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FOXO4 Induces Human Plasminogen Activator Inhibitor-1 Gene Expression *via* an Indirect Mechanism by Modulating HIF-1α and CREB Levels

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Abstract

The plasminogen activator inhibitor-1 (PAI-1) expression can be enhanced by hypoxia and various stimuli associated with oxidative stress. Among the FOXO transcription factors, FOXO4 appears to be crucial in the response against oxidative stress. Therefore, it was the aim of this study to investigate the role of peroxide-induced oxidative stress and FOXO4 on PAI-1 expression under normoxia and hypoxia. Treatment of cells with hydrogen peroxide increased PAI-1 mRNA, protein, and promoter activity, and knocking down FOXO4 abolished the peroxide-dependent PAI-1 induction. *PAI-1* promoter reporter gene assays revealed that the peroxide and FOXO4-dependent induction was mediated through the HIF-1 and CREB-binding HRE within the PAI-1 promoter. Western blot analyses then indicated that peroxide and FOXO4 downregulated HIF-1α levels, whereas CREB levels were increased. Chromatin immunoprecipitations showed that FOXO4 did not bind the PAI-1 promoter, whereas CREB binding was enhanced on FOXO4 overexpression. In addition, knockdown of CREB abolished the FOXO4-mediated PAI-1 induction. Together, these findings provide the first evidence that oxidative stress and FOXO4 induce PAI-1 expression through an indirect mechanism involving modulation of HIF-1α and CREB protein levels and that enhanced CREB binding to the *PAI-1* promoter is critical for the PAI-1 induction under oxidative stress. *Antioxid. Redox Signal.* 13, 413–424.

Introduction

MANY PATHOLOGIC CONDITIONS such as atherosclerosis, pulmonary and renal diseases, cancer, and even diabetes mellitus are associated with hypoxia, formation of reactive oxygen species (ROS), and oxidative stress [recently reviewed by (35)]. The pathologies and undesired cellular functions caused by oxidative stress are usually the result of an abnormal intracellular signal transmission and the resulting inability of the cell to cope with varying oxygen tensions or oxidative stress. Interestingly, a number of redox-sensitive transcription factors, among them FOXO family members, appear to play an important role in the cellular resistance to oxidative stress.

FOXO transcription factors form a subgroup within the large family of Forkhead box O (or Fox) transcription factors, which plays a major role in the control of cellular proliferation, cell-cycle arrest, DNA repair, apoptosis, and oxidative stress. In humans, four FOXO family members have been described: FOXO1 (previously known as FHKR), FOXO3 (previously FKHRL1), FOXO4 (previously AFX), and FOXO6 [reviewed by (23)].

The activity of FOXO proteins is regulated primarily by an active PI3K-PKB/Akt pathway. Thereby, FOXOs are inactivated because of PKB/Akt-dependent phosphorylation at three highly conserved serine and threonine residues. The phosphorylation at these sites suppresses transactivation and promotes nuclear exclusion and degradation of FOXO proteins [reviewed by (3, 23, 41)]. In addition to PI3K and PKB/Akt, several other kinases and posttranslational modifications, such as ubiquitylation, sumoylation, and acetylation, contribute to the regulation of FOXO function [for review, see (3, 23, 41)]. Although the four known human FOXOs appear to be highly redundant in their function and to play an important role in the defense against oxidative stress, a number of details of how FOXO proteins are regulated by oxidative stress have been obtained by investigating FOXO4. On generation of ROS by treatment of cells with TNF-α or hydrogen peroxide (H_2O_2), FOXO4 undergoes phosphorylation at two Jun N-terminal kinase (JNK) phosphorylation sites (Thr447 and

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Thr451), which results in its translocation from the cytosol to the nucleus (17). In addition, monoubiquitylation of FOXO4 in response to increased oxidative stress contributes to an increase in its transcriptional activity (42) and the induction of a negative-feedback mechanism to counteract ROS by inducing genes, like *MnSOD* (32) and catalase (17).

A number of hormones, growth and coagulation factors, cytokines, and conditions generating oxidative stress were also shown to activate plasminogen activator inhibitor-1 (PAI-1) synthesis [for review, see (13)]. PAI-1 is the primary physiologic inhibitor of both the tissue-type and the urokinase-type plasminogen activators (tPA and uPA). In addition to its ability to inhibit uPA and tPA, PAI-1 also is involved in other molecular interactions (1). It is associated with the extracellular matrix, mainly through binding to vitronectin, and may therefore block cell adhesion because of interference between vitronectin and $\alpha_v\beta_3$ integrin or the uPA receptor (uPAR) (10). Further, PAI-1 was found to induce uPA-uPAR internalization, because the ternary PAI-1-uPA-uPAR complex can be cleared from the cell surface after binding to scavenger receptors from the low-density lipoprotein receptor–related protein family (LRP). All these molecular interactions suggest a highly complex scenario in which PAI-1 may not only affect fibrinolysis, pericellular proteolysis, and cell adhesion but may also be involved in initiating or modulating intracellular signalling [for review, see (2, 13)].

The clinical importance of PAI-1 is emphasized by numerous studies. As an inhibitor of fibrinolysis, decreased plasma PAI-1 levels cause bleeding complications (36), and a high plasma PAI-1 level is a risk factor for myocardial infarction and coronary heart disease, deep vein thrombosis, as well as acute and chronic inflammatory lung disorders (10). Additionally, enhanced plasma PAI-1 levels are found in patients with metabolic syndrome and type 2 diabetes mellitus (26, 37). Further, high levels of PAI-1 in tumor cells are associated with a poor prognosis in patients with breast tumors, and now PAI-1 has been shown to be one of the most informative biochemical prognostic markers in several cancer types (21).

We previously reported that the expression of PAI-1 can be enhanced by both hypoxia and insulin through the PI3K/PKB pathway, which suppresses FOXO activity, and that thereby the transcription factor hypoxia-inducible factor-1 (HIF-1) binds to hypoxia-response elements (HREs) in the PAI-1 gene promoter (12, 29). By contrast, oxidative stress that activates FOXOs appeared to have an additive effect on the insulin-dependent PAI-1 induction (43). Together, these findings implicate a mechanism allowing a positive response of *PAI-1* gene expression under conditions that would activate or deactivate FOXOs at the same time. Thus, we became interested in the mechanisms by which oxidative stress exerted by hydrogen peroxide or FOXOs or both, especially FOXO4, may have an impact on *PAI-1* gene expression under normoxia and hypoxia.

Materials and Methods

All biochemicals and enzymes were of analytic grade and were purchased from commercial suppliers.

Plasmids

The pGl3hPAI-806 plasmid, containing the human *PAI-1* promoter from ′806 to +19 and the E-box–mutated plasmids pGl3hPAI-806-M4, pGl3hPAI-806-M5, as well as the plasmid pGl3hPAI-806-HREm, with a mutation in the HIF-1 binding site were described (14). The reporter plasmids pCRE-Luc (Stratagene), pGl3-Epo-HRE-Luc (27), and pTK-IRE-Luc (20) contain multiple transcription factor–binding elements; the pCRE-Luc contains four cAMP-responsive elements; pGl3-EpoHRE-Luc contains three hypoxia-inducible factor–binding sites from the erythropoietin gene; and pTK-IRE-Luc contains three FOXO-binding elements from the glucose 6-phosphatase promoter. The plasmids pMT2-HA-FOXO4 and pcDNA3-HA-CREB-1, allowing expression of wild-type FOXO4 and CREB, respectively, were described (11, 39).

Cell culture, transient transfections, and luciferase assays

The human hepatoma cell line, HepG2, was propagated in MEM supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 1% nonessential amino acids (Invitrogen), and 0.5% antibiotics. Primary rat hepatocytes (PHCs) were isolated by collagenase perfusion as described (29) and cultured in medium M199 containing 0.5 n*M* insulin and 100 n*M* dexamethasone as permissive hormones and 5% fetal calf serum for the initial 5 h of culture. All cells were cultured under normoxia (16% O_2, 79% N_2, and 5% CO_2, by volume) or under hypoxia (5% O_2, 90% N_2, 5% CO_2, by volume) or both. H_2O_2 was applied to the cell-culture medium at a concentration of 50 μM unless otherwise indicated; for *PAI-1* Northern analyses, cells were cultured under normoxia and hypoxia for 4 h; for PAI-1 Western analyses, cells were cultured under normoxia and hypoxia for 8 h.

HepG2 cells ($\sim 4\times10^5$ per 60-mm dish) were transfected as described (29), thereby controlling transfection efficiency by cotransfection of 0.25 μg *Renilla luciferase* expression vector (pRL-SV40). In brief, 2.5 μg of the appropriate PAI-1 promoter *Firefly* luciferase (Luc) constructs were transfected together with 500 ng of FOXO4 expression vector or in the controls with 500 ng of the control vector. After 5 h, medium was changed, and cells were cultured under normoxia for 18 h. Then the medium was changed again, and cells were further cultured for 24 h under normoxia or hypoxia. Every culture experiment was performed in duplicate. HepG2 cells were harvested 48 h after transfection, and Luc activity from 20-μl cell lysate was recorded in a luminometer (Berthold) by using the dual luciferase assay kit (Promega).

RNA preparation and Northern analysis

Isolation of total RNA and Northern analysis were performed as described (29). Digoxigenin (DIG)-labelled antisense RNAs served as hybridization probes; they were generated by *in vitro* transcription from pBS-PAI-1 by using T3 RNA polymerase and from pBS-βactin by using T7 RNA polymerase and RNA labelling mixture containing 3.5 m*M* 11-DIG-UTP, 6.5 m*M* UTP, 10 m*M* GTP, 10 m*M* CTP, and 10 m*M* ATP. Hybridizations and detections were carried out essentially as described (29). Blots were quantified by using the ImageJ program (NCBI).

Western blot analyses

Western blot analyses were carried out as described (29). In brief, medium or lysates from cells were collected, and 100 μg

of protein was loaded onto 10% SDS-polyacrylamide gels and, after electrophoresis, blotted onto a nitrocellulose membrane. The primary antibody against human and rat PAI-1 (1:100) (American Diagnostics, Pfungstadt, Germany), as well as a primary mouse antibody human HIF-1α (1:1,000) (Novus, Littleton, CO), was used (27). The monoclonal CREB (Santa Cruz, Heidelberg, Germany), polyclonal phospho-CREB (Ser133) (Santa Cruz), polyclonal FOXO4 (Santa Cruz), and the monoclonal HA-tag (Santa Cruz) antibody were used in a dilution of 1:1,000, 1:1,000, 1:1,000, and 1:500, respectively. The human β-actin antibody (Sigma) was used in a dilution of 1:10,000. The secondary antibody was either an anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase (1:5,000; Biorad, Munich, Germany). The enhanced chemiluminescence (ECL) system (Amersham, Freiburg, Germany) was used for detection. Blots were quantified by using the ImageJ program (NCBI).

shRNA-mediated gene silencing

The specific human *CREB-1* sequence 5′-ATACAGCT GGCTAACAATGG-3′ (8) was used to generate the CREB-1 shRNA expressing pSuper vector (Oligo Engine) according to the manufacturer's instructions. Annealed FoxO4 shRNA oligonucleotides with the sequence 5′-ACAAGGGTGACAG CAAC-3′ were cloned into the *Mlu*1 and *Cla*1 restriction sites of the vector pLVTHM (45). The oligonucleotide with the sequence 5′-GCACGTTAAGTGCTACACA-3′ was used to generate a scrambles shRNA control. Lentiviral particles expressing the respective shRNAs were generated by transfecting the three different plasmids into HEK 293T cells. These plasmids are the pLVTHM vector carrying the oligonucleotides for shRNA, the pMD2G vector encoding the envelope glycoprotein, and the second-generation packaging plasmid psPAX2 (45). The expression of shRNA was verified in HEK cells, and the multiplicity of infection (MOI) was determined by obtaining the optimal degree of target-gene knockdown. HepG2 cells were prepared as described earlier, and after 5 h, the fresh medium was replaced and infected with lentiviral vectors at $\sim$40 MOI for 14 h. After 14 h, the cells were washed twice with PBS, and fresh medium was given for $\leq$24 h, depending on the experiment.

Chromatin immunoprecipitation

ChIP analyses were carried out as described (30). In brief, confluent cells were crosslinked with formaldehyde, lysed, and sonicated to obtain DNA fragments in a size from 500 to 1,000 base pairs. Chromatin was then precipitated overnight with either preimmune serum, 3 μg FOXO4, or CREB antibody (Santa Cruz Biotechnology), respectively, in a total volume of 1 ml at 4°C. DNA from ChIP was analyzed by quantitative PCR by using Taqman Gene Expression Master Mix (ABI). PCR was performed with primers that amplify a 174-bp region of the human PAI-1 promoter containing the HIF-1–binding HRE (−194/−187) with the following primers [forward (−274/−265), 5′-ggcagagggcagaaaggtca-3′; and reverse (−120/−103), 5′-tgaacagccagcgggtcc-3′) at 58°C for 30 cycles. The mRNA levels of β-actin were measured by PCR for normalization and amplification of soluble chromatin before immunoprecipitation was used as an input control.

Results

Hydrogen peroxide induces PAI-1 gene expression

To investigate whether mild oxidative stress regulates PAI-1 expression, HepG2 cells were incubated with 50 μM H_2O_2 under normoxia (16%O_2) and hypoxia (5%O_2), respectively, and PAI-1 expression was measured with Northern and Western blotting. Hypoxia induced *PAI-1* mRNA expression about 3-fold, in line with previous studies (14, 18, 29). Interestingly, treatment of cells with 50 μM H_2O_2 for 4 h induced *PAI-1* mRNA by about 2-fold under normoxia. Although the *PAI-1* expression was induced by about 4-fold under hypoxia when compared with the normoxic control, the hypoxia-induced PAI-1 expression was quenched by H_2O_2 because the fold induction with respect to the hypoxic control was lower with peroxide (Fig. 1A and B). Further, the increase in PAI-1 mRNA mediated by hypoxia and H_2O_2 was followed by an increase in PAI-1 protein levels (Fig. 1A and B). Thus, mild oxidative stress exerted by H_2O_2 can induce PAI-1 expression primarily under normoxia.

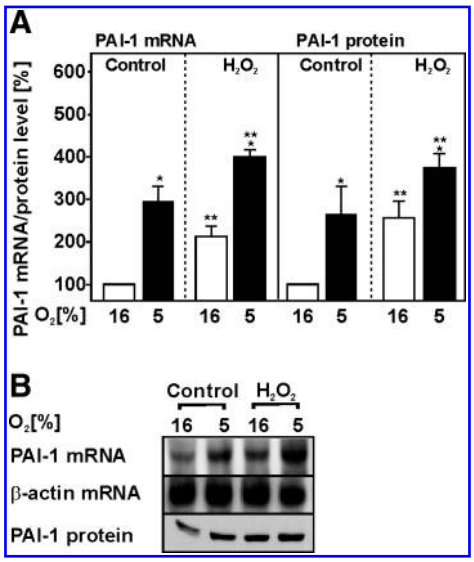


FIG. 1. H_2O_2 induces PAI-1 mRNA and protein expression. HepG2 cells or primary rat hepatocytes were cultured under normoxia (16% O_2) for 24 h and then treated with or without 50 μM H_2O_2 and further cultured under normoxia and hypoxia (5% O_2) for 4 h (mRNA) or 8 h (protein). (**A**) The PAI-1 mRNA expression and protein levels measured with Northern and Western blots, respectively, under normoxia (16% O_2) without H_2O_2 were set to 100%. Values represent mean $\pm$ SEM of three independent experiments. Statistics: Student's *t* test for paired values: *significant difference 16% O_2 versus 5% O_2; **significant difference 16% O_2 versus 16% $O_2 + H_2O_2$ and 5% O_2 versus 5% $O_2 + H_2O_2$; $p \leq 0.05$. (**B**) Representative Northern and Western blots: 20 μg of total RNA isolated from cultured hepatocytes was hybridized with DIG-labelled *PAI-1* and β-actin antisense RNA probes. Then 100 μg of protein from the culture medium was subjected to Western blotting with an antibody against PAI-1. Autoradiographic signals were detected by chemiluminescence and quantified by Image J (NCBI).

FOXO4 is critically involved in the H_2O_2-dependent induction of PAI-1

Previously, the triple FOXO1/3/4 knockout mouse studies (34) indicated that the FOXOs are largely redundant in their function. Therefore, we tested whether FOXO1, FOXO3, and FOXO4 have an impact on *PAI-1* transcription. To examine effects of FOXOs, we performed cotransfection studies by using FOXO1, −3, and −4 expression vectors and luciferase reporter gene constructs containing the human *PAI-1* promoter (-pGL3hPAI-806/+19) or the insulin-response element (IRE) from the glucose 6-phosphatase gene (IRE-TK-Luc), which contains several FoxO-binding sites and is known to respond positively to FoxO1 (20). We found that cotransfection of the *PAI-1* promoter Luc construct with expression vectors for FOXO1 had no effect on Luc activity, although FOXO1 induced the IRE-TK-Luc construct by ~20-fold in the same experiments. Although cotransfection of the PAI-1 promoter constructs with FOXO3 expression vectors induced Luc activity by about 3-fold, the cotransfection of FOXO4 exerted the most robust induction and increased the *PAI-1* promoter by ~10-fold (Fig. 2A). These results indicate that FOXO4 exerts the most dominant effect on the PAI-1 promoter and may thus play an important role in the regulation of PAI-1 by oxidative stress.

Therefore, we asked whether knocking down FOXO4 would counteract the effect of H_2O_2 on PAI-1 expression. To address this issue, HepG2 cells were infected with lentiviral particles expressing scrambled control shRNA or FOXO4 shRNA. Initial studies confirmed that lentiviruses expressing FOXO4 shRNA (but not a scrambled shRNA) reduce protein levels of FOXO4 in HepG2 cells by >80% (Fig. 2B and C). The control shRNA did also not have an effect on basal PAI-1 protein levels nor did it disrupt the effect of hypoxia and H_2O_2 on PAI-1 expression, whereas the transduction of cells with the FOXO4 shRNA-expressing virus slightly decreased basal PAI-1 levels but did not interfere with the induction by hypoxia (Fig. 2B and C). By contrast, the expression of FOXO4 shRNA disrupted both the effect of H_2O_2 and the induction of PAI-1 by hypoxia (Fig. 2C and D). Together, these results indicate that FOXO4 contributes to PAI-1 expression and is required for H_2O_2 to induce PAI-1 expression.

The hypoxia-responsive element in the PAI-1 promoter mediates the induction by H_2O_2 and FOXO4

To explore further the mechanisms by which H_2O_2 and FOXO4 modulate PAI-1 expression, we considered that H_2O_2 or FOXO4 may interfere directly with the activity of the PAI-1 promoter and performed functional luciferase (Luc) reporter gene analyses in HepG2 cells. Given the importance of the HRE in the hypoxia-dependent *PAI-1* gene expression (14, 18, 29), we included not only the wild-type human *PAI-1* promoter construct (pGl3PAI-806/+19) but also *PAI-1* promoter constructs that were mutated at the HRE (pGl3hPAI-806-HREm) and for comparison at the E-boxes 4 (pGl3hPAI-806-M4) and 5 (pGl3hPAI-806-M5). Hypoxia increased Luc activity in cells transfected with the wild-type human *PAI-1* promoter construct (pGl3PAI-806/+19), and mutation of the HRE in the construct pGl3hPAI-806-HREm abolished the hypoxic response in line with previous studies (11, 14, 18, 29). Treatment of cells with H_2O_2 increased Luc activity by about 2-fold under normoxia and by about 3-fold under hypoxia (Fig. 3A).

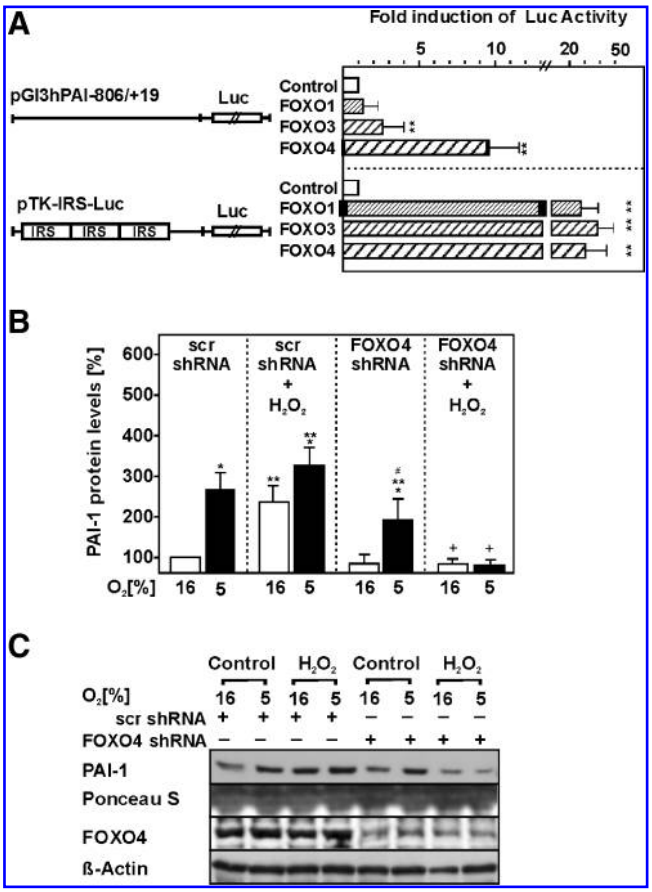


FIG. 2. The H_2O_2-dependent induction of *PAI-1* gene expression can be disrupted by FOXO4 silencing. (**A**) HepG2 cells were cotransfected with FOXO expression vectors or in the controls with empty vector and Luc gene constructs driven by the wild-type human PAI-1 promoter (pGL3-hPAI-806) or the FOXO-responsive pTK-IRS-Luc. The Luc activities were expressed as fold induction compared with the Luc activity, measured in the respective controls. Values represent mean SEM of three independent experiments, each performed in duplicate. Statistics, Student's *t* test for paired values: **significant difference, control versus FOXO; $p \leq 0.05$. (**B**) Cells were infected with lentiviral vectors at ~40 MOI for 14 h along with polybrene at a concentration of 1 μg/ml of medium. Then the medium was changed, and cells were exposed to H_2O_2 (50 μM) under normoxia and hypoxia for 8 h before Western blot analysis by using antibodies against PAI-1 or FOXO4. The PAI-1 protein levels measured under normoxia (16% O_2) without H_2O_2 were set to 100%. Values represent mean ± SEM of three independent experiments. Statistics, Student's *t* test for paired values: *significant difference, 16% O_2 versus 5% O_2; **significant difference, 16% O_2 versus 16% $O_2 + H_2O_2$ and 5% O_2 versus 5% $O_2 + H_2O_2$; #significant difference, 16% O_2 versus 16% O_2 + shFOXO4 and 5% O_2 versus 5% O_2 + FOXO4; +significant difference, 16% $O_2 + H_2O_2$ versus 16% $O_2 + H_2O_2$ + FOXO4 shRNA and 5% $O_2 + H_2O_2$ versus 5% $O_2 + H_2O_2$ + FOXO4 shRNA; $p \leq 0.05$; (**C**) Representative Western blots: 100 μg of protein from the culture medium was subjected to Western blotting with an antibody against PAI-1, FOXO4, and β-actin. Autoradiographic signals were detected with chemiluminescence and quantified with Image J (NCBI).

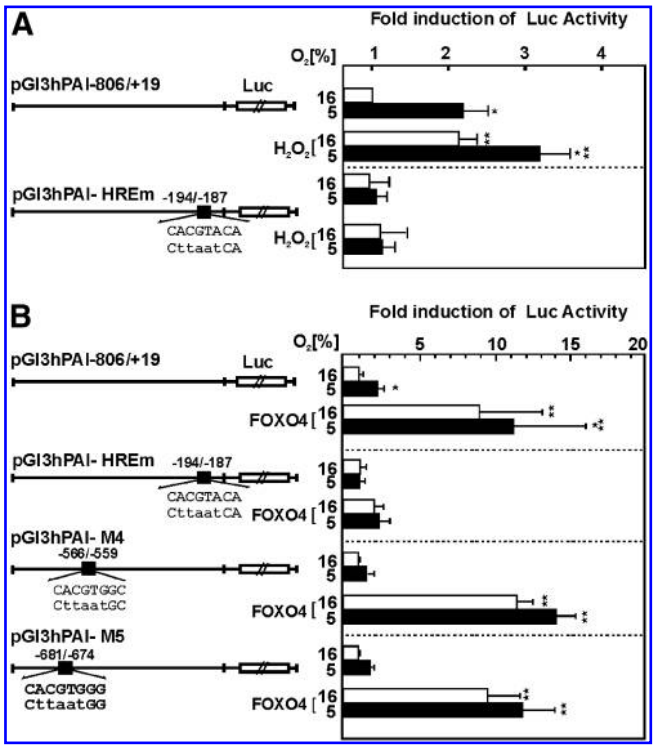


FIG. 3. The activation of the *PAI-1* promoter by H_2O_2 and FOXO4 is mediated by the hypoxia-responsive element (HRE). (**A**) HepG2 cells were transfected with Luc gene constructs driven by the wild-type human PAI-1 promoter (pGL3-hPAI-806) or the human PAI-1 promoter mutated at the HRE. After transfection, cells were cultured for an additional 16 h, and then they were treated with or without H_2O_2 (50 μM) and further cultured under normoxia (16% O_2) or hypoxia (5% O_2) for 8 h. (**B**) Cells were cotransfected with the FOXO4 expression vector or in controls empty vector and the wild-type human *PAI-1* promoter Luc construct (pGL3-hPAI-806) or respective promoter constructs mutated at the HRE, E4, or E5. The Luc activities were expressed as fold induction compared with the Luc activity, measured in the respective controls. Values represent mean ± SEM of three independent experiments, each performed in duplicate. Statistics, Student's *t* test for paired values: *significant difference, 16% O_2 versus 5% O_2, **significant difference, 16% O_2 versus 16% $O_2 + H_2O_2$ or FOXO4 and 5% O_2 versus 5% O_2 H_2O_2 or FOXO4; $p \leq 0.05$.

Interestingly, the ability of H_2O_2 to induce *PAI-1* promoter activity was disrupted completely by mutation of the HRE (Fig. 3A) but not with the other mutants (data not shown), indicating that this site is required for activation of the *PAI-1* promoter in response to H_2O_2.

When the wild-type human *PAI-1* promoter Luc construct (pGl3PAI-806/+19) was cotransfected with an expression vector for FOXO4, we found that FOXO4 could strongly transactivate the *PAI-1* promoter. The FOXO4-dependent induction of the *PAI-1* promoter was completely abolished on mutation of the HRE in pGl3hPAI-HREm. By contrast, Luc activity remained unaffected when the E-box–mutated constructs pGl3hPAI-M4 and pGl3hPAI-M5 were cotransfected with FOXO4 (Fig. 3B). Together, these data support the concept that H_2O_2 and FOXO4 act like hypoxia through the HRE within the human *PAI-1* promoter.

H_2O_2 and FOXO4 modulate the function and protein levels of HIF-1α and CREB

We previously reported that the HRE within the *PAI-1* promoter serves as a high-affinity binding site for hypoxia-inducible factor-1 (HIF-1) (14, 29). Thereby, stabilization of the HIF-1α subunit under hypoxia contributes to the formation of the HIF-1 dimer and permits the increased expression of PAI-1 under hypoxia. In addition, under normoxia (*i.e.*, in the absence of HIF-1α), the HRE can also be bound by the cAMP-responsive element–binding protein (CREB), which mediates the effects of enhanced cAMP levels (11). Accordingly, we asked whether H_2O_2 and FOXO4 affect the activity and protein levels of HIF-1α and CREB in HepG2 cells under normoxia and hypoxia.

To examine effects of H_2O_2 on FOXO4, HIF-1, and CREB function, we performed transfection studies by using luciferase reporter gene constructs containing either multiple FOXO-binding elements (IRS) from the glucose 6-phosphatase gene (TK-IRS-Luc), HIF-binding elements (HRE) from the erythropoietin gene (EPO-HRE-Luc), or cAMP-responsive elements from the somatostatin gene (CRE-Luc). Although hypoxia had no influence on Luc activity in TK-IRS-Luc transfected cells, treatment with H_2O_2 resulted in an enhancement of Luc activity by about 4-fold. By contrast, in EPO-HRE-Luc–transfected cells, H_2O_2 repressed Luc activity by ~50% under hypoxia. Further, H_2O_2 increased Luc activity in pCRE-Luc–transfected cells by about 3-fold under normoxia and hypoxia (Fig. 4A).

Next, we examined whether H_2O_2 has an impact on the HIF-1α, CREB, and FOXO4 protein levels. The FOXO4 protein levels were not affected under hypoxia or on H_2O_2 treatment, consistent with the results of reporter gene studies previously described and in line with previous studies indicating that H_2O_2 increases FOXO4 activity (17, 42). Treatment of cells with H_2O_2 reduced the HIF-1α protein levels by about twofold under normoxia and hypoxia, whereas the CREB protein levels were induced under hypoxia and on treatment with H_2O_2 (Fig. 4B and C).

Next, we asked whether FOXO4 itself can influence HIF-1α and CREB protein levels under normoxia and hypoxia. As expected, we found that hypoxia induced HIF-1α protein levels in HepG2 cells, but overexpression of FOXO4 decreased HIF-1α levels and especially disrupted the hypoxia-dependent induction of HIF-1α (Fig. 4D and E). By contrast, FOXO4 overexpression induced CREB protein levels to about the same levels under normoxia and hypoxia (Fig. 4D and E). Together, these findings indicate that H_2O_2 and FOXO4 downregulate HIF-1α protein levels, whereas at the same time, CREB protein levels and activity are induced.

CREB mediates the FOXO4-dependent induction of PAI-1 expression

The obtained results suggest that FOXO4 is crucially involved in the H_2O_2-dependent PAI-1 gene expression through downregulation of HIF-1 and induction of CREB activities. Because CREB can bind to the HRE, this would suggest that CREB might be the proper factor mediating the induction of PAI-1 by interacting with the *PAI-1* promoter. To address this issue, we examined the expression of PAI-1 in response to FOXO4 while knocking down CREB. We found that hypoxia enhanced PAI-1 levels by about threefold, and overexpression

of FOXO4 induced PAI-1 by about 2.5-fold under normoxia and by about fourfold under hypoxia (Fig. 5A and B). The FOXO4-mediated increase of PAI-1 expression was disrupted by knocking down CREB after transfection of HepG2 cells with a CREB shRNA-expressing vector (Fig. 5A and B).

Further, CREB shRNA reduced *PAI-1* promoter activity by ~20%, in line with previous studies indicating that CREB activates PAI-1 expression. However, the CREB shRNA did not influence the inducibility by hypoxia. Overexpression of FOXO4 induced the *PAI-1* promoter by about 8-fold and 12-fold under normoxia and hypoxia, respectively. Again, this induction of the PAI-1 promoter by FOXO4 was disrupted by knocking down CREB. Importantly, neither the basal activity of the IRE-TK-Luc construct nor its FOXO4-dependent induction was affected by CREB shRNA (Fig. 5C). Thus, these findings indicate that FOXO4 modulates PAI-1 gene expression through CREB.

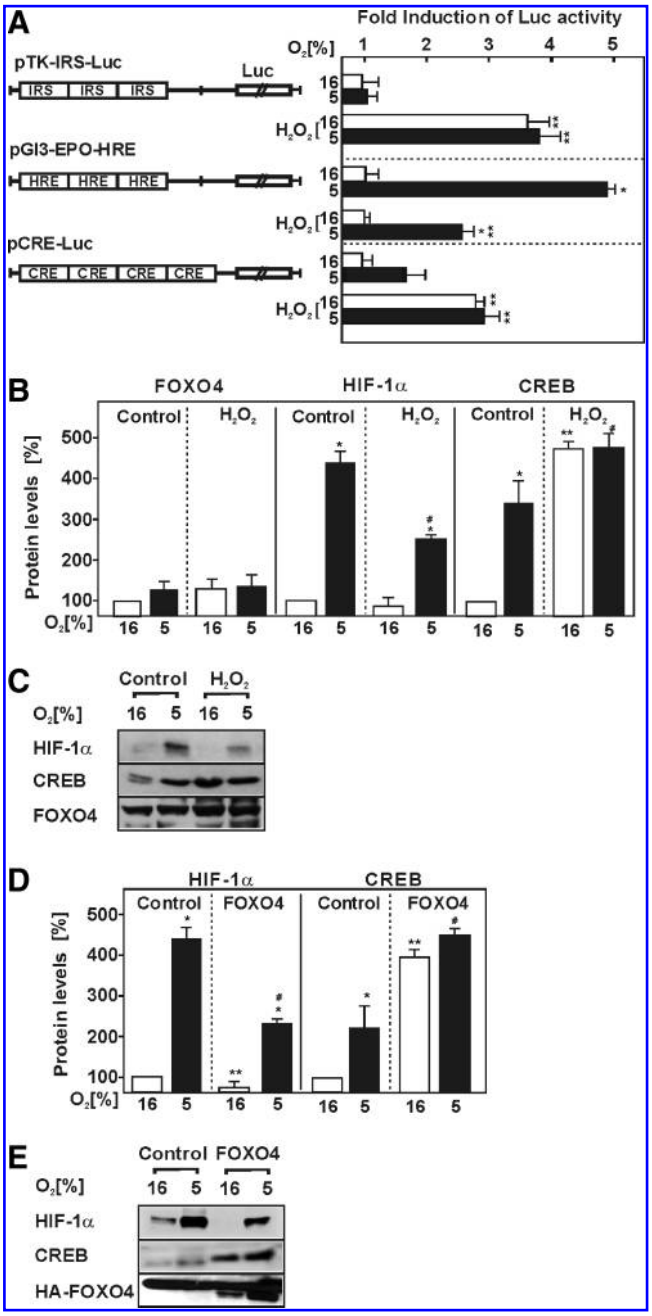


FIG. 4. H_2O_2 and FOXO4 modulate HIF-1α and CREB function. (**A**) HepG2 cells were transfected with the FOXO-responsive pTK-IRS-Luc, the HIF-responsive pGl3-EPO-HRE, and the CREB-responsive pCRE-Luc constructs and cultured in normoxia (16% O_2) or hypoxia (5% O_2) either with or without H_2O_2 (50 μM) for 8 h before luciferase assay. The Luc activities were expressed as fold induction compared with the Luc activity, measured in the respective controls. Values are expressed as mean ± SEM of three independent experiments. *Significant difference, 16% O_2 versus 5% O_2, and **significant difference, 16% O_2 versus 16% $O_2 + H_2O_2$ and 5% O_2 versus 5% $O_2 + H_2O_2$, $p \leq 0.05$. (**B**) HepG2 cells were treated with or without H_2O_2 and cultured under normoxia (16%) and hypoxia (5%) for 8 h. The HIF-1α, CREB, and FOXO4 protein levels were measured with Western blot analysis, and control protein levels under normoxia were set to 100%. Values are expressed as mean ± SEM of three independent experiments. *Significant difference, 16% O_2 versus 5% O_2; **significant difference, 16% O_2 versus 16% $O_2 + H_2O_2$ and # 5% O_2 versus 5% $O_2 + H_2O_2$, $p \leq 0.05$. (**C**) Representative Western blots. Proteins were detected with Western blot analysis with the HIF-1α, CREB, and FOXO4 antibodies. (**D**) HepG2 cells were transfected with expression vectors for FOXO4 and cultured under normoxia and hypoxia for 4 h. The HIF-1α and CREB protein levels were measured with Western blot analysis, and protein levels under normoxia were set to 100%. Values are expressed as mean ± SEM of three independent experiments. *Significant difference, 16% O_2 versus 5% O_2; **significant difference 16% O_2 versus 16% O_2 + FOXO4; and #5% O_2 versus 5% O_2 + FOXO4, $p \leq 0.05$. (**E**) Representative Western blots. Total cell proteins were detected with Western blot analysis with the HIF-1α, CREB, and HA-tag antibodies.

CREB, but not FOXO4, binds to the HRE within the human PAI-1 promoter

To analyze whether the FOXO4 effects on *PAI-1* gene expression are the result of a direct interaction of CREB with the *PAI-1* promoter, we performed chromatin immunoprecipitation (ChIP) assays with chromatin fragments prepared from control cells and cells overexpressing FOXO4. The ChIP was then performed with unspecific IgG or antibodies against either FOXO4 or CREB and PCR primers, which allowed amplification of the *PAI-1* promoter containing the HRE. The PCR after immunopreciptiation with antibodies against FOXO4 and CREB confirmed that CREB but not FOXO4 associates with this region of the PAI-1 promoter (Fig. 6A).

Next, we performed quantitative ChIP assays to determine whether overexpression of FOXO4 enhances the recruitment of CREB and reduces the recruitment of HIF-1α to the *PAI-1* promoter. Quantitative PCR after immunoprecipitation with antibodies against CREB demonstrated that overexpression of FOXO4 increases the association of CREB with the *PAI-1* promoter by about 3-fold (Fig. 6B). This positive effect of FOXO4 on CREB binding could then be counteracted by knocking down CREB, which reversed the effect of FOXO4 and decreased the binding of CREB (Fig. 6B). When HIF-1α was precipitated from cells cultured under hypoxia, it was found that overexpression of FOXO4 reduced binding of HIF-1α to the *PAI-1* promoter by ~50%, and this reduced binding

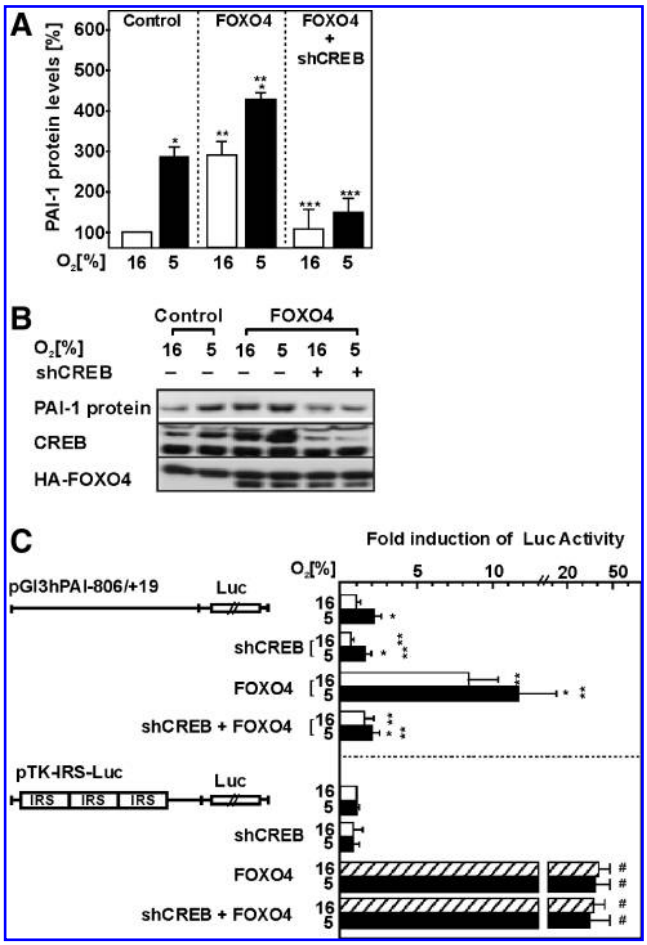


FIG. 5. FOXO4 induces PAI-1 expression through CREB. HepG2 cells were cotransfected with the FOXO4 expression vector and vectors for control shRNA or shRNA against CREB and cultured for 48 h under normoxia. Then cells were further cultured under normoxia (16% O_2) or hypoxia (5% O_2) for the next 24 h before Western blot analysis. (**A**) The PAI-1 protein levels under normoxia were set to 100%. Values represent mean ± SEM of three independent experiments. Statistics: Student's *t* test for paired values: *significant difference 16% O_2 versus 5% O_2; **significant difference 16% O_2 versus 16% O_2 + FOXO4 and 5% O_2 versus 5% O_2 + FOXO4; ***significant difference, 16% O_2 + FOXO4 versus 16% O_2 + FOXO4 + shCREB and 5% O_2 + FOXO4 versus 5% O_2 + FOXO4 + shCREB; $p \leq 0.05$. (**B**) Representative Western blot: 100 μg of total protein from HepG2 cells was analyzed by using antibodies against PAI-1, CREB-1, and HA-tag. (**C**) HepG2 cells were cotransfected with FOXO4 vectors, CREB shRNA expression vectors, or in the controls, with empty vector and scrambled shRNA and Luc gene constructs driven by the wild-type human *PAI-1* promoter (pGL3-hPAI-806) or the FOXO-responsive pTK-IRS-Luc. The Luc activities were expressed as fold induction compared with the Luc activity, measured in the respective controls. Values represent mean ± SEM of three independent experiments, each performed in duplicate. Statistics: Student's *t* test for paired values: *significant difference, 16% O_2 versus 5% O_2; **significant difference control versus FOXO4 and shCREB versus control, #FOXO4, FOXO4 + shCREB versus the respective control; $p \leq 0.05$.

was not counterregulated by CREB shRNA. Overexpression of CREB shRNA alone also did not affect recruitment of HIF-1α to the PAI-1 promoter. Further, when we performed ChIP analyses for the PAI-1 promoter after cells have been treated with H_2O_2 we showed that peroxide induced CREB binding. By contrast, less CREB was bound to the PAI-1–promoter fragment when peroxide-treated cells expressed FOXO4 shRNA (Fig. 6C).

Taken together, these results indicate that FOXO4 acts through an indirect mechanism not requiring its own binding to the PAI-1 promoter. Thereby, FOXO4 or oxidative stress decreases levels of HIF-1α and its recruitment to the PAI-1 promoter, while CREB expression and recruitment of CREB to its binding site in the *PAI-1* promoter are induced.

Discussion

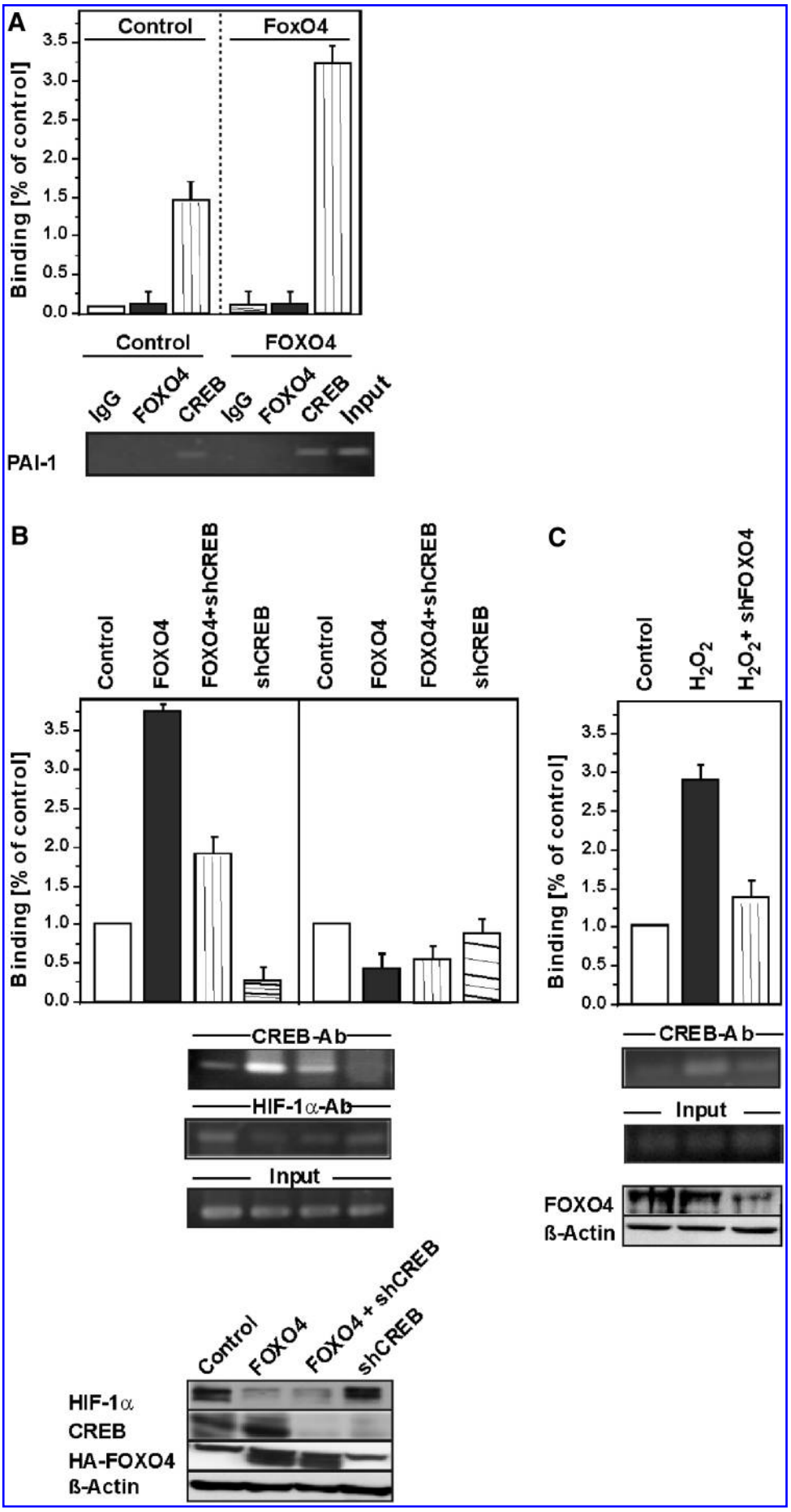
In this study, we investigated the regulation of PAI-1 expression in response to mild oxidative stress exerted by H_2O_2 under normoxia and hypoxia and identified a novel mechanism by which H_2O_2 can induce PAI-1 expression (*i.e.*, by modulating the function of FOXO4. FOXO4 in turn, acted indirectly by downregulation of HIF-1α and simultaneous upregulation of CREB, thus indicating that CREB is an FOXO4 target. The H_2O_2- and FOXO4-induced CREB proteins were then recruited to the *PAI-1* promoter.

FOXO transcription factors as mediators of oxidative stress

Although previous reports indicated that FOXOs are largely redundant in their function (34), our study indicated involvement of FOXO4 in the PAI-1 regulation by oxidative stress. This may be of importance for a number of processes, especially associated with high PAI-1 levels, but also for cell-growth or cell-death pathways, which are also influenced by FOXO-mediated oxidative-stress resistance. It was found that β-catenin can directly interact with FoxO proteins and enhance their transcriptional activity (16). This interaction is enhanced in cells exposed to oxidative stress, and because β-catenin possesses important regulatory functions for developmental processes or tumorigenesis, this may constitute a molecular bridge linking PAI-1 expression with tumorigenesis.

The link between FoxO proteins and oxidative stress appears to be evolutionarily conserved, because in yeast, worms, and *Drosophila*, oxidative stress causes nuclear relocalization of FoxOs, the induction of stress-defense genes like superoxide dismutase (*SOD*), and an increase in life span (41). In addition, combined deficiency of FoxO1, −3, −4 in mouse hematopoietic stem cells reduced ROS scavenging, leading to higher levels of ROS (40). Importantly, production of ROS in FoxO-deficient hematopoietic stem cells correlated with changes in the expression of genes that regulate their synthesis. Thus, members of the FoxO family function as a key component in the response to oxidative stress.

In the present study, the role of FOXO4 in PAI-1 gene regulation in response to mild oxidative stress is further underlined by the fact that knocking down FOXO4 abolishes the H_2O_2-mediated PAI-1 induction. The response of FOXO4 to changes in ROS concentrations appears to be transmitted through the cysteine residues within the protein, which are then involved in the formation of cysteine-thiol disulfide–dependent complexes between FOXO4 and the p300/CBP


A
Control
FoxO4
Binding [% of control]
3.5
3.0
2.5
2.0
1.5
1.0
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Control
FOXO4
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FOXO4
CREB
IgG
FOXO4
CREB
Input
PAI-1
B
Control
FOXO4
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shCREB
Control
FOXO4
FOXO4+shCREB
shCREB
Binding [% of control]
CREB-Ab
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acetyltransferase (9). Moreover, it has been shown that FOXO4 becomes monoubiquitylated on exposure of cells to H_2O_2, which results in its nuclear retention and an increase in its activity (42), whereas FOXO1 and FOXO3a are polyubiquitylated, which in turn initiates their degradation (22). In our study, this scenario is reflected by the enhancement of reporter gene activity from the FOXO-dependent luciferase reporter gene construct (IRS-TK-Luc) after treatment of cells with H_2O_2, which indicates the increase in FOXO4 activity.

In addition to FOXO4, other FOXO family members appear to act on *PAI-1* gene expression by indirect mechanism(s). We previously reported that FOXO1 acts rather indirectly on the *PAI-1* promoter in HepG2 cells (12), in line with other studies, additionally showing that FoxO3a and FoxC1 were indirectly involved in the induction of PAI-1 by insulin in GH4 cells (25, 43). By contrast, murine FoxC2 has been demonstrated to transactivate the *PAI-1* promoter in bovine arterial endothelial cells through direct binding to the promoter region between −736/−723 and −77/−46 (19). In addition, a recent article published while this article was in preparation indicated direct binding of FoxO3a to the *PAI-1* promoter region containing the proximal promoter region (−52/−43) and its involvement in the response to insulin in GH4 cells (25). Thus, FOXO proteins appear to be important for PAI-1 expression and, depending on the stimulus, different FOXO proteins may contribute by indirect or direct mechanisms.

ROS-dependent modulation of HIF-1α

Our studies indicated that H_2O_2 and FOXO4 decreased the level of the hypoxia-sensitive HIF-1α protein. Because the formation of ROS requires molecular oxygen, it has been suggested that ROS may be involved in the response to varying oxygen tensions. Several groups, including our own, previously reported that formation of ROS is decreased under hypoxia, whereas others reported that ROS are increased under hypoxia [for review, see (28)]. Earlier findings, consistent with the obtained data from the present study and the hypothesis that hypoxia decreased ROS, showed that the addition of H_2O_2 to cells grown under hypoxia resulted in destabilization of the HIF-1α protein in Hep3B cells (24) and of the HIF-2α protein in HeLa cells (44). Similarly, the xanthine/xanthine oxidase system decreased hypoxia-induced HIF-1α protein levels, and extracellular SOD decreased $CoCl_2$-induced HIF-1α levels in Hep3B cells [for review, see (28)]. By contrast, overexpression of Cu/Zn SOD promoted formation of H_2O_2 and increased HIF-1α levels in vascular cells (5). In addition, cell-permeable SOD mimetics enhanced HIF-1α levels under normoxia in rat renal medullary interstitial cells and human cerebral vascular smooth muscle cells [for review, see (28)]. Further, increased HIF-1α protein levels were found in H_2O_2-treated aortic and pulmonary artery smooth muscle cells (6) and Hep3B cells (7). At first glance, these studies were controversial; however, they clearly showed that a variation in ROS levels, which may be generated within different cellular compartments [for review, see (28, 31)], may contribute to the regulation of HIF-1α; therefore, the sensitivity of the HIF system to ROS may depend on the metabolic status, a threshold ROS concentration, the severity and duration of the hypoxic period, and the cell type [for review, see (28, 31)]. In addition, the sources of ROS generation, the exact kinetics, and the conditions of ROS production are not completely resolved; however, the data of the present study along with the previous findings support the concept that a modulation of ROS levels and also FOXO4 activity has an important impact on HIF-1α and the expression of PAI-1.

We found that FOXO4 downregulated HIF-1α protein levels, which is in line with an earlier study showing that FOXO4 decreased HIF-1α protein stability through a VHL-independent pathway involving the proteasome (39). Actually, FOXO4 appears not to be the only FOXO family member that acts on HIF-1α. FOXO3a has been shown to interfere with the C-terminal TAD of HIF-1α in a PTEN- and PI3K-dependent manner, resulting in an inhibition of the p300-dependent HIF-1 transcriptional activity (15). Intriguingly, FOXO3a did not affect protein stability, whereas the FOXO4 effects observed in our study clearly showed a downregulation of HIF-1α protein levels. Additionally, it was shown that the FOXO4-mediated downregulation of HIF-1α is responsible for the inhibition of the hypoxia-dependent induction of *VEGF*, *EPO*, and *GLUT-1* expression (39). By contrast, our study, in the context of PAI-1, shows that the FOXO4-dependent reduction in HIF-1α levels can be compensated for by enhanced CREB levels. This would allow an enhanced PAI-1 expression under conditions associated with oxidative stress or hypoxia, such as during tumorangiogenesis in which HIF-1 is required. Thus PAI-1 would be necessary to maintain the growth advantage of tumor cells, and these findings correlate with the possibility that high PAI-1 levels in tumors could be a marker for poor prognosis.

CREB: an old factor with a new function in the redox-dependent PAI-1 expression

Our results indicate for the first time that CREB is a FOXO target, because H_2O_2- and FOXO4-enhanced CREB levels

FIG. 6. FOXO4 increases recruitment of CREB to its binding site within the *PAI-1* promoter. (**A**) Chromatin fragments were immunoprecipitated with anti-FOXO4, anti-CREB, or control antibodies (nonspecific IgG) from cross-linked control cells or cells overexpressing FOXO4. Quantitative PCR was performed with primers for the *PAI-1* promoter fragment containing the HRE, as outlined in Materials and Methods. Amplification of soluble chromatin before immunoprecipitation was used as an input control. (**B**) Chromatin was precipitated from cells transfected with FOXO4 expression vectors and CREB shRNA expression vectors, and quantitative ChIP assays were performed by using the CREB antibody and PCR primers for the *PAI-1* promoter fragment containing the HRE. When performing the ChIP assays with the HIF-1α antibody, chromatin was precipitated from cells cultured under hypoxia for 4 h. Amplification of soluble chromatin before immunoprecipitation was used as an input control. Western blot analyses with antibodies against HIF-1α, CREB, FOXO4, and β-actin were performed to show the knockdown efficiency. (**C**) Chromatin was precipitated from H_2O_2-treated cells infected with scrambled or FOXO4 shRNA vectors, and quantitative ChIP assays were performed by using the CREB antibody and PCR primers for the *PAI-1* promoter fragment containing the HRE. Amplification of soluble chromatin before immunoprecipitation was used as an input control. Western blot analyses with antibodies against FOXO4 and β-actin were performed to show the knockdown.

were antagonized by FOXO4 shRNA. The involvement of CREB in the oxidative-stress response in general, and in the PAI-1 gene regulation under stress in particular, is a new concept that may have implications for several pathologic events associated with proliferation and apoptosis. Whether this induction involves a FOXO4-dependent action at the *CREB* promoter or involves CREB stability remains open and will be addressed in future studies. We also found that CREB levels were upregulated in response to hypoxia, in line with previous studies (4). The involvement of CREB in the PAI-1 response to H_2O_2 links oxidative stress and FOXO4 through CREB to the cAMP pathway, which has been shown to contribute to inhibition of cell proliferation in a variety of cell lines [for review, see (38)]. It was shown that cAMP can suppress cyclin D1 and induce the cell-cycle inhibitor p27, thereby arresting the cells in the G_1 phase. The antiproliferative effect of cAMP was then due to a transcriptional activation of FoxO4 (33). In addition, our own studies showed that enhanced cAMP levels caused by glucagon can induce PAI-1 expression through CREB (11). Thus, the induction of PAI-1 by CREB possibly represents a feedback during regeneration processes, proliferation, or diabetes, in which insulin levels are low, but ROS and cAMP levels are increased, indicating that the CREB-mediated PAI-1 induction may contribute to angiopathies occurring during diabetes.

Based on the findings of our study, we propose the following model by which oxidative stress may regulate PAI-1 expression (Fig. 7). Under hypoxic conditions, the HIF-1α/HIF-1β (ARNT) dimer binds to the *PAI-1* promoter at the HRE. Oxidative stress activates FOXO4, which in turn induces CREB expression and contributes to the VHL-independent proteasomal degradation of HIF-1α. At the same time, the FOXO4-induced CREB is recruited to the so-far unbound HRE of the proximal *PAI-1* promoter, where it again promotes induction of PAI-1. Taken together, our study extends our understanding of the mechanisms mediating effects of oxidative stress and shows that the response of the *PAI-1* gene to H_2O_2 involves the cooperative action of the two transcription factors, FOXO4 and CREB.

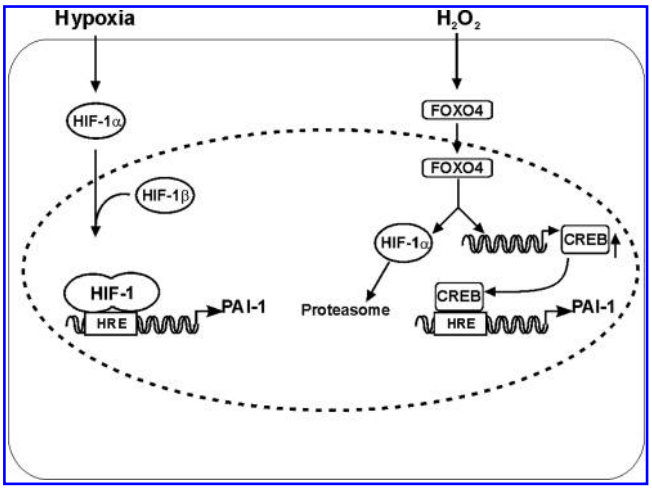

FIG. 7. Model of PAI-1 regulation in response to oxidative stress. Hypoxia induces binding of the HIF-1α/HIF-1β (ARNT) dimer to the PAI-1 promoter at the HRE. Oxidative stress activates FOXO4, which then induces CREB expression and contributes to the VHL-independent proteasomal degradation of HIF-1α and the release of HIF-1 from the HRE. At the same time, the FOXO4-induced CREB is recruited to the unbound HRE of the PAI-1 promoter, where it again induces PAI-1 expression.

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Author Disclosure Statement

The authors declare no competing financial interests.

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Abbreviations Used

CREB = cAMP-responsive element–binding protein
FOXO = Forkhead box O
HIF = hypoxia-inducible factor
HRE = hypoxia-responsive element
Luc = luciferase
PAI = plasminogen activator inhibitor
ROS = reactive oxygen species

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