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In vitro reoxygenation following hypoxia increases MMP-2 and TIMP-2 secretion by human umbilical vein endothelial cells

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Endothelial cells lining the inner blood vessel walls play a key role in the response to hypoxia, which is frequently encountered in clinical conditions such as myocardial infarction, renal ischemia and cerebral ischemia. In the present study we investigated the effects of hypoxia and hypoxia/reoxygenation on gelatinases (matrix metalloproteinase-2 and -9), their inhibitor (TIMP-2) and activator (MT1-MMP), in human umbilical vein endothelial (HUVE) cells. HUVE cells were subjected to 4 h of hypoxia or hypoxia followed by 4 and 24 h of reoxygenation. The pro- and active forms of MMP-2 and MMP-9 were analyzed by gelatin zymography; TIMP-2 protein level was assayed using ELISA, while MT1-MMP activity was measured using an activity assay. The secretion of MMP-2 proform increased significantly in cells subjected to 4 h of hypoxia followed by 4 or 24 h of reoxygenation, compared with the normoxic group. TIMP-2 protein level also increased significantly in the hypoxia/reoxygenation groups, compared with the normoxic group. There were no statistically significant differences in the levels of active MT1-MMP in all groups. This study indicates that MMP-2 and TIMP-2 could be regarded as important components of a mechanism in the pathophysiology of ischemic injury following reperfusion.

Keywords: hypoxia, reoxygenation, matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-2, membrane type-1 matrix metalloproteinase, endothelial cell

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INTRODUCTION

Hypoxia developed as a consequence of insufficient oxygen in the milieu is frequently encountered in pathological conditions such as myocardial infarction, renal ischemia, and cerebral ischemia. Endothelial cells lining the inner blood vessel walls play the key role in the response to hypoxia (Graven & Farber, 1998; Faller, 1999). The most important feature of endothelial cell hypoxia is its triggering of angiogenesis, which involves the successive steps of basal membrane degradation by endothelial cells, endothelial cell proliferation and migration, and neovascularization (Folkman & Shing, 1992; Carmeliet, 2000). The initial steps bring about the degradation of the extracellular matrix surrounding the endothelial cells by matrix metalloproteinases (MMPs). These are a group of over 20 enzymes which play an important role in extracellular matrix degradation (Stetler-Stevenson, 1999). They are divided into six classes according to their substrate specificity: interstitial collagenases, gelatinases (MMP-2, MMP-9), stromelysins, matrilysins, membrane-type MMPs (MT-MMP) and "others". All MMPs contain pre-, pro-, catalytic and hemopexin-like domains, matrilysins do not have the hemopexin-like domain. The pre-domain is required for the secretory pathway and plays a role in the cellular localization of MMPs. The pro-domain is responsible for enzyme latency and is lost during activation. In particular, two members of the MMP family, MMP-2 and MMP-9, known as gelatinases, can degrade important substrates such as collagen IV, laminin and fibronectin, which are major components of vascular basal lamina (Nguyen *et al.*, 2001). They are tightly regulated at the transcriptional and post-transcriptional levels. The post-transcriptional regulation depends on a balance between pro-enzyme activation and the level of an inhibitor protein (tissue inhibitor of metalloproteinases, TIMP). The endogenous inhibitor protein of MMP-2 is TIMP-2 (Visse & Nagase, 2003). Membrane type metalloproteinase-1 (MT1-MMP) is involved in proteolytic removal of the terminal propeptide domain during MMP-2 activation. TIMP-2, at low levels, promotes this activation by forming a membrane complex with MT1-MMP, anchoring proMMP-2 to the cell surface. However, inhibition of MMPs is associated with high levels of TIMP-2 (Hernandez-Barrantes *et al.*, 2000; Seiki & Yana, 2003).

Although numerous studies have been carried out on the effects of hypoxia and hypoxia/reoxygenation (H/R) on MMP activity and expression in tumour cells (Kunz & Ibrahim, 2003; Nakoman *et al.*, 2005; Ridgway *et al.*, 2005), only a limited amount of work has been published on the effects of hypoxia and H/R on endothelial cell MMP expression and activity. In those studies, different results were obtained depending on the cell type and the duration and conditions of hypoxia and reoxygenation (Yang *et al.*, 2002; 2005; Ben-Yosef *et al.*, 2002; 2005; Lee & Lo, 2004).

We were interested in investigating the effects of short hypoxia and H/R on gelatinases and their inhibitor (TIMP-2) and activator protein (MT1-MMP). In this study, we assessed the activities of MMP-2, MMP-9 and MT1-MMP, and TIMP-2 protein level, using human um-

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Abbreviations: bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; hEGF, human epidermal growth factor; H/R, hypoxia followed by reoxygenation; HUVE cell, human umbilical vein endothelial cell; IGF-I, insulin-like growth factor; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; rhVEGF, human recombinant vascular endothelial growth factor; TIMP, tissue inhibitor of metalloproteinase.

bilical vein endothelial (HUVE) cells as an *in vitro* model system.

MATERIALS AND METHODS

Cell culture. HUVE cells were obtained from Clonetics Corporation (Cambrex Inc., Walkersville, USA) and grown in endothelial basal medium supplemented with 2% fetal bovine serum (FBS), human recombinant vascular endothelial growth factor (rhVEGF), basic fibroblast growth factor (bFGF), human epidermal growth factor (hEGF), insulin-like growth factor (IGF-I), hydrocortisone, ascorbic acid, heparin, and GA-1000 (gentamicin and amphotericin B, 1 μg/ml) according to the instructions of the supplier. Cells were cultured in 75 cm^2 culture flasks (5000 cells/cm^2) under 5% CO_2/air at 37°C. When cells reached around 80% confluence, they were collected from the culture vessels using trypsin/EDTA solution and passaged at 1:4 ratio. For all experiments, cells were harvested after 3–5 passages. Since FBS contains endogenous MMPs, the culture medium was changed to EBM-2 complete medium without serum and growth factors prior to the assays for gelatinases, TIMP-2, and MT1-MMP.

Application of hypoxia and reoxygenation. HUVE cells were maintained at hypoxia for 4 h or for 4 h of hypoxia followed by 4 or 24 h of reoxygenation as previously described (Dhar-Mascareno *et al.*, 2005). Hypoxia was performed in a hypoxia system (Modular Incubator Chamber MIC-101, Billups-Rothenberg) which consisted of a hypoxia chamber and a gas-mixture inlet. The gas mixture contained 95% nitrogen and 5% carbon dioxide. Following the placement of the cell cultures in this chamber, this mixture was applied for 5 min. During the hypoxia, the oxygen concentration of the chamber was measured using an oxymeter (Oxygen Analyser, Billups-Rothenberg) and found to be 1%. At the end of the 5 min, the hypoxia chamber holding the cells was placed in an incubator for 4 h. For reoxygenation, the flasks were taken out of the hypoxia chamber and placed in the regular incubator (5% CO_2/air) for the 4 or 24 h reoxygenation period. At the end of the treatment, the cells were harvested and appropriate samples prepared for analysis. Cells incubated at normoxic conditions in the same incubator were used as controls.

Determination of protein content. Protein levels were determined by using bicinchoninic acid protein kit (BCA) (Sigma, Germany). Bovine serum albumin was used as standard (Wiechelman *et al.*, 1988).

Gelatin zymography. Culture media were centrifuged for 5 min at 4°C to remove cells and debris. To measure the activities of gelatinases present in the supernatants, gels containing 7.5% polyacrylamide, 0.1% type I gelatin, and 10% SDS were prepared (Kleiner & Stetler-Stevenson, 1994). Equal volumes of supernatants and a non-reducing sample buffer (0.5 M Tris/HCl, pH 6.8, 10% (w/v) SDS, 10% (v/v) glycerol and 0.02% (w/v) bromophenol blue) were mixed and applied to the wells so that each well contained 5 μg of protein. Electrophoresis was performed for 5 h, at +4°C, under a constant voltage of 125 V (30 mA/gel (18 cm (h) × 20 cm (w)). MMP marker (Chemicon, CC-073, Temecula, CA, USA), containing both pro and active forms of MMP-2 and MMP-9, was used as a positive control on each gel. The gels were washed two times with 2.5% Triton X-100 for 15 min to remove SDS, and incubated in developing buffer containing 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 10 mM $CaCl_2$, 0.5 mM $ZnCl_2$ and 0.02% Brij-35 for 16 h at 37°C. The following day, the gels were stained for 1 h with staining solution (0.5% Coomassie Blue R250, 40% methanol and 10% acetic acid) and destained in the same solution without Coomassie Blue R250. A clear zone in the blue background indicated the presence of gelatinolytic activity. Computerized densitometry was used to evaluate relative enzymatic activity (UVP BioImaging System with a Labworks 4.6 Image Acquisition Software, Cambridge, UK). The results were expressed in arbitrary units (AU) per micrograms protein.

TIMP-2 ELISA. TIMP-2 protein level was analysed by using an ELISA-based kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. The same supernatants obtained as for gelatin zymography were used for TIMP-2 analysis. Duplicate measurements were made for each sample. The absorbances were measured at 630 nm using a microplate reader (Synergy HT, BioTek Instrument Inc, Winooski, USA). Quantification was achieved by the construction of standard curves using known concentrations of TIMP-2. The results were represented as nanograms per micrograms protein.

MT1-MMP activity assay. MT1-MMP activity level was analysed by using MT1-MMP activity assay kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, MT1-MMP was extracted from cultured HUVE cells with extraction buffer (50 mM Tris/HCl buffer, pH 7.6, containing 1.5 mM sodium chloride, 0.5 mM calcium chloride, 1 μM zinc chloride, 0.01% (v/v) Brij 35 and 0.2% (v/v) Triton X-100) and its activity was measured through activation of the modified pro enzyme and the subsequent clevage of a chromogenic peptide substrate. The resultant colour was read at 405 nm in a microplate reader. The concentration of active MT1-MMP in a sample was determined by interpolation from a standard curve. The results were represented as nanograms per micrograms protein.

Statistical analysis. Statistical analysis was performed using the nonparametric Mann-Whitney *U* test. All data were expressed as means ± standard deviation. For the correlation between MMP-2, TIMP-2 and MT1-MMP levels, Spearman test was employed. A P value of <0.05 was considered to be statistically significant.

RESULTS

H/R-related changes in gelatinase activity

To establish whether short-duration hypoxia or hypoxia followed by reoxygenation affect gelatinase activities, HUVE cells were exposed to 4 h of hypoxia or hypoxia followed by reoxygenation (4 or 24 h). The HUVE cells were found to constitutively express only proMMP-2 (72 kDa), whereas MMP-9 was undetected (Fig. 1A). The data obtained for proMMP-2 from the computerized densitometric imaging of the gels (Fig. 1B) showed that there was a slight increase in proMMP-2 secretion after 4 h of hypoxia (200.77 ± 41.06 AU/μg protein); however, this difference was not statistically significant compared with the normoxic group (157.99 ± 24.33 AU/μg protein). The proMMP-2 secretion increased significantly after 4 h of hypoxia followed by 4 h or 24 h of reoxygenation (357.60 ± 63.18 and 837.39 ± 91.90 AU/μg protein, respectively), compared with the normoxic group.

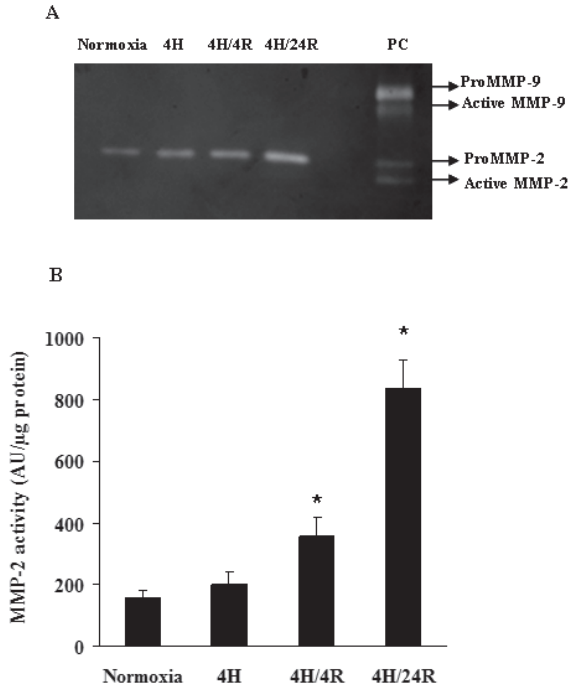

Figure 1. H/R increases MMP-2 but not MMP-9 secretion by HUVE cells

(A) Gelatin zymogram photograph representing MMP-2 activity detected as clear zones in conditioned medium collected from HUVE cells exposed to 4 h hypoxia (4H) and hypoxia followed by various periods of reoxygenation (H/R); 4R for 4 h, and 24R for 24 h reoxygenation. PC indicates positive control loaded with MMP-2 and MMP-9 standards. **(B)** Densitometric analysis of MMP-2 activity in conditioned media. Data are representative results from experiments repeated at least three times. *$P < 0.05$ *versus* normoxia.

H/R-related changes in TIMP-2 protein level

TIMP-2 data obtained from the supernatants of the HUVE cell cultured in different conditions (given in ng/mg protein) are shown in Fig. 2. Four hours of hypoxia led to a slight reduction in the TIMP-2 protein level (33.26 ± 15.51 ng/mg protein); however, this difference was not statistically significant compared with the normoxic group (49.42 ± 11.15 ng/mg protein). Four hours of hypoxia followed by 4 or 24 h reoxygenation caused statistically significant elevations of TIMP-2 level to 68.76 ± 16.29 ng/mg protein and 201.49 ± 34.24 ng/mg protein, respectively.

H/R-related changes in active MT1-MMP level

In order to assess the effect of H/R on MT1-MMP activity, it was extracted from cells and its activity was determined using an activity assay. As shown in Table 1, the level of active MT1-MMP increased after 4 h of hypoxia (69.77 ± 13.02 ng/mg protein), however, this increase was not statistically significant compared with the normoxic group (49.95 ± 12.02 ng/mg protein). The level of active MT1-MMP decreased slightly after 4 h of hypoxia followed by 4 h of reoxygenation (30.57 ± 10.62 ng/mg protein) and increased slightly after 4 h of hypoxia followed by 24 h of reoxygenation (60.61 ± 9.39 ng/mg protein). However, these results also were not statistically significant.

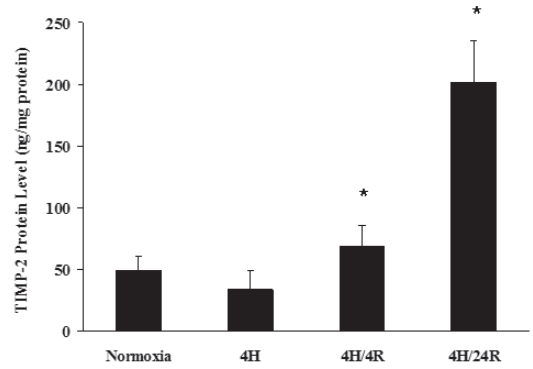

Figure 2. H/R increases TIMP-2 protein level in supernatants of HUVE cells

Cells were grown at following conditions: Normoxia, 4 h hypoxia (4H) and hypoxia followed by various periods of reoxygenation (H/R); 4R for 4 h, and 24R for 24 h reoxygenation. Data presented are the mean ± S.D. of n = 3 experiments. *$P < 0.05$ *versus* normoxia.

Correlation analysis

The only significant and strong positive correlations occurred between the levels of proMMP-2 and TIMP-2 ($P < 0.05$, $r = 0.635$).

DISCUSSION

Endothelial hypoxia triggers angiogenesis which involves the successive steps of basal membrane degradation by endothelial cells, endothelial cell proliferation and migration, and neovascularization (Folkman & Shing, 1992; Carmeliet, 2000). These biological events involve the degradation of the extracellular matrix and MMPs are the most important enzymes involved (Stetler-Stevenson, 1999).

In this study, MMP-2 activity was detected in HUVE cells (between passages 3–5) by using gelatin zymography, but MMP-9 (a member of gelatinase family) could not be detected. Gelatin zymography can show pro- and active-forms of gelatinases on the same zymogram, with a sensitivity down to 10 pg (Birkedal-Hansen & Taylor, 1982; Kleiner & Stetler-Stevenson, 1994). Previous studies reported that MMP-2 was expressed constitutively by endothelial cells, while MMP-9 was not expressed in basal conditions (Corcoran *et al.*, 1996). MMP-9 is an enzyme which originates from inflammatory cells (Coussens *et al.*, 2000) and can be induced by cytokines/growth factors (e.g., TNF-α) (Hanemaaijer *et al.*, 1993). It has been demonstrated that phorbol myristate acetate (PMA), a tumour-promoting chemical, increases the synthesis of MMP-9 by microvascular endothelial cells

Table 1. MT1-MMP activity in HUVE cells

Cells were grown at following conditions: Normoxia, 4 h hypoxia (4H) or hypoxia followed by various periods of reoxygenation (H/R); 4R for 4 h, and 24R for 24 h reoxygenation. The values represent mean ± S.D. of n = 3 experiments. *$P < 0.05$ *versus* normoxia.

Experimental conditions	MT1-MMP (ng/mg protein)	*P*
Normoxia	49.95 ± 12.02	
4H	69.77 ± 13.02	0.175
4H/4R	30.57 ± 10.62	0.251
4H/24R	60.61 ± 9.39	0.602

more than in macrovascular endothelial cells (Jackson & Nguyen, 1997). In contrast, Arkell *et al.* (2003) showed that MMP-9 is secreted also by HUVE cells at early passage numbers (0 and 1), but disappears in later passages. They suggested that the failure to detect MMP-9 in many studies may be related to passage number of the cells used in those studies. In fact, in our study MMP-9 was not detected in HUVE cells in normoxic condition. Even 4 h hypoxia or 4 h hypoxia followed by 4 or 24 h of reoxygenation could not induce MMP-9 secretion in HUVE cells. This finding is supported by a study which reported that hypoxia has no effect on MMP-9 in choroid-retinal endothelial cells (Ottino *et al.*, 2004). However, a significant increase was detected in proMMP-2 secretion and TIMP-2 protein levels after 72 h of hypoxia followed by 4 h or 24 h of reoxygenation, compared to normoxia, but there was no significant increase in MT1-MMP activity in our study.

We found significant increases in the proform of MMP-2 secretion in both H/R groups compared with the normoxic group, but no active form of MMP-2 was detected in normoxia, hypoxia or H/R conditions. This finding is supported by studies performed with endothelial cells (Yang *et al.*, 2002; 2005; Cao *et al.*, 2005; Kiran *et al.*, 2006). Our study showed that TIMP-2 protein levels also increased in the H/R groups, showing a positive correlation with proMMP-2 levels. Most studies of the effects of H/R on MMP activity and expression have been carried out with tumour cells (Kunz & Ibrahim, 2003; Nakoman *et al.*, 2005; Ridgway *et al.,* 2005). In contrast, few studies have analyzed the effects of H/R on MMP activity and expression in human endothelial cells (Yang *et al.*, 2002; 2005; Ben-Yosef *et al.*, 2002; 2005). Yang and coworkers (2002; 2005) showed that there were no differences in MMP release by liver sinusoidal endothelial cells grown in normoxia and hypoxia conditions. However, MMP-2 secretion increased after reoxygenation. The data in our study are similar to those results. Moreover, Yang *et al.* (2002; 2005) reported that liver sinusoidal endothelial cells were sensitive to H/R-induced apoptosis. They showed that prostaglandin E_1 and α-1 antitrypsin protected human liver sinusoidal endothelial cell from apoptosis induced by H/R (Yang *et al.*, 2002 and 2005, respectively). In another study performed by Ben-Yosef *et al.* (2002), EAhy 926 cells (a hybrid cell line constituted from a primary HUVE cells and human lung tumor cells) were exposed to hypoxia for different periods (6, 24 or 48 h) and 24 h of reoxygenation. They reported that a short-term hypoxia decreased the levels of MMP-2 and its regulator proteins (TIMP-2 and MT1-MMP), and while MMP-2 and MT1-MMP increased during the reoxygenation, TIMP-2 remained below the level for cells cultured in normoxic conditions. Our results are different than those of Ben-Yosef. This might be the result of the different cell types used in our study and by Ben-Yosef group. Actually, it has been reported that the heterogeneity among endothelial cells derived from different vascular beds resulted in different responses in the expression of genes regulating the dynamic behavior of the vascular system in response to changing oxygen tension (Nilsson *et al.,* 2004).

On the other hand, Ben-Yosef *et al.* (2002) interpreted the observation that no active MMP-2 could be detected by gelatin zymography by suggesting that *in vitro* conditions were not appropriate for the activation of MMP-2, or that the half-life of active MMP-2 was very short. In addition, the activation mechanism of MMP-2 involves the triple-complex of MMP-2–TIMP-2–MT1-MMP (Strongin *et al.,* 1995; Nagase, 1997). As known, MT1-MMP is the first MMP to be defined as specific activator of proMMP-2 on the cell surface. As for all MMPs, MT1-MMP should also be detached from its propeptide region in order to show proteolytic function on the cell surface. Then, the active MT1-MMP is transferred to the cell surface (Sato *et al.*, 1996). In this respect, the findings of MT1-MMP that we obtained are compatible with the finding that the active form of MMP-2 is not detected. As also known, the enzymatic activity of MT1-MMP located on the cell surface is specifically inhibited by TIMP-2, TIMP-3 and TIMP-4 (Yana & Weiss, 2000). The significantly increased TIMP-2 protein levels that we found during H/R compared to normoxia cells preventing the binding of MT1-MMP to MMP-2 and consequently preventing the activation of proMMP-2**,** may explain why active MMP-2 was not observed. This constitutes post-translational regulation of the MMPs. There is also the possibility that MT1-MMP could not be activated in the cell, or that the activated MT1-MMP could not be transferred to the cell surface, or that the active MT1-MMP could not be attached to proMMP-2, being affected by *in vitro* conditions (pH, Ca^{2+} level, etc.) (Yan *et al.*, 2000). In another study by Ben-Yosef (2005), only hypoxia was administered to HUVE cells (for 24 or 48 h), and its effects on MMP-2 expression and the roles of these effects in angiogenesis and cell death were investigated. They concluded that hypoxia-induced MMP-2 shows a dual effect, affecting both cell death and cell life. In the H/R model we used in our study, a significant increase was demonstrated both in MMP-2 activity and TIMP-2 protein levels by reoxygenation (for 4 or 24 h) following a short-term hypoxia (for 4 h). The increase in MMP-2 secretion caused by reoxygenation may be stimulated by the oxidative stress which occurs following reoxygenation. In a study on ischemic mouse brains by Gashe it was reported that the activation of MMP-9 and MMP-2 increased with oxidative stress (Gashe *et al.*, 2001). This report supports the MMP-2 increase which we observed upon reoxygenation. As we determined in our study the increase in TIMP-2 protein level in HUVE cells with the duration of reoxygenation may be interpreted as a compensation mechanism related to increased proMMP-2. In contrast to the findings of previous studies reporting that MMP-2 protein is expressed by endothelial cells, it has been stated that MMP-2 transcription also increases through hypoxia-induced AP-1 (activating protein-1) and hypoxia-inducible factor-1-α in recent studies which investigated the effect of MMP-2 on cardiac and colon cancer cells (Bergman *et al.*, 2003; Krishnamachary *et al.*, 2003). However, these responses may be cell-specific and depend on the experimental model and conditions used.

Both angiogenesis and cell death involve the degradation of basal membrane and extracellular matrix, as well as the impairment of cell–cell and cell–matrix interactions. It is reported that high levels of MMPs cause cells to become detached from the extracellular matrix, directing them toward apoptosis (Woessner, 1991; Frisch & Francis, 1994). Reperfusion was also shown to result in structural and biochemical changes in endothelial cells leading to damaged endothelium (Glyn and Ward, 2002). The present *in vitro* results, demonstrating for the first time H/R-induced MMP-2 secretion in HUVE cells, are in accordance with *in vivo* studies performed on ischemia/reperfusion injury and MMP activities (Zhang *et al.*, 2002; Lalu *et al.*, 2005, Catalyurek *et al.*, 2008; Lu *et al.*, 2008).

In summary, our results clearly show an enhancement of MMP-2 and TIMP-2 secretion during reoxygenation. Further studies are needed to explore the mechanism of

the increase of MMP-2 and TIMP-2 during *in vitro* reoxygenation of HUVE cells.

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