Erythropoietin Prevents Hypoxia-Induced GATA-4 Ubiquitination *via* Phosphorylation of Serine 105 of GATA-4

Ji Hae Jun,[a] Eun Jung Shin,[b] Ji Ho Kim,[b,c] Si Oh Kim,[d] Jae-Kwang Shim,[b,c] and Young-Lan Kwak*[,a,b,c]

[a] *Severance Biomedical Science Institute, Yonsei University College of Medicine;* [b] *Anesthesia and Pain Research Institute, Yonsei University Health System;* [c] *Department of Anesthesiology and Pain Medicine, Yonsei University College of Medicine; Seoul 120–752, Republic of Korea: and* [d] *Department of Anesthesiology and Pain Medicine, College of Medicine, Kyungpook National University Hospital; Daegu 702–701, Korea.*
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Erythropoietin (EPO), an essential hormone for erythropoiesis, can provide protection against myocardial ischemia/reperfusion (I/R) injury and hypoxic apoptosis. GATA-4 is a zinc finger transcription factor, and its activation and post-translational modification are essential components in the transcriptional response to hypoxia. GATA-4 has also been reported to play a role in the cellular mechanisms of EPO-induced myocardial protection against I/R injury. In this study, we aimed to investigate the influence of EPO on GATA-4 protein stability and post-translational modification under hypoxic conditions without reperfusion. EPO induced cell viability under long-term hypoxia. EPO significantly increased phosphorylation of GATA-4 *via* the extracellular signal-regulated kinase (ERK) signaling pathway and reduced hypoxia-induced GATA-4 ubiquitination, which enhanced GATA-4 stability under hypoxia. ERK activation by over-expression of constitutively active mitogen-activated protein kinase 1 (MEK1) strongly increased GATA-4 phosphorylation and its protein levels and decreased GATA-4 ubiquitination under hypoxia. Despite ERK activation, GATA-4 ubiquitination was not affected under hypoxia in a GATA-4-S105A mutant. Under hypoxic condition without reperfusion, EPO-induced ERK activation was associated with post-translational modification of GATA-4, mediated by enhancement of phosphorylation of GATA-4 at Ser-105. Subsequent attenuation of GATA-4 ubiquitination led to increases in GATA-4 protein stability, which resulted in increased cell viability under hypoxia.

Key words erythropoietin; hypoxia; Ser-105; GATA-4; phosphorylation; ubiquitination

Erythropoietin (EPO), a principle regulator of erythropoiesis,[1] can protect the myocardium against ischemia/reperfusion (I/R) injury[2,3] *via* various signal transduction pathways.[4,5] Even under hypoxic conditions, EPO attenuates apoptosis of cardiomyocytes *via* phosphatidylinositol 3 kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) 1/2 pathways.[6]

GATA-4, a zinc finger transcription factor, is a crucial regulator of cardiac development.[7,8] In the adult heart, GATA-4 mediates hypertrophic responses[9,10] through stimulation of gene expression including troponin C, troponin I, atrial natriuretic factor (ANF), myosin light chains, α-myosin heavy chain (α-MHC), and β-MHC.[11] GATA-4 also mediates anti-apoptotic protein expression and stimulates cell survival signals and stress-induced gene expression against myocardial I/R injury and hypoxic injury.[12,13] The GATA-4 protein is subject to post-translational modifications such as phosphorylation, acetylation, and ubiquitination, which modulate its DNA binding, transcriptional activities and nuclear localization in cardiomyocytes.[11,14] In cardiomyocytes, p38 mitogen-activated protein kinase (MAPK) and ERK increased GATA-4 phosphorylation.[10,15] Phosphorylation of GATA-4 at Ser-105 leads to induction of transcriptional activity[15,16] and an increase in its stability within the cells.[17] Additionally, p300, which possesses intrinsic histone acetyltransferase activity,[18,19] induces GATA-4 acetylation, thereby enhancing its DNA binding and transcriptional activities.[20,21] While much is known about phosphorylation and acetylation of GATA-4, the molecular mechanism of GATA-4 ubiquitination, one of the critical post-translational modification processes, remains elusive, despite the fact that ubiquitination is involved in regulating bioactivities of diverse proteins.

In several studies, EPO-induced cardioprotection against I/R injury has been shown to be associated with regulation of GATA-4 protein levels and post-translational modifications *via* various signaling pathways including the PI3K/AKT and ERK signaling pathways.[22] Unlike I/R injury, however, the influence of EPO on GATA-4 activity under hypoxic conditions without reperfusion has not been elucidated, while the protective role of EPO or GATA-4 on hypoxia-induced cardiomyocyte apoptosis and infarction has been demonstrated separately.[22,23] In this study, we investigated the influence of the EPO-ERK signaling pathway on GATA-4 stability in terms of post-translational modifications including the culprit amino acid residue under hypoxic conditions without reperfusion.

MATERIALS AND METHODS

Primary Culture of Rat Cardiomyocytes All animal procedures were performed with approval from the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.[24] Sprague-Dawley neonatal rat pups (1- to 3-d-old) were sacrificed by cervical dislocation. Neonatal rat cardiomyocytes were prepared as previously described.[25,26] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bo-

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: ylkwak@yuhs.ac

vine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Culture media and supplements were purchased from WelGENE (Seoul, Korea).

Cell Culture under Normoxic and Hypoxic Condition P19, embryonal carcinoma cells, were maintained in α-minimal essential medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 95% humidified air plus 5% CO_2. Cells were plated into 60 or 100 mm tissue culture dishes with culture medium and incubated for various times under normoxia (5% CO_2 in air) or hypoxia (1% O_2, 5% CO_2, and 94% N_2) conditions. Treatments were as follows: EPO (Calbiochem, La Jolla, CA, U.S.A.), MEK1/2 inhibitor U0126 (Cell Signaling Technology, Inc.), p38 inhibitor SB203580, and c-Jun N-terminal kinase (JNK) inhibitor II SP600125 (Calbiochem) and MG132 (Sigma, Saint Louis, MO, U.S.A.).

Cell Viability Assay P19 cells were plated 5×10^4 cells per well in 96-well plates, incubated overnight and pre-treated with EPO for 1 h under normoxic condition. Control cells did not receive EPO pretreatment. Cells were incubated for 24 h under normoxic or hypoxic conditions, and cell viability was analyzed using the EZ-CyTox 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Daeillab, Korea) according to the manufacturer's instructions.

Immunoprecipitation and Immunoblot Analysis After appropriate treatments, cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in cell lysis buffer supplemented with 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, a protease inhibitor mixture and phosphatase inhibitor cocktail-2 and -3 (Sigma). After measuring protein concentrations using bicinchoninic acid (BCA) reagents, 1 mg of protein from each cell lysate was immunoprecipitated with the appropriate primary antibodies and protein G-agarose beads for 16 h at 4°C with continuous rotation. The beads were collected and washed, and bead-bound proteins were eluted by boiling in 1×Laemmli sample buffer with 1 M dithiothreitol (DTT) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. The antibodies were as follows: anti-phospho GATA-4 at Ser 105 (Abcam, Cambridge, MA, U.S.A.), anti-phospho ERK and anti-ERK (Cell Signaling Technology, Inc.), GATA-4, actin, and horse-radish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.).

Ubiquitination Analysis P19 cells were transiently transfected with GATA-4, pcDNA, and caMEK1 expression vectors as indicated. Under hypoxic condition, the cells were treated with 20 μM MG132 (a proteasome inhibitor), 10 μM U0126, and 20 IU/mL EPO as indicated, for an additional 4 h. Cells were then washed and lysed in ice-cold cell lysis buffer. Immunoprecipitation with anti-GATA-4 antibody and subsequent immunoblot analysis with anti-ubiquitin antibody (Cell Signaling Technology, Inc.) were performed to detect ubiquitinated GATA-4.

Determination of GATA-4 Stability under Hypoxia P19 cells were transiently transfected with His-GATA-4 expression plasmids. Transfected cells were treated with 10 μg/mL cycloheximide (CHX) for protein synthesis inhibition (Sigma). After 1 h, cells were further incubated in the presence or absence of EPO (20 IU/mL) for the indicated time under hypoxic conditions. The level of GATA-4 was analyzed by immunoblot analysis.

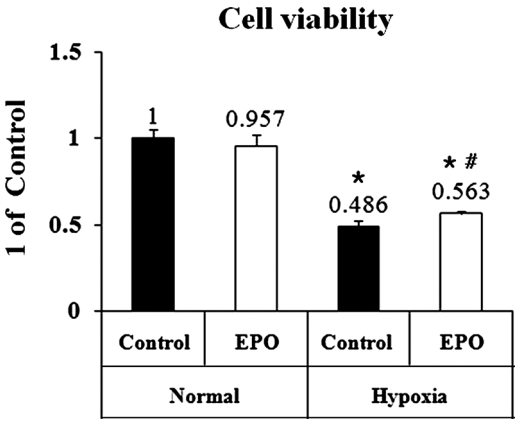

Fig. 1. EPO Increased Cell Viability under Hypoxia

P19 cells were pre-treated with or without EPO for 1 h under normoxia. Cells were exposed to normoxic or hypoxic conditions for 24 h. Cell viability was determined by MTT assay. EPO, erythropoietin. n=12 times. $^*p<0.01$ compared with control under normoxia. $^{\#}p<0.01$ compared with control under hypoxia.

DNA Constructs and Site-Directed Mutagenesis The plasmid constructs His-tag GATA-4 (wild type (WT)) and GATA-4 S105A mutant were previously described.[27] pcDNA and the constitutively active MEK1 plasmid (pFC-MEK1) were purchased from Stratagene (La Jolla, CA, U.S.A.).

Statistical Analyses All results were expressed as mean± S.D. Statistical significance was analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test following the Bonferroni correction. A *p*-value <0.01 was considered significant.

RESULTS

EPO Increased Cell Viability under Hypoxia EPO is known to attenuate cardiomyocytes apoptosis under hypoxia.[6] To confirm the effect of EPO on cell viability under hypoxia, we examined P19 cells cultured for 24 h in the presence or absence of EPO under normoxic or hypoxic condition. The reduced cell viability caused by hypoxia was increased by EPO treatment under hypoxic conditions (Fig. 1). EPO showed no effects under normoxic condition (Fig. 1).

EPO Increased the GATA-4 Protein Level under Hypoxia Previously, it was shown that EPO enhances GATA-4 stability *via* increasing GATA-4 phosphorylation and acetylation under normoxia.[27] To investigate the role of EPO on GATA-4 post-translational modification under hypoxic conditions, we examined whether EPO treatment affected GATA-4 protein levels in primary rat cardiomyocytes. EPO treatment increased endogenous GATA-4 protein levels at 2 h after exposure to hypoxic conditions with a maximal increase at 4 h after exposure to hypoxic conditions (Fig. 2A). EPO also increased exogenous GATA-4 protein levels, which were measured in P19 embryonal carcinoma cell lines transiently transfected with His-tagged GATA-4 expression plasmids under hypoxic condition (Fig. 2B). We also examined whether EPO-enhanced GATA-4 protein level was related to an increase in GATA-4 stability by EPO stimulation under hypoxia (Fig. 2C). EPO significantly delayed GATA-4 protein degradation under hypoxia.

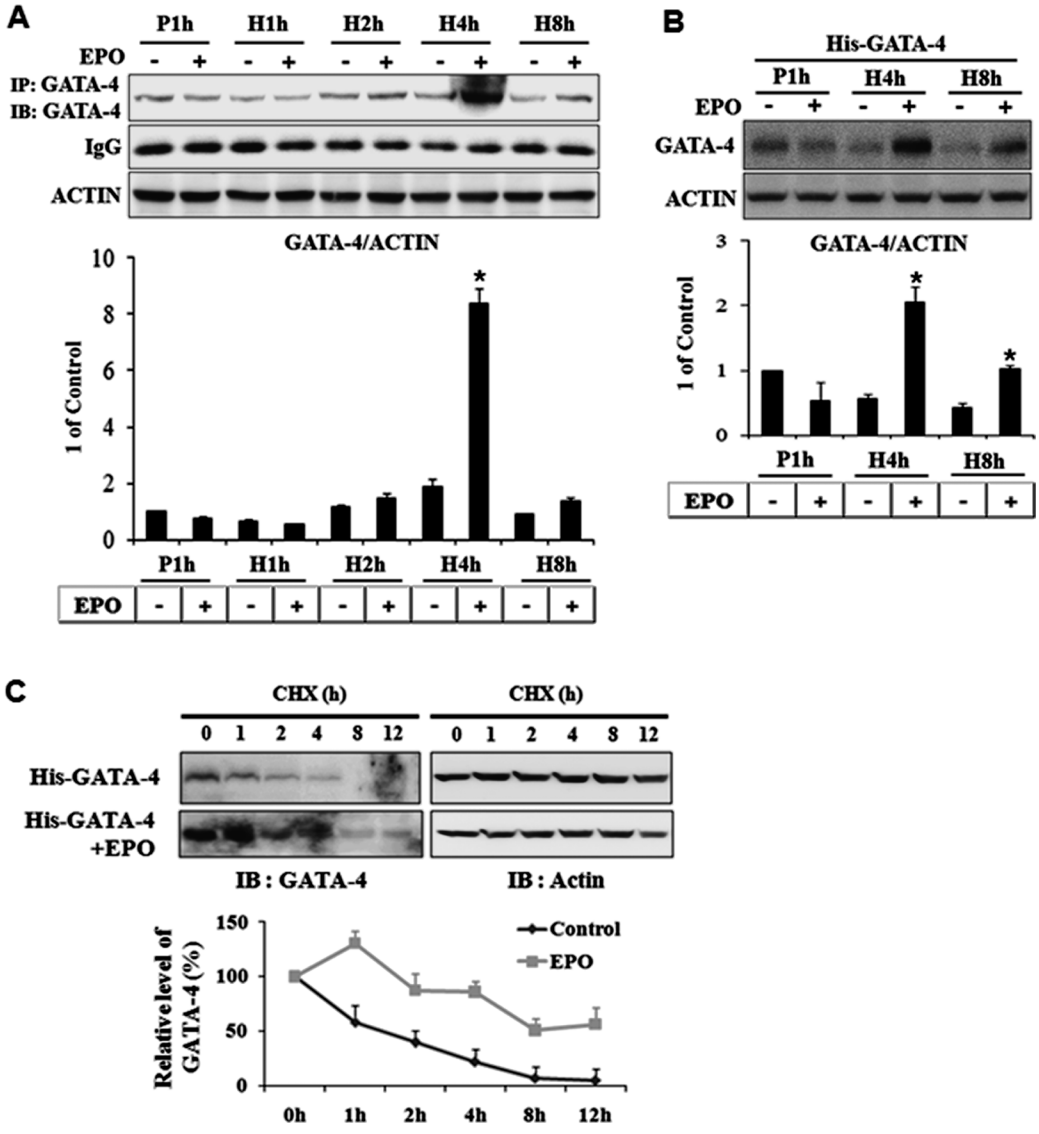

Fig. 2. EPO Increased GATA-4 Protein Level under Hypoxia

(A) EPO increased endogenous GATA-4 protein level in primary rat cardiomyocytes. Cells were treated with or without EPO (20 IU/mL) for indicated times under hypoxia. Endogenous GATA-4 protein level was determined by immunoprecipitation (IP) and immunoblot (IB) analyses. (B) EPO increased exogenously expressed His-GATA-4 protein level. P19 cells were transiently transfected with a His-GATA-4 expression plasmid, incubated in the presence or absence of EPO for indicated times under hypoxia and subjected to IB analysis. (C) EPO treatment stabilized GATA-4 protein under hypoxia. P19 cells were transiently transfected with a His-GATA-4 expression vector. Twenty-four hours after transfection, cells were pre-treated with cycloheximide (CHX, 10 μg/mL). After 1 h, cells were further incubated in the presence or absence of EPO (20 IU/mL) for predetermined durations under hypoxia. The levels of GATA-4 and actin were determined by IB analysis. EPO, erythropoietin. P, pre-treatment. H, hypoxia. $n=4$ times. *$p<0.01$ compared with control/each time group.

ERK Activation Was Involved in EPO-Induced Increase in GATA-4 Protein Stability under Hypoxia Since MAP kinases pathways, part of the downstream EPO signaling pathways, are known to contribute to GATA-4 activation *via* direct phosphorylation,[11] we evaluated which of the MAP kinases was responsible for EPO-induced increase in GATA-4 protein under hypoxia. In GATA-4-overexpressed P19 cells, the EPO-induced increase in GATA-4 protein level was significantly attenuated by U0126, an ERK specific inhibitor, at 4 h after exposure to hypoxia. SB 203580, a p38 inhibitor, had a minor effect on GATA-4 protein level following EPO stimulation (Fig. 3A). In GATA-4-overexpressed P19 cells, EPO treatment for 4 h under hypoxia increased ERK phosphorylation, which was maximal at a dose of 20 IU/mL (Fig. 3B). When cells were exposed to EPO (20 IU/mL) under hypoxia for 24 h, ERK phosphorylation increased until 8 h, with a peak increase at 4 h after starting EPO treatment (Fig. 3C), and disappeared at 24 h after EPO treatment (data not shown).

EPO-ERK Pathway Activation Increased GATA-4 Phosphorylation and Its Protein Level under Hypoxia Under hypoxic culture conditions, EPO (20 IU/mL) induced ERK phosphorylation and enhanced GATA-4 phosphorylation and its protein levels for 4 h after starting EPO treatment (Fig. 4A). These effects were inhibited by U1026 (Fig. 4A). Similar to EPO, overexpression of constitutively active MEK1 in P19 cells significantly increased ERK phosphorylation as well as GATA-4 phosphorylation and protein levels (Fig. 4B). These results demonstrate that, under hypoxia, EPO-induced ERK phosphorylation leads to increases in GATA-4 phosphorylation and protein levels.

EPO-Induced GATA-4 Phosphorylation Decreased GATA-4 Ubiquitination *via* the ERK Signaling Pathway

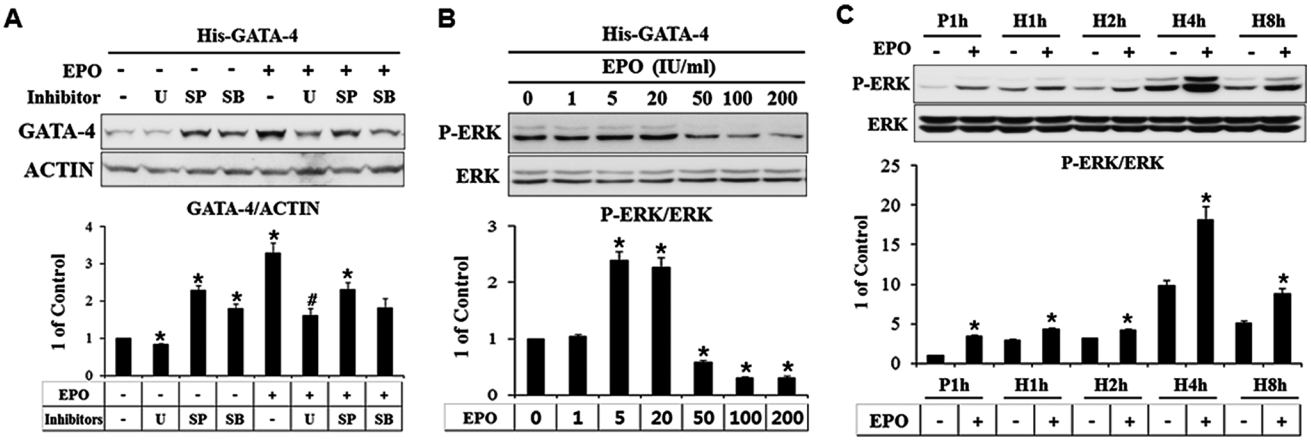

Fig. 3. ERK Activation Is Involved in EPO-Enhanced GATA-4 Protein Levels under Hypoxia

(A) U0126, an ERK inhibitor, suppressed GATA-4 protein levels under hypoxia. P19 cells were transiently transfected with His-GATA-4 expression vectors and incubated for 16 h. The cells were pretreated with vehicle (DMSO) or the indicated inhibitors for 1 h and further incubated in the presence or absence of EPO for an additional 1 h under normal conditions and then incubated for 4 h under hypoxic conditions. Exogenous GATA-4 protein level was determined by IB analysis. EPO, erythropoietin. (B and C) EPO induced ERK activation in GATA-4 overexpressed P19 cells. Cells were serum-starved for 16 h under normoxia and further incubated in the presence or absence EPO at the indicated dose for 4 h (B) and time (C) under hypoxia. Whole cell lysates were prepared and subjected to IB analysis. EPO, erythropoietin. P, pretreatment. H, hypoxia. $n=4$ times. $^{*}p<0.01$ compared with control/each time group. $^{\#}p<0.01$ compared with EPO treatment.

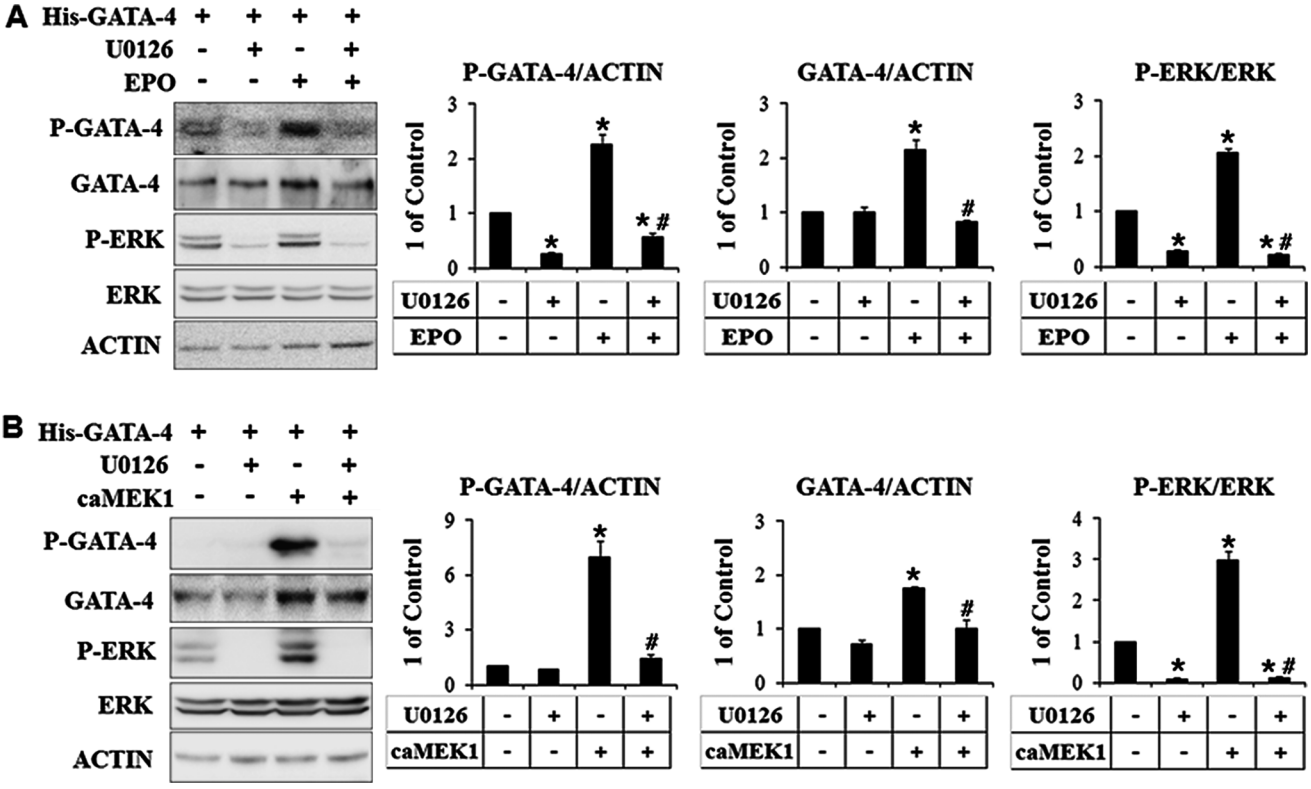

Fig. 4. EPO-Activated ERK Kinase Increased GATA-4 Phosphorylation and Its Protein Level

(A) P19 cells were transiently transfected with His-GATA-4 expression vectors and incubated for 16 h. Cells were pretreated with vehicle (DMSO) or U0126 for 1 h under normoxia and further incubated in the presence or absence of EPO for an additional 4 h under hypoxia. GATA-4 phosphorylation and protein levels were determined by immunoblot (IB) analysis. (B) P19 cells were transiently transfected with His-GATA-4 and constitutively active MEK1 expression vectors and incubated in the presence or absence of U1026 for 4 h under hypoxia. IB analysis was then performed. P-GATA-4, phosphorylation of Ser-105 of GATA-4. $n=4$ times. $^{*}p<0.01$ compared with control. $^{\#}p<0.01$ compared with EPO treatment.

under Hypoxia Previous studies have demonstrated that GATA-4 DNA binding and transcriptional activity are regulated through direct interactions and post-translational modifications.[28,29] Ubiquitination is one of these post-translational modifications involved in GATA-4 stability under hypoxia. To determine whether phosphorylation of GATA-4 by EPO under hypoxia could protect against protein degradation through the inhibition of proteasome-dependent ubiquitination, we performed a ubiquitination assay. In the presence of MG132 (a proteasomal inhibitor), the polyubiquitinated GATA-4 level was greater under hypoxia than under normoxia (Fig. 5A). EPO decreased hypoxia-induced GATA-4 ubiquitination, while U0126 alone increased GATA-4 ubiquitination under hypoxia (Fig. 5B). EPO did not affect GATA-4 ubiquitination

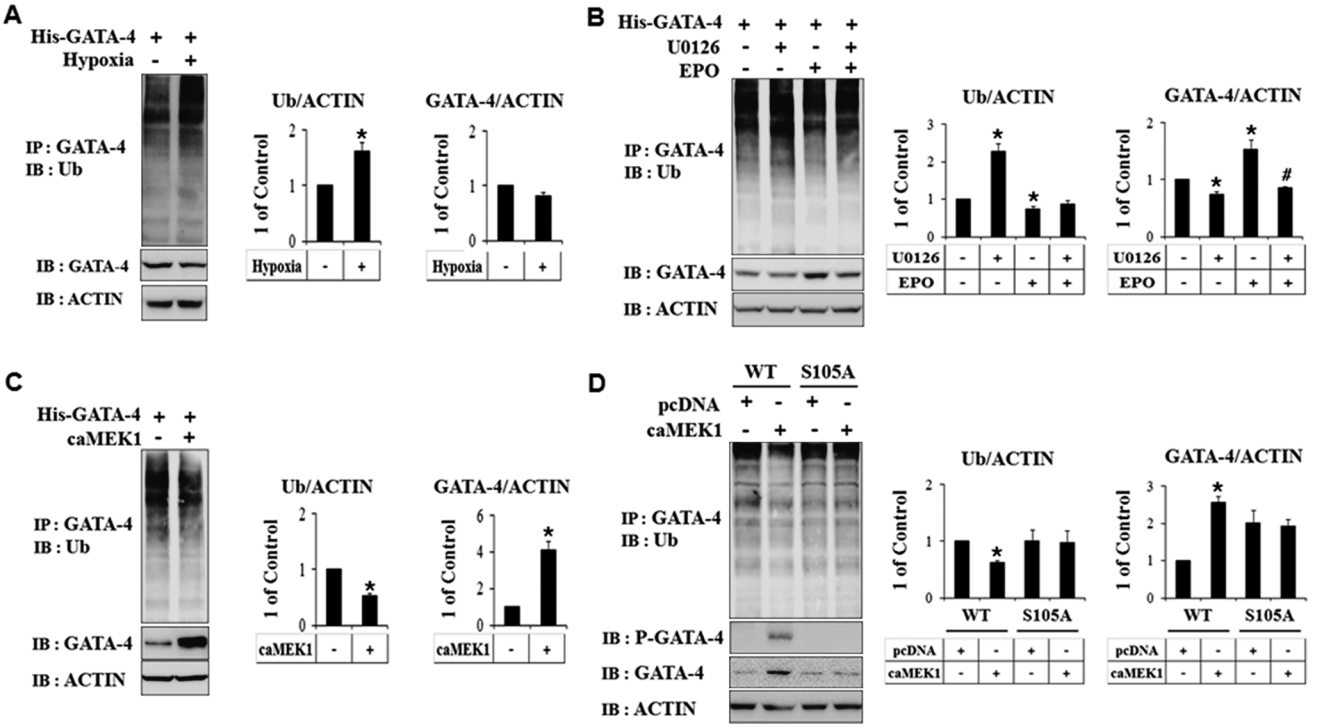

Fig. 5. EPO-Induced GATA-4 Phosphorylation Decreased GATA-4 Ubiquitination *via* the ERK Signaling Pathway under Hypoxia

(A) P19 cells were transiently transfected with His-GATA-4 expression vectors and incubated for 16 h under normoxia. Cells were treated with MG132 (20 μM) for an additional 4 h under hypoxia. (B) P19 cells were transiently transfected with His-GATA-4 expression vectors and incubated for 16 h. Cells were pretreated with MG132 (20 μM) and vehicle (DMSO) or U0126 for 1 h and further incubated in the presence or absence of EPO for an additional 4 h under hypoxia. (C) P19 cells were transiently transfected with His-GATA-4 and constitutively active MEK1 expression vectors and incubated in the presence MG132 (20 μM) for 4 h under hypoxia. (D) P19 cells were transiently transfected with His-GATA-4 (WT), GATA-4-S105A mutant, or constitutively active MEK1 expression vectors and incubated in the presence MG132 (20 μM) for 4 h under hypoxia. IP and IB analyses were then performed. P-GATA-4, phosphorylation of Ser-105 of GATA-4. Ub, anti-ubiquitin antibody. $n=4$ times. $^{*}p<0.01$ compared with control. $^{\#}p<0.01$ compared with EPO treatment.

under normoxic conditions (data not shown). MEK1 overexpression also decreased GATA-4 ubiquitination under hypoxia (Fig. 5C). These results indicate that ERK enhances GATA-4 stability *via* stimulation of GATA-4 phosphorylation and concomitant diminution of GATA-4 ubiquitination under hypoxia. The MEK1-ERK signaling pathway activated GATA-4 phosphorylation at Ser 105; thus, the effect of ERK activation on GATA-4 ubiquitination in GATA-4-S105A mutant was investigated. Reduced hypoxia-induced GATA-4 ubiquitination by ERK activation was not observed in a GATA-4-S105A mutant (Fig. 5D). The S105A mutant was confirmed by immunoblot analysis using a specific antibody for Ser 105-phosphorylated GATA-4. These results suggest that EPO-induced phosphorylation of GATA-4 at serine-105 plays an important role in attenuating hypoxia-induced GATA-4 ubiquitination, leading to stabilization of the GATA-4 protein.

DISCUSSION

The GATA family, possessing two highly conserved zinc finger DNA binding domains (Cys-X2-Cys-X17-Cys-X2-Cys),[30,31] is involved in the regulation of cell growth, differentiation, and survival. In particular, GATA-4 has been considered to be an important survival factor for postnatal cardiomyocytes.[32] GATA-4 overexpression mainly affects a functional group of genes related to cell signaling/communication, inflammatory/immune response, biosynthesis/metabolism, cell cycle/division, and protein synthesis/turnover/posttranslational modifications.[33] Considering the clinical prevalence and significance of myocardial infarction, GATA-4 activation has been proposed as an important component of the transcriptional response to hypoxia.[22] Indeed, GATA-4 could decrease the rate of apoptosis at an early time point and enhance angiogenesis in the infarcted myocardium.[33]

Although EPO is a principle regulator of erythropoiesis, EPO receptors are also expressed by other cell types, and EPO has been shown to protect the myocardium against I/R injury or hypoxia[3] *via* various signal transduction pathways.[4] In cardiomyocytes, EPO was reported to attenuate apoptosis *via* the PI3K/AKT and ERK 1/2 pathways.[6] In previous studies, EPO was shown to convey a cardioprotective effect against the regulation of GATA-4 protein level *via* various signaling pathways including the PI3K/AKT and ERK signaling pathways.[22,34] EPO increased GATA-4 phosphorylation and enhanced the transcriptional activation of GATA-4 after I/R injury.[22] Phosphorylation is a form of post-translational modification that plays an important role in the functional performance of GATA-4 in physiological as well as pathological cellular processes.[11] In a previous study performed under normoxic conditions, we observed that EPO-induced phosphorylation of GATA-4 serine-261 lead to enhanced acetylation, but not ubiquitination, of GATA-4.[27] Under hypoxic conditions, the cardioprotective effects of EPO treatment or GATA-4 activity have only been studied separately.[23] However, little is known about the influence of exogenous EPO treatment on GATA-4 stability and its post-translational modification under hypoxic conditions without reperfusion, which mimic the clinical scenario of myocardial infarction. The results of our

current study provide primary evidence that EPO-ERK signaling activation is involved in post-translational modifications of GATA-4 through the attenuation of hypoxia-induced GATA-4 ubiquitination, which is directly associated with phosphorylation of GATA-4 at the Ser-105 residue. No significant difference could be observed between the WT and S261A mutant on GATA-4 ubiquitination under hypoxia (data not shown).

To control the balance between cell death and survival, appropriate actions are critical in response to external stimuli such as exposure to hypoxia or oxygen deprivation.[35,36] Previous studies have established that diverse signaling pathways transmitted from external signals to the interior of a cell consist of tightly regulated complex cascades of events.[37] The molecular interactions within these cascades can affect conformational changes that are required for the activation of particular signaling molecules and enable proteins to modify both themselves and nearby substrates.[38] Since the function of the transcription factors is controlled by protein–protein interactions and/or post-translational modifications, numerous studies on post-translational modifications of GATA-4 have focused on phosphorylation, acetylation, and sumoylation. However, the molecular mechanisms of GATA-4 ubiquitination have not been fully elucidated.

Ubiquitination and subsequent proteasome-dependent protein degradation are involved not only in signal transduction, but also in other diverse cellular processes including cell cycle progression, transcriptional regulation, DNA repair, and apoptosis.[39] In addition to the attachment of a single ubiquitin molecule, chains consisting of several ubiquitin moieties can be attached to target proteins. The functional outcome of polyubiquitination depends on the lysine residue within the ubiquitin moiety that is used for chain elongation.[38] While phosphorylation of proteins is catalyzed by kinases, the covalent attachment of ubiquitin to lysine residues or to the N terminus of a target protein is regulated by the collaborated action of three different classes of proteins. These proteins consist of the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin-protein ligase (E3).[40,41] The two forms of post-translational modification, phosphorylation and ubiquitination, affect each other, with both being able to regulate, activate, or inactivate proteins within the signal transduction cascades.[42,43] With GATA-4, it was previously determined that phosphorylation at Ser-105[15] and acetylation at four lysine residues (K311, K318, K320, and K322) in the C-motif increased its activity.[20] Additionally, sumoylation of GATA-4 at Lys-366 by small ubiquitin-like modifier-1 led to an increase in its transcriptional activity.[29,44] However, the exact ubiquitination sites of GATA-4 are still unknown.

In the current study, EPO-induced ERK activation mitigated hypoxia-induced GATA-4 ubiquitination through enhanced phosphorylation of GATA-4 at the Ser-105 residue under hypoxic conditions. These events may result in reduction of GATA-4 degradation, leading to an increase in the stability of GATA-4 as in I/R. Considering that up-regulation of GATA-4 phosphorylation and down-regulation of GATA-4 ubiquitination are closely associated with cell growth and survival, EPO might mitigate myocardial damage under hypoxic conditions through this mechanism. In previous studies, EPO was reported to prevent hypoxia-induced apoptosis of rat ventricular myocytes[23] and to reduce the number of apoptotic myocytes and the extent of the infarct area after permanent coronary ligation in rats.[45,46] Interestingly, the mechanisms of these protective effects were investigated mainly in terms of Akt activation or Janus kinase-signal transducers and activator of transcription (JAK-STAT) pathways. As previously mentioned, GATA-4 activity is an essential component of the transcriptional response to hypoxia and regulates the JAK-STAT pathways.[33,47]

In the field of cardiovascular research, interest in EPO as a therapeutic rescue against I/R injury and cardiac remodeling after myocardial infarction has been raised. The fact that there is a significant time delay in the production of endogenous EPO following injury provides the rationale for the use of exogenous EPO.[48] The ability of exogenous EPO to protect an "area at risk" by inhibiting apoptosis and inflammatory reactions until reperfusion therapy is available, and the attenuation of post-infarction remodeling has been regarded as one of the promising therapeutic strategies to improve the prognosis of acute coronary syndrome,[23,47] although the clinical data are still controversial.[49,50] GATA-4 has been shown to play a role as a survival factor that can break the vicious cycle of post-infarction heart failure. That EPO-ERK activation stabilizes GATA-4 activity by activating phosphorylation and diminishing ubiquitination of GATA-4 provides a clue into a possible molecular mechanism of the previously reported cardioprotective effects of EPO. Based on our current results, further research should be performed to maximize the impact of exogenous EPO on locally induced myocardial protection, regeneration, and angiogenesis through a novel GATA-4-dependent molecular mechanism in the post-infarcted heart.

In conclusion, under hypoxic conditions without reperfusion, EPO-induced ERK activation was associated with post-translational modification of GATA-4 mediated by enhancement of phosphorylation of GATA-4 at Ser-105. Subsequent attenuation of GATA-4 ubiquitination led to an increase in GATA-4 protein stability.

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