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Production of Proteolytic Enzymes in Mast Cells, Fibroblasts, Vascular Smooth Muscle and Endothelial Cells Cultivated Under Normoxic or Hypoxic Conditions

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Summary

Matrix metalloproteinases (MMPs) is a family of proteolytic enzymes involved in remodeling of extracellular matrix. Although proteolytic enzymes are produced by many cell types, mast cells seem to be more important than other types in remodeling of pulmonary arteries during hypoxia. Therefore, we tested *in vitro* production of MMPs and serine proteases in four cell types (mast cells, fibroblasts, vascular smooth muscle cells and endothelial cells) cultivated for 48 h under normoxic or hypoxic (3 % O_2) conditions. MMP-13 was visualized by immunohistochemistry, MMP-2 and MMP-9 were detected by zymography in cell lysates. Enzymatic activities (MMPs, tryptase and chymase) were estimated in the cultivation media. Hypoxia had a minimal effect on total MMP activity in the cultivation media of all types of cells, but immunofluorescence revealed higher intensity of MMP-13 in the cells exposed to hypoxia except of fibroblasts. Tryptase activity was three times higher and chymase activity twice higher in mast cells cultivated in hypoxia than in those cultured in normoxia. Among all cell types studied here, mast cells are the most abundant source of proteolytic enzymes under normoxic and hypoxic conditions. Moreover, in these cells hypoxia increases the production of both specific serine proteases tryptase and chymase, which can act as MMPs activators.

Key words

Hypoxia • Metalloproteinases • Tryptase • Chymase • Immunofluorescence

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Introduction

Exposure of various tissues and organs to hypoxia involves remodeling of their extracellular matrix (ECM). An increase in collagenolytic activity in the walls of the peripheral pulmonary arteries results in the presence of small collagen cleavage products (Novotná and Herget 2001) and participates in the pathogenesis of hypoxic pulmonary hypertension (Herget 2000).

The initial step of ECM degradation involves the activity of interstitial collagenase, i.e. matrix metalloproteinase MMP-1, which corresponds to MMP-13 in rodents. This enzyme cleaves the native helix of collagen fibres in typical sites of single peptide bonds, generating fragments of approximately 1/4 and 3/4 of the size of the original molecule (Freije *et al.* 1994). Other metalloproteinases, i.e. gelatinases (MMP-2, MMP-9) cleave denatured forms of collagen (Krane *et al.* 1996). MMPs are produced by various cell types as proenzymes, i.e. proMMPs, and are activated extracellularly. The activation cascade involves plasmin, other types of

MMPs, tryptase (Gruber *et al.* 1989) and chymase (Tchougounova *et al.* 2005).

MMPs are secreted by many types of cells in all tissues; in the case of the lungs, these cell types include fibroblasts and smooth muscle cells (Karakiulakis *et al.* 2007), endothelial cells (Uchida *et al.* 2008), alveolar macrophages (Gibbs *et al.* 1999) and mast cells (Maxová *et al.* 2008, Thakker-Varia *et al.* 1998). Many previous and recent studies emphasized the role of mast cells, in spite of the relatively small amount of this cell type in the pulmonary tissue, usually ranging between 1-5 % of all cells under physiological conditions (van Overveld *et al.* 1988). However, under pathological conditions, namely in human coronary atheromas, the ratio between mast cells and smooth muscle cells, i.e. the most numerous cell population in the vessel wall, reached 1:5 (Kovanen *et al.* 1995). Exposure of mast cells to hypoxia changes their distribution in the pulmonary vasculature (Vajner *et al.* 2006), and inhibition of their degranulation attenuates the development of hypoxic pulmonary hypertension (HPH) (Bañasová *et al.* 2008).

In order to elucidate the location of collagenolytic proteases in specific cell types present in the pulmonary vasculature, we compared the production of collagenolytic enzymes, namely MMP-13, MMP-9, MMP-2, total MMPs, tryptase and chymase, in mast cells, fibroblasts, vascular smooth muscle cells and endothelial cells cultivated under normoxic and hypoxic conditions.

Methods

Cells

A rat mastocytoma cell line (RBL-2H3, ATCC Manassas, VA, U.S.A, CRL-2256) was used as a model of mast cells infiltrating the lung tissue. For the experiments, the cells were expanded in Eagle's Minimum Essential Medium (E-MEM; Sevapharma, Prague, Czech Republic) supplemented with 5 % of fetal bovine serum (FBS; J. Kysilka, Brno, Czech Republic).

The mouse fibroblast cells (line 3T3, ATCC, CCL-92) for the experiments were expanded in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N^o D5648) supplemented with 10 % FBS (Sebak GmbH, Aidenbach, Germany) and Gentamicin (40 μg/ml, LEK, Ljubljana, Slovenia).

Vascular smooth muscle cells (VSMC) were isolated from the intima-media complex of the thoracic aorta of 8-week-old male Wistar SPF rats (Inst. Physiol., Acad. Sci. CR) by an explantation method described earlier (Bačáková *et al.* 2001). The cells were expanded in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N^o D5648) supplemented with 10 % of FBS (Sebak GmbH, Aidenbach, Germany) and Gentamicin (40 μg/ml, LEK, Ljubljana, Slovenia), and were used in the 5th passage.

An endothelial cell line derived from bovine pulmonary artery (CPAE, CCL 209, American Tissue Culture Collection, Rockville, MD, U.S.A.) was used as a model of vascular endothelial cells. For the experiments, these cells were expanded in Minimum Essential Eagle's Medium supplemented with 2 mM L-glutamin, Earle's BSS with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate (all chemicals from Sigma), and 20 % of FBS.

Cell culture conditions

The cells of all types (RBL-2H3, 3T3, VSMC and CPAE) were seeded in the Lab-Tek Chamber Slide System (177402 Nunc) with eight wells. Each cell type was cultivated in eight sets of chambers. Each well contained 50 000 cells and 0.2 ml of Eagle's Minimum Essential Medium (E-MEM; Sevapharma, Prague, Czech Republic) supplemented with 5 % of FBS. After seeding, all groups of cells were allowed to recover in air with 5 % CO_2 for 24 h at 37 °C. Then the slides with cells were divided into two groups of four sets each, and were put in the Modular Incubator Chambers (Billups-Rothenberg, Inc.) purged with normoxic (21 % O_2 + 5 % CO_2) or hypoxic mixture (3 % O_2 + 5 % CO_2) for 10 min with flow rate 5 l/min and cultivated for the next 48 h at 37 °C. The gas mixtures were prepared by Linde a.s., Prague, Czech Republic. The oxygen concentrations inside the chambers were tested twice by MiniOX 3000 (Cheirón s.r.o., Plzeň, Czech Republic): before the closure of the outlet port of the chamber, and at the opening of the port when cultivation came to an end.

The cultivation media of all cell types were collected and frozen. Four sets of the normoxic groups and four sets of the hypoxic groups were divided in the following manner: two sets of each group were washed using phosphate-buffered saline (PBS) solution (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4), pH 7.35; Sigma), fixed by methanol and used for immunostaining. The remaining cells from the two sets were frozen in 0.15 ml of purified deionized water and assayed by zymography.

Immunocytochemistry and immunofluorescence microscopy

The cells were fixed in cold methanol (–20 °C) for 10 min, washed in PBS and permeabilized with 0.1 % Triton X100 (Sigma) for 20 min. Incubation with the primary antibody anti-MMP-13 (Ab-6) Mouse (Calbiochem), diluted in PBS (1:40), was performed for 2 h at room temperature. The slides were then rinsed with PBS three times for 5 min, and the cells were subsequently incubated with the secondary antibody Alexa Fluor 488-conjugated F(ab´)2 fragment of Goat Anti-Mouse IgG (Invitrogen), diluted in PBS (1:400) for 1 h and rinsed with PBS. Propidium iodide (Calbiochem) nucleic stain was added for 30 s, and after a final rinse with PBS, the slides were mounted under glass coverslips with Gel/Mount (Permanent Aqueous Mounting Medium; Biomeda, Foster City, CA, USA). In the control sample, the primary antibody was omitted, in order to exclude non-specific reactions.

The samples were studied using an Opton epifluorescence microscope (Zeiss, Germany). Microphotographs were taken using an Olympus DP-70 camera (Japan).

Zymography

The frozen cellular extracts were thawed at room temperature, and then the MMPs were analyzed by zymography (Novotná and Herget 1998). Zymography preferentially targeted MMPs with gelatinolytic activity (MMP-2 and MMP-9). There are lytic zones in the gel, due to the presence of the investigated enzymes in the cell extracts. A high molecular weight calibration kit (Pharmacia Biotech), proenzyme MMP-2 and proenzyme MMP-9 (Oncogene Research Product) were used as standards.

Activity of metalloproteinases and serine proteases

The activities of proteolytic enzymes were estimated in doublets of cultivation media pooled from six wells and related to the amount of proteins in each measured sample. The background activity of the cultivation media supplemented with 5 % FBS was subtracted from each sample. Mca-P-L-G-Ldpa-A-R-NH_2 fluorogenic peptide substrate I (R&D Systems, Inc.) activities were used to measure the total MMPs activity, and the tryptase activity was assayed using the peptide substrate BAPNA (N-α-benzoyl-DL-arginine p-nitro-anilide hydrochloride, Sigma), as has been described previously (Maxová *et al.* 2008). Activities are expressed in μU/mg protein. Chromogenic substrate S-2586 was used for determination of chymotrypsin activity. The substrate non-split by trypsin was added to Trisma buffer (pH 8.3). Absorbance was measured at 405 nm (fluorimeter Genios Taecan, Switzerland) and the activity was expressed in ng/mg protein).

Statistics

The results are presented as mean ± SEM. The statistical analyses were performed using StatView 5.0 (SAS Institute, Cary, NC). Comparison procedures were made by the unpaired t-test, and $p<0.05$ values were considered significant.

Results

Immunofluorescence of MMP-13

All studied cell types, i.e. rat mastocytoma RBL-2H3 cells, mouse 3T3 fibroblasts, rat aortic smooth muscle cells, as well as bovine pulmonary artery endothelial CPAE cells, showed positive immuno-fluorescence staining against MMP-13 (Fig. 1). However, the intensity of the fluorescence was much greater in the RBL-2H3 cells than in other cell types. It was also much greater in RBL-2H3 cells cultivated under hypoxic conditions than in those cultivated under normoxic conditions. Under normoxic conditions, the cytoplasm of RBL cells was stained with heterogeneous intensity, i.e. with brighter and less bright regions, while under hypoxic conditions the RBL cells were stained homogeneously and very brightly (Fig. 1A,B).

Similarly, in the other cell types, apart from the 3T3 fibroblasts, the intensity of the MMP-13 immuno-fluorescence was higher in cells incubated under hypoxic conditions (Fig. 1C-H). The enzyme was located in small dot-like granules, which seemed to be more numerous in cells cultured in hypoxia, and filled the entire cytoplasm. Only in 3T3 cells cultured in hypoxia was the fluorescence of MMP-13 relatively low, and without granular distribution (Fig. 1D).

Zymography of proteolytic enzymes

Zymography performed on cell lysates showed lytic zones corresponding with proMMP-9 and active MMP-9, which were well apparent in RBL-2H3 mast cells and slightly apparent in CPAE endothelial cells, and displayed a similar intensity in the normoxic and hypoxic groups. No MMP-9 in active and proenzymatic form were detected in 3T3 fibroblasts and VSMC. ProMMP-2

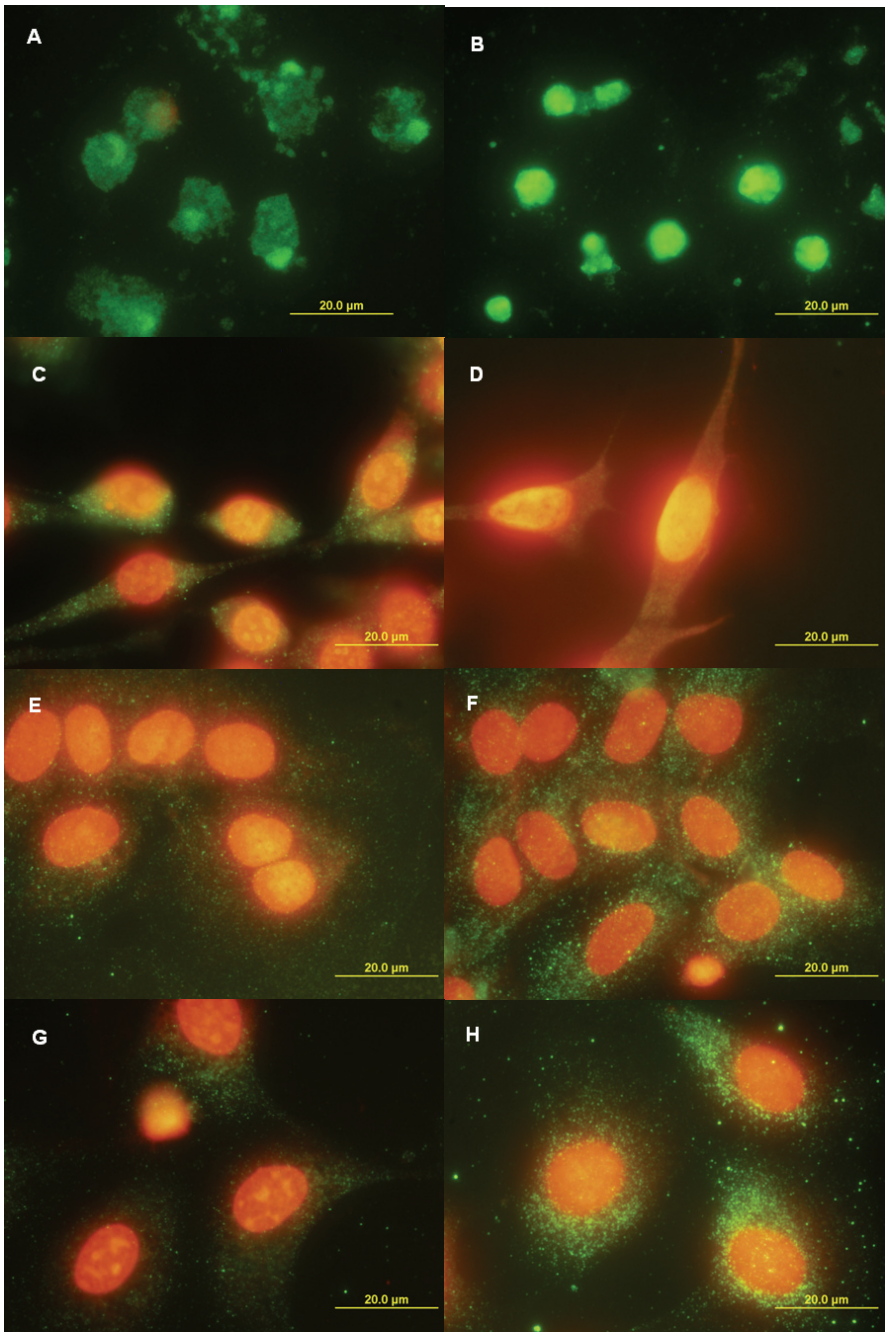

Fig. 1. Immunofluorescence staining of MMP-13 in rat mastocytoma RBL-2H3 cells (A, B), 3T3 fibroblasts (C, D), rat aortic smooth muscle cells (E, F) and bovine pulmonary artery endothelial CPAE cells (G, H) cultured for 48 h under normoxic (A, C, E, G) and hypoxic (B, D, F, H) conditions. The cell nuclei were counterstained with propidium iodide (C-H).

was present in all studied cell types, predominantly in RBL-2H3 and 3T3 cells, and was more transparent on gels obtained using extracts from cells cultured under normoxic conditions (Fig. 2). MMP-2 was slightly active only in normoxic 3T3 fibroblasts.

Activity of proteolytic enzymes

Figures 3A-C demonstrate the activities of tryptase, chymase and total MMPs in the cultivation media after subtracting the activity of the control medium. The activities of serine proteases were higher by one or two orders of magnitude in RBL-2H3 mast cells than in other cell types. The tryptase and chymase activities (Figs 3A,B) in the media from RBL-2H3 cells cultivated in hypoxic conditions were significantly higher than those cultivated in normoxic conditions (unpaired t-test, $p=0.027$ and $p=0.0009$, respectively).

MMP activities (Fig. 3C) were found in the cultivation media obtained from RBL-2H3 and CPAE cells. The activity of MMPs was four times higher in

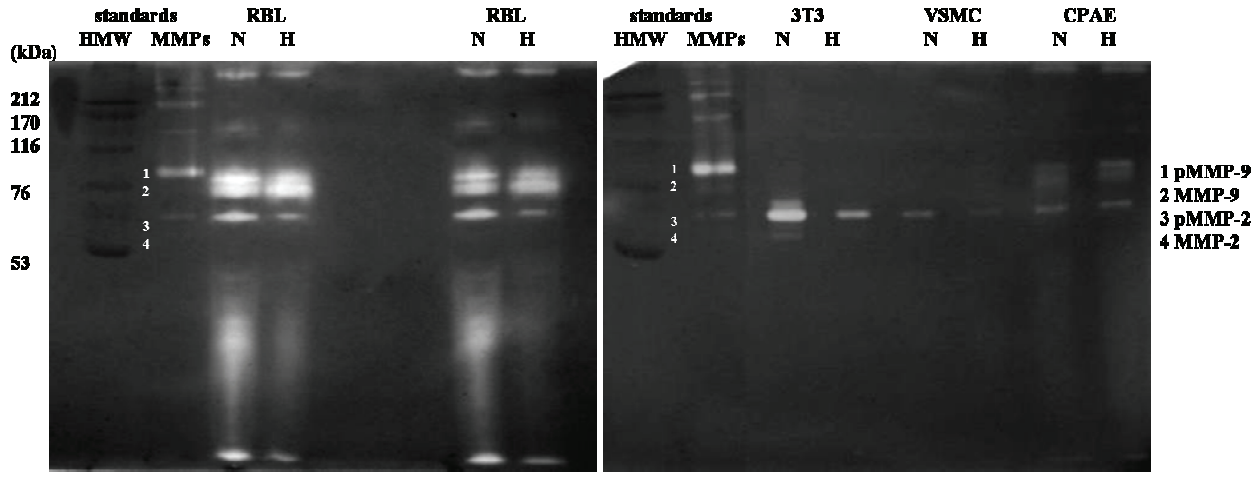

Fig. 2. Zymogram from the cell lysates prepared from rat mastocytoma RBL-2H3 cells, (RBL) mouse 3T3 fibroblasts (3T3), rat aortic smooth muscle cells (VSMC) and bovine pulmonary artery endothelial CPAE cells (CPAE) cultured for 48 hours under normoxic (N) and hypoxic (H) conditions. Lytic zones correspond to metalloproteinases MMP-2 and MMP-9 in their proenzymatic (p) and activated forms.

RBL-2H3 cells, and did not differ between the normoxic and hypoxic groups. The activity of the released MMPs was significantly lower in CPAE cells cultivated in hypoxia than in those cultivated in normoxia (p=0.0049). 3T3 fibroblasts and VSMC showed no activity of MMPs in the cultivation media.

Discussion

The aim of our *in vitro* study was to compare the ability to produce and to release proteolytic enzymes under normoxic and hypoxic conditions in four types of cells that are commonly present in lung tissue. We found that rat mastocytoma RBL-2H3 cells are more efficient in production and activation of proteolytic enzymes under hypoxia than all other cell types investigated in this study, i.e. vascular endothelial cells, vascular smooth muscle cells and fibroblasts.

As a model of mast cells in this study, a rat basophilic leukemia (mastocytoma) cell line RBL-2H3 was used. Although these cells were derived from tumor tissue, they posses most characteristics of mature mast cells in peripheral tissues *in situ* as well as of freshly isolated serosal or perivascular mast cells *in vitro*. For example, they can be activated to produce hydrogen peroxide, to degranulate and to release various mediators, such as histamine, cytokines, growth factors, prostanoids and proteases (Jeong *et al.* 2003, Kido *et al.* 1986, Lee *et al.* 2006). Therefore, these cells have been extensively used for a wide range of pharmacological, immunological, allergological and biochemical studies (Bodinier *et al.* 2008, Jeong *et al.* 2003, Lee *et al.* 2006). Similarly, rat aortic smooth muscle cells and bovine pulmonary endothelial CPAE cells can be considered as representative models for the given cell types. Our earlier studies revealed that these cells contained differentiation markers (alpha-actin for VSMC and von Willebrand factor for endothelial cells) which are typical for VSMC and endothelial cells in general, i.e. irrespective of the donor species, gender, postnatal age or vascular region (Filová *et al.* 2009, Skalli *et al.* 1986). In addition, rat aortic smooth muscle cells, endothelial CPAE cells and 3T3 fibroblasts are capable of producing proteases, including MMP-2 and MMP-9 (Droppelmann et al. 2009, Lee *et al.* 1998, Vosgerau *et al.* 2010). These cell types were also responsive to hypoxic conditions, e.g. by expression of HIF-1, production of cytokines and growth factors, hypertrophy and hyperplasia (Pak *et al.* 2007) and also modulation of protease secretion (Palmer *et al.* 2000, Saed *et al.* 2000).

We used various methods targeted at determining the production or release of proteolytic enzymes. The total MMPs activity, tryptase and chymase were measured in the cell lysates. Thus, the results represent only enzymes released from the cells during normoxic or hypoxic conditions and cannot provide any information about their amount inside the cells. Zymography was done from cell lysates and shows the ability of MMP-2 and MMP-9 to split gelatin substrate. Immunofluorescence of MMP-13 directly visualized the enzyme content in the granules of the cells.

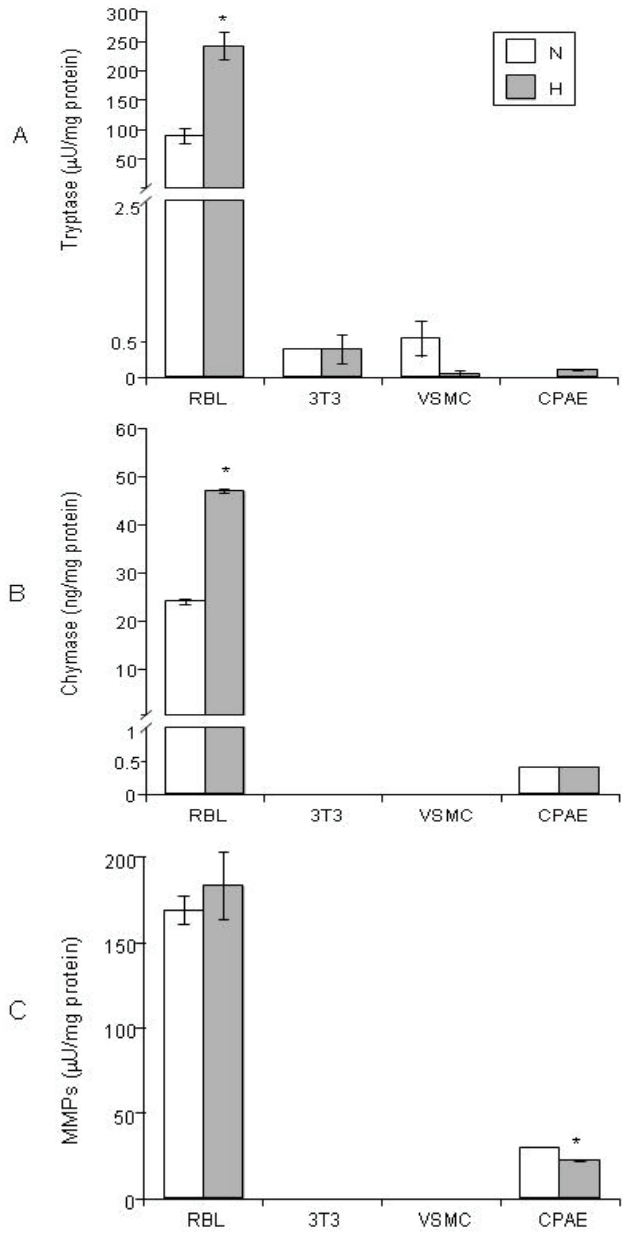

Fig. 3. Activity of proteolytic enzymes tryptase (**A**), chymase (**B**) and total MMPs activity (**C**), measured in the cultivation media obtained from rat mastocytoma RBL-2H3 cells (RBL), mouse 3T3 fibroblasts (3T3), rat aortic smooth muscle cells (VSMC) and bovine pulmonary artery endothelial CPAE cells (CPAE) after 48 h of cultivation in a normoxic atmosphere (N) and in a hypoxic atmosphere (H). $*$ $p<0.05$, H group vs. N group.

As shown by immunofluorescence, RBL-2H3 cells were intensively stained with an antibody against MMP-13, and the staining intensity was further increased by exposing these cells to hypoxia. As shown by zymography, RBL-2H3 cells also contained MMP-9 in both proenzymatic and active form, as well as MMP-2 proenzyme. In addition, the RBL-2H3 cells released active forms of tryptase, chymase as well as MMPs into their cultivation medium, and the activities of tryptase and chymase were further enhanced by hypoxia. Chymase is known as a proteolytic enzyme which activates proMMP-2 as well as proMMP-9 (Tchougounova *et al.* 2005), tryptase stimulates fibroblast proliferation and collagen I production (Cairns and Walls 1997), activates latent pro-MMP-3 (Gruber *et al.* 1989), and acts as a gelatinase (Fajardo and Pejler 2003).

In our earlier study (Maxová *et al.* 2008), similar behavior was observed in mast cells isolated from rat lungs and cultured under hypoxic conditions. In response to hypoxia, there was an increase in the number of rat lung mast cells positively labeled with an anti-MMP-13 antibody, and also an increase in the activity of tryptase and total MMPs in both culture medium and cellular extract (Maxová *et al.* 2008). Under the *in vivo* conditions, i.e. in rats exposed to hypoxia, a significant increase in the number of mast cells and their MMP-13 expression was found within the walls of pulmonary blood vessels (Vajner *et al.* 2006). Thus, RBL-2H3 cells can be considered as a good model of pulmonary mast cells, especially in cases of low availability of pulmonary mast cells.

It is known that hypoxia leads to the development of hypoxic pulmonary hypertension, which is characterized by an increase in the numbers and activation of other cell types. 3T3 fibroblasts, VSMC and CPAE cells were capable of producing proteolytic enzymes, although in substantially lower amounts and activities in our study. There are many clinical studies describing the production of proteolytic enzymes by these cell types in inflammatory diseases. The levels of human interstitial collagenase MMP-1 (an enzyme which corresponds to MMP-13 in rodents), as well as MMP-8 and MMP-9, were increased in the sputum from patients with chronic obstructive pulmonary disease (COPD) (Culpitt *et al.* 2005). MMP-13 was expressed in the human aorta affected by aneurysm or atherosclerosis (Mao *et al.* 1999). Increased expression of MMP-13 was demonstrated in the endothelial and smooth muscle cells of human varicose leg veins and venous aneurysms, compared to normal saphenous vein (Irwin *et al.* 2008). MMP-13 was detected in arterial smooth muscle cells and fibroblasts in primary cultures established from lung samples taken from patients undergoing lobectomy or pneumonectomy. However, this enzyme was increased by hypoxia (3 % O_2) only in fibroblasts (Karakiulakis *et al.* 2007). This finding was not confirmed in our study using 3T3 fibroblasts, in which the intensity of immunofluorescence staining for MMP-13 in hypoxic 3T3 cells was

somewhat lower than in cells cultured under normoxia. In addition, minimal activity of tryptase was found in the culture media in both normoxic and hypoxic 3T3 fibroblasts and normoxic VSMC, although this enzyme has been considered as a mast cell-specific protease and has often been used as a marker of mast cell activation.

In addition, all types of cells in our study were able to release pro-MMP2 into the medium, RBL-2H3 and CPAE were more active in MMP-9 production in both active and proenzymatic form, and showed activity of total MMPs in the culture media. In accordance with these findings, human macrovascular endothelial cells (line EAhy 926) constitutively expressed latent MMP-2, although MMP-9 was undetected in serum-free media after 48 h of incubation with these cells (Ben-Yosef *et al.* 2002). On the other hand, after 48-h exposure to hypoxia, EAhy 926 endothelial cells enhanced MMP-2 mRNA and enzyme secretion, whereas in our experiments, hypoxia usually did not increase (or even attenuated) the production and activity of MMP-9, MMP-2 as well as total MMPs in both CPAE and VSMC. Similarly, hypoxia down-regulated the secretion of MMP-2 and MMP-9 in endothelial and smooth muscle cells in cultures derived from porcine pulmonary artery (Ye *et al.* 2005). In cultured human pulmonary VSMC and fibroblasts, hypoxia up-regulated the expression of MMP-1, MMP-2 and MMP-9 precursors, but without their subsequent activation (Karakiulakis *et al.* 2007). In a study performed on fetal rat lung explants cultured at 3 % of O_2, hypoxia inhibited the degradation of MMPs and decreased the activity of MMP-2, although it did not change the synthesis of mRNA for this enzyme during lung branching morphogenesis (Gebb *et al.* 2005).

Tissue remodeling would involve degrading the extracellular matrix, weakening the cell-matrix adhesion, increased migration and proliferation of vascular smooth muscle cells and thickening of the vessel wall (Bačáková *et al.* 2002). Burke *et al*. (2009) showed that sustained hypoxia promotes the development of a pulmonary artery specific inflammatory microenvironment with progressive accumulation of macrophages (Greenlee *et al.* 2007), high expression of chemokines, cytokines and fibrosis-associated molecules in the tissue of pulmonary arteries.

It can be concluded that all cell types studied here showed considerable amounts or activities of proteolytic enzymes (MMP-2, MMP-9, MMP-13, tryptase, chymase), and thus they can act synergetically "*in vivo*" in degrading and remodeling extracellular matrix molecules in both pulmonary and systemic blood vessels under pathological conditions, including hypoxia.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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