

“In vitro” hypoxia increases production of matrix metalloproteinases and tryptase in isolated rat lung mast cells

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Short title:

Hypoxia stimulates MMPs production in rat lung mast cells

Summary

Chronic hypoxia results in hypoxic pulmonary hypertension characterized by fibrotization and muscularization of the walls of peripheral pulmonary arteries. This vessel remodelling is accompanied by an increase in the amount of lung mast cells (LMC) and the presence of small collagen cleavage products in the vessel walls. We hypothesize that hypoxia activates LMC, which release matrix metalloproteinases (MMPs) cleaving collagen and starting increased turnover of connective tissue proteins. This study was designed to determine whether “in vitro” hypoxia stimulates production of MMPs in rat LMC and increases their collagenolytic activity. The LMC were separated on the Percoll gradient and then were divided into two groups and cultivated for 24 h in 21% O₂+ 5% CO₂ or in 10% O₂ + 5% CO₂. Presence of the rat interstitial tissue collagenase (MMP-13) was in LMC visualized by immunohistological staining and confirmed by Western blot analysis. Total MMPs activity and tryptase activity were measured in both cultivation media and cellular extracts. Exposure to hypoxia “in vitro” increased the amount of cells positively labeled by anti-MMP-13 antibody as well as activities of all measured enzymes. The results therefore support the concept that LMC are an important source of increased collagenolytic activity in chronic hypoxia.

Key words

hypoxia in vitro; rat lung mast cells; tissue metalloproteinases; tryptase; remodeling of pulmonary vessels

Introduction

Chronic hypoxia induces structural remodeling of pulmonary vessels. Connective tissue fibers are deposited and vascular smooth muscle cells are accumulated in the walls of pulmonary prealveolar vessels. We previously suggested that an increase in collagenolytic activity in the walls of the peripheral pulmonary arteries, which results in a presence of small collagen cleavage products (Novotná and Herget 1998), is one of the important triggering mechanisms of vascular remodeling induced by hypoxia (Hampl and Herget 2000, Novotná and Herget 2002). It is known that low molecular weight cleavage products of matrix proteins (“matrikines”) initiate the fibroproduction and mesenchymal cell proliferation (Li *et al.* 2000). Collagen breakdown is a result of increased activity of matrix metalloproteinases (MMPs). In the rat species, the cleavage of native collagen starts by MMP-13 (i.e., a rodent-like interstitial collagenase) (Freije *et al.* 1994), and then continues by the activation of MMPs cascade including MMP-2, MMP-9 and other collagenolytic enzymes (Novotná and Herget 2002). Although the source of MMPs is not clear, the lung mast cells (LMC) are the most likely candidates. The number of LMC increases in chronic hypoxia (Kay *et al.* 1974, Vajner *et al.* 2006) and they express collagenolytic enzymes (Tozzi *et al.* 1998).

In order to elucidate the role of LMC in the early phase of chronic hypoxia, we tested whether “in vitro” hypoxia stimulates production of MMPs and tryptase in the mast cells isolated from rat lungs.

Materials and Methods

Mast cell donors and experiment setting

Twenty adult male Wistar specific pathogen-free rats (Anlab, Prague, Czech

Republic) weighing from 320 to 360 g were used. Experiments were performed in accordance with the European Community and NIH guidelines for using experimental animals. All procedures were approved by our institution's Animal Studies Committee.

LMC were obtained in five separate isolations (lungs from four animals were used on single isolation). Each set of cells was divided in two portions. The cells of the first part were cultivated for 24 h in 21% O₂ + 5% CO₂ (control normoxic group N), while the cells of the second portion were kept for the same time in hypoxia (10% O₂ + 5% CO₂, group H). At the end of the exposure we determined the change in MMP-13 formation as the change in relative count of the MMP-13 positive staining cells. Presence of MMP-13 and gelatinases (MMP-2 and MMP-9) were assayed in the cellular extracts by Western blot and zymography respectively. Total MMPs and tryptase activities were measured in both cultivation media and in extracts from the cells.

Chemicals for mast cells isolation

Reagents and solutions: Phosphate-buffered saline (PBS) solution (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄; 1.47 mM KH₂PO₄), adjusted to pH 7.3; Eagle Minimum Essential Medium (E-MEM; Sevapharma, Prague, Czech Republic); cultivation medium RPMI-1640 (Sigma, Prague, Czech Republic) supplemented with 5% fetal calf serum, 10mM HEPES and 50 µg/ml Gentamicin (Lek Pharmaceuticals, Ljubljana, Slovenia); Trypan blue and Toluidine blue (Sigma); Thiopental (ICN Czech Republic, Roztoky, Czech Republic); Heparin (Léčiva, Prague, Czech Republic).

Enzymes: 2% trypsin (Sevapharma), collagenase (type I), elastase (type I, porcine pancreatic), hyaluronidase (type Is) and DNase were all obtained from Sigma.

Isotonic Percoll solution with a desired density of 1.11 g/ml was prepared by dissolving 20 ml of Percoll (300 mOsm/ kg H₂O) (Sigma), 2.5 ml of distilled water and 2.5 ml of 1.5 M NaCl, pH was adjusted to 7.4. Preformed continuous Percoll gradient was prepared by centrifugation (running conditions: rotor JA 25.50, 30 000 x g for 30 min, 7°C). The density marker beads (Sigma) were used as external markers in a centrifuge tube containing identical gradient material to the one used for the experiment sample.

Mast cell isolation procedure

Rats were anesthetized by intraperitoneal injection of Thiopental (40 mg/kg) intubated and ventilated with positive pressure. Heparin (0.2 ml, 1250 UI/ml), was administered intracardially and lungs were perfused via pulmonary artery with 50 ml PBS with a peristaltic perfusion pump at a rate of 4 ml/100 g of rat body weight/min. The lungs and trachea were excised en block, washed in PBS and lavaged 6 times by 4 ml PBS. The trachea and major bronchi were removed and lungs were cut into 1-2 mm fragments. The minced tissue was 3 times washed by 60 ml PBS. Tissue with 18 ml PBS and 2 ml 2 % trypsin was shaken for 15 min and washed out by MEM. Then it was incubated for 45 min at 37°C in an enzymatic mixture containing collagenase (0.01 g), hyaluronidase (0.005 g) and elastase (0.25 ml) in 20 ml MEM. Remaining intact tissue was disrupted by repeat passage through a syringe and DNase (0.001 g in 10 ml MEM) was added for 10 min. Then the digested tissue in 70 ml MEM with 10 ml serum was stepwise filtered through the mesh and 70 µm

pore size sifter. All harvested cells were twice centrifuged at 2000 x g for 10 min. The embedded cells were resuspended at 4 ml of PBS, layered onto the preformed continuous Percoll gradient and centrifuged at 400 x g at 7°C for 30 min. A centrifugation fraction (1.102 – 1.119 g/ml densities) containing mast cells was collected. This fraction was diluted in 15 ml of cultivation medium RPMI-1640 and centrifuged at 300 x g for 30 min. The sedimented cells were dispersed in 1 ml of cultivation medium, and their number and vitality were assessed by trypan blue staining. To identify the mast cells (red-purple metachromatic heparin and histamine granules) toluidine blue staining was used.

Mast cells cultivation and exposure to “in vitro” hypoxia

Cells were seeded in Lab-Tek Chamber Slide System (177402 Nunc) with eight wells (50 000 cells per well), and 0.2 ml of cultivation medium supplemented with 5% fetal calf serum was added into each well. Both groups of cells were allowed to recover at 37°C in air + 5 % CO₂ for 24 h in a cell incubator. Then the cells were put in the glass bottles (2 l) purged with normoxic (21 % O₂ + 5 % CO₂) or hypoxic mixture (10 % O₂ + 5 % CO₂) for 10 min with flow rate 3l/min (Gas Mixer Pegas 4000 MF, Columbus Instruments, Ohio, USA) and cultivated for next 24 h at 37°C. Partial pressures pO₂ and pCO₂ were measured in liquid samples by a radiometer (Radiometer Medical A/S IDK-2700, Copenhagen, Denmark) at the end of 24 h normoxic or hypoxic period.

The cultivation media were collected and frozen. Remaining cells were processed in three ways. One portion of the cells was fixed and used for immunohistochemical examination. Second one was frozen in PBS and stored for further experiments. The

third portion served for obtaining mediators from the granules of the mast cells. For this purpose, the mast cell degranulation was stimulated by calcium ionophore A23187 (Sigma; 5 µg/ml in serum-free culture medium for 2 hours at 37°C). Yielded cellular extracts were then collected and frozen, too.

Histology and immunohistochemistry

The mast cells were cultured on chamber slides, and fixed with methanol. After blocking the endogenous alkaline phosphatase (AP) with levamisole and the non-specific binding with 10% bovine serum albumin (BSA) in Tris-buffered saline (TBS), the slides were incubated with the monoclonal anti-MMP-13 antibody 24 h at the room temperature in a humid chamber. Anti-MMP-13 monoclonal antibody was prepared in our laboratory. Briefly, the antibody was obtained as the ascitic fluid of mice BALB/c. The antigen, i.e. rat MMP-13, was prepared using affinity chromatography (Tyagi and Cleutjens 1996). Its identity was confirmed by immunoblotting using a standard of MMP-13 proenzyme (His. Tag, Human, Recombinant, *Spodoptera frugiperda*; Calbiochem). The antigen was diluted with TBS (1:50). As a secondary antibody, rabbit anti-mouse polyclonal AP-labelled (Sigma), diluted 1:50 with TBS, was used in the second step for 30 minutes. The binding reaction was visualised using Fast Red TR/Naphthol AS-MX Sigma FAST tablets (Sigma). Cells were counterstained with haematoxylin and mounted in gelatine. In staining control samples, the primary antibody was omitted. MMP-13 positive and negative cells ratios (expressed as percentage share of positive cells) was counted in 36 assays from 5 separate isolations.

Immunofluorescence microscopy

Cells fixed in methanol stored at -20°C were washed for 10 min in PBS. Incubation with primary antibodies Anti-MMP-13 (Ab-6) Mouse (Calbiochem) diluted in PBS was carried out for 2 h at a room temperature. The slides were then rinsed by PBS three times for 5 min and cells were subsequently incubated with secondary antibodies Alexa Fluor 488 F(ab')₂ fragment of Goat Anti-Mouse IgG (Invitrogen) for 30 min and rinsed. Propidium Iodide (Calbiochem) nucleic stain was added for 1 min and after a final rinse with PBS, the slides were mounted under glass coverslips with Gel/Mount (Permanent Aqueous mounting Medium) (Biomedex, USA). As a control, the primary antibody was omitted to exclude non-specific reactions.

The samples were then studied using the Nikon Eclipse E 400 microscope with the Nikon Y-FL epifluorescence attachment with the B-2A filter (excitation filter 450 – 498 nm, dichroic mirror 505 nm and barrier filter 520 nm; Nikon, Tokyo, Japan). Microphotographs were taken by the Nikon digital camera DS-5MC using the NIS-elements AR image-analyser software (Laboratory Imaging, Prague, Czech Republic).

Western blot analysis of MMP-13

The cellular extracts (described above) were used for this analysis. After electrophoretic separation, proteins were electrotransferred to a nitrocellulose membrane in 15 mM sodium borate buffer (pH 9.3) in Mini Trans-Blott Electrophoretic Transfer Cell (Bio-Rad Laboratories, USA) for 24 h at 4°C. Starting transfer power conditions were 25 V/ 250 mA, finishing conditions were 25 V/350

mA. Monoclonal antibodies to MMP-13 (prepared in our laboratory, see above) were diluted 1:50 in 2 % skimmed milk-PBS and nitro-cellulose membrane was incubated in this solution for 1 h at room temperature, and washed with 2% skimmed milk-PBS. The membrane was then incubated in a horseradish peroxidase (HRP)-conjugated swine anti-rat antibody solution diluted 1:500 for 1 h at room temperature. After washing, the membrane was stained with HRP substrate, 4-chloro-1-naphthol (15 mg in 5 ml of methanol, 20 ml of 10 mM Tris-HCl, 0.04% H₂O₂). The reaction was allowed to proceed in the dark for 15 min until all bands were visualised. The membrane was then air-dried.

Zymography

Cellular extracts with released enzymes were thawed at the room temperature and then the MMPs were analyzed by zymography (Novotná and Herget 1998). Gelatin substrate was used; therefore the zymography preferentially targeted MMPs with gelatinolytic activity (MMP-2 and MMP-9). Proenzyme MMP-2, proenzyme MMP-9 (Oncogene Research Product) and high molecular weight calibration kit (Pharmacia Biotech) were used as standards.

Enzymatic activity

Mca-P-L-G-Ldpa-A-R-NH₂ fluorogenic peptide substrate I (R&D Systems, Inc.) was used to measure the total MMPs activity. This peptide substrate can be cleaved by MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13. Measurement of fluorescence was done by plate filter fluorimeter Genios (Tecan) with excitation at 340nm wavelenght and emission at 400nm. The final substrate concentration of

reaction mixture was 1 μ M in 50mM HEPES buffer pH 7.4 supplemented with 10mM CaCl_2 and 10 μ M ZnCl_2 . Enzymatic activity was measured in both the cultivation medium as well as in cellular extracts. Enzymatic reaction was performed at 37°C and fluorescence was measured. Enzymatic activity was expressed in pmol/min/ml. Trypsase activity was assayed in the cultivation media and thawed cell lysates using the peptide substrate BAPNA (N- α -benzoyl-DL-arginine p-nitroanilide hydrochloride, Sigma). Fifty μ l of sample was added to 20 mM Tris buffer (pH 8.0) containing 1M glycerol and 7.77 nM BAPNA. Absorbance was measured at 405 nm and trypsin activity was expressed in μ U/min.

Statistics

Non-parametric Mann – Whitney test was used for statistical evaluation of the MMP-13-positive mast cells. Enzymatic activities were compared by ANOVA (StatView 5.0, SAS Institute Inc., Cary, NC, USA).

All data are presented as mean \pm SE (Standard Error), differences were considered significant when $p < 0.05$.

Results

LMC isolation

We harvested from 0.65×10^6 to 1.2×10^6 mast cells with 90 – 95 % vitality during one isolation. Mast cells purity assessed by toluidine blue staining was 70-80 %. The major contaminating cell type was erythrocytes, which had the similar density as the mast cells.

Immunohistochemistry

We found significantly higher amount of cells labeled with monoclonal anti-rat MMP-13 antibody in group exposed to hypoxia (H: 19 ± 3 %; N: 3 ± 1 %; $p < 0.05$). Anti-MMP-13 immunofluorescence staining is shown on Figs. 1A-D.

Western blot

Western blot analysis confirmed the presence of MMP-13 in the mast cells (Fig. 2).

Zymography

Zymography showed the lytic zones corresponding with standards of proMMP-9 and proMMP-2. Proenzymes outweighed the active forms, of which only MMP-9 was visible. The lytic zones of proenzymes were more transparent in cellular extracts obtained from LMC exposed to hypoxia (Fig. 3).

Enzymatic activities

Fig. 4 demonstrates MMPs activity in cultivation media and in cellular extracts (i.e., supernatants). While MMPs activities of cultivation media in normoxic and hypoxic groups did not differ after 24 hours of cultivation, the MMPs activity of cellular extracts was in hypoxic group significantly higher than in normoxic group.

Fig. 5 shows tryptase activity in cultivation media and in cell lysates. Tryptase activity of cultivation media did not differ between normoxic and hypoxic groups.

Cell lysates from hypoxia exposed cells showed significantly higher activity of tryptase than lysates from cells cultivated in normoxia.

Both MMPs and tryptase activities in cultivation media are expressed after deduction

of activity of the control medium.

Discussion

In this study we found that rat lung mast cells exposed to 24 hours of “in vitro” hypoxia showed higher activities of MMPs and tryptase than cells cultivated in normoxia. The results suggest that enzymes synthesized by lung mast cells can participate in the collagenolysis at the onset of remodeling of pulmonary vessels in chronic hypoxia.

The LMC were previously isolated from guinea pigs (Pele *et al.* 1989, Undem *et al.* 1985), bovines (Stahl *et al.* 1996), humans (Van Overveld *et al.* 1988) and rats with pulmonary fibrosis (Tomioka *et al.* 1989). The technique of LMC isolation was modified by Stahl *et al.* (Stahl *et al.* 1996, Enerback and Svensson 1980). The yield and vitality of isolated rat LMC in our present work are comparable with data described by other groups (Stahl *et al.* 1996).

Lung mast cells are an important source of collagenolytic and proteolytic enzymes as was described by others (Edwards *et al.* 2005, Tozzi *et al.* 1998). It has been repeatedly shown that their number in the lung tissue increases in chronic hypoxia (Kay *et al.* 1974, Mungall 1976, Tucker *et al.* 1977, Williams *et al.* 1981). In an earlier quantitative morphological study we found the highest accumulation of LMC expressed MMP-13 in the wall of peripheral pulmonary arteries at the beginning of hypoxic exposure (Vajner *et al.* 2006).

Hypoxic pulmonary hypertension is adaptation and not continuously developing process. In the rat species the changes of pulmonary vasculature develop in the first few days and after one week of exposure to hypoxia the hypoxic pulmonary

hypertension fades into stable state with no signs of tissue radical injury (Hampl and Herget 2000). After two weeks of exposure the hypoxic pulmonary hypertension does not progress (Herget *et al.* 1978), and changes of collagen metabolism concern more conductive part of pulmonary vasculature (transmural vascular stress) than pathogenetically important resistance vessels. Treatment of hypoxic pulmonary hypertension with antioxidant is effective at the beginning of exposure and without affect in steady state (Lachmanová *et al.* 2005).

The mast cells in the pulmonary tissue are under the control of many substances that may regulate their secretion. Our finding that hypoxia stimulates MMP-13 formation also in isolated LMC suggests that a mechanism mediating increase of MMP-13 has to be intrinsic to mast cells. Two mechanisms or their combination may be considered. Hypoxia can stimulate release of a regulatory substance (e. g. cytokine) that would stimulate LMC by autocrine mechanism. The second alternative is that primary stimulus is hypoxia-induced oxidant stress. Siwik *et al.* (Siwik *et al.* 2001) described increase MMP-13, MMP-2 and MMP-9 activities in rat cardiac fibroblasts exposed to hydrogen peroxide and superoxide. We showed inhibitory effect of antioxidant N – acetylcysteine on MMP-13 formation in the mast cells line RBL-2H3 exposed to hypoxia “in vitro” in our preliminary experiments (Maxová *et al.* 2004).

We demonstrated that hypoxia simultaneously stimulates a higher formation of tryptase in the mast cells exposed to hypoxia. Tryptase is a neutral protease considered as a marker of the mast cells activation (Hallgren and Pejler 2006). It also seems to be an important factor inducing mast cell chemotaxis (Berger *et al.* 2003). Tryptase can activate pro-MMPs but it may also have direct effects on the

extracellular matrix (Hallgren and Pejler 2006). Its fibrogenic action (Cairns and Walls 1997, Zhang *et al.* 1999) and gelatinolytic activity (Fajardo and Pejler 2003) in pulmonary vascular remodelling were described.

We selected the period of 24 hours hypoxia, when LMC were still not degranulated, for two reasons: 1. From our recent study (Hodyc *et al.* 2007) we know that biochemical changes (oxidant tissue stress) develop within the first day of sojourn in hypoxia. 2. It is technically impossible to harvest LMC from animals exposed to chronic hypoxia as the LMC before and after degranulation cannot be all at once separated by fractionation on Percoll column. However, mast cell degranulation seems to be “in vivo” an important process in hypoxia-induced pulmonary vascular remodeling, because prevention of mast cell degranulation (disodium cromoglycate administration) reduced development of hypoxic pulmonary hypertension (Kay *et al.* 1981, Maxová *et al.* 2006). The degranulation therefore requires longer hypoxic exposure, and presence of other cell type or humoral factors.

We conclude that lung mast cells exposed to hypoxia “in vitro” increase the MMPs and tryptase production. Enhanced MMPs and tryptase production may be related to the mechanism of pulmonary vascular remodeling in hypoxic pulmonary hypertension.

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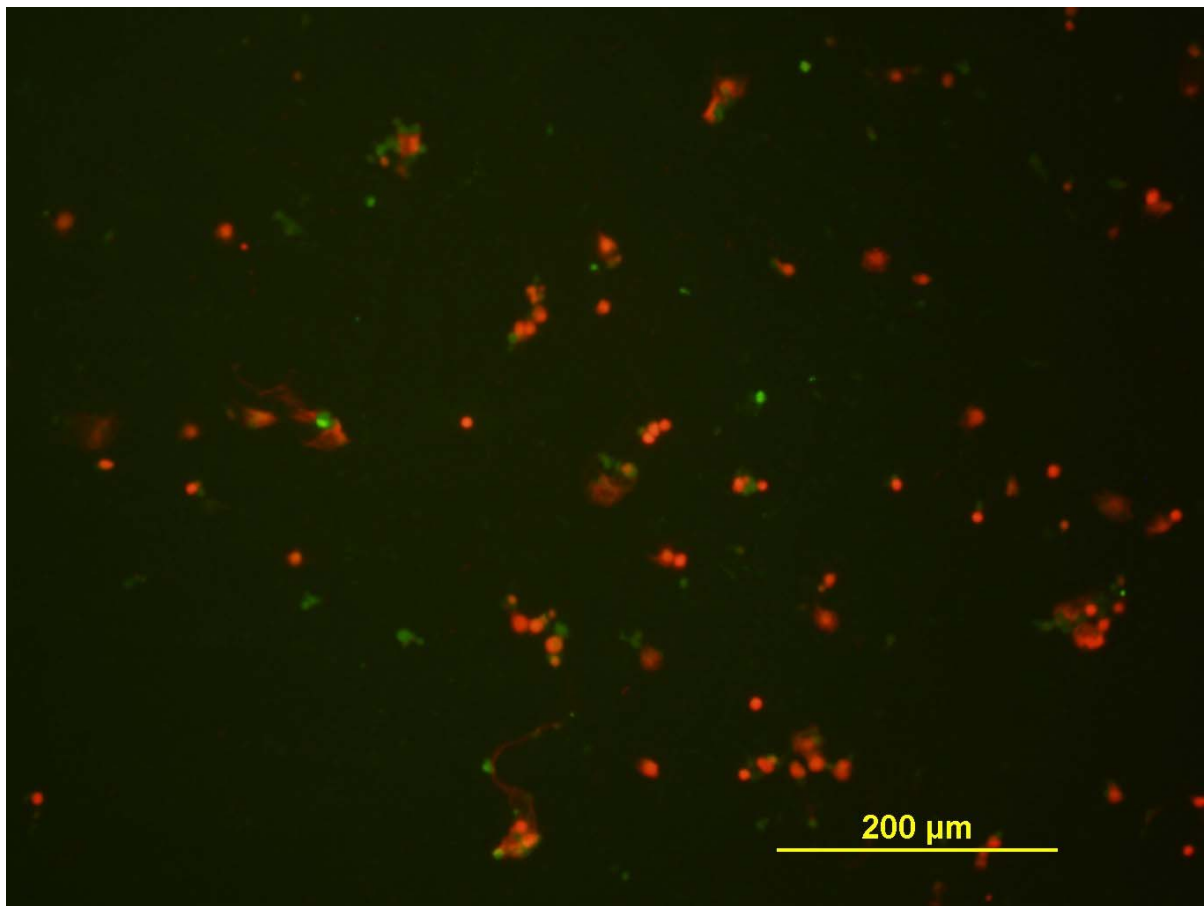
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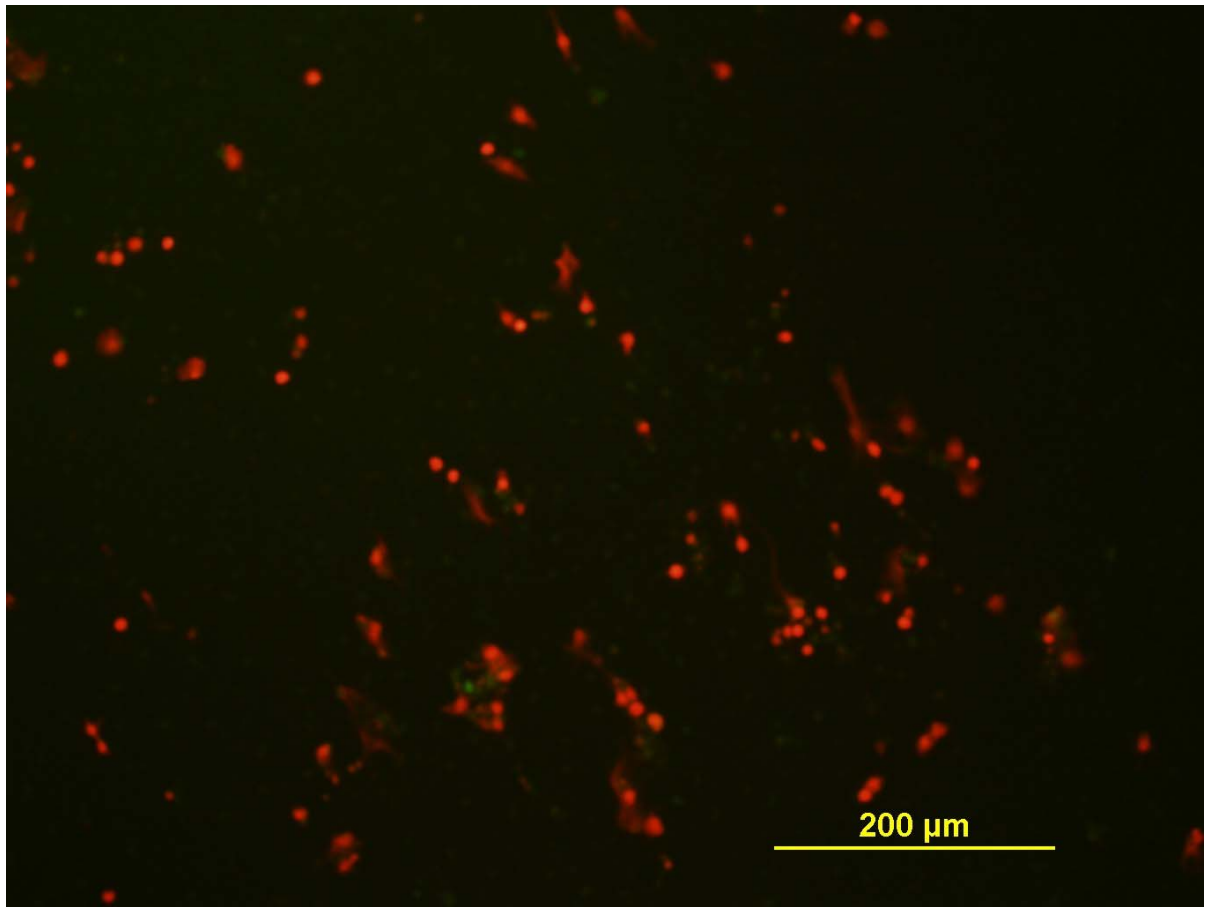
Fig. 1

Immunohistological characterization of MMP-13 (green) in isolated lung mast cells (red nuclei) using immunofluorescence labeling. LMC exposed for 24 hour to hypoxia (A) show higher MMP-13 positivity against cells cultured in normoxic conditions (B). Details of two markedly positive MMP-13 labeled hypoxic LMC (C) and one positive MMP-13 labeled normoxic LMC (D).

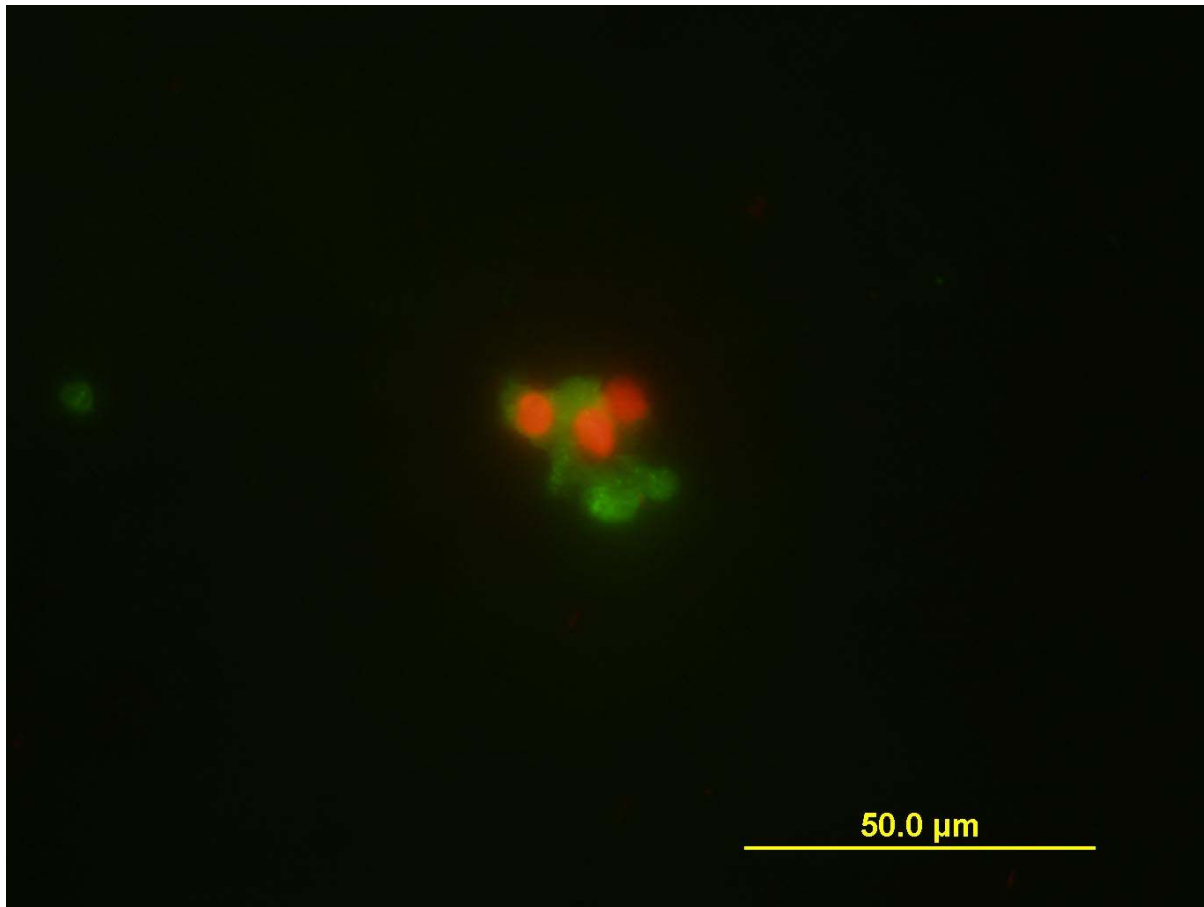
A



B



C



D

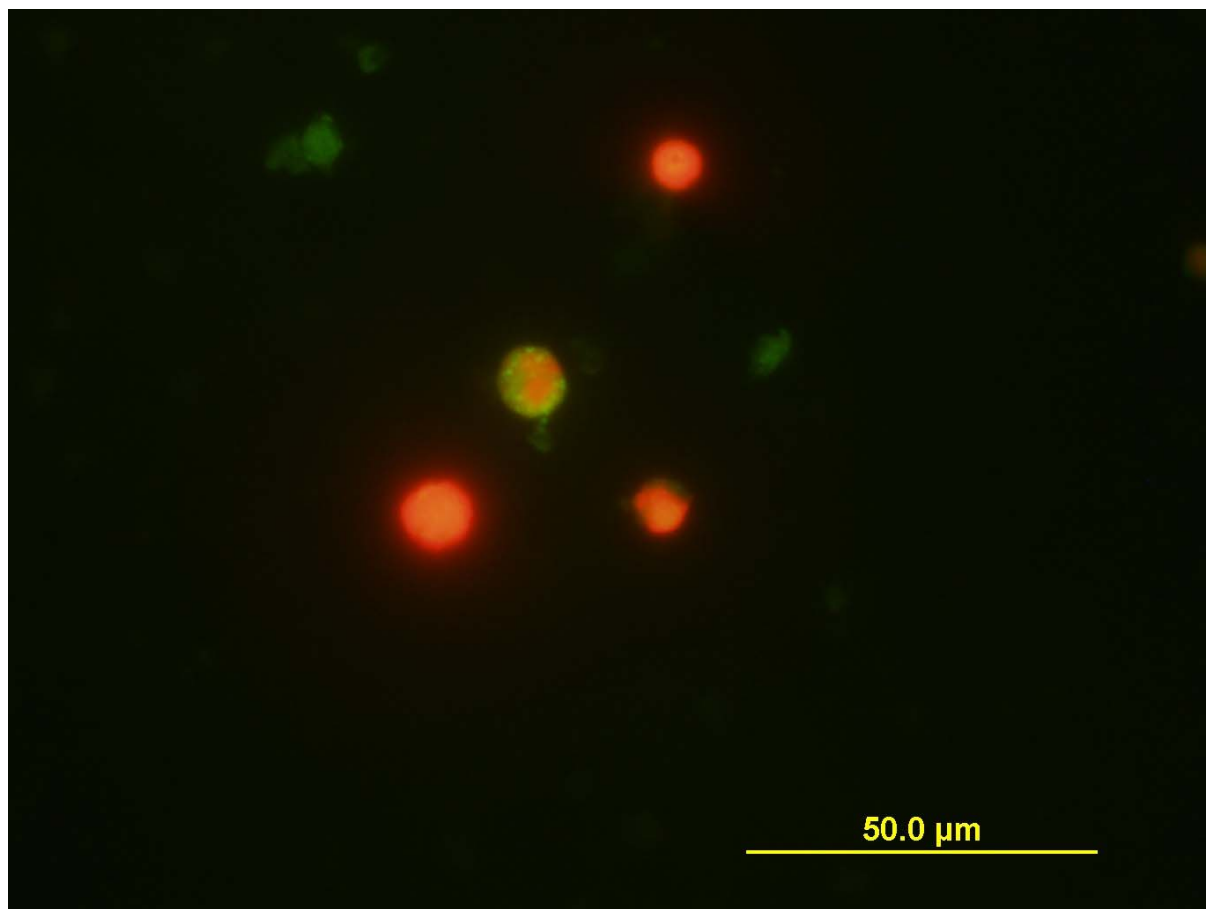


Fig. 2

MMP-13 detected by immunoblotting prevails in cellular extracts from LMC exposed to 24 h hypoxia.

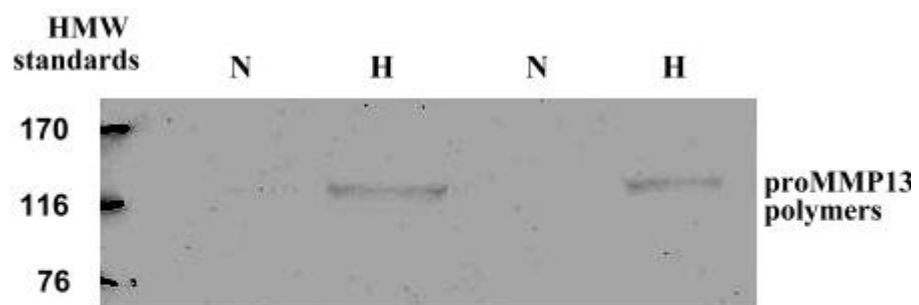


Fig. 3

Typical zymogram from the cellular extracts in groups of LMC cultivated 24 h in normoxic (N) and hypoxic (H) conditions.

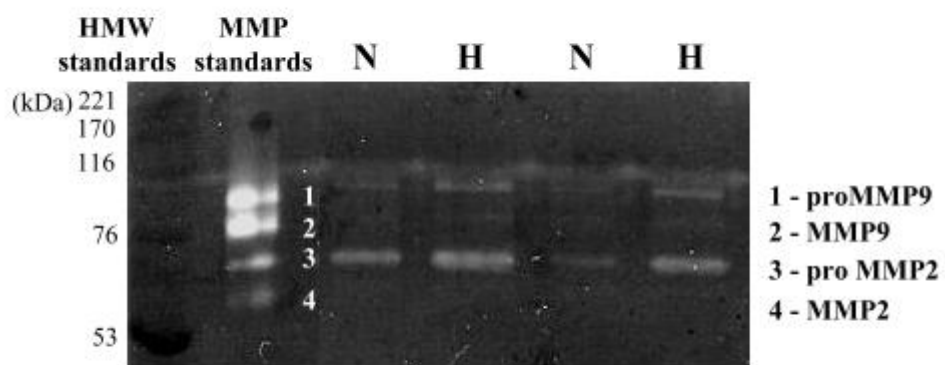


Fig. 4

Total MMPs activity (means \pm SE) assigned in cultivation media and cellular extracts (supernatant) in groups of cells cultivated 24 h in normoxic and hypoxic conditions (ANOVA, $p = 0.0005$).

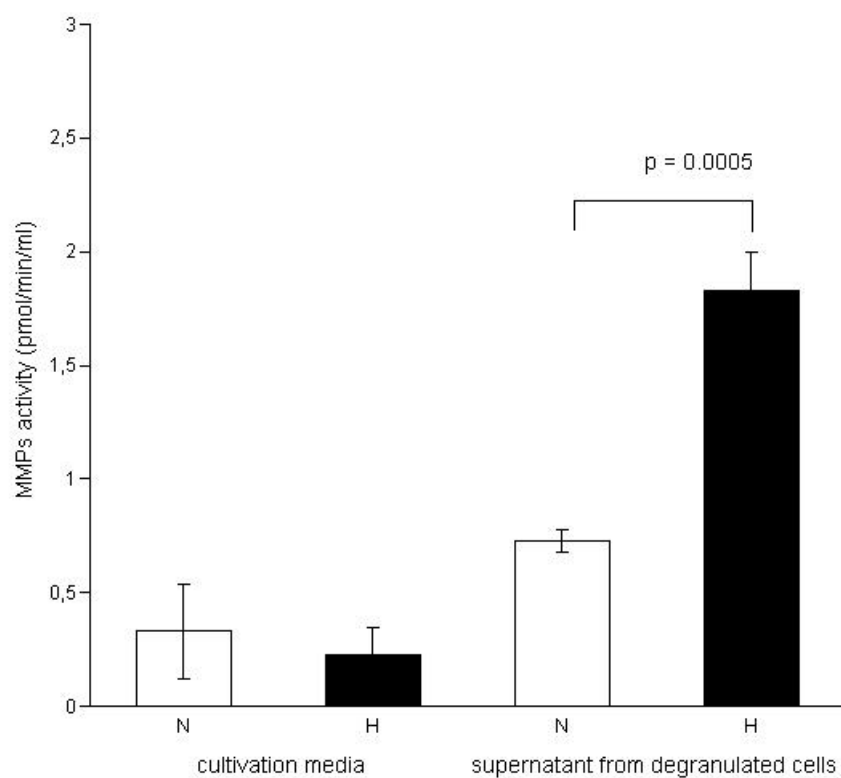


Fig. 5

Tryptase activity (means \pm SE) assigned in cultivation media and cell lysates in groups of cells cultivated 24 h in normoxic and hypoxic conditions (ANOVA, $p = 0.004$).

