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Hypoxia promotes fibrogenesis in human renal fibroblasts

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Hypoxia promotes fibrogenesis in human renal fibroblasts.

Background. The mechanisms underlying progressive renal fibrosis are unknown, but the common association of fibrosis and microvascular loss suggests that hypoxia per se may be a fibrogenic stimulus.

Methods. To determine whether human renal fibroblasts (HRFs), the primary matrix-producing cells in the tubulointerstitium, possess oxygen-sensitive responses relevant to fibrogenesis, cells were exposed to 1% O_2 in vitro.

Results. Hypoxia simultaneously stimulated extracellular matrix synthesis and suppressed turnover with increased production of collagen α1(I) (Coll-I), decreased expression of collagenase, and increased tissue inhibitor of metalloproteinase (TIMP)-1. These effects are time dependent, require new RNA and protein synthesis, and are specific to hypoxia. The changes in Coll-I and TIMP-1 gene expression involve a heme-protein O_2 sensor and protein kinase- and tyrosine kinase-mediated signaling. Although hypoxia induced transforming growth factor-β1 (TGF-β1), neutralizing anti–TGF-β1-antibody did not block hypoxia-induced Coll-I and TIMP-1 mRNA expression. Furthermore, hypoxic-cell conditioned-medium had no effect on the expression of these mRNAs in naive fibroblasts, suggesting direct effects on gene transcription. Transient transfections identified a hypoxia response element (HRE) in the TIMP-1 promoter and demonstrated HIF-1–dependent promoter activation by decreased ambient pO_2.

Conclusions. These data suggest that hypoxia co-ordinately up-regulates matrix production and decreases turnover in renal fibroblasts. The results support a role for hypoxia in the pathogenesis of fibrosis and provide evidence for novel, direct hypoxic effects on the expression of genes involved in fibrogenesis.

Progressive renal disease (PRD), characterized by interstitial fibrosis, represents a significant clinical problem with poor outcome and limited therapeutic options [1, 2]. Although many progressive diseases in the kidney are glomerular in origin, it is now well established that tubulointerstitial involvement provides the best prognostic indicator of progression [3]. Tubulointersititial fibrosis is characterized by tubular dilation and atrophy, an increase in interstitial cell number, activation of fibroblasts to myofibroblasts with increased expression of α-smooth muscle actin (α-SMA), obliteration of the microvasculature, and accumulation of extracellular matrix (ECM) [1–5]. Despite the large number of studies documenting changes in a variety of ECM proteins and growth factors, most notably transforming growth factor-β1 (TGF-β1) [5, 6], in models of PRD and in human pathologies, the mechanisms underlying accumulation of ECM remain obscure. In trying to understand the association of glomerular damage, interstitial fibrosis, and tubular atrophy in PRD, we have proposed a sequence of events that ascribes a central role to microvascular compromise and hypoxia in the pathogenesis of interstitial fibrosis [7, 8].

Hypoxia can regulate expression of a wide variety of genes that may be induced or suppressed by either transcriptional and post-transcriptional mechanisms [reviewed in 9–11]. Hypoxia response elements (HREs) have been identified in the regulatory regions of a number of genes and contain consensus binding sites for the transcription factor hypoxia-inducible factor-1 (HIF-1) [12–14]. A variety of other transcription factors are induced by low O_2, including hepatocyte nuclear factor-4 (HNF-4), nuclear factor–interleukin-6 (NF-IL-6), nuclear factor-κB (NF-κB), and members of the *fos* and *jun* (AP-1) families [9–11, 15, 16], many of which have been shown to be involved in the regulation of expression of genes relevant to fibrogenesis. The question of how the signal of decreased oxygen is transduced to changes in gene expression remains open, but studies on a number of hypoxia-regulated genes have implicated a cellular heme-protein oxygen sensor [9–11, 17] and have shown that hypoxia activates a variety of signal transduction pathways, including protein kinase C (PKC)-, protein kinase A (PKA), and tyrosine kinase (TK)-mediated pathways [9–14].

Several studies have demonstrated changes in collagen gene expression in various mesenchymal cell types in response to low O_2 [18–21], and our previous study showed that hypoxia increased *Coll-I* gene expression

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and collagen production in human proximal tubular epithelial cells (PTEs) [20]. In fibrosis, both increased production and decreased turnover contribute to ECM accumulation [5]. The matrix metalloproteinases (MMPs) [reviewed in 22, 23] and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [reviewed in 24, 25], are important regulators of ECM turnover. TIMP-1 mRNA and protein are consistently increased in renal fibroses in vivo, suggesting an important role for this molecule in disease pathogenesis [5]. Relatively little is known about the effects of hypoxia on matrix turnover, although our recent study in PTE showed that low oxygen suppressed activity of at least one MMP (MMP-2) via a post-transcriptional mechanism(s) [20].

Interstitial fibroblasts are the major matrix-producing cells in the tubulointerstitium and as such are likely to be the major effector cells in fibrosis. The present study examined the effect of hypoxia on growth, activation, and ECM metabolism in human renal fibroblasts (HRFs). In this cell type, hypoxia promotes a fibrogenic phenotype increasing production of interstitial collagens and decreasing turnover via TGF-β1–independent mechanisms involving a heme-protein O_2 sensor and activation of PKC- and TK-mediated signaling pathways. Regulation of at least one of the genes, *TIMP-1*, is dependent on binding of HIF-1 to a HRE in the 5′ promoter, revealing a novel regulatory mechanism for this family of molecules and identifying *TIMP-1* as a new hypoxia-inducible gene. Together, these data support the hypothesis that hypoxia plays an important role in the pathogenesis of PRD.

METHODS

Human renal cortical fibroblasts

To circumvent the limited in vitro lifespan of renal fibroblasts, cells with a fibroblastic morphology (passage 2), obtained as an outgrowth from an explanted biopsy of histologically normal renal cortex, were conditionally immortalized by stable integration of amphoBAGγ.U19 retroviral construct encoding a thermolabile Simian Virus 40 (SV40) T-antigen plus a neomycin-resistance gene (a gift from Dr. P. Jat, Ludwig Institute, London, UK) [26]. Transfectants (tsHRF) were selected in Dulbecco's modified Eagle's:Ham's F12 (1:1) medium (DME:F12; LTI, Paisley, Scotland, UK) containing 10% fetal calf serum (FCS; LTI), 100U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin-B (1% antibiotic-antimycotic; Sigma Chemical Co., Poole, UK), and 0.5 mg/mL G418 (Geneticin; LTI) at the permissive temperature, 33°C, in a humidified atmosphere of 5% CO_2. Integration of the vector into cellular DNA was confirmed by Southern blotting. tsHRFs were grown in medium containing G418 for several passages and then maintained in the absence of G418. At confluence, cells were passaged using trypsin:ethylenediaminetetraacetic acid (0.5:0.2 mg/mL; LTI). Every 5 to 10 passages, tsHRF were reselected in G418 to check for reversion and loss of antibiotic resistance. Comparison of morphological and immunocytochemical markers in tsHRF at the nonpermissive temperature (37°C) with nontransformed parental cells showed that tsHRF retained parental characteristics. Cells displayed a typical fibroblastic morphology and formed confluent monolayers with no evidence of the "hill and valley" growth characteristic of mesangial cells. Immunocytochemistry for selected cytoskeletal and cell surface proteins showed that both parental HRF and tsHRF were positive for vimentin, fibroblast surface antigen (FSA), and SMA, with occasional desmin-positive cells. Cells were negative for cytokeratin and myosin and for alkaline phosphatase activity, a marker of PTE [20]. At 33°C, all tsHRF showed positive nuclear staining for T antigen, which decreased with time at 37°C and was further suppressed by incubation in serum-free medium. Nontransformed cells were uniformly negative. For experiments, confluent tsHRF were made quiescent by incubation in serum-free medium (quiescence medium) for 48 hours at 37°C. All experiments were performed at 37°C.

Immunocytochemistry

Immunocytochemistry was performed as described previously [20] using the Vectastain-*Elite* biotin-streptavidin-immunoperoxidase system (Vector Labs., Peterborough, UK) with aminoethylcarbazole as a chromogenic substrate giving a red-brown deposit. Cells were counterstained with hematoxylin for nuclear identification.

Antibodies (dilution). These included: monoclonal anti-T antigen (pAb412, undiluted; a gift from Dr. P. Jat); monoclonal anti-desmin (1:1000; Sigma); polyclonal rabbit anti-bovine myosin (1:10; Sigma); monoclonal anti-pan cytokeratin (1:100; Sigma); monoclonal anti-vimentin (Vim 3B4, 1:250; Roche Molecular Biologicals, Lewes, UK); monoclonal anti-human FSA (1:500; Sigma); and monoclonal anti-SMA (1:1000; Sigma). SMA staining was quantitated by counting the number of stained compared with unstained cells in five random high-power fields.

Hypoxia

Confluent quiescent cells were exposed to 1% O_2/5% CO_2/94% N_2 (British Oxygen Co. Ltd., Luton, UK) in a Billups-Rothenburg™ Chamber (ICN-Flow, High Wycombe, UK) in open dishes for up to 72 hours. Cells were overlaid with medium to a depth of 3 mm sufficient to prevent dehydration. To examine the effects of reoxygenation, cells were exposed to hypoxia (24 to 48 hours) and then returned to normoxia (21%O_2; up to 48 hours). The degree and duration of hypoxia used was based on previous studies in PTE [20, 27]. Measurement of the pO_2 of the medium indicated that O_2 levels fell rapidly

as the incubator was flushed with 1% O_2. At the end of the incubation, O_2 concentration in the chamber was checked using a Radiometer ABL4 (Radiometer, Copenhagen, Denmark), and medium pH was also measured [without cells, normoxia pH 7.05; hypoxia (48 hours), pH 6.75]. Control cells were maintained under normoxic conditions (21% O_2) for equivalent time periods. Conditioned medium (CM; serum free) was collected at each time point, clarified by centrifugation at $1000 \times g$ for 15 minutes at 4°C and stored at −80°C. Protein concentration was measured using a modified Bradford assay [20]. All experiments were repeated at least three times with reproducible results.

Cell viability, proliferation, and protein synthesis

Cell viability was assessed by qualitative evaluation of detached cells, trypan blue exclusion, and LDH release (LDH Assay Kit; Sigma). DNA synthesis was measured by incorporation of tritiated thymidine (^{3}H-Thy) into DNA [20]. Total protein synthesis was measured by incorporation of tritiated phenylalanine, L-[4-^{3}H]-Phe (specific activity 28Ci/mmol; Amersham, Arlington Heights, IL, USA). ^{3}H-Phe, 2 μCi/well was added 24 hours prior to harvesting the cells. Protein was precipitated with ice-cold 10% trichloroacetic acid (TCA) and solubilized, and radioactivity was counted. Data are presented as ^{3}H-Phe dpm/well.

Collagen production

Total collagen production was measured by reverse-phase high-performance liquid chromatography (RP-HPLC; System Gold; Beckman Instruments Ltd., Palo Alto, CA, USA) according to the method of Campa, McAnulty, and Laurent [28], as described previously [20]. Procollagen production is expressed as nmol/L hydroxyproline/well.

Northern blot analysis of mRNA expression

Total RNA was extracted using Trizol™ (LTI), and Northern blot analysis was performed as described in Orphanides, Fine, and Norman [20]. Photographs of ethidium bromide-stained gels and autoradiograms were scanned and quantified using ImageMaster Software (Pharmacia, St. Albans, UK). Variations in loading were normalized to the ethidium bromide staining of rRNA, and changes in mRNAs were calculated by comparison to normoxic values assigned an arbitrary value of 1 [20].

Probes. cDNAs for human MMP-2, MMP-9, TIMP-1, and TIMP-2 were a gift from Dr. G. Murphy (University of East Anglia, Norwich, UK). Human TIMP-3 cDNA was obtained from Dr. D. Edwards (University of East Anglia). cDNAs for interstitial collagenase (MMP-1), collagen α1(I) (Coll-I; Hf677), and α2(III) (Coll-III; Hf934) were from ATCC (Rockville, MD, USA). cDNA for TGF-β1 was a gift from Dr. R. Derynk (Genentech, South San Francisco, CA, USA). HIF-1α cDNA was a gift from Dr. G. Bell (University of Chicago, Chicago, IL, USA).

Effect of cycloheximide (Cyc) and actinomycin-D (Act-D) on gene expression

To examine the effect of inhibition of mRNA and protein synthesis on hypoxia-induced changes, 0.25 to 1.0 μg/mL Act-D (Sigma) or 0.25 to 1.0 μg/mL Cyc (Sigma) was added to the cells immediately prior to exposure to hypoxia. Under normoxic conditions, 0.1 to 1 μg/mL Cyc caused a dose-dependent inhibition of protein synthesis (^{3}H-Phe-incorporation). One μg/mL inhibited synthesis by >90%, and concentrations >1 μg/mL (3.55 nmol/L) were cytotoxic. After 48 hours, CM was collected, and cells were harvested for RNA extraction and Northern analysis.

ELISA for TIMP-1 and MMP-1

Levels of TIMP-1 and MMP-1 protein in CM were measured using the Biotrak ELISA systems (Amersham) according to the manufacturer's instructions using 100 μL aliquots of undiluted CM (MMP-1) or CM diluted 1:5 in assay buffer (TIMP-1). Absorbance was read on a Multiskan MCC/340plate reader (Titertek Labsystems, Huntsville, IL, USA), and the amount was calculated per microgram of secreted protein.

Gelatin-substrate gel zymography for gelatinase expression

To measure secreted gelatinase activity, equal amounts of CM protein were electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) impregnated with 1 mg/mL gelatin and zymography performed as described previously [20]. Gelatinase activity was visualized as cleared zones in Coomassie Blue G250-stained gels. Equal loading of protein was confirmed by silver staining of parallel gels. Zymograms were scanned and quantified by densitometry [20].

Western blot analysis

HIF-1α protein. Nuclear protein was prepared from cells exposed to normoxia or hypoxia for two, four, six, or eight hours. Cells were harvested onto ice, washed twice with cold phosphate-buffered saline (PBS) and lysed in hypotonic buffer (10 mmol/L Tris, pH 7.8, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl), and the nuclei were pelleted. Nuclear protein was extracted by incubation in hypertonic buffer (20 mmol/L Tris, pH 7.8, 1.5 mmol/L $MgCl_2$, 420 mmol/L KCl, 20% glycerol), and the lysate was dialyzed against 20 mmol/L Tris, pH 7.8, 100 mmol/L KCl, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), and 20% glycerol. All buffers contained protease inhibitors (Complete inhibitor cocktail; Roche) and 1 mmol/L sodium vanadate (Sigma). Protein concentration was mea-

sured using the bicinchoninic acid (BCA) assay reagent (Pierce, Rockford, IL, USA). Proteins (10 μg/sample) were electrophoresed through a 12% SDS-PAGE under denaturing conditions. Proteins were transferred to nitrocellulose membrane (0.2 μm pore size; Schleicher & Schuell, Dassel, Germany) for 1.5 hours at 180 mA and stained with Ponceau-S (Sigma) to confirm equal loading and transfer. Membranes were blocked in 5% dried milk (Marvel) in 10 mmol/L Tris, pH 7.6, 150 mmol/L NaCl containing 0.1% Tween 20 (TBS-Tween) for 30 minutes at ambient temperature, followed by incubation in anti–HIF-1α monoclonal antibody (Hα28b; a generous gift from Dr. P. Ratcliffe, Institute of Molecular Medicine, Oxford, UK) [29] diluted 1:100 in TBS-Tween overnight at 4°C with gentle agitation. Control lanes were incubated without primary antibody. Membranes were washed three times, for five minutes each, in TBS-Tween followed by incubation in horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Dako) diluted 1:2000 in TBS-Tween for 30 minutes at ambient temperature. Membranes were washed three times three to four minutes each, and positive signals were visualized by ECL (Amersham).

Coll-I protein. Secreted and cellular protein from normoxic and hypoxic (24 and 48 h) cells was precipitated with 67% ethanol overnight at 4°C. The dishes were scraped, and the precipitate was collected by centrifugation and solubilized in SDS-PAGE sample buffer. Equal loading of the samples was confirmed by Coomassie staining of parallel lanes and by Ponceau-S staining of the membrane after transfer. Western blot analysis was performed as described previously in this article using a goat anti-human collagen type I polyclonal antibody (1:1000; Southern Biotechnology Associates Inc., Birmingham, AL, USA) and a horseradish peroxidase-conjugated rabbit anti-goat secondary antibody. The anti-Coll-I antibody detects a doublet at approximately 200 kD.

Effect of cellular stress on TIMP-1 and Coll-I mRNA expression

To examine the specificity of the effects of hypoxia on gene expression, cells were exposed to a variety of cellular stressors under normoxic conditions, and TIMP-1 and Coll-I mRNAs were examined by Northern blotting.

Oxidant stress. Cells were treated with 0 to 200 μmol/L H_2O_2 for 24 to 48 hours, with H_2O_2 replenished every 8 hours.

Heat shock. Cells were exposed to either heat shock alone, 42 to 44°C for 15 minutes, 30 minutes, and 1, 2, 6, 8, and 16 hours or heat shock followed by up to 48 hours at 37°C.

Hypoglycemia. Cells were grown to near confluence in growth medium (DME:F12 basal glucose concentration 17 mmol/L D-glucose), transferred to growth medium containing 5.5 mmol/L D-glucose (Glutamax; LTI) for three days, and then made quiescent in serum-free, low-glucose medium for 48 hours. mRNA expression was compared with cells in 17 mmol/L glucose medium.

Hyperlactemia. Fifteen mmol/L lactic acid was added for 24 to 48 hours.

Effect of TGF-β1 on proliferation and expression of SMA, TIMP-1, Coll-I, Coll-III mRNA and secreted MMP-1 and MMP-2

To examine the effect of TGF-β1 on cell proliferation, confluent, quiescent tsHRF were exposed to 0 to 10 ng/mL TGF-β1 (Genzyme, West Malling, Kent, UK) for 24 hours, and DNA synthesis was measured as described previously in this article. For studies of gene expression, cells were treated with 0 to 10 ng/mL TGF-β1 for 24 hours under normoxic conditions. CM was collected and cells harvested for RNA. Cells treated with TGF-β1 for 24 hours were stained for SMA.

Measurement of TGF-β production

The mink lung epithelial cell, CCL64, bioassay was used to measure the concentration of TGF-β (all isoforms) in CM [20]. CM (with or without heat activation) was diluted 1:1 in medium, and the concentration of growth factor was determined by comparison with the inhibition of ^{3}H-Thy induced by 0 to 10 ng/mL exogenous TGF-β1.

Effect of neutralizing antibody to TGF-β1 on hypoxia-induced Coll-I and TIMP-1 mRNA expression

To establish whether the effects of hypoxia are mediated by autocrine TGF-β1, neutralizing antibody to TGF-β1 (1 to 2 μg/mL; R&D Systems Europe Ltd., Abingdon, UK) was added to the cells immediately prior to hypoxia. Nonimmune IgG was added to parallel cultures to confirm the specificity of the antibody. The efficacy of the antibody was confirmed by the effect of the simultaneous addition of antibody and exogenous TGF-β1 on Coll-I and TIMP-1 gene expression in normoxic cells.

Effect of hypoxic fibroblast conditioned medium on gene expression in naive fibroblasts

To establish whether hypoxia induces secreted factors that stimulate TIMP-1 and Coll-I mRNA expression, confluent quiescent tsHRF were treated with CM (containing equivalent amounts of protein) from either normoxic tsHRF or from tsHRF exposed to hypoxia for up to 72 or 48 hours of hypoxia plus 24 to 48 hours of reoxygenation. Naive fibroblasts were incubated with CM from cells for 48 hours under normoxic conditions, and mRNA levels were examined.

Effect of desferrioxamine or $CoCl_2$ on Coll-I and TIMP-1 gene expression

To investigate the effect of iron chelators and a transition metal ion on TIMP-1 and Coll-I mRNA expression,

cells were exposed to 0 to 200 μmol/L desferrioxamine (DFO) or cobalt chloride ($CoCl_2$) for 24 or 48 hours under normoxic conditions, and mRNA expression was analyzed by Northern blotting.

Transient transfection with TIMP-1 promoter-CAT reporter constructs

The human TIMP-1 promoter-reporter constructs in pBLCAT3 were as described previously [30]. DNAs were prepared using Qiagen columns (QIAGEN Ltd., Crawley, UK). Cells (either tsHRF or NRK-49F rat kidney fibroblasts; ATCC) were plated at 5×10^4 cells per well in six-well plates, grown overnight in complete medium, and transfected with 2 μg DNA per well using Lipofectin (LTI) 6 μL/well in Opti-Mem-I medium (LTI) for 7 hours at 33°C (tsHRF) or 37°C (NRK). Control wells were transfected with the empty vector pBLCAT3. DNA-containing medium was replaced with DME:F12, 0.5% FCS for one hour. Transfected cells were exposed to either normoxia or hypoxia for up to 48 hours and were harvested either immediately after hypoxia or after 24 hours of reoxygenation. Cells were washed three times with cold PBS. Lysates were prepared according to the manufacturer's instructions, and CAT activity was measured by CAT-ELISA (Roche). Reporter gene activity was corrected for total cell protein and for efficiency of transfection as monitored by the Hirt's assay [30]. Data are presented as the % increase in CAT activity above normoxic cells.

Mutation of HIF binding site

The HIF binding-site core sequence 5′-RCGTG-3′ at −26/–23 in the −102/+95 bp TIMP-1 promoter construct was replaced by the sequence 5′-RAAAC-3′ using oligonucleotide-based polymerase chain reaction (PCR) methodology (QuikChange Site-directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA), and the mutation was confirmed by sequencing. Activity of the original and mutated constructs was compared in transfected cells after 48 hours of hypoxia.

Effect of PKC and TK inhibitors on TIMP-1 and Coll-I gene expression

To establish whether PKC activation mimicked the effects of hypoxia on mRNA expression, cells were treated with phorbyl 12-myristate 13-acetate (PMA; 0 to 25 ng/mL) for 24 and 48 hours to activate PKC and mRNAs examined by Northern blotting. PKC inhibitors (Calbiochem, San Diego, CA, USA), calphostin-C (Cal-C; 0.05, 0.1 μmol/L), and bisindolylmaleimide (BI; 5, 20 μmol/L) or the TK inhibitors (Calbiochem) genistein (Gen; 10, 30 μmol/L) and lavendustin-A (Lav-A; 1, 10 μmol/L) were added under subdued lighting conditions immediately prior to hypoxia. Cells were incubated for 24 to 48 hours, and TIMP-1 and Coll-I mRNA expression was analyzed by Northern blot. These concentrations of inhibitors had no qualitative effect on cell viability. At the concentrations used, both PKC inhibitors blocked PMA (10 ng/mL)-induced changes in gene expression (data not shown).

Statistics

All values are expressed as mean ± SD. Statistical significance ($P < 0.05$) was evaluated using Student's *t*-test.

RESULTS

Effect of hypoxia/reoxygenation on cell morphology, viability, proliferation, and protein synthesis

No differences in cell morphology or numbers of floating cells were observed between normoxic and hypoxic cultures, suggesting that up to 48 hours at 1% O_2 followed by up to 48 hours of reoxygenation had no marked effect on cell viability. LDH release was similar in normoxic (N) and 48-hour hypoxic (H) cells (N, 31.22 ± 4.24%; H, 35.28 ± 9.43%). Hypoxia (24 h) stimulated a more than threefold increase in DNA synthesis (N, 2.09 ± 0.17; H, 6.47 ± 1.1 × 10^2 ^{3}H-Thy dpm/μg protein; $P <$ 0.005) which remained elevated after 48 hours of hypoxia, (N, 1.75 ± 0.44; H, 2.77 ± 0.82 × 10^2 ^{3}H-Thy dpm/μg protein). During reoxygenation (24 to 48 h), DNA synthesis was similar to normoxic controls (N, 0.43 ± 0.08; H/R, 0.41 ± 0.06). Total protein synthesis decreased after 24 hours of hypoxia (to 63% of control), but by 48 hours, hypoxia returned to normoxic values and increased slightly (37% above normoxia) during reoxygenation.

Effect of hypoxia on SMA expression

Smooth muscle actin expression is a marker of fibroblast activation to a myofibroblastic phenotype, and interstitial cell SMA expression is increased in fibrotic kidneys in vivo [5, 31]. Quiescent tsHRF showed variable basal levels of SMA expression (37 to 68% cells) with ′ominent intracellular fibrillar staining. Hypoxia (48 h) stimulated SMA up to 1.8-fold with a more marked increase in cultures with lower basal expression (Fig. 1). Immunostaining suggested that hypoxia increases both the number of myofibroblastic cells and the intensity of staining.

Effect of hypoxia on collagen production

The mRNA for α1(I) collagen (Coll-I) appears as a doublet of 5.2 and 4.8 kb. Hypoxia increased steady-state Coll-I mRNA levels, which remained elevated after 24 hours of reoxygenation (Fig. 2A, B). mRNA expression initially increased between 6 and 12 hours of hypoxia and was increased further at 48 hours, the longest time point examined (Fig. 2B). There was some variation in the magnitude of the increase in expression, but hypoxic cells consistently showed higher levels of expression than normoxic controls ($N = 16$ experiments). The addition

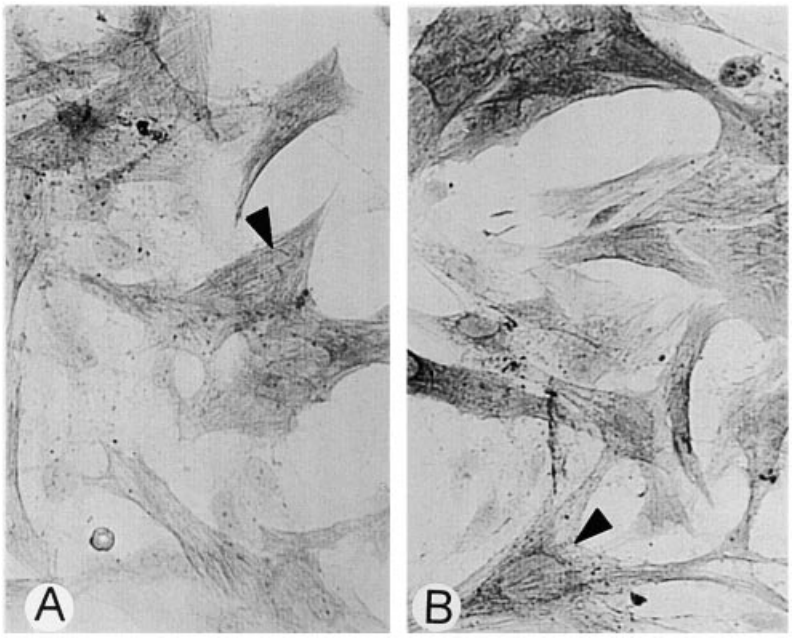

Fig. 1. Effect of hypoxia on smooth muscle actin (SMA) expression in tsHRF. SMA appears as prominent intracellular fibrillar staining (arrowheads). (*A*) Normoxia. (*B*) Hypoxia (48 hours; ×400).

of either Cyc or Act-D immediately prior to hypoxia suppressed the hypoxia-induced increase in Coll-I mRNA (Fig. 2C), which was completely blocked at inhibitor concentrations >0.5 μg/mL, indicating a requirement for both de novo protein and new RNA synthesis. In parallel with the increase in gene expression, total collagen production (by HPLC) and Coll-I protein (Western blotting) increased (Fig. 2D). Coll-III mRNA was expressed at lower levels than Coll-I mRNA in normoxic cells but was induced by hypoxia with a similar time course (data not shown).

Effect of hypoxia on MMP and TIMP expression

Zymography of CM showed that tsHRF secrete predominantly latent MMP-2 (72 kD Gelatinase-A) with only low levels of active enzyme (68 kD). MMP-2 was expressed at relatively high levels in confluent quiescent cells. No concentration of CM was required prior to zymography. Neither hypoxia (24 to 48 h) nor hypoxia/reoxygenation had any effect on the amount or activation of secreted MMP-2 (Fig. 3A) or *MMP-2* gene expression (Fig. 3B). tsHRF also secreted lower and more variable amounts of MMP-9 (92 kD Gelatinase-B), which was unaffected by hypoxia. MMP-1 was produced at very low levels by confluent quiescent tsHRF, precluding accurate comparison of levels of secreted protein (even after concentration of the medium), although slightly lower values were obtained in medium from hypoxic cells compared with normoxic controls (24 hours N, 3.44 ng/mL; H, 3.13 ng/mL). By Northern blot analysis, MMP-1 mRNA levels were decreased by low O_2 (Fig. 3B).

tsHRF expresses mRNAs for TIMP-1 (~0.9 kb), TIMP-2 (~1, 3.5 kb) and TIMP-3 (~2.4, 4.5 kb), with TIMP-1 as the most abundant inhibitor. Hypoxia increased TIMP-1 mRNA levels, which declined slightly on reoxygenation, although expression remained above normoxic controls (Fig. 4A, B). The increase in mRNA was first apparent between 6 and 12 hours of hypoxia (Fig. 4B), following a similar time course to the hypoxia-induced increase in Coll-I mRNA. Likewise the hypoxia-induced increase in TIMP-1 mRNA was blocked by Act-D or Cyc (Fig. 4C), indicating a requirement for de novo RNA and protein synthesis. The increase in TIMP-1 occurred in cells exposed to hypoxia in serum-free medium or medium containing 0.5 to 2% FCS and was independent of passage number. Hypoxia also increased TIMP-1 protein (after 48 hours; N, 50 ng/mL; H, 110 ng/mL), which remained slightly elevated during reoxygenation. Hypoxia differentially affected expression of the mRNAs for the three TIMPs, increasing expression of TIMP-1 and -3 mRNA, while TIMP-2 mRNA levels were unaffected, although TIMP-2 mRNA could be induced by other stimuli, for example, 10% FCS (twofold after 48 hours). For quantitation, all transcripts for TIMP-2 or -3 were combined (Fig. 4D). To investigate the mechanisms of hypoxia-induced changes in ECM metabolism, changes in Coll-I and TIMP-1 mRNA were used as indicators of increased matrix production and decreased turnover respectively.

Effect of cellular stress on TIMP-1 and Coll-I mRNA expression

As shown in Figure 5, the effect of hypoxia on the expression of either Coll-I or TIMP-1 in tsHRF was not mimicked by a variety of cellular stresses. The concentra-

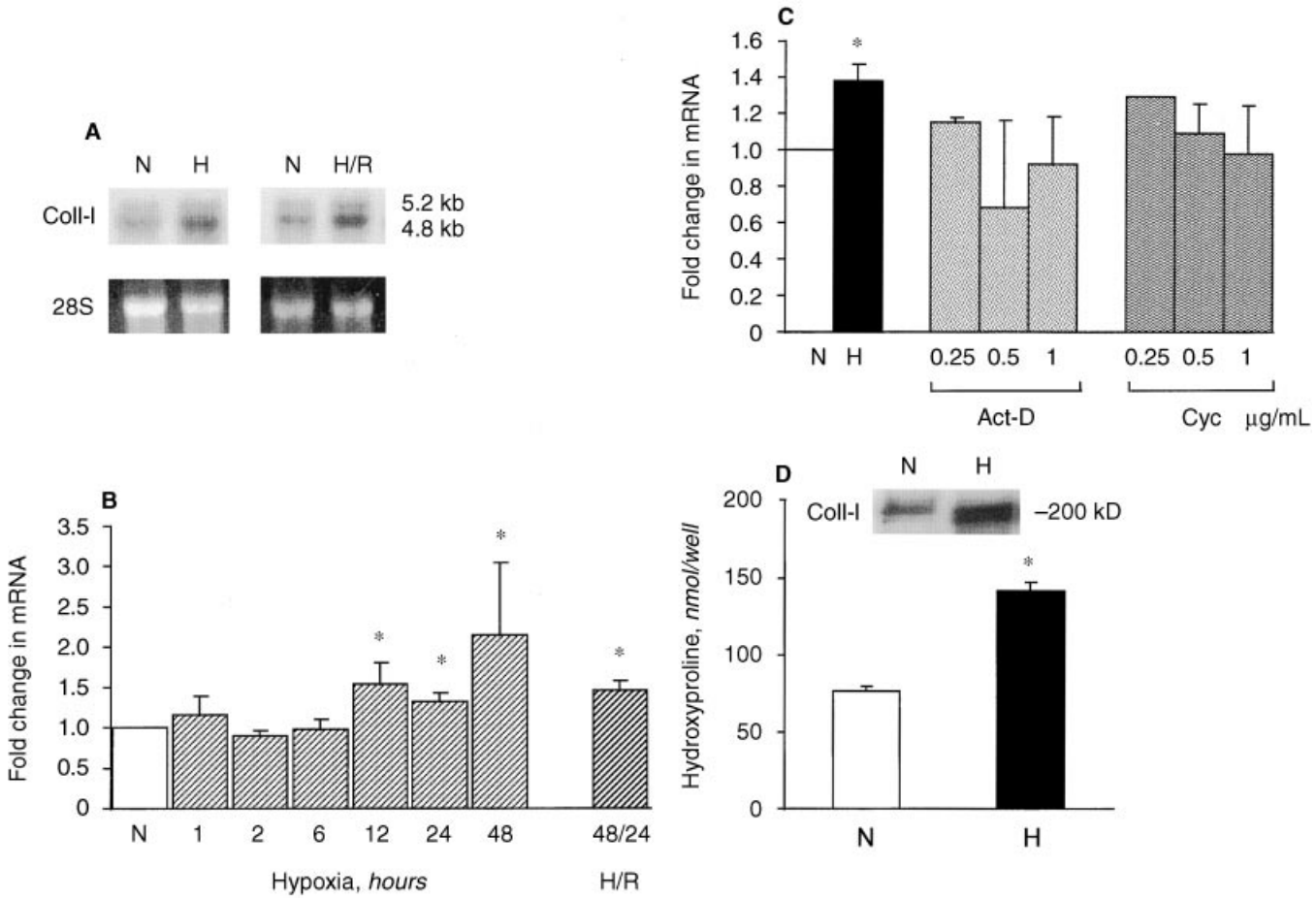

Fig. 2. Effect of hypoxia on collagen α1(I). (*A*) Northern blot of Coll-I mRNA expression after 48 hours of hypoxia (H) and 48 hours of hypoxia/24 hours of reoxygenation (H/R). Ethidium bromide staining of the 28S rRNA is shown to demonstrate equal loading. (*B*) Time course of hypoxia-induced Coll-I mRNA expression. Signals were normalized to the 28S rRNA, normoxic cells (N; □). Each time point was assigned an arbitrary value of 1, and the fold increase with hypoxia (H; ▨) or hypoxia followed by reoxygenation (H/R) was calculated. $N = 4$ experiments. $^*P < 0.05$ vs. N. (*C*) Effect of Act-D to 1 μg/mL (▩) or Cyc 0 to 1 μg/mL (■) on hypoxia-induced Coll-I mRNA expression. Inhibitors were added immediately prior to hypoxia, and cells were harvested after 48 hours. Other symbols are: (□) normoxia, N; (■) hypoxia, H. $^*P < 0.05$ vs. N. $N = 3$ experiments. (*D*) Total collagen production measured by high-performance liquid chromatography. Symbols are: (□) normoxia; (■) 48 hours of hypoxia. Data are from a representative experiment with triplicate wells/sample. $^*P < 0.05$ versus N. (*Insert*) Western blot of Coll-I protein in normoxic (N) and hypoxic (H) cells after 48 hours of hypoxia.

tions of H_2O_2 were selected to impose oxidative stress but not induce apoptosis. Lower concentrations of H_2O_2 (0 to 50 μmol/L) had no effect on either TIMP-1 or Coll-I mRNA levels, but higher concentrations (100 to 200 μmol/L) suppressed both mRNAs in a concentration-dependent manner with a more marked effect on Coll-I mRNA. The various periods of heat shock alone or followed by normothermia (37°C) had no effect on the expression of Coll-I and TIMP-1 mRNA. Coll-I mRNA levels appeared slightly decreased by 16 hours of heat shock followed by incubation at 37°C. Reduced glucose concentration (48 hours) had no effect on Coll-I and TIMP-1 mRNA, suggesting that the profibrogenic effects of hypoxia are not an artifact promoted by the in vitro bias to anaerobic respiration. Since lactate accumulates with anaerobic respiration, exogenous lactic acid might be expected to mimic the hypoxia induced-changes in gene expression. However, 15 mmol/L lactic acid for up to 48 hours had no marked effect on either TIMP-1 or Coll-I mRNA levels.

TGF-β1 as a mediator of hypoxia-induced changes in ECM metabolism

Exogenous TGF-β1 (5 ng/mL, 48 h) stimulated expression of mRNAs for TIMP-1 (1.2-fold), Coll-I (2.1-fold), and Coll-III (2-fold) in quiescent tsHRF. TGF-β1 also increased secreted MMP-2 levels and stimulated SMA expression (~15%) but had no effect on cell number (data not shown).

tsHRF constitutively expresses TGF-β1 mRNA and secretes TGF-β protein, predominantly the latent form. Hypoxia (48 hours) increased the expression of TGF-β1

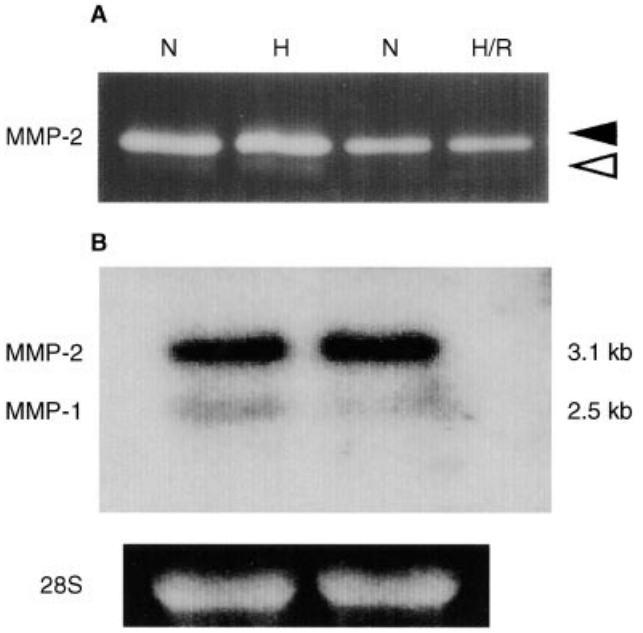

Fig. 3. Effect of hypoxia on matrix metalloproteinases (MMPs). (*A*) Zymography of conditioned medium (CM) shows tsHRF produce predominantly MMP-2 (inactive ~72 kD, filled arrow; active ~68 kD, open arrow). Abbreviations are: N, normoxia; H, 48 hours of hypoxia; H/R, 48 hours of hypoxia/24 hours of reoxygenation. (*B*) Northern blot of MMP-2 (3.1 kb) and MMP-1 (2.5 kb) mRNA in normoxia (N) compared with 48 hours of hypoxia (H).

mRNA, which declined slightly during reoxygenation (24 h) but remained above normoxic levels (Fig. 6A). The amount of TGF-β, measured by the CCL64-bioassay, also increased slightly after hypoxia (48 h, 3.57 ± 1.05 ng/mL vs. 2.83 ± 1.32 ng/mL in normoxic cells). In normoxia, neutralizing anti–TGF-β1 antibody (1 to 2 μg/mL, sufficient to block the effect of 5 to 10 ng/mL exogenous TGF-β1 on TIMP-1 and Coll-I mRNAs by >50%) induced a small increase in expression of TIMP-1 mRNA and slightly suppressed Coll-I mRNA levels (Fig. 6B), suggesting a possible role for TGF-β1 in the basal regulation of these genes. The antibody had no effect on the hypoxia-induced increase in TIMP-1 mRNA either immediately after hypoxia (48 hours; Fig. 6B) or during reoxygenation. Although the antibody caused a slight decrease (maximum 20%) in Coll-I mRNA levels induced by 48 hours of hypoxia (Fig. 6B) or hypoxia/reoxygenation, mRNA levels remained elevated above normoxic controls.

Effect of hypoxic cell-conditioned medium on gene expression

Incubation of confluent, quiescent tsHRF for 48 hours in CM from normoxic cells exposed to 12, 24, 48, 72 hours of hypoxia, 48 hours of hypoxia/24 hours or 48 hours of reoxygenation did not induce expression of either TIMP-1 or Coll-I mRNA; rather, 48 hours' hypoxic-cell CM appeared to suppress mRNA levels (Fig. 7). This decrease did not appear to be a nonspecific effect of increased protein in the medium, since incubation of cells in quiescence medium containing equivalent amounts of bovine serum albumin (BSA) did not alter mRNA levels (data not shown).

Effect of DFO and $CoCl_2$ on MMP, TIMP-1, and Coll-I

Iron chelators and transition metal ions have been shown to mimic the effect of hypoxia on the expression of a number of genes, suggesting that the cellular oxygen sensor may be a heme protein [9–11, 17]. In tsHRF, neither compound (0 to 100 μmol/L) had any effect on MMP-2 activity, and both increased the expression of TIMP-1 and Coll-I mRNA (Fig. 8), mimicking the effect of hypoxia on these two genes. At 50 μmol/L, DFO appeared to be a more potent inducer of gene expression than $CoCl_2$. However, $CoCl_2$ induced a concentration-dependent increase in the two mRNAs expression, while the effect of DFO appeared maximal at 50 μmol/L.

Expression of HIF-1α mRNA and protein

Hypoxia can directly regulate gene transcription via binding of HIF-1, an αβ heterodimer, to *cis*-acting DNA sequences. Since the α subunit of HIF-1 confers hypoxia inducibility [32], HIF-1α expression was examined in this study. The cDNA probe for HIF-1α hybridized to a single mRNA transcript approximately 3.2 kb, which was constitutively expressed by tsHRF. Hypoxia rapidly increased HIF-1α mRNA, apparent by one hour (20 to 25% above control), which peaked between 1 and 2 hours and then declined to below control levels by 6 hours, reached a nadir at around 18 hours, and remained suppressed up to 72 hours of hypoxia, although there was a trend back toward normoxic levels (Fig. 9A). In reoxygenated cells, HIF-1α mRNA remained below control for up to 48 hours of reoxygenation. Exposure of tsHRF to a short period (6 hours) of hypoxia, encompassing the peak of HIF-1α mRNA expression, followed by 42 hours normoxia, did not mimic the effect of hypoxia to increase TIMP-1 or Coll-I mRNA expression, which remained at nomoxic levels (data not shown). Localization of HIF-1α protein was analyzed by Western blotting. The monoclonal antibody detects several proteins between 120 and 130 kD with a predominant band at approximately 120 kD [29]. HIF-1α protein accumulated in the nucleus of hypoxic cells with a peak around four hours and declining thereafter (Fig. 9B). No HIF-1α protein was detected prior to exposure to hypoxia or in normoxic cell nuclei.

Regulation of the TIMP-1 promoter by hypoxia

In tsHRF transiently transfected with a TIMP-1 promoter-CAT reporter construct [−738/+95, containing 738 bp of 5′ flanking sequence, exon 1 (48 bp) plus 47 bp of

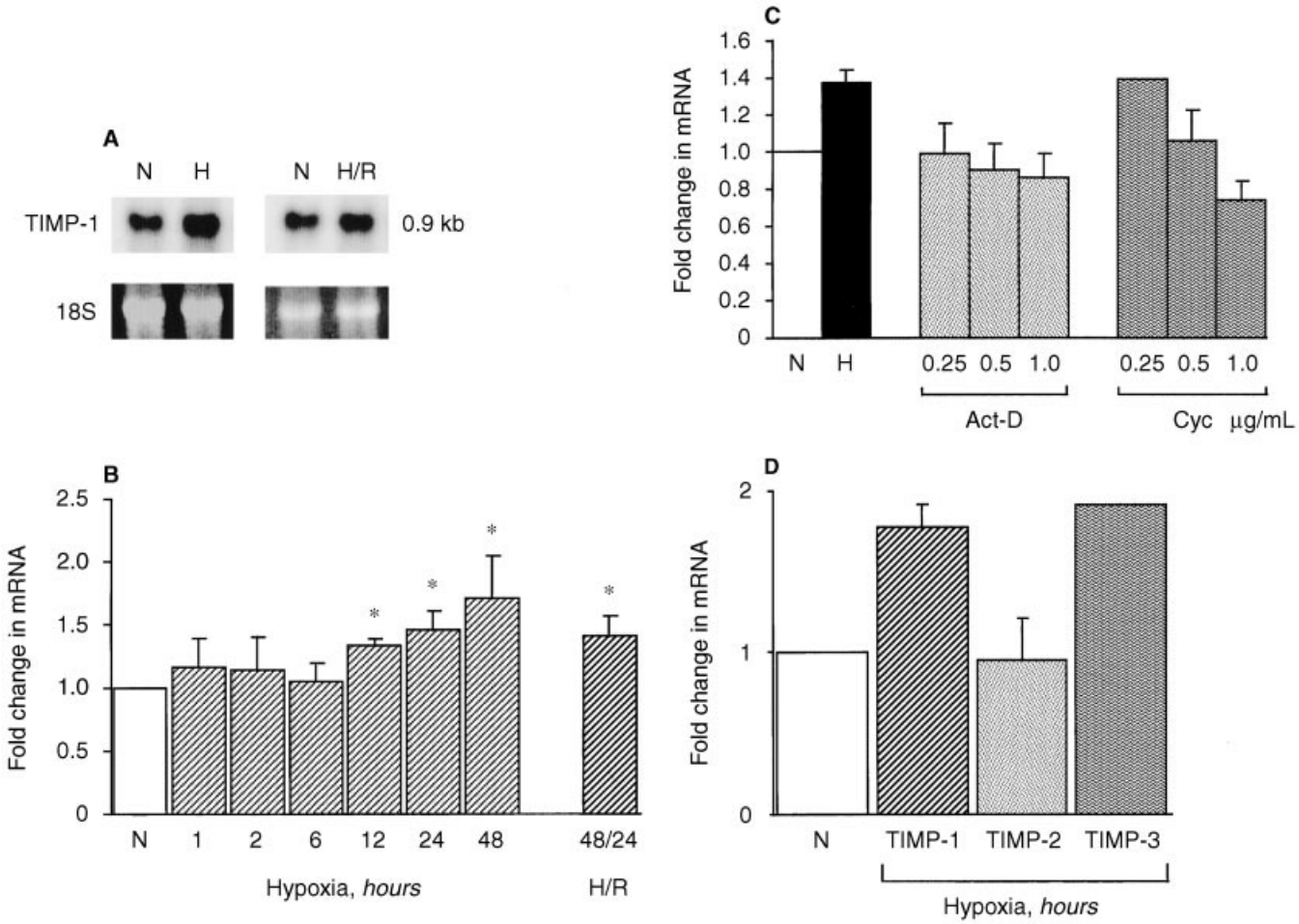

Fig. 4. Effect of hypoxia on tissue inhibitor of metalloproteinases (TIMPs). (*A*) Northern blot of TIMP-1 mRNA expression after 48 hours of hypoxia (H) or 48 hours of hypoxia/24 hours of reoxygenation (H/R) compared with normoxic cells (N). TIMP-1 probe detects a single transcript of 0.9 kb. Ethidium bromide stained 18S rRNA is also shown. (*B*) Time course of hypoxia-induced TIMP-1 mRNA expression. Signals were normalized to 18S rRNA; normoxic cells (□) at each time point were assigned a value of 1, and the fold change in hypoxia (▨) was calculated. H/R indicates 48 hours of hypoxia followed by 24 hours of reoxygenation. *$P < 0.05$ versus N. (*C*) Effect of actinomycin-D (Act-D) 0 to 1 μg/mL (▩) or cycloheximide (Cyc) 0 to 1 μg/mL (■) on the hypoxia-induced increase in TIMP-1 mRNA. Inhibitors were added prior to hypoxia and cells harvested after 48 hours. Symbols are: (□) normoxia; (■) hypoxia. *$P < 0.05$ vs. N. (*D*) Differential effect of hypoxia (48 h) on TIMP-1 (▨), TIMP-2 (▩), and TIMP-3 (■) mRNA expression compared with normoxic cells (□).

intron 1] [30] hypoxia increased CAT activity (Fig. 10A), which remained elevated during reoxygenation, suggesting that increased TIMP-1 mRNA levels are due, at least in part, to increased gene transcription. A more detailed promoter analysis to identify putative HREs used the rat renal fibroblast cell line, NRK-49F, in order to provide the large numbers of cells required. NRK fibroblasts display a similar hypoxia-induced increase in TIMP-1 mRNA expression (data not shown). All of the promoter constructs tested were active in normoxic fibroblasts and were induced by hypoxia with some differences in the magnitude of the response (Fig. 10B). A comparison of the various deletion constructs localized the hypoxia-inducible region to between −59/+8 bp. Sequence analysis of this region revealed a putative HIF-binding-site consensus sequence (5′-RCGTG-3′ at −27/−23). Mutation of this site, to 5′-RAAAC-3′, in a randomly-selected construct (−102/+95) suppressed hypoxia-induced reporter gene activity (Fig. 10C).

Effect of PKC and TK inhibitors on hypoxia-induced changes in gene expression

Both PKC- and TK-mediated signal transduction pathways have been implicated in hypoxic-regulation of various genes in different cell types [9–11]. In tsHRF, stimulation of PKC by PMA (5 to 25 ng/mL, 24 to 48 hours) dose dependently stimulated expression of TIMP-1 mRNA but suppressed Coll-I mRNA levels (data not shown), suggesting reciprocal effects of PKC activation on these two genes. Two PKC inhibitors, Cal-C and BI (at concentrations at least twice the IC_{50} which also blocked PMA-induced changes in gene expression), were tested for

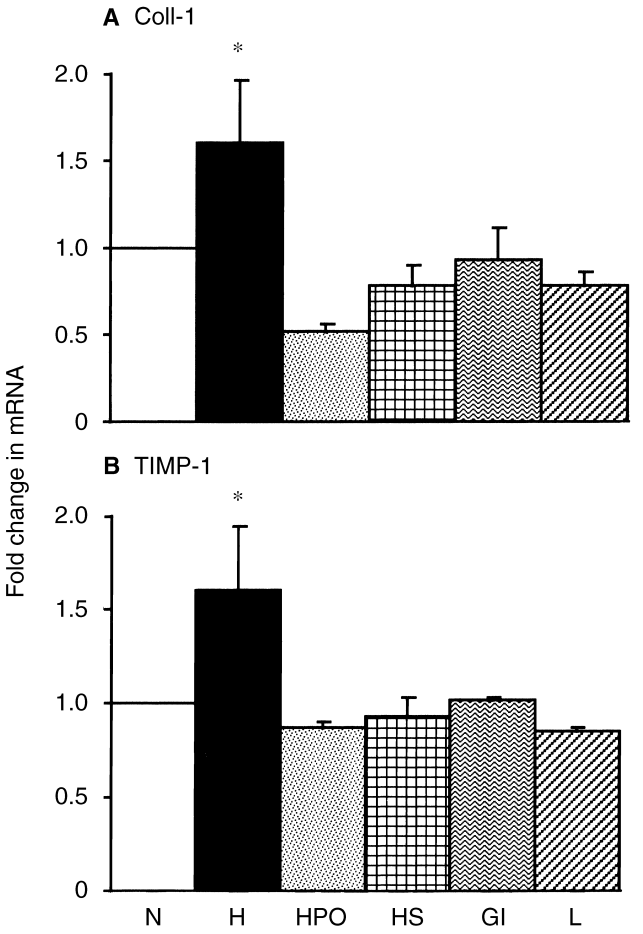

Fig. 5. Effect of cellular stress on Coll-I (*A*) and TIMP-1 (*B*) mRNA expression in tsHRF. Cells were exposed to hypoxia (H; ■); H_2O_2 (HPO, 200 μmol/L; ▩); heat shock (HS; 16 h at 42 to 45°C/32h at 37°C; ⊞); low glucose (Gl; 5.5 mmol/L; ▨); or lactate (L; 15 mmol/L; ▨) for 48 hours. Gene expression was analyzed by Northern blotting and was quantitated by densitometry. *$P < 0.05$ versus N.

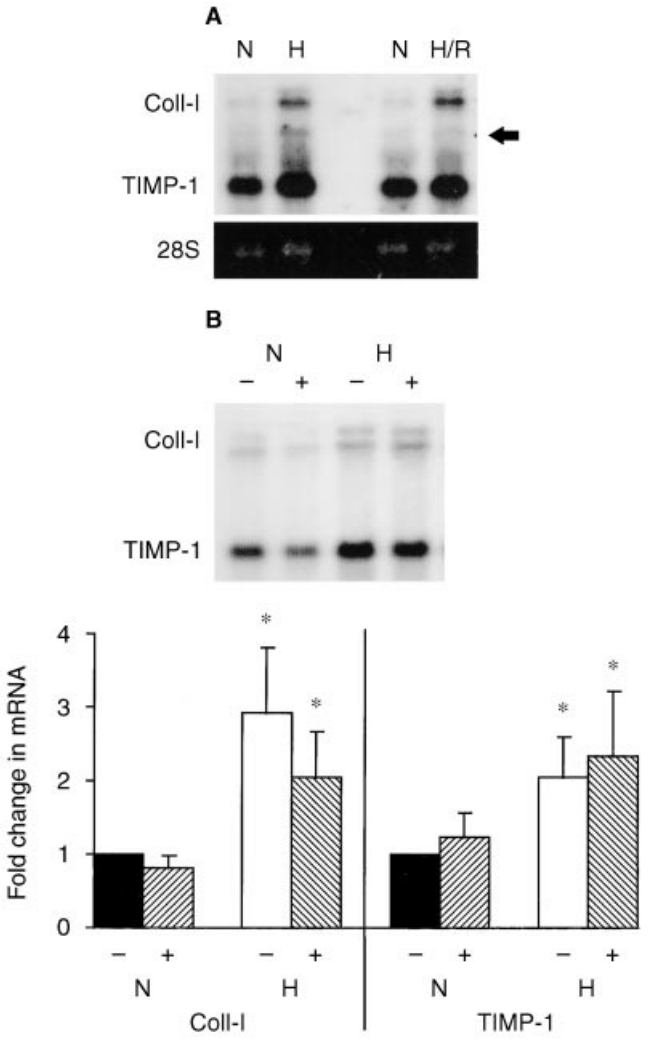

Fig. 6. (*A*) Effect of hypoxia on expression of transforming growth factor-β1 (TGF-β1) mRNA (2.5 kb, arrow). tsHRF were exposed to hypoxia for 48 hours (H) or 48 hours of hypoxia followed by 24 hours of normoxia (H/R) compared with normoxic controls (N). A representative autoradiogram is shown, including the signals for Coll-I and TIMP-1 relative to the 28S rRNA. (*B*) Effect of neutralizing anti–TGF-β1 antibody on the hypoxia-induced increase in Coll-I (left panel) and TIMP-1 (right panel) mRNA levels. Antibody was added immediately before exposure to hypoxia or normoxia, and cells were incubated for 48 hours. mRNA expression was measured by Northern blotting and quantitated by densitometry: (−) nonspecific IgG (■, normoxia; □ hypoxia); (+)2 μg/mL anti-TGF-β1 antibody (▨, normoxia; ▧, hypoxia). N = 3 experiments. *$P < 0.05$ versus N. Insert shows Northern blot analysis.

their effect on the hypoxia-induced increase in TIMP-1 and Coll-I mRNA. Cal-C blocked the hypoxia-induced increase in both TIMP-1 and Coll-I mRNA (Fig. 11). BI (up to 10 μmol/L) slightly decreased Coll-I mRNA, but levels remained significantly increased above normoxic controls, while the increase in TIMP-1 mRNA in the presence of inhibitor was no longer significant (Fig. 11). Both TK inhibitors, Gen and Lav-A, blocked the increase in TIMP-1 mRNA induced by 48 hours of hypoxia, while only Lav-A suppressed the expression of Coll-I mRNA (Fig. 11).

DISCUSSION

The mechanisms underlying PRD have not been established. We have previously suggested that hypoxia, a result of microvascular compromise, may play an important role in the pathogenesis of fibrosis [7, 8, 20, 27]. The strong correlation between progression of end-stage renal disease and tubulointerstitial fibrosis has highlighted the importance of interstitial fibroblasts, the major matrix-producing cells, in this disease [5]. Relatively little is known about the biology of this cell type; however, a subpopulation of interstitial fibroblasts produces erythropoietin (EPO) and thus possess an O_2-sensing mechanism [33]. To determine whether hypoxia induces changes relevant to fibrogenesis in renal fibroblasts, the in vitro effect of 1% O_2 on cell proliferation, myofibroblastic differentiation, and ECM metabolism was evaluated.

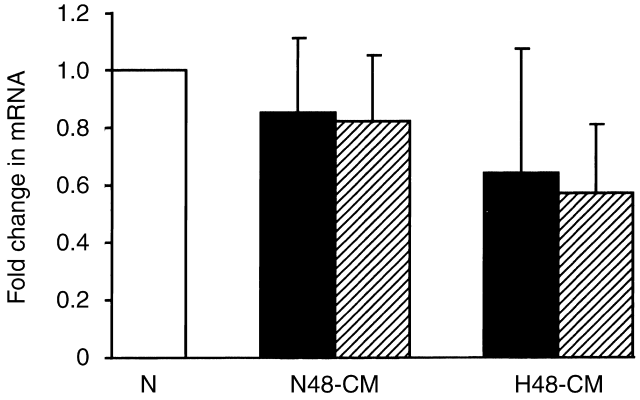

Fig. 7. Effect of conditioned medium (CM) from tsHRF exposed to 48 hours of hypoxia (H48-CM) or 48 hours of normoxia (N48-CM) on Coll-I (■) and TIMP-1 (▨) mRNA in naive tsHRF after 48 hours. mRNA levels in cells under normoxic conditions in basal medium (□) were assigned a value of 1, and the fold change in response to CM was calculated. $N = 4$ experiments.

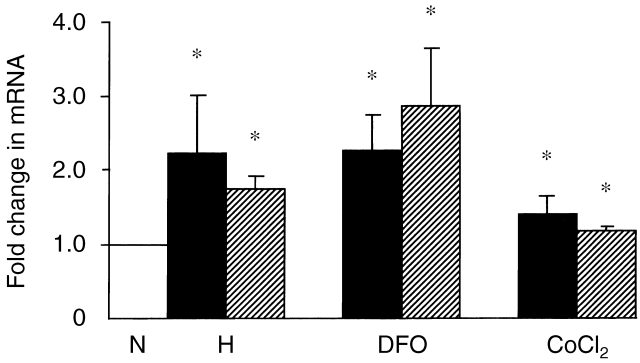

Fig. 8. Effect of hypoxia (H), DFO (50 μmol/L), or $CoCl_2$ (50 μmol/L) on Coll-I (■) and TIMP-1 (▨) mRNA expression in tsHRF after 48 hours. Fold change in mRNA calculated relative to normoxic controls (N; □) was assigned an arbitrary value of 1. $^*P < 0.05$ vs. N. $N = 3$ experiments.

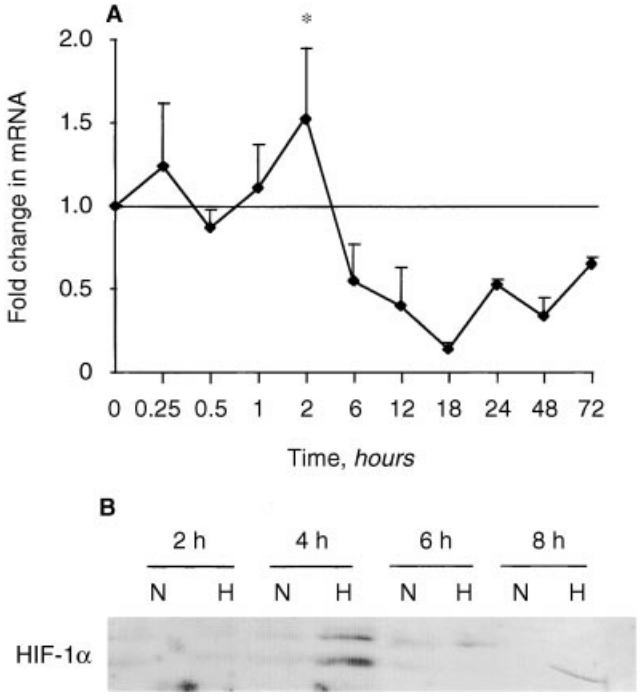

Fig. 9. Effect of hypoxia on hypoxia inducible factor-1 (HIF-1) expression. (*A*) Time course of expression of HIF-1α mRNA in tsHRF exposed to hypoxia compared with normoxic cells (assigned a value of 1, indicated by the horizontal line). $^*P < 0.05$ vs. N. $N = 3$ experiments. (*B*) Western blot analysis of HIF-1α protein accumulation in the nuclei of hypoxic cells with time (h).

In vivo interstitial fibrosis is marked by an increase in interstitial cell number caused, at least in part, by an increase in cell proliferation [5], together with an increase in cells expressing a myofibroblastic phenotype [5, 31]. Consistent with a role as a fibrogenic stimulus, hypoxia stimulated fibroblast proliferation and induced SMA expression. The cardinal feature of interstitial fibrosis is ECM accumulation, which occurs as a result of both increased synthesis and decreased turnover [5]. In the present study, Coll-I and -III were examined as representative ECM components. Coll-I is the predominant collagen representing 80% of collagen produced by fibroblasts, with Coll-III comprising 15 to 20%, and these are the predominant components of the fibrotic matrix [34]. Consistent with reports in other mesenchymal cells [18, 19, 21] and with our previous study in renal PTE [20], hypoxia stimulated an early and time-dependent increase in Coll-I mRNA and collagen production in tsHRF. However, none of the previous studies have addressed the mechanisms of hypoxic regulation of collagen gene expression.

In parallel with changes in ECM production, hypoxia also altered ECM turnover suppressing expression of MMP-1, the collagenase primarily responsible for degradation of fibrillar collagens [22, 23]. In contrast to PTE in which hypoxia suppressed MMP-2 [20], there was no change in MMP-2 expression in hypoxic tsHRF demonstrating cell type-specific regulation of this enzyme by low O_2. MMP-9 was also unaffected by hypoxia in tsHRF, consistent with a recent report on expression of this enzyme in hypoxic tumor cell lines [35]. The decrease in MMP-1 mRNA in hypoxic tsHRF suggests negative regulation of this gene via either decreased transcription or decreased mRNA stability; this is in contrast to the translational or post-translational suppression of MMP-2 in hypoxic PTE [20]. Although there are examples of genes that are suppressed by low O_2 [20, 36], relatively little is known about negative regulation of genes by hypoxia and the mechanisms of hypoxic suppression of *MMP-1* gene expression remain to be investigated.

tsHRFs express three of the four known TIMPs, two of which, TIMP-1 and -3, were induced by hypoxia, while TIMP-2 mRNA expression was unaffected, demonstra-

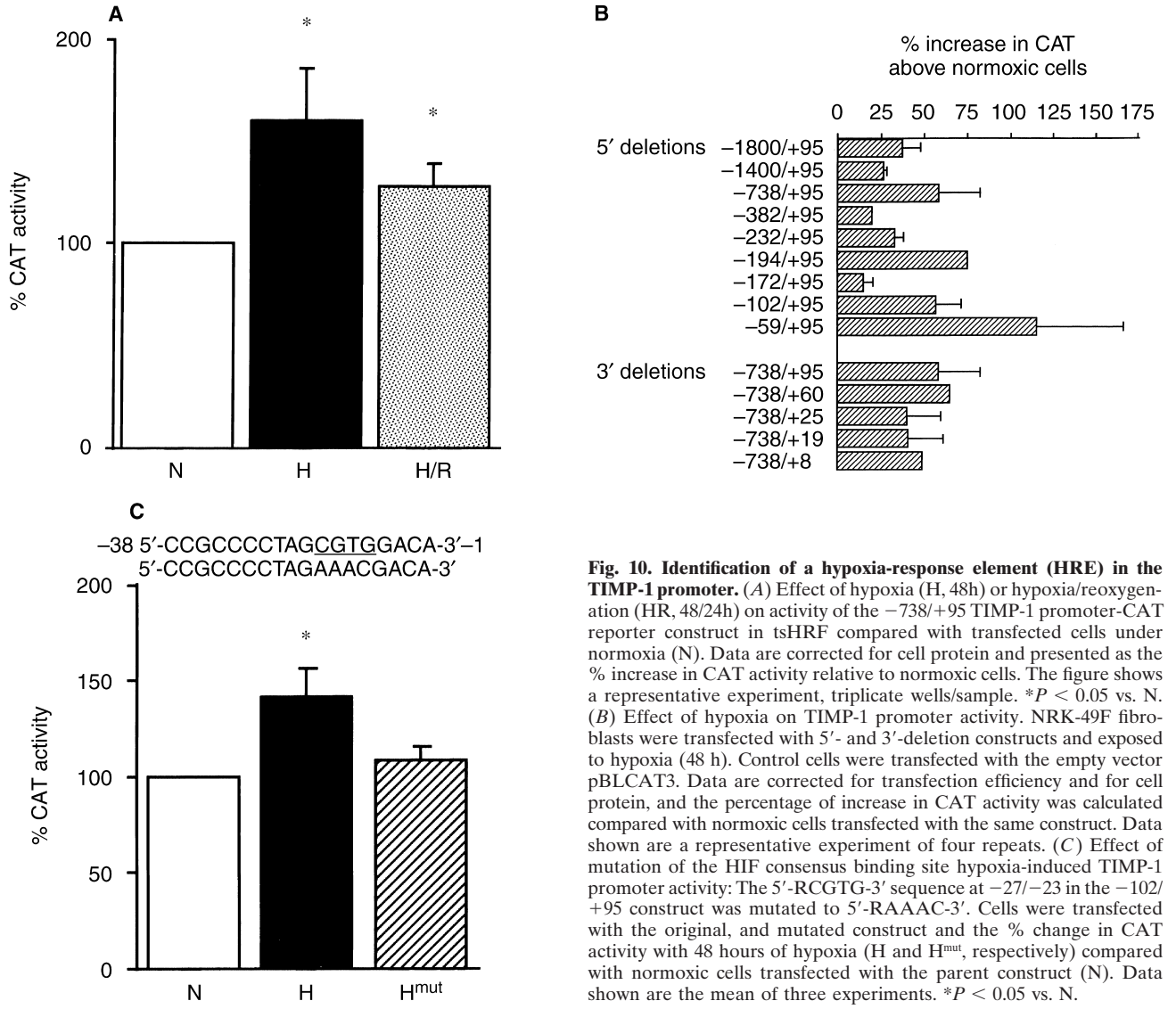

Fig. 10. Identification of a hypoxia-response element (HRE) in the TIMP-1 promoter. (*A*) Effect of hypoxia (H, 48h) or hypoxia/reoxygenation (HR, 48/24h) on activity of the −738/+95 TIMP-1 promoter-CAT reporter construct in tsHRF compared with transfected cells under normoxia (N). Data are corrected for cell protein and presented as the % increase in CAT activity relative to normoxic cells. The figure shows a representative experiment, triplicate wells/sample. $*P < 0.05$ vs. N. (*B*) Effect of hypoxia on TIMP-1 promoter activity. NRK-49F fibroblasts were transfected with 5′- and 3′-deletion constructs and exposed to hypoxia (48 h). Control cells were transfected with the empty vector pBLCAT3. Data are corrected for transfection efficiency and for cell protein, and the percentage of increase in CAT activity was calculated compared with normoxic cells transfected with the same construct. Data shown are a representative experiment of four repeats. (*C*) Effect of mutation of the HIF consensus binding site hypoxia-induced TIMP-1 promoter activity: The 5′-RCGTG-3′ sequence at −27/−23 in the −102/+95 construct was mutated to 5′-RAAAC-3′. Cells were transfected with the original, and mutated construct and the % change in CAT activity with 48 hours of hypoxia (H and H^{mut}, respectively) compared with normoxic cells transfected with the parent construct (N). Data shown are the mean of three experiments. $*P < 0.05$ vs. N.

ting specific effects of hypoxia on different members of the same family of inhibitors. Hypoxic induction of TIMP-1 mRNA followed a similar time course to that of Coll-I mRNA, suggesting coordinate regulation of these two genes. An increase in TIMP-1 mRNA appears to be a widespread response to hypoxia in that up-regulation of mRNA levels was observed in a variety of mesenchymal cells including hepatic stellate cells, skin fibroblasts, and lung fibroblasts (unpublished observations) possibly pointing to a common, hypoxia-induced fibrogenic pathway(s) in different tissues that succumb to fibrosis [37]. Although the primary consequence of increased TIMP-1 would be decreased matrix degradation, TIMPs are multifunctional molecules with effects of cell growth, differentiation, and apoptosis [24, 25, 38, 39] independent of their MMP-inhibitory activity and so may have other autocrine or paracrine effects relevant to fibrosis. For example, TIMP-1 inhibits proliferation of some epithelial cells and inhibits angiogenesis, which may be important in tubular regeneration and capillary renovation and replacement after injury. In terms of potential autocrine effects, exogenous recombinant TIMP-1 had no effect on tsHRF proliferation or myofibroblast differentiation (unpublished observations). TIMPs also have both pro- and anti-apoptotic effects [38, 39], which if they occur in the kidney in vivo, would lead to inappropriate cell accumulation or removal. Indeed, changes in apoptosis have been implicated in the pathogenesis of fibrosis of a number of organs [40].

To investigate the mechanisms underlying the fibrogenic response of tsHRF to hypoxia, Coll-I and TIMP-1 mRNA were used as indicators of ECM synthesis and turnover, respectively. The effects of hypoxia on these two genes appeared to be specific to low O_2 rather than

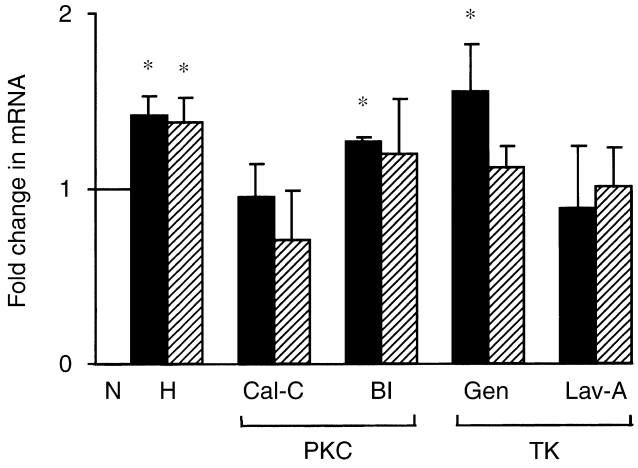

Fig. 11. Effect of protein kinase C (PKC) and tyrosine kinase (TK) inhibitors on hypoxia-induced (H) Coll-I (■) TIMP-1 (▨) mRNA levels. Inhibitors Cal-C (0.1 μmol/L), BI (10 μmol/L), Gen (10 μmol/L), or Lav-A (1 μmol/L) were added prior to hypoxia and the cells incubated under hypoxic conditions for 48 hours. The change in mRNA in response to hypoxia was calculated relative to untreated, normoxic cells (N; □) assigned a value of 1. $*P < 0.05$ vs. N. $N = 3$ experiments.

a general stress response in that a variety of cellular stresses failed to elicit a similar response. Although as increasing literature supports a contributory role for oxidative stress in the pathogenesis of fibrosis [41, 42], the lack of effect of H_2O_2 is consistent with one model of hypoxic regulation of gene expression in which specific genes are normally suppressed by H_2O_2; decreased O_2 releases this suppression and induces the genes [9], in which case exposure of cells to H_2O_2 would not be expected to induce a similar pattern of gene expression to that induced by low O_2. With regard to other cell stresses, the lack of effect of heat shock on Coll-I or TIMP-1 mRNA expression in tsHRF supports the suggestion from studies on the *EPO* gene that hypoxia and heat shock act via independent pathways [9–11, 33]. The lack of response to hypoglycemia and hyperlactemia, both of which have been reported to stimulate collagen production in some mesenchymal cells [43–45], also suggests hypoxia-specific regulation of Coll-I and TIMP-1.

Hypoxia can alter gene expression via a variety of mechanisms: by increasing gene transcription via binding of inducible transcription factors to HREs in 5′- or 3′-regulatory sequences, increasing mRNA stability [9–14], or by indirectly inducing autocrine/paracrine mediators. One of the growth factors induced by hypoxia is TGF-β1 [9–11], perhaps the premier renal "fibrokine" [5, 6], and the stimulatory effects of hypoxia on collagen production in mesangial cells and in dermal fibroblasts have been attributed to the autocrine actions of this factor (abstract, Sahai et al, *J Am Soc Nephrol* 13:910, 1995) [18]. Although TGF-β1 mRNA increased in response to hypoxia in HRF, neutralizing anti–TGF-β1 antibody studies and CM transfer experiments both mitigate against a hypoxia-inducible autocrine mediator and suggest direct effects on *TIMP-1* and *Coll-I* gene expression.

To address this question, regulation of TIMP-1 was examined in more detail. Transient transfection assays showed that the TIMP-1 promoter contains *cis*-acting sequences between −59/+8 that respond to O_2. The hypoxia-induced increase in reporter activity was of similar magnitude to the increase in endogenous steady-state mRNA levels, suggesting that increased transcription (rather than increased stability) accounts for the hypoxia-induced increase in mRNA consistent with transcriptional regulation of TIMP-1 [24, 25, 30].

Binding of HIF-1 to *cis*-acting HREs is a major regulatory mechanism by which hypoxia alters gene transcription. HIF-1α mRNA is constitutively expressed in tsHRF in vitro with a rapid transient increase in response to hypoxia, following a time course similar to that reported in Hep3B cells [12] and preceding the increase in Coll-I and TIMP-1 mRNAs. The increase in HIF-1 mRNA is followed by nuclear localization of HIF-1 protein. The hTIMP-1 promoter sequence [30] contains several potential HIF binding-sites (5′-RCGTG-3′) [9–11, 32–34], one of which (−26/−23) lies within the putative HRE. Mutation of this site abrogated the hypoxia-induced increase in promoter activity implicating HIF-1 in activation of the TIMP-1 promoter. HIF-1 binding is necessary but not sufficient for transcription of hypoxia-inducible genes, and other factors are required [9–11, 13–15]. The −59/+8 fragment of the TIMP-1 promoter contains binding sites for a number of other transcription factors that can be activated by hypoxia, including AP-2, AP-4, Sp-1, NF-1, and Elk-1, but the accessory factors for TIMP-1 promoter activation remain to be identified. Furthermore, other factors or indirect mechanisms must be invoked in increased TIMP-1 mRNA levels during reoxygenation, since HIF binding declines rapidly on return to normoxia [12–14]. It is of interest that the TIMP-3 promoter also contains HIF-1 binding motifs, which are absent from the TIMP-2 promoter [46, 47], consistent with the hypoxic inducibility of TIMP-3 but not TIMP-2.

Regulation of gene expression by O_2 requires an O_2 sensor that detects changes in pO_2 and activates intracellular signal transduction pathways [9–11, 33]. Many of the actions of molecular oxygen are mediated by heme-containing proteins, and a number of lines of evidence suggest the cellular O_2 sensor is a heme-protein, although the protein(s) remains to be isolated and characterized. In tsHRF, the mimetic effect an iron chelator and a transition metal on *Coll-I* and *TIMP-1* gene expression implicates a heme-protein O_2 sensor similar to that involved in *EPO* gene regulation [9–11, 33, 48] in the hypoxic-regulation of these two genes. One model of the sensor proposes that the heme-protein converts O_2 to H_2O_2 altering the redox status of the cell and activating

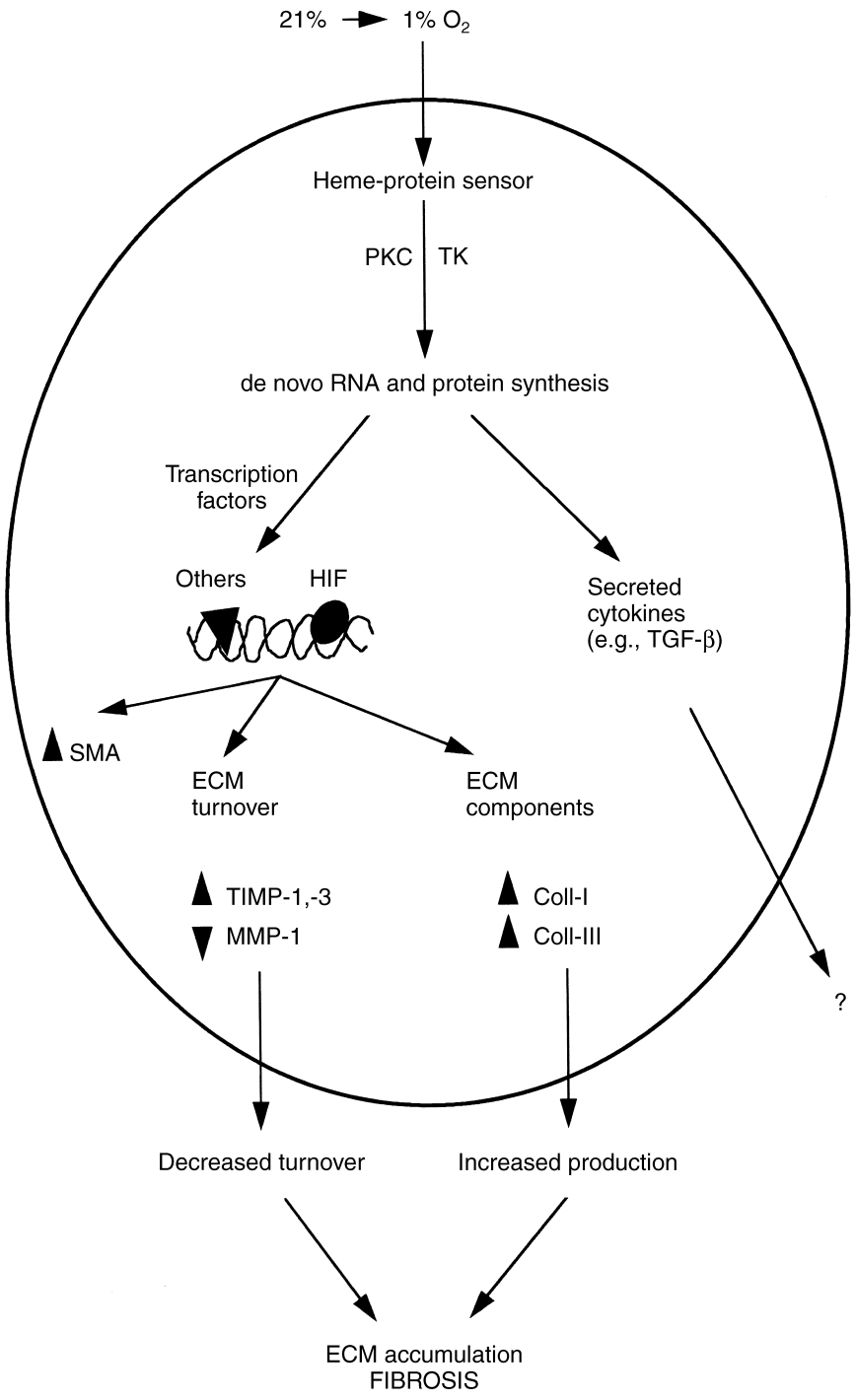

Fig. 12. Schematic summary of the mechanisms of hypoxia-induced fibrogenesis.

intracellular signaling pathways [9]; however, the fact that H_2O_2 does not mimic the effect of hypoxia on TIMP-1 and Coll-I mRNA argues against a redox-sensitive pathway in the hypoxic induction of these genes.

Although numerous signaling pathways have been implicated in hypoxic signal transduction, direct evidence linking specific pathways to transcriptional activation of particular hypoxia-induced genes is sparse, and there are considerable cell type- and gene-dependent differences [9–11]. The use of protein kinase inhibitors provides information on the signaling pathways involved in gene activation, and data in this study implicate both PKC- and TK-mediated pathways in the hypoxic induction of TIMP-1. Of the PKC inhibitors tested, BI is considered more specific than Cal-C, which targets all cysteine-rich proteins [49]. The lack of inhibition of hypoxic-induction

of Coll-I mRNA by BI implies that induction of Coll-I mRNA does not require activation of PKC. Indeed, activation of PKC with PMA down-regulates Coll-I mRNA. The differential response of TIMP-1 and Coll-I mRNA expression to TK inhibitors also suggests that alternate intracellular pathways act to regulate these genes in response to a common stimulus. In other cell types, PKC signaling increases HIF, and several protein kinase inhibitors, including genistein, staurosporine and 2-aminopurine, have been reported to inhibit HIF-1–dependent gene expression or HIF-1 DNA binding [32–34]. If hypoxic induction TIMP-1 mRNA is HIF-1–dependent and HIF-1 is blocked by these inhibitors, the same inhibitors would be expected to block TIMP-1 mRNA expression, which is, indeed, the case. In contrast, Coll-I mRNA induction is not blocked by genistein, suggesting HIF-independent regulation.

In summary, our study demonstrates that hypoxia induces a fibrogenic response in renal fibroblasts, simultaneously stimulating production of ECM and decreasing turnover (Fig. 12). These effects appear to independent of secreted autocrine mediators and to be mediated via a heme-protein O_2 sensor and PKC- and TK-signaling pathways. The hypoxic-induction of TIMP-1 is dependent on binding HIF-1 to a 5′ HRE, revealing a novel regulatory mechanism for this inhibitor and expanding the spectrum of genes regulated by changes in low O_2 to include those involved in ECM turnover. Taken together, the data suggest an important role for hypoxia in the initiation and progression of fibrosis. Understanding the molecular mechanisms by which decreased oxygen induces a fibrogenic program may open new avenues to therapy for what are, currently, intractable diseases.

APPENDIX

Abbreviations used in this article are: Act-D, actinomycin-D; CM, conditioned medium; $CoCl_2$, cobalt chloride; Coll-I, collagen α1(I); Coll-III, collagen α1(III); Cyc, cycloheximide; DFO, desferrioxamine; ECM, extracellular matrix; EPO, erythropoietin; FSA, fibroblast surface antigen; H, hypoxia; HIF, hypoxia-inducible factor; HPLC, high-pressure liquid chromatography; HRE, hypoxia response element; HRF, human renal fibroblasts; ^{3}H-Phe, tritiated phenalaline; ^{3}H-Thy, tritiated thymidine; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; N, normoxia; PKA, protein kinase A; PKC, protein kinase C; PRD, progressive renal disease; PTE, proximal tubular epithelial cells; SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinases; TK, tyrosine kinase; tsHRF, conditionally immortalized human renal fibroblasts.

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REFERENCES

1. Jacobson H: Chronic renal failure: Pathophysiology. *Lancet* 338:419–427, 1991
2. Bohle A, Kressel G, Müller C, Müller G: The pathogenesis of chronic renal failure. *Pathol Res Pract* 185:421–440, 1989
3. Bohle A, Strutz F, Müller G: On the pathogenesis of chronic renal failure in primary glomerulopathies: A view from the interstitium. *Exp Nephrol* 2:205–210, 1994
4. Bohle A, Gise HV, Mackensen-Haen S, Stark-Jacob B: The obliteration of the post-glomerular capillaries and its influence upon the function of both glomeruli and tubuli. *Klin Wochenschr* 59:1043–1051, 1981
5. Eddy A: Molecular insights into renal fibrosis. *J Am Soc Nephrol* 7:2495–2508, 1996
6. Border WA, Noble N: TGF-β in kidney fibrosis: A target for gene therapy. *Kidney Int* 51:1388–1396, 1997
7. Fine LG, Ong ACM, Norman JT: Mechanisms of tubulo-interstitial injury in progressive renal diseases. *Eur J Clin Invest* 23:259–265, 1993
8. Fine LG, Orphanides C, Norman JT: Progressive renal disease: The chronic hypoxia hypothesis. *Kidney Int* 53(Suppl 65):S74–S78, 1998
9. Bunn HF, Poynton R: Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* 76:839–885, 1996
10. Bauer C, Kurtz A (eds): Forefronts in Nephrology: Oxygen sensing on the cellular and molecular levels *Kidney Int* 51:371–608, 1997
11. Pugh CW: Oxygen and genes in health and disease. *Q J Med* 90: 307–310, 1997
12. Wood SM, Ratcliffe PJ: Mammalian oxygen sensing and hypoxia-inducible factor-1. *Int J Biochem Cell Biol* 29:1419–1432, 1997
13. Semenza GL: Hypoxia-inducible factor-1 and the molecular physiology of oxygen homeostasis. *J Lab Clin Med* 131:207–214, 1998
14. Wenger RH, Gassman M: Oxygen(es) and the hypoxia-inducible factor-1. *Biol Chem* 378:609–616, 1997
15. Rupec RA, Baeuerle PA: The genomic response of tumor cells to hypoxia and reoxygenation: Differential activation of transcription factors AP-1 and NF-κB. *Eur J Biochem* 234:632–640, 1995
16. Müller JM, Krauss B, Kaltschmidt C, Baeuerle PA, Rupec RA: Hypoxia induces c-fos transcription via a mitogen-activated protein kinase-dependent pathway. *J Biol Chem* 272:23435–23439, 1997
17. Ratcliffe PJ, Maxwell PH, Pugh CW: Beyond erythropoietin: The oxygen sensor. *Nephrol Dial Transplant* 12:1842–1848, 1997
18. Falanga V, Matrin TA, Tagaki H, Kirsner RB, Helfman T, Pardes KR: Low oxygen tension increases mRNA levels of alpha1(I) procollagen in human dermal fibroblasts. *J Cell Physiol* 157:408–412, 1994
19. Durmowicz AG, Parks WC, Hyde DM, Mecham RP, Stenmark KR: Persistence, re-expression and induction of pulmonary arterial fibronectin, tropoelastin and type I procollagen mRNA expression in neonatal hypoxic pulmonary hypertension. *Am J Pathol* 145: 1411–1420, 1994
20. Orphanides C, Fine LG, Norman JT: Hypoxia stimulates proximal tubular cell matrix production via a TGF-β1-independent mechanism. *Kidney Int* 52:637–647, 1997
21. Tamamori M, Ito H, Hiroe M, Marumo F, Hata R-I: Stimulation of

collagen synthesis in rat cardiac fibroblasts by exposure to hypoxic culture conditions and suppression of the effect by natriuretic peptides. *Cell Biol Int* 21:175–180, 1997
22. Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, Decarlo A, Engler JA: Matrix metalloproteinases: A review. *Crit Rev Oral Biol Med* 4:197–250, 1993
23. Stetler-Stevenson WG: Dynamics of matrix turnover during pathologic remodeling of the extracellular matrix. *Am J Pathol* 148:1345–1350, 1996
24. Denhardt DT, Feng B, Edwards DR, Cocuzzi ET, Malyankar UM: Tissue inhibitor of metalloproteinases (TIMP, aka ERP): Structure, control of expression and biological functions. *Pharmacol Ther* 59:329–341, 1993
25. Gomez DE, Alonso DF, Yoshiji H, Thorgiersson UP: Tissue inhibitors of metalloproteinases: Structure, regulation and biological functions. *Eur J Cell Biol* 74:111–122, 1997
26. Jat PS, Sharp PA: Cell lines established by a temperature-sensitive Simian Virus 40 large T-antigen gene are growth restricted at the non-permissive temperature. *Mol Cell Biol* 9:1672–1681, 1989
27. Ong ACM, Jowett TP, Firth JD, Burton S, Karet FE, Fine LG: An endothelin-1 mediated autocrine growth loop involved in human renal tubular regeneration. *Kidney Int* 48:390–401, 1995
28. Campa JS, McAnulty RJ, Laurent GJ: Application of high pressure liquid chromatography to studies of collagen production by isolated cells in culture. *Anal Biochem* 186:257–263, 1990
29. Weisener MS, Turley H, Allen WE, Willam C, Eckhardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH: Induction of endothelial PAS domain protein-1 by hypoxia: Characterisation and comparison with hypoxia inducible factor-1 alpha. *Blood* 92:2260–2268, 1998
30. Clark IM, Rowan AD, Edwards DR, Bech-Hansen T, Mann DA, Bahr MJ, Cawston TE: Transcriptional regulation of the human tissue inhibitor of metalloproteinases-1 (TIMP-1) gene in fibroblasts involves elements in the promoter, exon 1 and intron 1. *Biochem J* 324:611–617, 1997
31. Boukhalfa G, Desmoulière A, Rondeau E, Gabbiani G, Sraer J: Relationship between alpha-smooth muscle actin expression and fibrotic changes in the human kidney. *Exp Nephrol* 4:241–247, 1996
32. Pugh CW, O'Rourke JF, Nagao M, Gleadle JM, Ratcliffe PJ: Activation of hypoxia-inducible transcription factor-1: Definition of regulatory domains within the α subunit. *J Biol Chem* 272:11205–11214, 1997
33. Ratcliffe P: Molecular biology of erythropoietin. *Kidney Int* 44:887–904, 1994
34. Kovacs EJ, Dipietro LA: Fibrogenic cytokines and connective tissue production. *FASEB J* 8:854–861, 1994
35. Himelstein BP, Koch CJ: Studies of type IV collagenase regulation by hypoxia. *Cancer Lett* 124:127–133, 1998
36. McQuillan LP, Leung GK, Anderson PA, Kostyk SK, Kourembas S: Hypoxia inhibits expression of eNOS via transcriptional and post-transcriptional mechanisms. *Am J Physiol* 267:H1921–H1927, 1994
37. El Nahas AM (ed): Renal scarring: A multi-organ approach to fibrosis. *Exp Nephrol* 3:65–148, 1995
38. Baker AH, Zaltsman AB, George SJ, Newby AC: Divergent effects of TIMP-1-2 or -3 overexpression in rat vascular smooth muscle cell invasion, proliferation and death in vitro. TIMP-3 promotes apoptosis. *J Clin Invest* 101:1478–1487, 1998
39. Alexander CM, Howard EW, Bissell MJ, Werb Z: Rescue of mammary epithelial cell apoptosis and entactin degradation by tissue inhibitor of metalloproteinases-1 transgene. *J Cell Biol* 135:1669–1677, 1996
40. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawlet S, Hovell C, Arthur MJ: Mechanisms of spontaneous resolution of rat liver fibrosis: Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest* 102:538–549, 1998
41. Poli G, Parola M: Oxidative damage and fibrogenesis. *Free Radic Biol Med* 22:287–330, 1997
42. Hernández-Muñoz R, Díaz-Muñoz M, Chagoya de Sánchez V: Possible role of cell redox state on collagen metabolism in carbon tetrachloride-induced cirrhosis as evidenced by adenosine administration to rats. *Biochim Biophys Acta* 1200:93–99, 1994
43. Breborowicz A, Martis L, Oreopoulos DG: In vitro influence of lactate on function of peritoneal fibroblasts. *Adv Perit Dial* 10:225–229, 1994
44. Cerbón-Ambriz J, Cerbón-Solórzano J, Rojkind M: Regulation of collagen production in freshly isolated cell populations from normal and cirrhotic rat liver: Effect of lactate. *Hepatology* 13:551–556, 1991
45. Hunt TK, Banda MJ, Silver IA: Cell interactions in post-traumatic fibrosis, in *Fibrosis, Ciba Foundation Symposium*, London, Pitman, 1985, pp 127–149
46. Declerk YA, Darville MI, Eeckhout Y, Rousseau GG: Characterisation of the promoter of the gene encoding human tissue inhibitor of metalloproteinase-2 (TIMP-2). *Gene* 139:185–191, 1994
47. Wick M, Härönen R, Mumberg D, Bürger C, Olsen BR, Budarf M, Apte SS, Müller R: Structure of the human TIMP-3 gene and its cell cycle-regulated promoter. *Biochem J* 311:549–554, 1995
48. Gleadle JM, Ebert BL, Firth JD, Ratcliffe P: Regulation of angiogenic growth factor expression by hypoxia, transition metals and chelating agents. *Am J Physiol* 268:C1362–C1368, 1995
49. Nixon JS: The biology of protein kinase C inhibitors, in *Protein Kinase C*, edited by Parker PJ, Dekker LV, London, RG Landes Company, 1997, pp 205–236