

The adaptor protein Ruk/CIN85 activates plasminogen activator inhibitor-1 (PAI-1) expression via hypoxia-inducible factor-1 α

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Summary

Increased levels of plasminogen activator inhibitor-1 (PAI-1) indicate an enhanced risk of ischaemic/hypoxic cardiovascular events and a poor prognosis. The expression of PAI-1 can be induced by various stimuli including hypoxia, insulin and insulin-like growth factor 1 (IGF-1). The hypoxia-inducible factor-1 (HIF-1) is critical for hypoxia or insulin/IGF-1 mediated PAI-1 induction, but the components involved in merging the signals are not known so far. The adaptor/scaffold protein Ruk/CIN85 may be a candidate since it plays important roles in the regulation of processes associated with cardiovascular and oncological diseases such as downregulation of receptor tyrosine kinases, apoptosis, adhesion and invasion. Therefore, it was the aim of this study to investigate the involvement of Ruk/CIN85 in the regulation of PAI-1 expression. It was found that Ruk/CIN85 induced PAI-1 mRNA and protein expression both under normoxia and hypoxia. The induction of PAI-1 expression by Ruk/CIN85 occurred at the transcriptional level since the

half-life of PAI-1 mRNA was not affected in cells overexpressing Ruk/CIN85 and reporter gene assays using wild-type and mutant human PAI-1 promoter luciferase constructs showed that the hypoxia responsive element was responsible for Ruk/CIN85 effects. Further, knocking down HIF-1 α abolished not only the hypoxia-dependent but also the Ruk/CIN85-dependent PAI-1 induction. In addition, transient or stable overexpression of Ruk/CIN85 also induced HIF-1 α protein levels and HIF-1 activity and knocking down Ruk/CIN85 reversed these effects. Thereby, Ruk/CIN85 interfered with the proline hydroxylation-dependent HIF-1 α protein destabilisation. Together, these results provide the first evidence that Ruk/CIN85 induces PAI-1 expression via modulation of HIF-1 α stability.

Keywords

Ruk/CIN85, PAI-1, hypoxia, HIF-1

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Introduction

The broad-spectrum serine protease plasmin is generated from plasminogen by tissue-type (tPA) and urokinase-type (uPA) mediated proteolysis (1). The action of tPA and uPA is primarily antagonised by plasminogen activator inhibitor-1 (PAI-1). PAI-1 is produced by platelets, vascular endothelial cells, vascular smooth muscle cells, and several non-vascular cell types (2–5). It is primarily measured in blood but is also found associated with the extracellular matrix, mainly by binding to vitronectin. Although mainly known due to its role in fibrinolysis, *in vitro*- and *in vivo*-data point out that PAI-1 is also involved in proliferation and tissue

remodeling processes occurring in cancer and in cardiovascular diseases. Indeed, transgenic mice that overexpress a stable form of human PAI-1 develop spontaneous macrovascular coronary thrombosis and myocardial infarction (6). In addition, a number of clinical studies showed that high levels of PAI-1 indicate a poor prognosis for cancer patients and an increased risk of ischaemic cardiovascular events (7, 8). The cardioprotective and renoprotective properties of some currently available drugs might be attributable in part to inhibition of PAI-1 (9).

PAI-1 expression could be induced by a variety of environmental, metabolic and hormonal factors. In particular, PAI-1 is upregulated by hypoxia, insulin and insulin-like growth factor type-1

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(IGF-1) which are often dysregulated in cardiovascular diseases, obesity and diabetes mellitus (10, 11). Interestingly, hypoxia as well as the insulin and IGF-1-dependent induction of PAI-1 expression occurs via a transcriptional mechanism involving binding of hypoxia-inducible factor-1 (HIF-1) to hypoxia response elements (HREs) within the PAI-1 promoter (12–15).

While the existing body of work provides a general picture of gene regulation by hypoxia and insulin/IGF-1, the roles played by individual molecular components which may merge these different signals are uncertain. Adaptor proteins which link protein-binding partners together and stimulate formation of signalling complexes (16) play an important role in the regulation of intracellular signalling pathways. The adaptor/scaffold protein CIN85 (c-Cbl-interacting protein of 85 kDa also known as Ruk, SETA and CD2BP3) is a member of a distinct, evolutionary conserved family of SH3-containing proteins. Proteins of this family are characterised by the presence of three SH3 domains (A, B and C) located at the N-termini, followed by proline- and serine-rich sequences and a C-terminal coiled-coil region. Due to their modular organisation, Ruk/CIN85 and its splice variants play important roles in various physiological processes such as apoptosis (17), ligand-induced endocytosis of receptor tyrosine kinases (RTK) (18), membrane trafficking (19) and cell adhesion (20).

Transcriptional profiling in mice revealed that Ruk/CIN85 expression was strongly upregulated by feeding, a process associated with high insulin levels, but normalised to control levels by fasting/weight reduction (21). Ruk/CIN85 expression was also shown to be induced in human tumors (22). These findings led to the hypothesis that Ruk/CIN85 may have an impact on the hypoxia or insulin/IGF-1-induced PAI-1 expression. Therefore, it was the aim of the present study to investigate the involvement of Ruk/CIN85 in the regulation of PAI-1 expression.

Materials and methods

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

Cell culture

Human breast adenocarcinoma MCF-7 cells were maintained in a normoxic atmosphere of 16% O₂, 79% N₂, and 5% CO₂ (by vol.) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 100 µg/ml streptomycin. For studies of protein expression, MCF-7 cells were plated on 6 cm dishes and cultured for two days in full serum. Then the medium was changed and the cells were further cultured either under normoxia or hypoxia (5% O₂, 90% N₂, 5% CO₂ [by vol.]) for 4 hours (h), 16 h or 24 h, as indicated.

Plasmid constructs

The PAI-1 promoter luciferase reporter gene constructs used here pGL3PAI-806, pGL3PAI-806M5, pGL3PAI-806M4, pGL3PAI-806M45, pGL3PAI-806HREm and pGL3PAI-806 HREmM45 have been already described (14) and the mutations are indicated in Figure 3. The pGL3-EPO-HRE, the Ruk/CIN85 encoding vector pRc/CMV2-Ruk₁ and constructs for pG₅E1B-LUC, pSG424 as well as Gal4-HIF-1α-TADN, Gal4-HIF-1α-TADNM, Gal4-HIF-1α-TADC and Gal4-HIF-1α-TADCM were already described (17, 23). In Gal4-HIF-1α-TADNM and Gal4-HIF-1α-TADCM proline P564 or asparagine N803 were mutated to alanine, respectively.

Cell transfection and luciferase assay

4x10⁵ MCF-7 cells per 60 mm dish were transfected essentially as described (24). In brief, 2 µg of the appropriate PAI-1 promoter Firefly luciferase (Luc) constructs were co-transfected in duplicate together with 500 ng of the expression vector for Ruk/CIN85 or empty vector and, to control transfection efficiency, with 0.25 µg of a *Renilla* luciferase expression vector (pRLSV40) (Promega, Heidelberg, Germany). Transfection with 2 µg pGL3-Epo-HRE-Luc together with the above mentioned expression vectors served as a positive control. After 12 h the medium was changed and the cells were cultured under normoxic and hypoxic conditions for 24 h. The detection of luciferase activity was performed with the Luciferase Assay Kit (Berthold, Pforzheim, Germany).

To investigate HIF-1α transactivation, 2 µg of reporter construct pG₅-E1B-Luc was co-transfected with 500 ng of each the Ruk/CIN85, the Gal4-HIF-1α-TADN, Gal4-HIF-1α-TADC or respective mutant constructs.

For Western blot experiments, cells were transfected with 0.5 µg Ruk/CIN85 expression vector or empty control vector. After 12 h the medium was changed, and the cells were cultured under normoxia for 16 h. Then, the medium was changed again and the cells were cultured under normoxia or hypoxia for additional 4 h.

Generation of MCF-7 cells that stably overexpress wild-type Ruk/CIN85

MCF-7 cells were transfected either with pRc/CMV2-Ruk or empty vector by using the calcium phosphate precipitation method. After transfection the cells were subjected to selection with 2 mg/ml geneticin (G418) (Sigma, St. Louis, MO, USA). Geneticin-resistant cells were sub-cloned and screened for Ruk/CIN85 expression by Western blot analysis.

RNA preparation and northern analysis

Isolation of total RNA and northern analysis were performed as described (14). When mRNA stability was measured, confluent control cells and cells overexpressing Ruk/CIN85 were cultured under normoxic and hypoxic conditions for 4 h prior and after the addition of actinomycin D (5 mg/ml). Digoxigenin (DIG)-labelled antisense RNAs served as hybridisation probes; they were generated by *in vitro*-transcription from pBS-PAI-1 using T3 RNA polymerase and pBS- β -actin using T7 RNA polymerase and RNA labelling mixture containing 3.5 mM 11-DIG-UTP, 6.5 mM UTP, 10 mM GTP, 10 mM CTP, 10 mM ATP. Hybridisations and detections were carried out essentially as described (14). Blots were quantified by using Gel-Pro analyzer software (Media Cybernetics, Silver Spring, MD, USA).

RNA interference (short hairpin RNA)

The plasmids expressing a 19mer short hairpin RNA (shRNA) against HIF-1 α or control siRNA were described (25) and 100 nM shRNA encoding vector was used essentially as described (25). The oligonucleotides with the sequence 5'-CCAGCAGAAACGAGAG-ATTAA-3' and 5'-GCACGTTAAGTGCTACACA-3' were used to generate Ruk/CIN85 shRNA and scrambled shRNA control expressing lentivirus, respectively. Preparation of lentiviral particles was performed essentially as described (26). MCF-7 cells were prepared as above and after 5 h the medium was replaced and cells were infected with lentiviral vectors at about 40 MOI for 14 h. After 14 h the cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was given for up to 24 h depending on the experiment.

Protein preparation and Western blot analysis

Cells were scraped in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, complete protease inhibitor cocktail tablet [Roche, Mannheim, Germany]), mechanically triturated through a 1-ml syringe, kept on ice for 20 minutes (min) and centrifuged at 12,000 g for 20 min at 4°C. Protein content was determined using bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (50 μ g per sample) were separated by electrophoresis on 10–12.5% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with rabbit anti-Ruk/CIN85 (1:3,000) (27), mouse anti-PAI-1 (reactive with PAI-1 and tPA/PAI-1 complexes) (1:100) (American Diagnostics, Pfungstadt, Germany), mouse anti-HIF-1 α (1:1,000) (Novus Biologicals, Littleton, CO, USA), or rabbit anti- β -actin (1:5,000) (Sigma) antibodies overnight at 4°C. Appropriate secondary antibodies (peroxidase-conjugated IgG [Promega]) were used in 1:5,000 dilutions. The enhanced chemiluminescence kit (Amersham Pharma-

cia Biotech, Freiburg, Germany) was used for detection of immunoreactive bands. Densitometric analysis was performed using the Gel-Pro analyzer software (Media Cybernetics).

Statistical analysis

Densitometry data were plotted as percent induction of relative density units, with the zero value absorbance in each figure set arbitrarily at 1. Statistical comparisons of absorbance differences were performed by the Mann-Whitney test (Statview 4.5, Abacus Concepts, Berkeley, CA, USA), and p values $p \leq 0.05$ were considered significant. Luc values presented are means \pm SEM. Results were compared by ANOVA for repeated Luc measurements followed by the Newman-Keuls test. A probability level $p \leq 0.05$ was accepted as significant.

Results

Ruk/CIN85 stimulates PAI-1 expression under normoxia and hypoxia

In order to analyse whether Ruk/CIN85 may contribute to PAI-1 expression we aimed to use a cell line almost lacking endogenous Ruk/CIN85 versus a cell line of the same genetic background but stably expressing Ruk/CIN85 as a model system. Since MCF-7 breast adenocarcinoma cells do not display evident Ruk/CIN85 expression, we generated control cells which were stably transfected with an empty expression vector and cells stably overexpressing Ruk/CIN85. When control cells were exposed to hypoxia (5% O₂) PAI-1 protein levels were induced by about 1.7-fold and two-fold after 16 h and 24 h (Fig. 1A, B), respectively, in line with previous studies (12, 14). In the cells stably overexpressing Ruk/CIN85 an induction of PAI-1 levels by about two-fold was seen already under normoxia. Exposure of these cells to hypoxia enhanced PAI-1 expression by about three-fold after 16 h and by about six-fold after 24 h (Fig. 1A, B).

Ruk/CIN85 increases PAI-1 expression at the transcriptional level

To find out whether the Ruk/CIN85 effects occur at the transcriptional level we measured PAI-1 mRNA levels under normoxia and hypoxia in control and Ruk/CIN85 overexpressing cells. In addition, we determined whether Ruk/CIN85 affects PAI-1 mRNA stability by measuring the half-life in the presence of the transcriptional inhibitor actinomycin D. Confluent control and overexpressing Ruk/CIN85 MCF-7 cells were cultured under normoxia and hypoxia for 4 h and then actinomycin D (5 mg/ml) was added to the medium. The total RNA was harvested at selected intervals

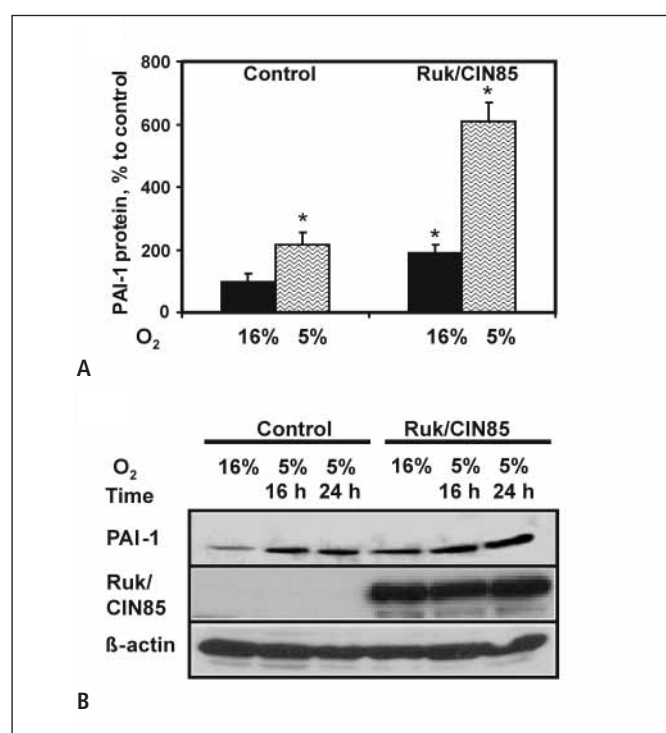


Figure 1: Ruk/CIN85 stimulates human PAI-1 expression. A) Western blot analysis of PAI-1 expression. Mock-transfected cells (control) and MCF-7 cells that stably overexpress Ruk/CIN85 were first cultured for 24 h at normoxic conditions and then further cultured either under normoxia (16% O₂) or hypoxia (5% O₂) for 24 h. PAI-1 protein levels determined from the cell lysates were quantified by densitometry and normalised to β-actin levels as loading control. The PAI-1 level in control cells under normoxia was set equal to 100%. Values are means ± SEMs of three independent culture experiments. *significant difference 16% O₂ vs. 5% O₂ and Ruk/CIN85 overexpressing cells versus control, $p < 0.05$. B) Representative immunoblots of PAI-1, Ruk/CIN85 and β-actin in MCF-7 cells cultured either under normoxia (16% O₂) or hypoxia (5% O₂) for 16 h or 24 h.

and Northern blot analyses were performed with PAI-1 and β-actin probes. The results shown on Figure 2A and B indicate that hypoxia and Ruk/CIN85 induce PAI-1 mRNA levels by about two-fold when compared to the normoxic controls. Further, Ruk/CIN85 induced PAI-1 expression by about five-fold under hypoxia. The half-life of the 3.2 kb and 2.2 kb PAI-1 mRNA transcripts were ~60 min and ~150 min, respectively, under control conditions and the exposure of the cells to hypoxia did not affect their half-life which is in line with previous publications (Fig. 2A, data not shown for 150 min) ([28, 29] and citations therein). Similarly, overexpression of Ruk/CIN85 had no effect on the half-life of both PAI-1 transcripts suggesting that Ruk/CIN85 induces PAI-1 transcriptionally. To further confirm whether Ruk/CIN85 induces the expression of PAI-1 at the transcriptional level, we performed luciferase reporter gene studies using constructs containing 806 bp of the PAI-1 promoter. We found that hypoxia induced Luc activity by about two-fold in line with previous studies (14) (Fig. 2C). Over-

expression of Ruk/CIN85 increased Luc activity of the PAI-1 promoter construct by about two-fold under normoxia. Exposure of the Ruk/CIN85 overexpressing cells to hypoxia had the strongest effect and Luc activity was enhanced by about five-fold (Fig. 2C). Together, these data indicate that Ruk/CIN85 increases PAI-1 expression at the transcriptional level.

Ruk/CIN85 stimulates PAI-1 expression via the hypoxia response element in the PAI-1 promoter

We have previously reported that hypoxia contributes to the regulation of PAI-1 expression via binding of HIF-1 to a specific hypoxia response element (HRE) in the PAI-1 promoter (12). Accordingly, we asked whether Ruk/CIN85 also may contribute to PAI-1 expression via regulation of HIF-1 binding to the HRE. To further explore this, we used human PAI-1 promoter Luc constructs mutated at the HRE and the E-boxes 4 and 5 alone or in combination and co-transfected those together with a Ruk/CIN85 expression vector (Fig. 3A). We found that the Ruk/CIN85-dependent induction of luciferase activity in the pGL3PAI-806M4, pGL3PAI-806M5, or pGL3PAI-806M45 transfected cells was not significantly different from the Luc activity measured in the cells transfected with the wild-type PAI-1 promoter construct pGL3PAI-806. By contrast, mutation of nucleotides -194/-187 encompassing the HRE in the constructs pGL3PAI-806HREm and pGL3PAI-806 HREmM45 abolished the Ruk/CIN85-dependent induction of Luc activity (Fig. 3A). These data indicate that the HRE (-194/-187) in the human PAI-1 promoter is responsible for Ruk/CIN85 effects.

Involvement of HIF-1α in the Ruk/CIN85-dependent PAI-1 gene expression

The data from the PAI-1 promoter reporter gene assay point out that Ruk/CIN85 effects on PAI-1 expression are dependent on HIF-1α activity. In order to further investigate this, we exposed control cells and Ruk/CIN85 overexpressing cells to normoxia and hypoxia and measured the PAI-1 protein levels after HIF-1α knockdown with shRNA. Depletion of HIF-1α by shRNA abolished hypoxia-dependent PAI-1 expression not only in the control cells but also in the cells overexpressing Ruk/CIN85 and PAI-1 expression levels were restored to those found under normoxia (Fig. 3B, C). When we measured HIF-1α protein levels in the Ruk/CIN85 overexpressing cells we found that HIF-1α was upregulated under both normoxia and hypoxia by about two-fold and five-fold, respectively, in comparison with the control cells (Fig. 3B, C). Next, we examined PAI-1 expression in Ruk/CIN85 overexpressing cells in response to hypoxia while knocking down Ruk/CIN85. The infection of Ruk/CIN85 overexpressing cells with lentiviral particles expressing scrambled control shRNA or Ruk/CIN85 shRNA reduced not only PAI-1 but also HIF-1α protein levels both under normoxia and hypoxia (Fig. 3B, C). Together, these data indicate

that HIF-1 α is involved in the regulation of Ruk/CIN85-dependent PAI-1 expression.

Ruk/CIN85 affects HIF-1 α protein stability

We next analysed whether overexpression of Ruk/CIN85 has a direct effect on HIF-1 activity and HIF-1 α stability. To investigate this in more detail, cells were co-transfected with a luciferase reporter gene construct containing three copies of the HRE from the erythropoietin gene in front of the SV40 promoter, and a Ruk/CIN85 expression vector. It was found that, similar to the PAI-1 promoter-driven luciferase activity, Ruk/CIN85 overexpression induced Epo-HRE-dependent luciferase activity by about 3.5-fold and 4.5-fold under normoxia and hypoxia, respectively, and again, hypoxia and Ruk/CIN85 overexpression had an additive effect on reporter gene expression (Fig. 4A). These data suggest that the relatively rapid increase of HIF-1 α activity and protein levels upon Ruk/CIN85 overexpression are mediated via HIF-1 α transactivation or increased stability.

There are two transactivation domains (TADs) present in HIF-1 α , referred to as amino-terminal (TADN) and carboxy-terminal (TADC) TAD. To investigate whether Ruk/CIN85 may interfere with HIF-1 α transactivation, cells were co-transfected with the luciferase reporter construct pG5-E1B-Luc that contains five copies of a Gal4 response element and vectors allowing expression of fusion proteins consisting of the Gal4-DNA binding domain (Gal4) and either HIF-1 α TADN or TADC along with the Ruk/CIN85 expression vector.

In line with previous studies we found that HIF-1 α TADN and TADC transactivity could be induced by hypoxia (30, 31). Overexpression of Ruk/CIN85 also increased HIF-1 α -TADN activity by about 2-fold (Fig. 4B, C) but had no effect on TADC activity. Previous studies convincingly demonstrated that the amino acid proline 564 in the TADN plays a key role in HIF-1 α stabilisation (30, 32–34). It can be hydroxylated by HIF prolyl hydroxylases (PHDs) under normoxia, thereby mediating the interaction between HIF-1 α and the von Hippel-Lindau (VHL) tumour suppressor protein which then targets HIF-1 α for proteasomal degradation. The mutation of the critical amino acid proline (P564) in the construct Gal4-HIF-1 α -TADNM, which led to a hydroxylation resistant fusion protein, caused an increase in transactivity under normoxia and a loss of the response to hypoxia and Ruk/CIN85 (Fig. 4B). Furthermore, asparagine (N803) in HIF-1 α -TADC can also be hydroxylated by another hydroxylase named FIH and thus block the interaction of HIF-1 α and p300/CBP (35). While mutation of asparagine 803 to alanine enhanced transactivation it also abolished hypoxic induction. Again, no effect of Ruk/CIN85 could be detected with the TADC mutant (Fig. 4B). Thus, the induction of HIF-1 α -TADN transactivity by Ruk/CIN85 indicated an effect on HIF-1 α protein stabilisation. To test this, we measured the Gal4TADN and -TADC protein levels. In line with the transfection assays, hypoxia and Ruk/CIN85 enhanced Gal4-HIF-1 α -TADN levels, while the levels of the degradation resistant Gal4-HIF-

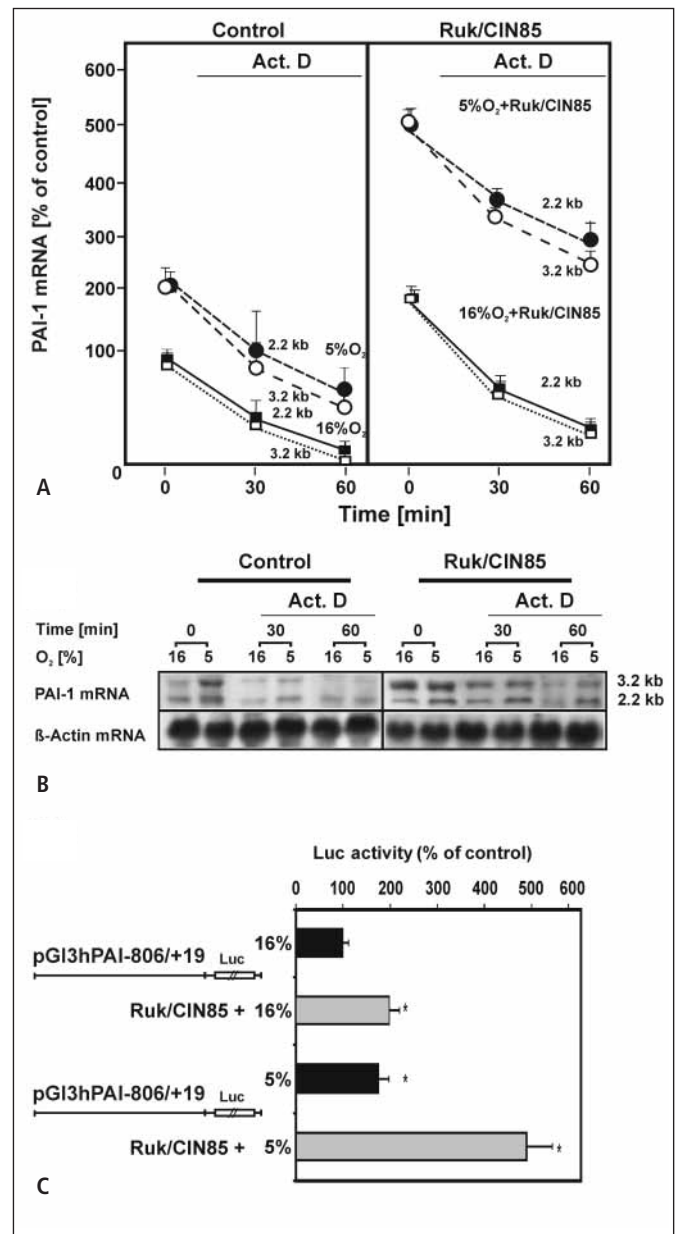


Figure 2: Hypoxia and Ruk/CIN85 do not affect PAI-1 mRNA stability. Confluent control cells and cells overexpressing Ruk/CIN85 were cultured under normoxic and hypoxic conditions for 4 h prior to addition of actinomycin D (5 mg/ml). A) Time-course of PAI-1 mRNA degradation. Results are presented as percentage of PAI-1 mRNA levels at time 0, i.e. after 4 h exposure to hypoxia. Values represent means \pm SEM of three independent experiments. B) Representative northern blot. Total RNAs were isolated at time point zero (0), 30 min and 1 h after actinomycin D (Act. D) treatment and analysed by Northern blot using probes for PAI-1 and β -actin. C) MCF-7 cells were transiently co-transfected with either Ruk/CIN85 expression plasmid or the empty control vector and a Luc gene construct driven by the wild-type human PAI-1 promoter (pGI3PAI-806). The cells were cultured either under normoxia (16% O₂) or hypoxia (5% O₂) for 24 h. The percentage of Luc activity was determined relative to the pGI3PAI-806 control that was set equal to 100%. Values are means \pm SEMs of four independent culture experiments. *significant difference 16% O₂ vs. 5% O₂ and Ruk/CIN85 overexpressing cells vs. control.

1 α -TADNM (P564A) were enhanced but no longer regulated by hypoxia or Ruk/CIN85. In contrast, the Gal4-HIF-1 α -TADC and Gal4-HIF-1 α -TADCM (N803A) protein levels were not regulated by hypoxia in line with other studies (36, 37) and by Ruk/CIN85 (Fig. 4C).

Discussion

In the present study we investigated the role of the adaptor protein Ruk/CIN85 in the regulation of human PAI-1 gene expression and identified a novel mechanism by which Ruk/CIN85 induces PAI-1 expression via HIF-1 α , i.e. by interfering with the function of the hypoxia responsive element in the PAI-1 promoter and by increasing the stability of the HIF-1 α protein.

The increased incidence of obesity and diabetes observed during the last decades is one of the major causes of increased arterial and venous thrombosis. The role of PAI-1 in the development of acute thrombotic disorders, including deep vein thrombosis and myocardial infarction, as well as fibrotic disorders (atherosclerosis, renal and pulmonary fibrosis) has been well recognised (38).

Although these diseases are characterised by dysregulated levels of a number of hormones, cytokines and growth factors, hypoxia appears to be a common phenomenon associated with almost all vascular disorders displaying high PAI-1 levels. The induction of PAI-1 expression by hypoxia was confirmed by a number of studies (12, 14, 39, 40) and it was shown that hypoxia stimulates binding of HIF-1 to specific hypoxia response elements within the PAI-1 promoter (12, 14, 40). Thereby the HRE acted as a high affinity binding site and E5 as a low affinity binding site (14) and this explains also that the inducible effect of Ruk/CIN85 is slightly reduced upon mutation of E5 in the PAI-1 promoter. Later it was shown that insulin and IGF-1 can contribute to an increase in PAI-1 by activating the transcription factor HIF-1 (13, 14). HIF-1 acts as a dimer consisting of the HIF-1 α subunit which carries the sensitivity towards oxygen and a constitutively expressed HIF-1 β subunit.

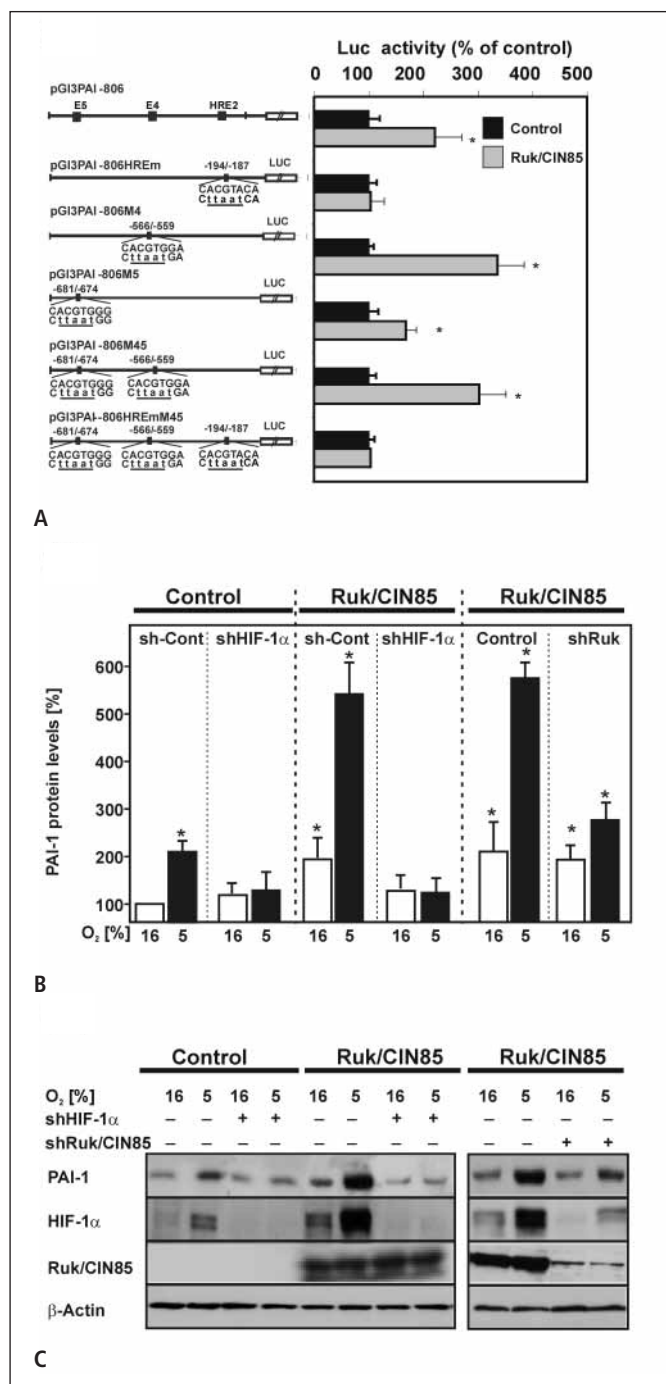


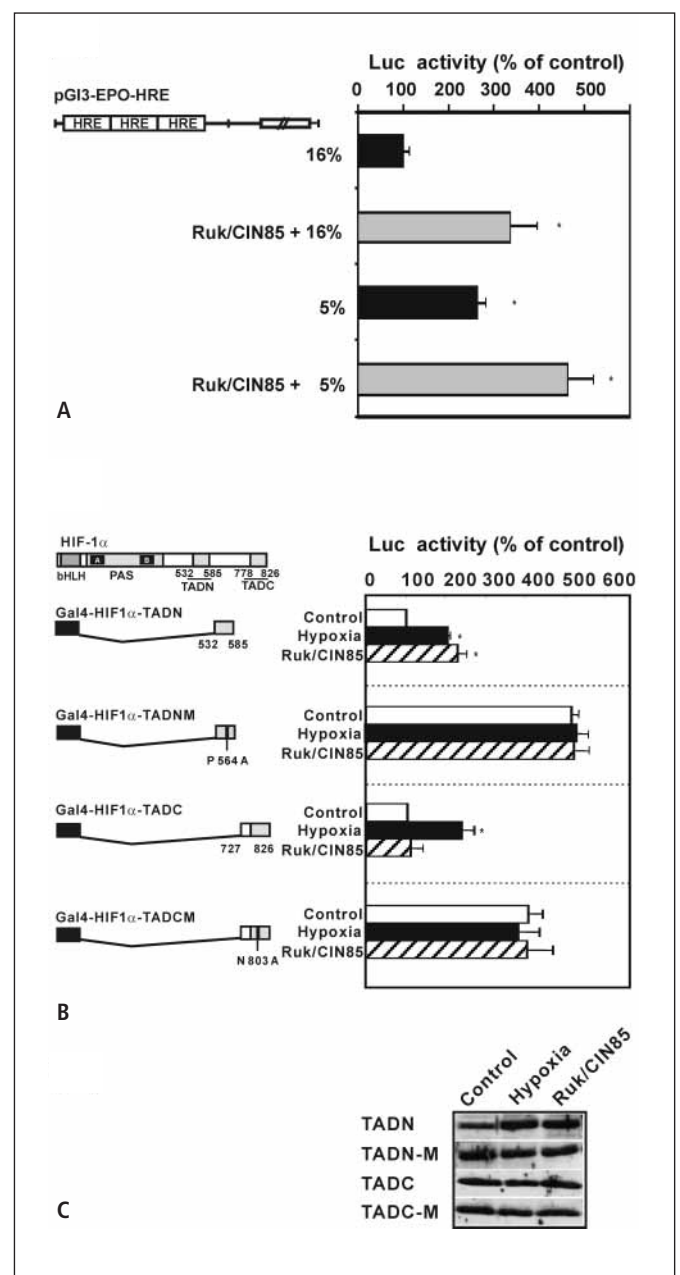
Figure 3: Ruk/CIN85 induces PAI-1 gene expression via induction of HIF-1 α protein levels. A) MCF-7 cells were transiently co-transfected with either Ruk/CIN85 expression plasmid or the empty control vector and Luc gene constructs driven by the wild-type human PAI-1 promoter (pGI3PAI-806), or the 806-bp promoter mutated at either HRE (pGI3PAI-806HREm), E-box 4 (pGI3PAI-806M4), E-box 5 (pGI3PAI-806M5), E-box 4 and 5 (pGI3PAI-806M45) or HRE, E-boxes 4 and 5 (pGI3PAI-806 HREmM45). The wild-type HRE and E-box sequences are shown on the upper strand and the respective mutant residues are below. The cells were cultured under normoxia for 48 h. The percentage of Luc activity was determined relative to corresponding empty vector-transfected controls that were set equal to 100%. Values are means \pm SEM of four independent culture experiments. *significant difference Ruk/CIN85 overexpressing cells vs. control. B) Western blot analysis of PAI-1 expression. Control and/or MCF-7 cells that stably overexpress Ruk/CIN85 were transfected with an expression vector for HIF-1 α shRNA, scrambled shRNA or were infected with lentiviral particles expressing Ruk/CIN85 shRNA or scrambled shRNA for 14 h. Then, the medium was changed and cells were cultured under normoxia and hypoxia for 24 h prior to Western blot analyses using antibodies against PAI-1, HIF-1 α or Ruk/CIN85. The PAI-1 level in control cells under normoxia was set equal to 100%. Values are means \pm SEMs of three independent culture experiments. *significant difference 16% O₂ vs. 5% O₂ and Ruk/CIN85 overexpressing cells vs. control. C) Representative immunoblots of PAI-1, HIF-1 α , Ruk/CIN85 and β -actin in MCF-7 cells cultured either under normoxia (16% O₂) or hypoxia (5% O₂).

The HIF-1 α protein stabilisation is conferred by the O₂-dependent degradation domain (ODD) which overlaps in part with the N-terminal transactivation domain (TADN). Under normoxia two proline residues (P402 and P564) within the ODD are subject to hydroxylation by prolyl hydroxylases (PHD) (41). The hydroxylation enables the binding of the VHL tumor suppressor protein, a component of an E3 ubiquitin ligase complex that targets the HIF-1 α subunit for degradation by the ubiquitin-proteasome pathway (32).

The mechanisms by which hypoxia activates gene expression are not comprehensively understood. Especially, the function and mode of action of individual proteins involved in this process need to be further investigated. Neither the involvement of adaptor protein Ruk/CIN85 in the regulation of plasminogen/plasmin system components nor its role in the regulation of HIF-1 α activity in general was elucidated previously. Ruk/CIN85 together with another protein known as CD2AP/CMS constitutes a novel family of ubiquitously expressed adaptor/scaffold proteins that take part in various cellular functions such as apoptosis (17), several transport processes (18), rearrangement of actin cytoskeleton, cell motility and adhesion (20, 42) and cell division (43). Since several heart-specific transcripts have been identified (44), Ruk/CIN85 may be important for heart and vessel homeostasis. Our data showed that the Ruk/CIN85-dependent induction of a Gal4-HIF-1 α -TADN construct was abolished upon mutation of the proline 564 residue within the N-TAD of HIF-1 α . The obtained results indicate that Ruk/CIN85 positively affects hypoxia-dependent gene expression via protection of HIF-1 α from hydroxylation by PHDs and subsequent protein stabilisation. The involved molecular mechanism is so far not known, but two different scenarios can be envisaged. First, Ruk/CIN85 may bind directly to the TADN and mask proline 564 since Ruk/CIN85 contains three SH3 domains

known to mediate protein-protein interactions by binding to proline-rich motifs (43, 45). Second, Ruk/CIN85 interferes with the function of the proline hydroxylases via direct or indirect binding. The latter mechanism might be much more likely since similar regulatory phenomena have been described. For example, Morg1 (MAPK organiser 1), a WD-repeat protein, acts as a molecular scaffold that directly binds prolyl hydroxylase-3 (PHD3) *in vitro* and *in vivo* and may link PHD3 to different signalling pathways (46). HIF-mediated reporter gene activity was shown to be decreased by Morg1 while suppression of *Morg1* led to a marked increase of HIF-1 activity. There are also data showing that the adaptor protein Shc which possess two distinct domains that bind phosphotyrosine containing sequences (PTB and SH2) and a cen-

Figure 4: Ruk/CIN85 enhances HIF-1 α transactivity and protein stability via the N-terminal transactivation domain. A) MCF-7 cells were transfected with a Luc gene construct containing three copies of the EPO HRE element in front of the SV40 promoter (pG13-EPO-HRE) together with the Ruk/CIN85 expression vector. The transfected cells were further cultured 24 h under normoxia (16% O₂) or hypoxia (5% O₂). In each experiment the Luc activity of pGL3-EPO-HRE at 16% O₂ was set to 100%. Values are means \pm SEM of three independent culture experiments. *significant difference 16% O₂ vs. 5% O₂ and Ruk/CIN85 overexpressing cells vs. control. B) MCF-7 cells were co-transfected with the Ruk/CIN85 expression plasmid along with a luciferase reporter construct pG5-E1B-LUC and different fusion gene constructs in which the Gal4 DNA binding domain was fused to either the HIF-1 α region from amino acid 532–585 containing TADN or 727–826 containing TADC, respectively, as shown on the left. The mutations in the constructs are indicated. After 24 h in culture the transfected cells were exposed to normoxia (16% O₂) or hypoxia (5% O₂) for 8 h. Values are means \pm SEM of three independent culture experiments. *significant difference 16% O₂ vs. 5% O₂ or Ruk/CIN85. C) Western blot analysis. Fifty μ g of protein from cells transfected with wild-type and mutated Gal4-HIF-1 α -TADN and Gal4-HIF-1 α -TADC constructs and the Ruk/CIN85 encoding vector were subjected to Western blot analysis with an antibody against Gal4DBD. Autoradiographic signals were obtained by chemiluminescence.



Abbreviations

CIN85, c-Cbl-interacting protein of 85 kDa; FCS, fetal calf serum; HIF-1 α , hypoxia-inducible factor-1 α ; HRE, hypoxia responsive element; Luc, luciferase; PA, plasminogen activator; PAI-1, plasminogen activator inhibitor-1; Ruk, Regulator for ubiquitous kinase; SETA, SH3 domain-containing expressed in tumorigenic astrocytes; SH3, Src-homology type 3 domain; SH3KBP1, SH3 domain kinase binding protein 1; shRNA, short hairpin RNA.

tral region that contains critical tyrosine phosphorylation sites (47) and its downstream effectors, Ras and Raf-1, are involved in hypoxia-induced HIF-1 stabilisation in human umbilical vein endothelial cells (48). In these cells overexpression of a dominant-negative Shc mutant resulted in significantly reduced HIF-1 α protein levels as compared with control.

Besides their role in cardiovascular diseases, the components of the plasminogen activation system are involved also in a number of processes that facilitate tumour progression such as extracellular matrix degradation, cell proliferation, migration and adhesion. High levels of PAI-1 in primary breast tumours are correlated with shortened disease-free interval and poor overall survival (49). Thus, our study demonstrating the role of Ruk/CIN85 in PAI-1 upregulation seems to be in line with the data concerning Ruk/CIN85 involvement in the process of carcinogenesis, especially in the stimulation of cancer cell invasiveness. In line, hypoxia and enhanced levels of HIF-1 α are also associated with a number of tumors (for review see [50]) and thus the data of the present study may not only be important for pathogenesis of cardiovascular diseases but also for oncological diseases.

In summary, we have demonstrated for the first time that Ruk/CIN85 plays a role in PAI-1 induction via a transcriptional mechanism involving stabilisation of the HIF-1 α protein.

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