

Regulation of Endothelial Matrix Metalloproteinase-2 by Hypoxia/Reoxygenation

Yaara Ben-Yosef, Nitza Lahat,* Sarah Shapiro, Haim Bitterman, Ariel Miller*

Abstract—Among the consequences resulting from the exposure of endothelial cells (ECs) to ischemia/reperfusion is angiogenesis, involving degradation of vascular basement membrane and extracellular matrix. Matrix metalloproteinase (MMP)-2, a member of the MMP family, partakes in this process. MMP-2, secreted as a proenzyme, undergoes activation through interaction with membrane type (MT)1-MMP and the endogenous tissue inhibitor of MMPs (TIMP)-2. Although hypoxia and reoxygenation (H/R) are major constituents of ischemia/reperfusion processes, their direct effects on endothelial MMP-2 have been scarcely investigated. This study examined the in vitro effects of H/R on human macrovascular ECs (EAhy 926). The level of MMP-2 mRNA (Northern blot) and protein (zymography, ELISA) and the mRNA of its activator (MT1-MMP) and inhibitor (TIMP-2) were analyzed. Short (6-hour) hypoxia inhibited the mRNA expression of MMP-2, MT1-MMP, and TIMP-2, culminating in reduced latent and active MMP-2 protein. Prolonged (24-hour) hypoxia further suppressed MT1-MMP and TIMP-2 mRNA, whereas it enhanced MMP-2 mRNA and enzyme secretion (after 48-hour hypoxia). Reoxygenation did not influence the inhibited TIMP-2 but upregulated MMP-2 and MT1-MMP mRNA expression, leading to enhanced secretion of active MMP-2 protein. These results demonstrate H/R-mediated modulation of EC MMP-2 at both transcriptional and posttranscriptional levels. Prolonged hypoxia of ECs appears to enhance MMP-2 production and secretion, whereas reoxygenation further increases its level. These H/R-mediated effects on MMPs have the potential of enabling EC migration and possible angiogenesis. (*Circ Res.* 2002;90:784-791.)

Key Words: endothelium ■ hypoxia ■ reoxygenation ■ matrix metalloproteinases ■ tissue inhibitors of matrix metalloproteinases

Extensive research has been carried out in recent years in the field of ischemic injury relating to distinct responses by different cell types as well as specific organs to hypoxic insult. Endothelial cells (ECs), which form the inner coating of the blood vessel wall, are central participants in the response to ischemic and ensuing reperfusion processes, which may lead to either repair or further injury, depending on the duration and severity of the primary insult.^{1,2} Among the substances produced by the endothelium during ischemia are vasoactive mediators, superoxide radicals, proinflammatory cytokines, and angiogenic factors.³ An important consequence of endothelial ischemia/reperfusion (I/R) events is angiogenesis, which includes a number of sequential steps, among them, induction of vascular discontinuity, proliferation and migration of ECs, and structural reorganization of the new vasculature.⁴⁻⁶ These steps necessitate the focal degradation of extracellular matrix (ECM) components surrounding the endothelium. Proteolysis of the ECM involves substrate-specific enzymes, among which members of the

matrix metalloproteinase (MMP) family are of major importance.⁶⁻⁹ These proteases are produced by the ECs themselves, by other cells forming the vascular wall, and by activated immune cells in the microenvironment.⁹

MMPs participate in a variety of physiological remodeling processes as well as in pathological processes associated with excessive tissue degradation. Identified to date are ≈20 members of this family, all of which share structural and functional features, including a Zn²⁺-dependent catalytic site. MMPs are produced and generally secreted in a latent form that requires stepwise activation through interactions with a variety of proteins, including membrane-type (MT)-MMPs and adhesion molecules.^{8,9} The potentially deleterious nature of MMPs is tightly regulated at different levels, including transcriptional and posttranscriptional levels. Posttranslational regulation involves binding to specific endogenous tissue inhibitors of MMPs (TIMPs), which may prevent proteolytic cleavage of the proenzyme, as well as function of the active enzyme.¹⁰ It is currently accepted that the balance

Original received August 30, 2001; revision received January 31, 2002; accepted March 6, 2002.

From The Neuroimmunology Unit (Y.B.-Y., A.M.), Immunology Research Unit (Y.B.-Y., N.L., S.S.), and the Department of Internal Medicine A (H.B.), Carmel Medical Center; the Faculty of Medicine (N.L., H.B., A.M.), Technion-Israel Institute of Technology; and the Rappaport Institute for Research in Medical Science (H.B., A.M.), Haifa, Israel.

*Both authors contributed equally to this study.

Correspondence to Ariel Miller, MD, PhD, The Neuroimmunology Unit, Department of Neurology, Carmel Medical Center, 7 Michal St, Haifa 34362, Israel. E-mail millera@tx.technion.ac.il

© 2002 American Heart Association, Inc.

Circulation Research is available at <http://www.circresaha.org>

DOI: 10.1161/01.RES.0000015588.70132.DC

between MMP and TIMP activity in a particular environment may determine their net degrading potential.⁸⁻¹⁰

MMP-2 (72 kDa, gelatinase A) participates in the breakdown of collagen type IV, a major component of subendothelial basement membranes.¹¹ Thus, in this context, MMP-2 production by endothelial or surrounding cells may be vital to the formation of new functional blood vessels, in either the early process of degradation or the later reconstruction of the underlying basement membrane. Like most MMPs, MMP-2, secreted as an inactive proenzyme, requires proteolytic removal of a terminal propeptide domain for its activation. MT-MMPs, in particular MT1-MMP, are involved in this process. Paradoxically, the specific physiological MMP-2 inhibitor, TIMP-2, at low levels, promotes this activation by forming a membrane complex with MT1-MMP, anchoring the pro-MMP-2 to the cell surface.^{8,9,11}

Hypoxia and reoxygenation (H/R) are the main constituents of I/R conditions, and a variety of their effects on different characteristics of ECs have been reported.^{1,2,12} However, the direct effects of H/R on MMPs, including MMP-2, produced by ECs have been scarcely investigated. In the present study, we provide evidence that exposure of ECs to varying durations of hypoxia and consecutive reoxygenation differentially affects the expression of MMP-2 as well as that of MT1-MMP and TIMP-2, the molecules involved in the modulation of its proteolytic activity. These interactions may culminate in the enhancement of MMP-2 proteolytic activity, enabling the migration of ECs and angiogenesis.

Materials and Methods

Cell Culture

Human endothelial cells (EAhy 926, a generous donation of Dr C.J. Edgell, University of North Carolina, Chapel Hill)¹³ were maintained in DMEM supplemented with 10% FCS, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mmol/L glutamine, and HAT (Hypoxanthine, Aminopterin, Thymidine; Biological Industries) at 37°C in a humidified atmosphere containing 5% CO₂. For evaluation of enzymatic activity, the cells were cultured in microtiter plates (2×10⁴ per well, Nunc A/S), and RNA evaluation was performed in 10-cm dishes (2×10⁶ per plate, Nunc A/S). For zymography and ELISA evaluations, cells were cultured in a serum-free medium containing 0.1% BSA.

Cells were incubated with phorbol 12-myristate 13-acetate (PMA, 5 to 50 ng/mL, Sigma Chemical Co) as a positive control for MMP-2 activation (24 hours for protein evaluation or 12 hours for mRNA evaluation). Actinomycin D (ActD, Sigma Chemical Co) at 2 µg/mL (determined in preliminary experiments) was added for a period of 6 hours or 24 hours to inhibit mRNA synthesis.

Hypoxic Conditions

Hypoxia was performed in a sealed Plexiglas custom-built incubator kept at 37°C by a thermostat-controlling unit (Fried Electronics) that was equipped with gloves, enabling access to cultures without opening the chamber. Containers with water allowed for the maintenance of constant relative humidity inside the incubator. The gas mixture used in the present study contained 95% N₂ and 5% CO₂. The adjustment of the gas mixture in the incubator was attained by a fast initial "flow-through" at an accelerated rate, followed by a steady continuous flow of gas. The PO₂ level was measured continuously by an oxygen analyzer connected to the outlet. Final evaluation of PO₂ pressure and pH in the culture medium, assessed at the end of each experiment by using a standard blood gas analyzer (ABL510, Radiometer), demonstrated PO₂ pressure of 23±4 mm Hg. Hypoxic conditions were maintained for 6, 24, or 48 hours. Cells

were reoxygenated by incubation in a standard 5% CO₂ incubator (PO₂ 158 mm Hg) for an additional 24 hours. Cells incubated under normoxic conditions in a standard incubator for the same length of time as either hypoxia or H/R exposure served as controls.

Cell Viability

Thymidine uptake was used to determine cell proliferation and possible cytotoxicity after H/R experiments. After exposure to hypoxia or H/R, cells were pulsed with [³H]thymidine, and the level of radioactivity was evaluated after 24 hours by using standard liquid scintillation techniques. This assay demonstrated that cell proliferation was similar in hypoxic and normoxic conditions, and no cytotoxic or cytostatic effects were observed. Viability of cells in culture was determined by trypan blue exclusion and found to be >99% in all conditions.

Gelatinolytic Activity

Supernatants (22.5-µL volume per sample) collected from cells exposed to hypoxia or H/R were analyzed by zymography, which enables detection of both activated and zymogenic forms of the gelatinases MMP-9 and MMP-2. Secreted proteins were separated by electrophoresis in SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were renatured by incubation in 2.5% Triton X-100 for 30 minutes, incubated overnight in substrate buffer (50 mmol/L Tris-HCl, pH 7.5, containing 10 mmol/L CaCl₂, Sigma Chemical Co) at 37°C, and stained with 0.5% Coomassie brilliant blue. Molecular weight markers (Bio-Rad Laboratories) and positive controls and recombinant MMP-2 and MMP-9 (Chemicon) were run with each gel. A clear zone in the blue background indicated the presence of gelatinolytic activity. Computerized densitometry was used to evaluate relative enzymatic activity (BioImaging Gel Documentation System, Dingo and Renium; TINA Software, Raytest).

Evaluation of MMP-2 Protein by ELISA

Commercial ELISA (Amersham) was carried out according to the manufacturer's instructions. This ELISA, based on MMP-2 activity, enables measurement of only the activated form of MMP-2. To measure the total potential activity of MMP-2 (latent and active forms) in the culture medium, supernatants were treated with *p*-aminophenyl mercuric acetate before ELISA. Duplicate evaluations were performed for each sample. Absorbance at 450 nm was measured by using an automated reader (Anthos Instruments).

Evaluation of mRNA by Northern Blot Analysis

Total cellular RNA was extracted from ECs with the use of Tri-Reagent (Medical Research Center) according to the manufacturer's instructions. The RNA (20 µg) was then separated by electrophoresis on 1% agarose gels containing formaldehyde, and its integrity was visualized after ethidium bromide staining. The RNA was capillary-transferred to Hybond membranes (Amersham) and hybridized to radiolabeled MMP-2, MT1-MMP, and TIMP-2 oligomer probes (Biognostik) at 65°C in 50 mmol/L Tris-HCl (pH 7.5), 10% dextran sulfate, 1N NaCl, 1% SDS, and 100 µg/mL sheared herring sperm DNA. After autoradiography, membranes were stripped and exposed to G3PDH (Clontech) for PMA experiments or to 28S rRNA probe for hypoxia experiments to normalize for loading errors. Relative levels of MMPs or TIMPs to G3PDH or 28S rRNA signal were determined after computerized densitometric assessment.

Statistical Evaluation

Statistical significance of results was determined by using the Student *t* test when hypoxia or reoxygenation was compared with its respective control group. ANOVA, followed by the Tukey-Kramer test, was used for the mRNA analysis after H/R treatments. A value of *P*<0.05 was considered to be significant. Mean±SD values of 3 to 5 experiments are presented.

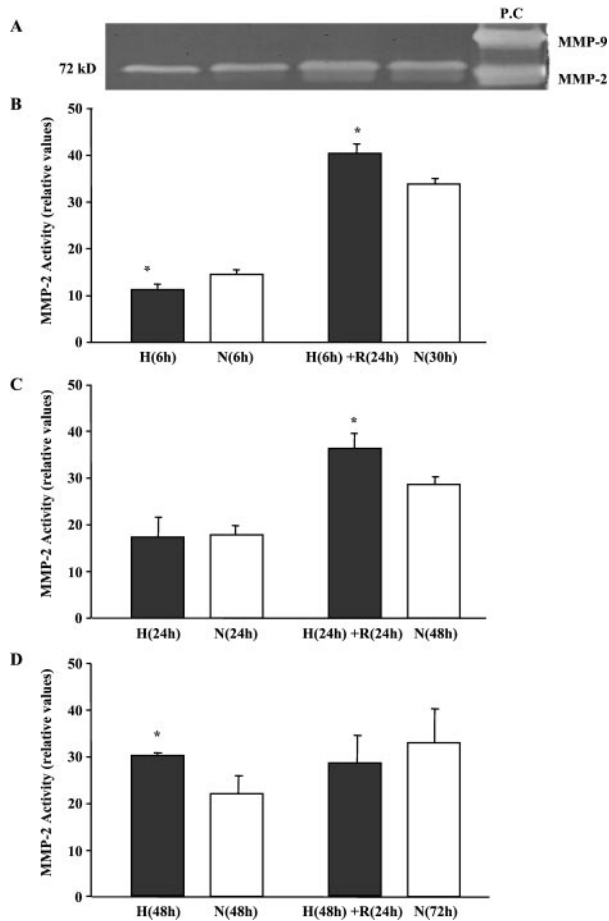


Figure 1. H/R modulate MMP-2 secreted by ECs. Supernatants from cultured ECs (EAhy 926) exposed to 6-hour (A and B), 24-hour (C), or 48-hour (D) hypoxia (H) followed by reoxygenation (H+R) were assessed by gelatin zymography (see Materials and Methods). Cells cultured in normoxic conditions (N) for the same length of time served as controls. Results of a typical zymogram, demonstrating bands of protease activity are shown in panel A. PC indicates positive control for MMP-2 and MMP-9 activity. Computerized relative densitometric analyses of the mean \pm SD of at least 3 experiments are presented in panels B through D. * $P < 0.05$ for H/R cells vs cells cultured in normoxic conditions for the same length of time.

Results

H/R-Mediated Modulation of MMP-2 Activity

Zymography was used to evaluate MMP-2 secreted by ECs after exposure to different periods of hypoxia (6, 24, and 48 hours) and to hypoxia followed by 24-hour reoxygenation (Figures 1A through 1D). The ECs were found to constitutively express only latent MMP-2 (72 kDa), whereas MMP-9 gelatinase activity was undetected (Figure 1A, lane 2). Hypoxia for 6 hours (Figure 1B) led to a slight, although significant, reduction (23%, $P = 0.02$) in the level of MMP-2 activity, and after 24 hours of hypoxia (Figure 1C), its level was similar to the constitutive level. After 48 hours of hypoxia, an elevation of 37% above the constitutive level of MMP-2 activity was observed ($P = 0.014$) (Figure 1D). Reoxygenation after 6 or 24 hours of hypoxia led to an increment (20% and 27%, respectively; $P < 0.003$) in MMP-2 activity compared with levels in cells under normoxic conditions,

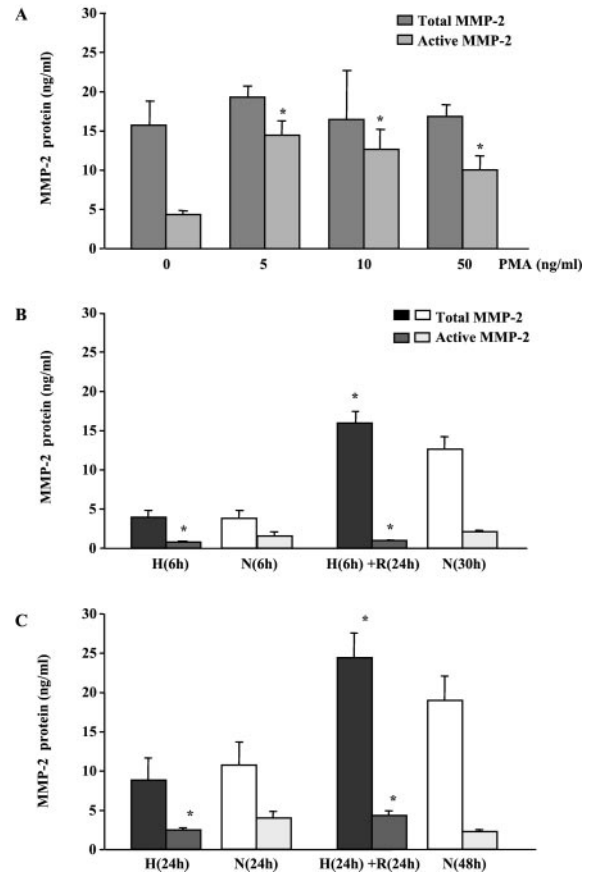


Figure 2. H/R modulate total and activated MMP-2 protein. ELISA was used to determine both total and activated forms of MMP-2 protein secreted after 6- or 24-hour hypoxia (H) and after 6- or 24-hour hypoxia followed by 24-hour reoxygenation (H+R; panels B and C, respectively). Cells cultured in normoxic conditions (N) for the same length of time and cells incubated with PMA for 24 hours (A) served as controls. Data shown are the mean \pm SD of 3 experiments. * $P < 0.05$ for H-, H+R-, or PMA-treated cells vs cells incubated in normoxic conditions for the same length of time.

whereas no increment was observed following reoxygenation after 48 hours of hypoxia. Although zymography is capable of separately detecting the higher molecular zymogen form (72 kDa) and the proteolytically active forms (62 to 68 kDa) of MMP-2, the lower molecular forms were barely visible, preventing their densitometric assessment. Therefore, ELISA was used to evaluate the level of activated MMP-2.

H/R-Mediated Modulation of Total and Active MMP-2 Protein

Because PMA has been reported to activate MMP-2 in ECs and in other cell types,^{14,15} this reagent was used as a positive control for the ELISA evaluation of the active MMP-2 level after H/R. Figure 2A demonstrates that PMA (5 ng/mL) led to a 3.3-fold increment ($P < 0.001$) in the level of active MMP-2 in relation to its constitutive level, whereas no change was observed in the total MMP-2 level, which included all its molecular weight forms (72 to 62 kDa).

The ELISA demonstrated relatively low constitutive levels of active MMP-2, $< 20\%$ of total MMP-2 (Figures 2B and 2C) in contrast to the results obtained after activation by

PMA. After 6-hour hypoxia (Figure 2B), the amount of active MMP-2 was significantly reduced (50%, $P=0.039$). Although consecutive reoxygenation significantly elevated the total MMP-2 level over the level observed in normoxic cells (25%, $P=0.009$), it did not alter the hypoxia-mediated suppression of the active form of MMP-2. After 24-hour hypoxia (Figure 2C), the level of active protein was still reduced (by 40%, $P=0.017$) compared with the level in cells cultured in normoxic conditions, and no significant change was observed in the total protein level.

Reoxygenation after 24-hour hypoxia significantly enhanced both total and active forms of MMP-2 (30% [$P=0.01$] and 90% [$P=0.03$], respectively) compared with corresponding controls. Of note, unlike the level of total MMP-2, whose concentration depended on the duration of culture (Figures 2B and 2C; 6-, 24-, and 48-hour normoxia), the active form of MMP-2 did not accumulate in the supernatants.

H/R Differentially Modulate the mRNA

Expression of MMP-2, MT1-MMP, and TIMP-2

In light of the modulation of MMP-2 protein by H/R, its mRNA level was evaluated by using Northern blot analysis. In parallel, the expression of MT1-MMP and TIMP-2, the physiological modulators of MMP-2, was also examined after H/R, whereas exposure of the cells to PMA served as a control.

Modulation of MMP-2

Exposure of ECs to PMA (10 to 50 ng/mL) for 12 hours did not alter the level of constitutive MMP-2 mRNA expression (Figure 3A). Short hypoxia of 6 hours (Figure 3B) led to the suppression of MMP-2 mRNA expression by 33% ($P<0.001$) compared with the MMP-2 mRNA level observed under normoxic conditions. The effects of 3-hour hypoxia were also evaluated, and the findings were similar to those obtained after 6 hours (data not presented). After reoxygenation following 6 hours of hypoxia, MMP-2 mRNA expression was 40% higher than its level after hypoxic conditions ($P=0.01$), thus returning to the constitutive level. Prolonged hypoxia (24 hours) had an opposite effect on the expression of MMP-2 mRNA, leading to a 60% elevation ($P=0.01$) compared with normoxic values (Figure 3C). Reoxygenation after 24 hours of hypoxia did not lead to any further change in the elevated level of MMP-2 mRNA expression, which remained higher (57%, $P=0.05$) than that observed in normoxic conditions.

Modulation of MT1-MMP

Exposure of ECs to PMA led to a significant elevation in the level of MT1-MMP mRNA expression (Figure 4A). At a concentration of 20 ng/mL PMA, the expression of MT1-MMP was 5.4-fold above the constitutive level ($P<0.001$). Compared with normoxia, short hypoxia (6 hours, Figure 4B) led to 35% ($P<0.001$) suppression of MT1-MMP mRNA. However, reoxygenation after short hypoxia led to an 83% elevation of MT1-MMP mRNA expression compared with its level after hypoxia alone ($P<0.001$). Prolonged hypoxia (Figure 4C) led to a further suppression in MT1-MMP mRNA expression (50% of normoxic level, $P<0.0001$), whereas subsequent reoxygenation led to a 2.8-fold elevation of MT1-MMP mRNA ($P<0.001$) compared with its level

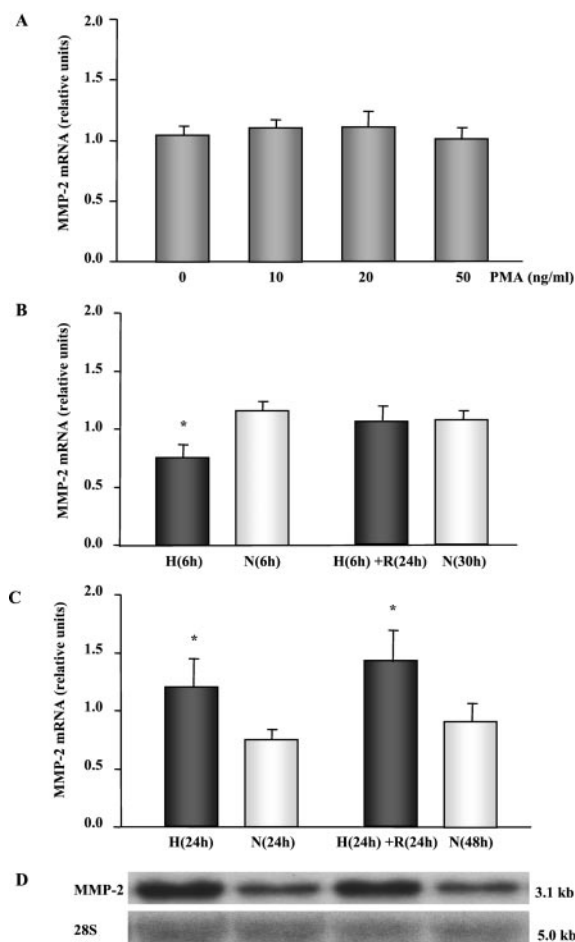


Figure 3. H/R modulate level of MMP-2 mRNA expression in ECs. Northern blot analysis of total cellular RNA was performed for cells exposed to 6- or 24-hour hypoxia (H) and for cells exposed to 6- or 24-hour hypoxia followed by 24-hour reoxygenation (H+R; panels B and C, respectively). Cells cultured in normoxic conditions (N) for the same length of time and cells incubated with PMA for 12 hours (A) served as controls. The histograms (A through C), each based on at least 4 experiments, display mean \pm SD of relative RNA values of MMP-2/G3PDH or MMP-2/28S rRNA. Representative Northern blot assay for 24-hour hypoxia followed by 24-hour reoxygenation is demonstrated in panel D. * $P<0.05$ for H-, H+R-, or PMA-treated cells vs cells cultured in normoxic conditions for the same length of time.

after hypoxia alone, resulting in a level similar to that observed for normoxic conditions.

Modulation of TIMP-2

PMA did not alter the constitutive level of TIMP-2 mRNA expressed in ECs in any of the concentrations used (Figure 5A). The effect of 6 hours of hypoxia was an 18% reduction ($P=0.003$) in the level of TIMP-2 mRNA expression compared with its level under normoxic conditions. Twenty-four hours of hypoxia further reduced the level of TIMP-2 mRNA expression (30% compared with cells cultured under normoxic conditions, $P=0.007$). The mRNA levels observed after reoxygenation following 6 or 24 hours of hypoxia remained lower than the levels for cells cultured in normoxic conditions (20% lower, $P\leq 0.002$) (Figures 5B and 5C).

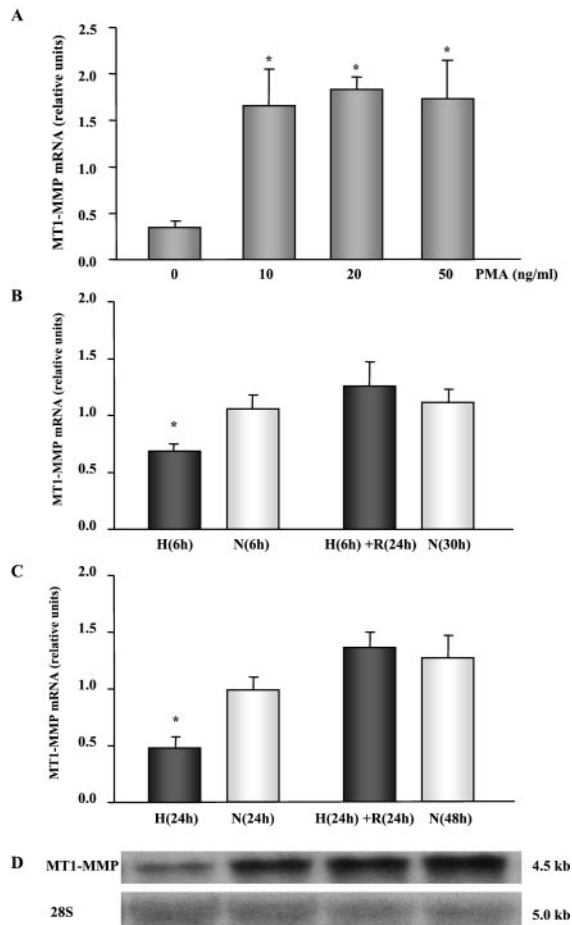


Figure 4. H/R modulate level of MT1-MMP mRNA expression in ECs. Northern blot analysis of total cellular RNA was performed for cells exposed to 6- or 24-hour hypoxia (H) and for cells exposed to 6- or 24-hour hypoxia followed by 24-hour reoxygenation (H+R; panels B and C, respectively). Cells cultured in normoxic conditions (N) for the same length of time and cells incubated with PMA for 12 hours (A) served as controls. The histograms (A through C), each based on at least 4 experiments, display mean \pm SD of relative RNA values of MT1-MMP/G3PDH or MT1-MMP/28S rRNA. Representative Northern blot for 24-hour hypoxia and 24-hour reoxygenation is demonstrated in panel D. * P <0.05 for H-, H+R-, or PMA-treated cells vs cells cultured in normoxic conditions for the same length of time.

Influence of H/R on the Stability of MMP-2, MT1-MMP, and TIMP-2 Transcripts

ActD, an inhibitor of mRNA synthesis, was added to cultures to determine whether the H/R-induced modulation of the mRNA expression was the result of changes in mRNA stability of the genes examined, MMP-2, MT1-MMP, and TIMP-2.

MMP-2 mRNA Stability

The addition of ActD (Figure 6A) to cultures did not influence the baseline level of MMP-2 mRNA expression under normoxic conditions. The addition of ActD to cultures exposed to hypoxia (24 hours) prevented the significant elevation of MMP-2 mRNA expression observed without ActD ($P=0.003$), resulting in a level similar to that observed in cells under normoxic conditions (Figure 6A). The addition of ActD to cells during the consecutive reoxygenation re-

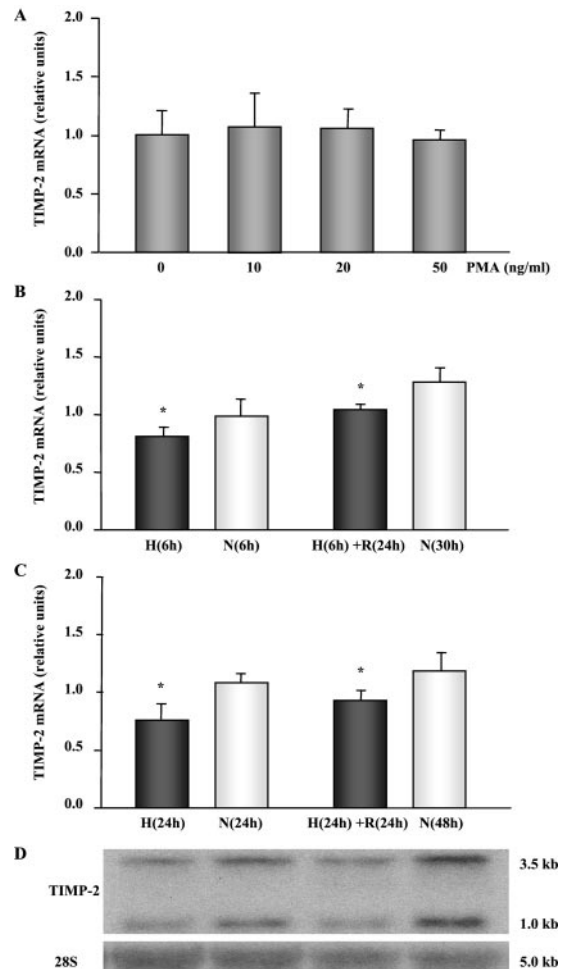


Figure 5. H/R inhibit TIMP-2 mRNA expression in ECs. Northern blot analysis of total cellular RNA was performed for cells exposed to 6-hour (B) or 24-hour (C) hypoxia (H) and for cells exposed to 6- or 24-hour hypoxia followed by 24-hour reoxygenation (H+R; panels B and C, respectively). Cells cultured in normoxic conditions (N) for the same length of time and cells incubated with PMA for 12 hours (A) served as controls. The histograms (A through C), each based on at least 4 experiments, display mean \pm SD of relative RNA values of TIMP-2/G3PDH or TIMP-2/28S rRNA. Representative Northern blot for 24-hour hypoxia and 24-hour reoxygenation is demonstrated in panel D. * P <0.05 for H-, H+R-, or PMA-treated cells vs cells maintained in normoxic conditions for the same length of time.

sulted in an elevation of 34% ($P=0.008$) of MMP-2 mRNA expression compared with cells cultured without ActD (Figure 6B) and a 2.2-fold increment ($P<0.001$) compared with normoxic cells, with or without ActD.

Experiments performed with ActD during short hypoxia demonstrated that ActD abolished the hypoxia-induced reduction in MMP-2 mRNA expression (data not shown); this finding was similar to that for MT1-MMP and TIMP-2 mRNA expression after the addition of ActD for 24-hour hypoxia (see below).

MT1-MMP mRNA Stability

The addition of ActD to ECs during 24 hours of hypoxia led to a significant enhancement (2.3-fold, $P<0.001$) in MT1-MMP mRNA compared with its expression without ActD,

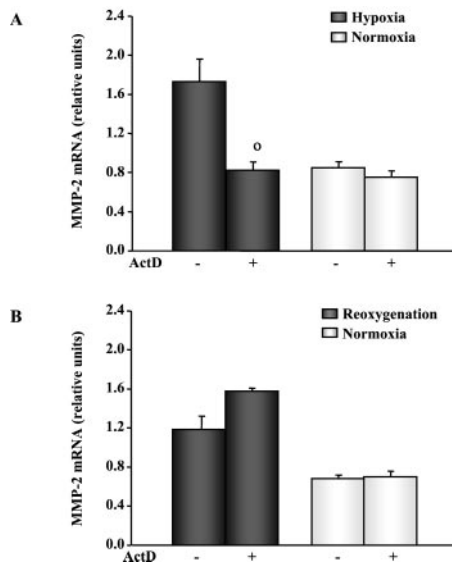


Figure 6. Prolonged hypoxia but not reoxygenation modulates transcription of MMP-2 mRNA. ActD (2 $\mu\text{g}/\text{mL}$) was added to cultured ECs either for 24-hour hypoxia (A) or after 24 hours of hypoxia for 24 hours of reoxygenation (B). Cells under normoxic conditions, with (+) or without (-) ActD, cultured for the same length of time, served as controls. Northern blot analysis was performed to evaluate total cellular RNA. The histograms, each based on 3 experiments, display mean \pm SD of relative values of MMP-2/28S rRNA. ° $P < 0.05$ for cells similarly treated with vs without ActD.

completely reversing the hypoxia-induced inhibition of the MT1-MMP mRNA (Figure 7A). The addition of ActD during reoxygenation abolished the enhancement of MT1-MMP mRNA expression observed without ActD, leading to a 65%

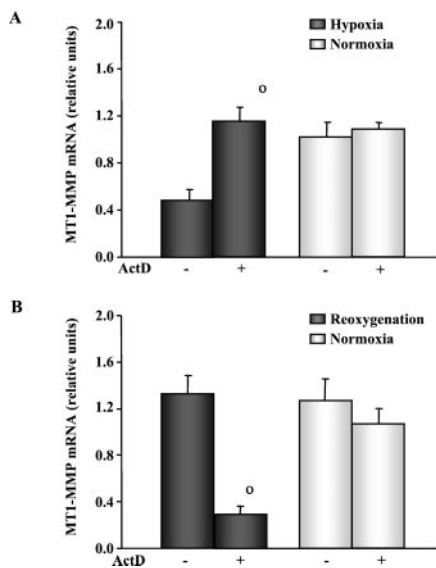


Figure 7. Prolonged H/R differentially modulate transcription of MT1-MMP mRNA. ActD (2 $\mu\text{g}/\text{mL}$) was added to cultured ECs either for 24-hour hypoxia (A) or after 24-hour hypoxia for 24 hours of reoxygenation (B). Cells under normoxic conditions, with (+) or without (-) ActD, cultured for the same length of time, served as controls. Northern blot analysis was performed to evaluate total cellular RNA. The histograms, each based on 3 experiments, display mean \pm SD of relative values of MT1-MMP/28S rRNA. ° $P < 0.05$ for cells similarly treated with vs without ActD.

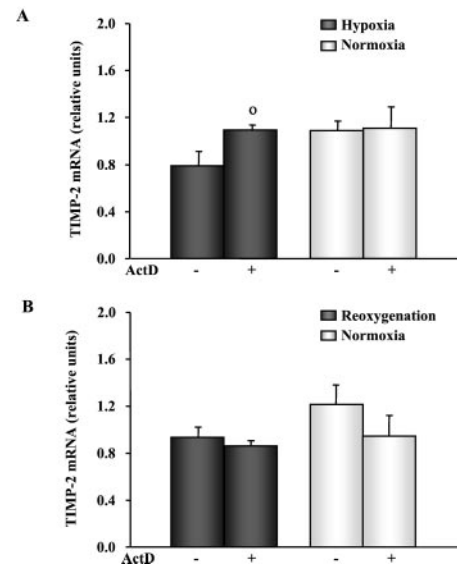


Figure 8. Prolonged H/R indirectly modulate transcription of TIMP-2 mRNA. ActD (2 $\mu\text{g}/\text{mL}$) was added to cultured ECs either for 24-hour hypoxia (A) or after 24-hour hypoxia for 24 hours of reoxygenation (B). Cells under normoxic conditions, with (+) or without (-) ActD, cultured for the same length of time, served as controls. Northern blot analysis was performed to evaluate total cellular RNA. The histograms, each based on 3 experiments, display mean \pm SD of relative values of TIMP-2/28S rRNA. ° $P < 0.05$ for cells similarly treated with vs without ActD.

reduction ($P < 0.001$) compared with nontreated reoxygenated cells (Figure 7B).

TIMP-2 mRNA Stability

The addition of ActD during 24-hour hypoxia led to a significant enhancement (40%, $P = 0.003$) of TIMP-2 mRNA expression compared with that of untreated cells, abolishing the hypoxia-induced suppression (Figure 8A). The addition of ActD during reoxygenation also reverted the suppression of the TIMP-2 mRNA expression observed without ActD (Figure 8B).

Discussion

Hypoxia is a strong inducer of angiogenesis-promoting factors that orchestrate complex interactions between ECs and the ECM, leading to neovascularization.¹⁶ Endothelial MMPs have been shown to play a pivotal role in these interactions because of their ability to degrade basement membrane components, a primary step in the process of angiogenesis.¹⁷ The human differentiated macrovascular endothelial cell line (EAhy 926) has been reported to constitutively express an array of MMPs, including MMP-2, MT1-MMP, and TIMP-2.¹⁸ Reports from previous *in vitro* studies relating to hypoxic modulation of MMPs^{2,19,20} and our preliminary results led us to use 6 hours as representative of acute hypoxia and 24 to 48 hours as representative of prolonged hypoxia.

Exposure of ECs to short hypoxia led to the inhibition of MMP-2, MT1-MMP, and TIMP-2 mRNA expression accompanied by a reduction in MMP-2 protein secretion. The mechanisms and biological significance of this reduction in MMP-2 and its regulators are not clear. The selective elevation of only the MMP-2 gene, observed after prolonged

hypoxia, resulted in elevated zymogen secretion but not in the increased active form of the enzyme, in accordance with our finding of a persistent decrease in the MT1-MMP mRNA expression. Additional activators of MMP-2, such as $\alpha_v\beta_3$ integrin,²¹ although not examined in the present study, are reported to be influenced by hypoxia.²² However, the observed lack of elevation in the MMP-2 active form suggests that these molecules did not contribute to the catalytic processing of secreted pro-MMP-2 during hypoxia. The enhanced production of the active form of MMP-2 after prolonged H/R is in agreement with the elevation of MMP-2 and MT1-MMP mRNAs as well as with the continued reduction of TIMP-2 mRNA. Of note, attempts to evaluate the level of TIMP-2 activity by reverse zymogram found that the sensitivity of the assay did not allow detection of this activity. The data demonstrated in the present study suggest that prolonged hypoxia of ECs upregulates “potential” MMP-2 activity, whereas reoxygenation, which induces elevated MT1-MMP mRNA, may be necessary for “full” MMP-2 activity.

ELISA and the zymographic assay used were complementary, rather than directly comparable, to each other. ELISA enabled us to detect the low quantity of active MMP-2, whereas zymography enabled the assessment of the latent form of the protein. Of note, however, are the seemingly conflicting findings: the low levels of the activated form of MMP-2 as opposed to the accumulation of the proenzyme detected in the supernatants. These low levels of activated MMP-2 may be the result of the isolated in vitro conditions, the existence of in vitro mechanisms preventing its activation, or its short half-life. MMP-2, because of its constitutive expression and its relatively poor response to exogenous regulators, was considered, until recently, to be a “housekeeping” gene.^{7,23} However, the changes in MMP-2 gene expression and its mild, but significant, protein activity suggest that MMP-2 is of prime importance in the response of ECs to hypoxia/ischemia.

The present in vitro results, demonstrating for the first time the distinct influences of H/R on MMP-2 expression in ECs, are in accordance with the few in vivo I/R studies performed.^{24–27} Enhanced activity of MMPs, primarily MMP-2 and MMP-9, coupled with a reduction in TIMP expression have been demonstrated in a variety of brain and heart ischemic models, whereas reperfusion aggravated the damage observed in ischemic rat brain, in correlation with the enhanced level of MMP expression. However, although elevated levels of MMPs may contribute to deleterious tissue degradation, they may also contribute to “repair processes,” such as angiogenesis and wound healing.^{24–27}

A different phenomenon was reported for ischemic kidneys. Ischemia in this organ led to a decrease in MMPs and an elevation in TIMP expression accompanied by fibrosis.²⁸ In vitro experiments with hypoxic renal epithelial cells demonstrated reduced MMP-2 (72-kDa) secretion, no change in TIMP-2, and enhanced TIMP-1 mRNA expression, which was associated with the in vivo ischemia-related kidney fibrosis.¹⁹ A different response to hypoxia was also observed in ovarian tumor cell lines, in which no changes in MMP-2 expression were found.²⁰ Taken together, the activity and role

of MMPs and TIMPs in ischemic processes seem not to be uniform and may depend on the tissue and cell type, as well as the duration and severity of the ischemia.

ECs of different origin may respond differently to environmental stimuli and, accordingly, express divergent MMPs.^{15,29} The distinct effects of H/R concerning MMP-2 and the molecules regulating its activity, as described in the present study, may be representative of macrovascular endothelium. Hypoxia of macrovascular endothelium is of relevance to ischemic processes occurring in a variety of vasculopathies.³⁰ It should also be noted that the complexity of the physiological in vivo I/R events might not be fully reflected in isolated cultured cells subjected to H/R. Nevertheless, in vitro studies of H/R are essential to appraise the basic mechanisms involved in I/R.

The present study also investigated whether the H/R-induced modulation of MMP-2, MT1-MMP, and TIMP-2 was the result of induction of transcriptional activity or secondary to changes in mRNA stability. The absence of changes in the mRNA levels of the three genes examined, after the addition of ActD to cells in normoxia, reflects their mRNA stability. Of interest was the ActD-mediated reversal of the hypoxia-induced inhibition of the genes (MMP-2 at 6 hours [data not shown] and MT1-MMP and TIMP-2 at 24 hours). These findings point to hypoxia-mediated enhancement of mRNAs encoding for proteins that induce MMP/TIMP mRNA degradation. The observed elevation of MMP-2 mRNA expression during 24-hour hypoxia was abolished in the presence of ActD, supporting de novo MMP-2 mRNA synthesis during prolonged hypoxia. The addition of ActD during reoxygenation did not alter the enhanced mRNA expression of MMP-2 or the reduced expression of TIMP-2 mRNA, implying that the changes in the MMP-2 and TIMP-2 mRNA levels were the result of transcription altered during hypoxia. In contrast, the enhanced expression of MT1-MMP mRNA observed after reoxygenation appears to be the result of upregulated transcription. Taken together, these experiments demonstrate that short hypoxia and prolonged hypoxia, as well as reoxygenation, induce distinct transcriptional regulation of MMP/TIMP genes in ECs.

Studies have identified specific molecules and transcription factors modulated by H/R,^{2,16,22,31} whereas other studies have implicated some of these factors in the regulation of MMP-2, MT1-MMP, and TIMP-2 gene expression.^{32–36} The common activator protein (AP)-1 was reported to play a dominant role in PMA-induced transcription of MMPs,^{8,9,37} and its expression is upregulated by hypoxia.³ Because the influence of PMA on the three genes studied has been well documented, it was chosen as a control in the present study, and the results observed are in accordance with reported data relating to PMA-mediated modulation of MMPs. However, the presence of an AP-1 binding site on the MMP-2 promoter is disputed, and no PMA-induced elevation in MMP-2 mRNA or protein has been reported.^{15,37} Nonetheless, PMA activates pro-MMP-2 through increased synthesis of its activator, MT1-MMP.¹¹ Likewise, PMA appears not to induce TIMP-2 transcription¹⁵ despite the identification of an AP-1 binding sequence in the TIMP-2 promoter.³⁸ Further studies are needed to elucidate the molecular mechanisms and factors

involved in the H/R-induced changes in transcription and activation of endothelial MMPs/TIMPs.

The present research demonstrates the complex regulation of endothelial MMP-2 expression after exposure to H/R. The results suggest important influences of both transcriptional and posttranscriptional mechanisms involved in the MMP-2 activity in ECs. Prolonged hypoxia, through the elevation of MMP-2, appears to have the potential to promote ECM degradation, and reoxygenation, through MT1-MMP elevation, may lead to a further increase in ECM breakdown. These changes associated with ECM remodeling by endothelial MMPs/TIMPs may play a central role in the response of the vascular compartment to H/R. Future research may have important implications toward modulating physiological and pathological angiogenesis, with the aim of developing new therapeutic strategies based on regulating endothelial MMPs. Such strategies may be relevant to promoting wound healing and hypoxic tolerance or to preventing atherosclerosis, tumor growth, and metastasis.

Acknowledgment

This work was supported by funds from the Rappaport Institute for Research in Medical Sciences, Haifa, Israel.

References

- Graven KK, Farber HW. Endothelial cell hypoxic stress proteins. *J Lab Clin Med.* 1998;132:456–463.
- Faller DV. Endothelial cell response to hypoxic stress. *Clin Exp Pharmacol Physiol.* 1999;26:74–84.
- Bandyopadhyay RS, Phelan M, Faller DV. Hypoxia induces AP-1-regulated genes and AP-1 transcription factor binding in human endothelial and other cell types. *Biochim Biophys Acta.* 1995;1264:72–78.
- Folkman J, Shing Y. Angiogenesis. *J Biol Chem.* 1992;267:10931–10934.
- Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med.* 2000;6:389–395.
- Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest.* 1999;103:1237–1241.
- Yu AE, Hewitt RE, Connor EW, Stetler-Stevenson WG. Matrix metalloproteinases, novel target for directed cancer therapy. *Drugs Aging.* 1997;11:229–244.
- Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and tissue inhibitors of metalloproteinases. *Crit Rev Eukaryot Gene Expr.* 1997;7:159–178.
- Nagase H. Activation mechanisms of matrix metalloproteinases. *Biol Chem.* 1997;378:151–160.
- Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol.* 1997;74:111–122.
- Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. *J Biol Chem.* 1995;270:5331–5338.
- Pohlman TH, Harlan JM. Adaptive responses of the endothelium to stress. *J Surg Res.* 2000;98:85–119.
- Edgell CS, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A.* 1983;80:3734–3737.
- Park MJ, Park IC, Hur JH, Rhee CH, Choe TB, Yi DH, Hong SI, Lee SH. Protein kinase C activation by phorbol ester increases in vitro invasion through regulation of matrix metalloproteinases/tissue inhibitors of metalloproteinases system in D54 glioblastoma cells. *Neurosci Lett.* 2000;290:201–204.
- Hanemaaijer R, Koolwijk P, Le-Clercq LL, De-Vree WJ, Van Hinsbergh VW. Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. *Biochem J.* 1993;296:803–809.
- Michiels C, Arnould T, Remacle J. Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions. *Biochim Biophys Acta.* 2000;1497:1–10.
- Haas TL, Joseph AM. Extracellular matrix metalloproteinase production in endothelial cells: implications for angiogenesis. *Trends Cardiovasc Med.* 1999;9:70–77.
- Nelimakka LO, Nikkari ST, Ravanti LS, Kahari VM, Jarvelainen HT. Collagenase-1, stromelysin-1 and 92 kDa gelatinase are associated with tumor necrosis factor- α induced morphological change of human endothelial cells in vitro. *Matrix Biol.* 1998;17:293–304.
- Orphanides C, Fine LG, Norman JT. Hypoxia stimulates proximal tubular cell matrix production via TGF- β independent mechanism. *Kidney Int.* 1997;52:637–647.
- Krtolica A, Ludlow JW. Hypoxia arrests ovarian carcinoma cell cycle progression, but invasion is unaffected. *Cancer Res.* 1996;56:1168–1173.
- Deryugina EI, Bourdon MA, Reisfeld RA, Strongin A. Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2. *Cancer Res.* 1998;58:3743–3750.
- Walton HL, Corjay MH, Mohamed SN, Mousa SA, Santomenna LD, Reilly TM. Hypoxia induces differential expression of the integrin receptors $\alpha_5\beta_3$ and $\alpha_v\beta_3$ in cultured human endothelial cells. *J Cell Biochem.* 2000;78:674–680.
- Polette M, Birembaut P. Membrane-type metalloproteinases in tumor invasion. *Int J Biochem Cell Biol.* 1998;30:1195–1202.
- Mun-Bryce S, Rosenberg GA. Matrix metalloproteinases in cerebrovascular disease. *J Cereb Blood Flow Metab.* 1998;18:1163–1172.
- Planas AM, Sole S, Justicia C, Farre ER. Estimation of gelatinase content in rat brain: effect of focal ischemia. *Biochem Biophys Res Commun.* 2000;278:803–807.
- Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation.* 2000;101:1833–1839.
- Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. *Stroke.* 1998;29:2189–2195.
- Eddy AA. Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol.* 1996;7:2495–2508.
- Jackson CJ, Nguyen M. Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. *Int J Biochem Cell Biol.* 1997;29:1167–1177.
- Shreenivas R, Ogawa S, Cozzolino F, Torcia G, Braunstein N, Butura C, Brett J, Lieberman HB, Furie MB, Joseph-Silverstein J. Macrovascular and microvascular endothelium during long-term hypoxia: alterations in cell growth, monolayer permeability, and cell surface coagulant properties. *J Cell Physiol.* 1991;146:8–17.
- Dachs GU, Tozer GM. Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. *Eur J Cancer.* 2000;36:1649–1660.
- Haas TL, Davis SJ, Madri JA. Three-dimensional type I collagen lattices induce coordinate expression of matrix metalloproteinases MT1-MMP and MMP-2 in microvascular endothelial cells. *J Biol Chem.* 1998;273:3604–3610.
- Bian J, Sun Y. Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. *Mol Cell Biol.* 1997;17:6330–6338.
- Zucker S, Mirza H, Conner CE, Lorenz AF, Drews MH, Bahou WF, Jesty J. Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. *Int J Cancer.* 1998;75:780–786.
- Lamoreaux WJ, Fitzgerald ME, Reiner A, Hasty KA, Charles ST. Vascular endothelial growth factor increases release of gelatinase A and decreases release of tissue inhibitor of metalloproteinases by microvascular endothelial cells in vitro. *Microvasc Res.* 1998;55:29–42.
- Benbow U, Brinckerhoff CE. The AP-1 site and MMP regulation: what is all the fuss about? *Matrix Biol.* 1997;15:519–526.
- Foda HD, George S, Conner C, Drews M, Tompkins DC, Zucker S. Activation of human umbilical vein endothelial cell progelatinase A by phorbol myristate acetate: a protein kinase C-dependent mechanism involving a membrane-type matrix metalloproteinase. *Lab Invest.* 1996;74:538–545.
- Hammani K, Blakis A, Morsette D, Bowcock AM, Schmutte C, Henriot P, DeClerck YA. Structure and characterization of the human tissue inhibitor of metalloproteinase-2 gene. *J Biol Chem.* 1996;271:25498–25505.

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Regulation of Endothelial Matrix Metalloproteinase-2 by Hypoxia/Reoxygenation Yaara Ben-Yosef, Nitza Lahat, Sarah Shapiro, Haim Bitterman and Ariel Miller

Circ Res. 2002;90:784-791; originally published online March 14, 2002;
doi: 10.1161/01.RES.0000015588.70132.DC

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:

<http://circres.ahajournals.org/content/90/7/784>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation Research* is online at:
<http://circres.ahajournals.org/subscriptions/>