p53* gene in response to $CoCl_2$. A: The endogenous *p53* mRNA is gradually repressed by increasing the concentration of $CoCl_2$ in HeLa cells. The *p53* mRNA was rarely detected in the treatment of 100 μM. B: The p53-Luc vector contains the luciferase reporter gene (Luc) driven by a basic promoter element (TATA box) joined to multiple repeats (15×) of p53 binding element (p53RE). Luciferase activity was determined in HeLa cell lysates 16 h after treatment of $CoCl_2$. Error bars indicate standard errors.

presence of c-Myc (about four-fold) than in its absence (about three-fold). The activity of the p53mEbox was neither activated by c-Myc, nor repressed by $CoCl_2$ in the presence of c-Myc (Fig. 3B, lanes 5–8). These results suggest that $CoCl_2$ also represses the transcriptional activation of the *p53* promoter by the c-Myc, which binds to the E-box element.

In order to determine whether the endogenous *p53* gene is repressed by treatment of $CoCl_2$, the *p53* mRNA level was measured by RT-PCR in HeLa cells. As shown in Fig. 4A, the expression of the *p53* gene was repressed by the treatment of $CoCl_2$ in a dose-dependent manner. In particular, the *p53* mRNA evidently disappeared at a concentration of 100 μM (Fig. 4A, lane 3). We also investigated whether $CoCl_2$ affects the transactivating function of p53 protein. When p53-Luc vector was transfected into HeLa cells to determine if $CoCl_2$ affects p53 function as a transcription factor, $CoCl_2$ repressed luciferase activity from p53-Luc reporter vector (Fig. 4B). These results indicated that the endogenous *p53* gene was repressed by $CoCl_2$, which is known to be a hypoxia-mimicking agent, and that functionally, at least as a transcription factor, p53 was also inactivated by $CoCl_2$.

4. Discussion

Recognition of the importance of angiogenesis for the growth and metastasis of cancers has raised fundamental questions regarding the molecular mechanisms of the angiogenic switch during tumor progression. The genetic alterations involved in tumorigenesis are also responsible for the phenotypic characteristics of cancer cells. The p53 tumor suppressor gene is one of the most frequently mutated genes in human cancers [29]. In addition to p53 mutations, which occur in $\sim 50\%$ of all cancers (involving >50 tissue types), p53 is also inactivated by viral oncoproteins such as the E6 protein of cervical cancer-associated human papillavirus 16 and 18, adenovirus E1A, X protein of hepatitis B virus, core protein of hepatitis C virus, and SV40 large T antigen [29,33]. These observations indicated that the loss of p53 function, via somatic mutations, transcriptional inactivation or expression of viral oncoproteins, contributes to activation of the angiogenic switch during tumorigenesis.

p53 has been known to inhibit HIF-stimulated transcription and promote Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α [30,39]. Loss of *p53* in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of *VEFG* gene in response to hypoxia, and forced expression of HIF-1α in p53-expressing tumor cells increases hypoxia-induced *VEGF* expression and augments neovascularization and growth of tumor xenografts [30]. Expression of HIF-1α protein, HIF-1 DNA binding activity, and *VEGF* mRNA are increased in p53 −/− knockout colon carcinoma cells as compared with the parental p53 +/+ cells, and in renal clear cell carcinoma cell lines, the loss of von Hippel–Lindau tumor suppressor function results in constitutive high-level expression of HIF-1α [22,40]. Thus, in addition to hypoxia, both oncogene activation and tumor suppressor gene inactivation seem to be associated with increased HIF-1α expression.

Here we demonstrated that $CoCl_2$ and HIF-1α repressed the transcription of the human *p53* gene through the E-box element and that the p53 protein was functionally, at least as a transcription factor, inactivated by $CoCl_2$. HIF-1α is also known to interact with the p53 protein, which inhibits the functions of HIF-1α [22,30,39]. The angiogenic switch is regulated by changes in the relative balance between inducers, such as VEGF, and inhibitors, such as TSP-1, of endothelial cell proliferation and migration [3]. The p53-mediated inhibition of *VEFG* expression, together with the ability of p53 to upregulate TSP-1, indicated that p53 provides dual functions that regulate angiogenesis [24,30]. Thus, the loss of p53 functions, such as somatic mutation, inactivation by viral oncoproteins, and especially transcriptional repression, during tumorigenesis deregulates both arms of the balance, providing a potent stimulus for neovascularization and tumor progression.

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