Title

Changes of Extracellular Matrix of Rat Cornea after Exposure to Hypoxia

Authors

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Short title

Changes of Rat Cornea after Exposure to Hypoxia

Summary

Purpose: To check whether hypoxia of corneal tissue increases the collagenolytic activity due to release of reactive oxygen and nitrogen species.

Methods: The rats were exposed to hypoxia 10%O_2 for 4, 14 and 21 days. The radical tissue injury was measured by level of nitro tyrosine and changes in the lipoperoxide related fluorophores. Collagen protein composition was analysed by slab gel electrophoresis. The activity of gelatinolytic enzymes was

studied using the zymography. The vascularization of the corneas was measured.

Results: We found no differences in the corneal tissue in the gel electrophoretic profile of collagenous proteins and gelatinolytic activity between the normoxic and hypoxic rats. We did not find any sign of radical tissue injury. There were no changes in vascularization of corneas after exposition to hypoxia.

Conclusions: The environmental 10% hypoxia does not induce radical tissue injury and increase of collagenolytic activity in rat cornea.

Key words

Cornea – hypoxia – metalloproteinase – oxidative damage – nitrotyrosine

Introduction

Corneal metabolism depends on a critical level of atmospheric oxygen. Below this level a series of specific metabolic events occurs (Klyce 1981; Bonanno and Polse 1987) . Glycogen depletion and alterated adenosine triphosphate concentrations in epithelium have been described (Thoft and Friend 1975). The lactate level is enhanced (Klyce 1981) , due to increased anaerobic glycolysis and altered endothelial transport (Barr and Schoessler 1980) . The acute metabolic responses to hypoxia are reversible. Chronic exposure to low oxygen concentration, however, may induce structural changes in all the cornea layers that may take several months to revers, or they even may be permanent (Polse *et al.* 1990; Madigan and Holden 1992).

The critical oxygen requirement to avoid structural corneal changes in human is still a debated subject. Exposure to pO_2 76mmHg (10% O_2, hypobaric hypoxia) for 30 days showed in species to induce structural changes in corneal stroma, neovascularization with advanced vessel differentiation and active vessel proliferation (Mastropasqua *et al.* 1998) .

Hypoxia causes free radical tissue injury that results in biochemical alterations of the extracellular matrix and in extracellular matrix remodelling. The tissue remodelling is probably directly linked to the production of reactive oxygen species (ROS) and other radicals (e.g. NO derived radicals) (Cadenas *et al.* 1977; Misra and Fridovich 1972; Fried *et al.* 1973; Herget *et al.* 2000). The increase in radical production is well documented in exposure to hypoxia (Hampl and Herget 2000) . A very reactive substance, peroxynitrite and its derivatives, originate from superoxide and nitric oxide interaction (Muijsers *et al.* 1997) . The

increase of nitrotyrosine, the stable product of peroxynitrite reaction, within proteins is often used as a marker of peroxynitrite production (Beckman 1996; Beckman and Koppenol 1996) . Plasma concentration of nitrotyrosine increases in the first days of exposure to hypoxia (Herget *et al*. 2000) .

Aldehydic products of free radical-initiated lipid peroxidation interact with proteins to form fluorescent compounds with distinguished spectral features. Also these fluorophores are used as markers of protein free radical damage (Gardner 1979; Fukuzawa 1985; Wilhelm and Herget 1999; Itakura 2000).

The reactive oxygen and nitrogen species react with the catalytic site of the metalloproteinases (Rajagopalan *et al.* 1996) . It was shown that chronic hypoxia initiates an increase in lung collagenolytic activity in rat lungs which results in the presence of low molecule weight collagen type I cleavages (Novotna and Herget 1998; Novotna and Herget 2002).

The aim of our study was to test the hypothesis that chronic hypoxia of cornea causes an increase in collagenolytic activity, which results in stromal remodelling. The putative role of increased production of radicals during the exposure to hypoxia was tested.

Materials and Methods

Exposure to hypoxia

Adult male Wistar rats were exposed to hypoxia (F_{i02} = 0,1) for 4 days, 14 days and 3 weeks in an isobaric hypoxic chamber (Hampl and Herget 1990). Oxygen concentration was continuously monitored and regulated KOH and soda lime adsorbed CO_2 and excess humidity was condensed in a refrigerator and adsorbed by silicagel. The each experimental group had its own control group.

Measurement of perilimbal vascularization

6 control normoxic rats and 8 rats exposed to hypoxia for 21 days were anesthetized by thiopental and the photographs of their corneas were taken using the slit lamp and the digital camera system. The photographs were analysed using a computer picture analysing system. The vessels ingrowth was measured from the limbus towards the center of corneas.

Preparation of the cornea tissue

The eyes were removed and the corneas were dissected immediately after sacrifying the rats with overdosing of thiopental. The central clear part of cornea was used for the experiments avoiding the limbal zone (diameter 4mm). The tissue was cut to small pieces and frozen.

Analysis of collagenous proteins in cornea

Six rats exposed to hypoxia for 4 days and 6 control normoxic animals, 6 rats exposed to hypoxia for 14 days (6 controls) and 6 rats exposed to hypoxia for 21 days (6 controls) were used for the analysis of collagenous proteins in the cornea and the zymography. For the analysis of collagenous proteins, after preparation of the corneal tissue and washing in distilled water, the tissue was pepsinized by 1:10 ratio between the weight of pepsin and dry weight of the tissue in 0,5 M CH_3COOH, pH 2.5, for 4 h at room temperature and then for 20 h at 4 ^oC and centrifuged (8000xg, 30min) (Novotna and Herget 1998) . The supernatant was lyophilized.

Gel electrophoretic separations (SDS-PAGE) of collagenous fraction were performed by the method of Laemli (1970) on discontinuous slab gel using 4% stacking gel and 7,5% separating gel. Samples were dissolved in a sample buffer in the concentration 4g/ml, and 12μg of the collagenous fraction was loaded per line. The collagen type I standard from Sigma was loaded in a concentration of 8 μg per line. The electrophoretic separation was run in a Tris-glycine buffer system without reduction in Mini-PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, USA). The gels was stained for proteins with 0,25% Coomassie Brilliant Blue R in methanol-acetic acid-water (40:10:50 v/v/v). Destaining was performed with methanol-acetic acid-water (40:10:50 v/v/v).

Zymography

The collagenolytic enzymes from the lyophilised corneal tissue were extracted using the nonreducing sample buffer (1,5% SDS; 15%glycerol; 0,025% bromphenol blue). 50μl/mg of the buffer per dry tissue weight was let to extract for 22 h at 4^oC and then pure extract was separated on 10% SDS-PAGE containing 0,1% gelatine. To remove SDS the gels were washed for 30 min in 2,5% (v/v) Triton X-100 and Triton was removed by washing the gels with distilled water and with incubation buffer (50 mM Tris-HCl, pH 7,8; 10 mM $CaCl_2$; 10mM NaCl). The gels were then incubated (17 h, 37^oC) in incubation buffer. Gels were stained with 0,25% Coomassie Brilliant Blue R in methanol-acetic acid -water (40:10:50 v/v/v). Destaining was performed with methanol-acetic acid -water (40:10:50 v/v/v). High molecular weight calibration kit (Pharmatia Biotech, USA) was used as standards. The lytic zones were analysed densitometrically using software for measurements of electrophoregram densities designed by ing. Jiří Semecký, Prague, CR.

Measurement of 3-nitrotyrosine

Mouse monoclonal antibody NO-60-E3 against 3-nitrotyrosine was prepared in our laboratory. Peroxynitrite was prepared by reaction of acidified solution of H_2O_2 with sodium nitrite followed by

stabilisation of peroxynitrous acid by sodium hydroxide (Koppenol *et al.* 1996) . Standard of nitrated BSA for ELISA was prepared by reaction with peroxynitrite (Beckman 1996).

Highly nitrated proteins were prepared by reaction with 1mM tetranitromethane (TNM) (Sokolovsky *et al.* 1966) and reaction was stopped by addition of free tyrosine and subsequent gel filtration on column of Sephadex G-25. 3-nitrotyrosine content of nitrated proteins was determined by difference of absorbance at 430nm in acid solution and under alkaline pH (Sokolovsky *et al*. 1967) .

Cornea was minced by scissors and homogenised by sonication four times for 1s (100W) with 250µl TBS pH 8,4. Extraction was performed for three hours at 4°C and after centrifugation supernatants were used for the estimation of concentration of 3-nitrotyrosine by inhibition ELISA (described below). Concentration of proteins was done by bicinchonic acid method (Smith *et al.* 1985) .

Method for estimation of 3-nitrotyrosine was slightly modified method of Herget et al. (Herget *et al.* 2000) . Briefly polystyrene 96-well plates (Maxisorp, Nunc) were coated with highly nitrated BSA dissolved in PBS at concentration 5nM nitrotyrosine. The plates were blocked by three 5 minutes incubation with PBS plus 0,05%(v/v) Twen-20 (TPBS). Then 50µl per well of 0,2% gelatin in TBS pH 8,4 was pipetted and standard solution of nitrated BSA (by peroxynitrite) or tested samples in triplicates were serially diluted. Further 50µl of diluted ascites of our monoclonal antibody NO-60-E3 (1:125 000) in the same buffer was added and mixture was incubated under gentle shaking at laboratory temperature for 90 minutes. The plates were three times washed with PBS and incubated with 100µl of antimouse Ig rabbit antibody conjugated with peroxidase (SwAR/Px, Sevapharma, Czech Republic; diluted 1:1000 in 1%BSA in PBS) for 90 minutes. After five washings with TPBS the plates were developed with o-phenylenediamine and reaction terminated by addition of sulphuric acid. Absorbance was read at 492nm using microplate reader. Standard curves and concentration of samples were calculated by Rodbard's four parameters equation (Rodbard and McClean 1977) .

Measurement of protein free radical damage

5 animals exposed to hypoxia for 4 days and 5 control animals, 7 animals exposed to hypoxia for 14 days and 6 control animals, and 6 animals exposed to hypoxia for 21 days and 6 control animals were used. The concentration of proteins modified by free radical products were measured on the basis of fluorescent protein adducts in the soluble protein fraction (Gardner 1979; Fukuzawa 1985; Itakura 2000). Briefly, cornea was homogenized in 50 mM phosphate buffer, pH 7,4, homogenate was cleared by centrifugation (10 000g, 10 min.) and tridimensional fluorescence spectra were measured in supernatant. Thus found excitation (360

nm) and emission (446 nm) maxima were used for quantitative measurements. The values are expressed in relative fluorescence units (RFU) per mg of protein, determined according to Lowry (Lowry 1951).

Chemicals

All chemicals were highest available purity. If not mentioned other the chemicals were obtained from Sigma-Aldrich Chemie GmbH (Diesenhofen, Germany).

Statistical analyses

The results were statistically evaluated by ANOVA. Differences were considered significant at $P<0.05$.

Results

Three weeks exposure to hypoxia did not induced an increase of perilimbal vascularization in rats that were exposed to hypoxia for 21 days (Figure 1).

Similarly, we did not find any differences in collagenous proteins isolated from corneas of hypoxic and control rats. Figure 2 shows typical gel with collagenous proteins isolated from cornea of control and hypoxic rat (21 days of hypoxia). No differences are apparent. Figures 3 A, B and C show densitometric analysis of electrophoretic profiles of the collagen type I standard and the collagenous fractions isolated from corneas of control and experimental rats exposed to hypoxia for 4, 14 and 21 days. The relative density of the main peaks of collagen chains did not significantly differ.

Zymographic analyses of the corneas on gelatine substrate showed lytic zones in the area of active enzyme of gelatinase A (MMP-2) and another lytic zone in the position of 72kDa, that corresponds with the area of the pro-enzyme of gelatinase A (pro-MMP-2). Figure 4 shows typical zymogram of extracts from cornea in control and hypoxic rat (21 days of hypoxia). We did not find significant differences in the density of the lytic zones between normoxic rats and the groups of rats exposed to 4, 14 and 21 days of hypoxia (data not shown).

As a measure of radical injury to corneal proteins we measured the tissue concentration of 3-nitrotyrosine and concentration of lipoperoxide related fluorophores. There was no difference in nitrotyrosine concentration in extracts of corneas of the animals exposed to hypoxia for 4 days, 14 days (data not shown) and 21 days, and the control animals (Figure 5).

Similarly, there was no difference in the concentration of lipoperoxide related fluorophores measured

in the corneas of the control animals (0,128±0,003 RFU (relatives fluorescence unit)/g) and the corneas of animals exposed to hypoxia for 4 days (0,127±0,003) RFU/g, 14 days (0,127±0,003 RFU/g), and 21 days(0,129±0,002 RFU/g).

Discussion

Tissue hypoxia is known to induce neovascularization and extracellular matrix remodelling in many organs. Radical tissue injury induced by lack of oxygen plays probably an important part in this process. Cornea, avascular organ, is physiologically supplied by diffusion of oxygen directly from ambient atmosphere. During the eye closure the oxygen comes mostly by diffusion from palpebral conjunctiva. PO_2 in conjunctival capillaries is about 60 mmHg and PO2 decreases to approximately 40mmg Hg at the corneal surface. Prolonged eyelid closure triggers a cascade of biochemical, cellular and microbial events, culminating in inflammation, hypoxia, and dry-eye states, though significant individual variations in the of open-eye and closed-eye responses to hypoxia were described (Liesegang 2002). The issue of corneal hypoxia is clinically relevant because it is believed that epithelial oedema, microcyst formation and decrease in epithelial mitotic rate and neovascularization after contact lenses application results from reduction of oxygen supply of corneal tissue (Ladage *et al.* 2001; Donnenfeld *et al.* 1991) . Contact lenses, however, may induce also trauma of corneal surface and some studies document that trauma alone may be responsible for a significant metabolic alteration in epithelial cells and may induce oedema (Thoft and Friend 1975; Cejkova *et al.* 1992). Local hypercapnia and increase of temperature under the contact lens should be taken in the consideration also (Mastropasqua *et al.* 1998) .

In the present study we addressed the question whether hypoxia in ambient air induce in avascular cornea the neovascularization and remodelling of matrix proteins similar to that which is known from the studies on vascular organs.

We hypothesized the hypoxia induces an increase in production of reactive oxygen and nitrogen species that accentuates collagenolytic activity within the corneal tissue which results in extracellular matrix proteins degradation (Fini *et al.* 1998). The ectracellular matrix remodelling may then participate in neovascularisation of the cornea.

The chronic hypoxia increases production of reactive oxygen species (ROS) (Wilhelm and Herget 1999) . It is supposed that the activation of collagenolysis in the early phases of hypoxic exposure is related to the production of radical oxygen species (ROS) and the other radicals. One of the supposed mechanism of activation of MMPs is by non-proteolytic compounds such as ROS or peroxynitrite (Novotna and Herget

2002) . We have found no increase in nitrotyrosine concentration (marker of peroxynitrite production, (Beckman 1996)) and in concentration of lipoperoxide related fluorophores (the marker of oxidative tissue damage) in the corneas of hypoxic rats.

Stromal extracellular matrix degradation is mediated by battery of enzymes including matrix metalloproteinases (MMP). MMP-2 (gelatinase A) is responsible for homeostatic remodelling of stroma in normal corneas (Fini *et al.* 1998; Matsubara *et al.* 1991) . During corneal repair after an injury, other MMPs take part in tissue remodelling and the rate of collagen turnover is much higher. Expression of collagenase and stromelysin is induced within the initiating phase of the repair process after superficial or penetrating injury to stroma in the rabbit model. The activity of gelatinase A (proenzyme and the active form) increases. Collagenase concentration increases within the repair tissue between 1 and 4 weeks after injury and then decreases between 4 and 8 weeks after injury. The expression pattern of other MMPs followed similar kinetics. The activities of collagenolytic enzymes peak at 1 week after wounding and then began decline; however, MMP expression is increased even at 7-9 months after wounding (Fini *et al*. 1998). In a rat model of inflammation-associated corneal neovascularization the MMP-2 mRNA concentration correlates with the development of new vessels (Kvanta *et al.* 2000) . Neutrophils, stromal fibroblast, and epithelial cells were identified as the possible source of stroma - degrading enzymes (Fini *et al*. 1998) .

We have found no difference in collagenolytic activity in the corneas dissected from the animals that were exposed to hypoxia and from the normoxic controls in any of the time interval studied. The electrophoretic profile of the collagenous proteins isolated from the corneas of hypoxic rats was not altered and limbal vascularisation or vascularisation of the cornea was not enhanced. Exposure to similar level of hypoxia in the same isobaric hypoxic chamber, however, induces increase in the collagenolytic activity in lung tissue and peripheral pulmonary arteries which results in collagen type I cleavage (Novotna and Herget 1998) . Our results disagree with the findings of Mastropasqua and collaborators (Mastropasqua *et al*. 1998) . They described corneal stroma reorganization and Descemet´s membrane thickening on corneas of rats exposed to hypoxia (pO_2 76 mmHg) for 30 days. Neovascularization with advanced vessel differentiation and active vessel proliferation were clearly detectable in their experiments. They found polymorphonuclear, lymphocytes and occasional macrophages scattered throughout the corneal stroma. Their findings therefore may be attributed to the presence of corneal inflammation, which may or may not be directly related to tissue hypoxia. We did not performe histologic analysis of the corneas and we cannot comment possible presence of some polymorfonuclear, lymphocytes or macrophages within the corneal stroma in our experiments**.** In addition the aim of the current study was to analyse the homogenous non-vascular central part of cornea only. We cannot, therefore, exclude possible hypoxia-induced inflammatory changes in limbal part of cornea.

We did not find any signs of advanced neovascularization of the hypoxic corneas, the extent of perilimbal vascularization did not differ significantly between the normoxic corneas and the corneas exposed to hypoxia. Therefore, the absence of vascularization of the cornea may explain the difference between absence of collagenolysis in cornea and rapidly developing collagen degradation in lung tissue at the same level of hypoxia. The possible gender differences also should be taken in focus as Mastropasqua (Mastropasqua *et al.* 1998) found the histological changes in corneas of female Wistar rats in contrast to male rats used in our experiments. The gender difference in susceptibility to tissue hypoxia is well known (Griffin *et al.* 2000; Zhao and Eghbali-Webb 2002).

We found that exposure to 10 % of oxygen does not induce collagenolysis and angiogenesis in central cornea. We conclude that hypoxia in ambient air severe enough to induce the lung structural remodelling does not affect collagen tissue proteins in central cornea and does not induce neovascularization. The possible explanation is that the level of hypoxia was not severe enough to induce corneal injury. The same level of hypoxia was efficient in inducing proliferative changes in the lung tissue (Herget *et al.* 2000; Novotna *et al.* 2001). The difference between the lungs and central cornea is that cornea remains avascular during the exposure to hypoxia. Therefore we hypothesize that presence of blood cell releasing radicals is needed to start extracellular matrix remodelling in chronic hypoxia. To prove our hypothesis, it would be interesting to clear the effect of hypoxia on the perilimbal part of cornea. We did not study the possible changes of the composition of non-collagen extracellular matrix proteins. The changes in production of proteoglycans were described in relation to oxygen tension in different connective tissues including cornea. Keratan sulphate replaces chondroitin sulphate (in terms of tissue concentrations) in condition of poor oxygen supply (Scott 1992). More studies are necessary to clear all possible effects of hypoxia on the corneal tissue.

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Legends to figures

Fig. 1. Perilimbal corneal vascularization of normoxic rats (grey column) and hypoxic rats after 21 days of exposure to hypoxia (shaded column); in mm.

Fig. 2. Gel electrophoresis profile of the collagenous fraction isolated from corneas of rats exposed to

normoxic condition (N) and exposed to hypoxia 10% O_2 for 21 days (H); Collagen I – collagen type I standard from rat tail (Sigma), HMW – high molecule weight standard (Pharmacia Biotech, USA), γ – γ-fraction (chain polymers), β – β-fraction (chain dimers), α1 – mixture of individual α1 chains, α2 - α2 chains

Fig. 3. Relative density of the main peaks separated by gel electrophoresis of collagenous proteins isolated from cornea of animals exposed to 4 days hypoxia (3A), 14 days hypoxia (3B) and 21 days hypoxia (3C); hypoxic animals (N = 6, grey column) and control animals (N= 6, shaded column)**;** gamma = γ -fraction (polymers of collagen chains); beta = β -fraction (dimers of collagen chains); alpha 1 = α1 collagen chains; alpha 2 = α2 chains

Fig. 4. Zymographic analysis of MMPs extracted from corneas of rats exposed to normoxic condition (N) and exposed to hypoxia 10% O_2 for 21 days (H).The lytic zones in the area of active gelatinase A (MMP-2) and in the position of 72 kDa (pro MMP-2) ; MMP-2 - standard of MMP-2 enzyme, MMP-9 – standard of MMP-9 enzyme, HMW - high molecule weight standard (Pharmacia Biotech, USA)

Fig. 5. Nitrotyrosine concentration in the rat cornea after 21 days of hypoxia (shaded column), and in the cornea of the control animals (grey column); in pmol/g.

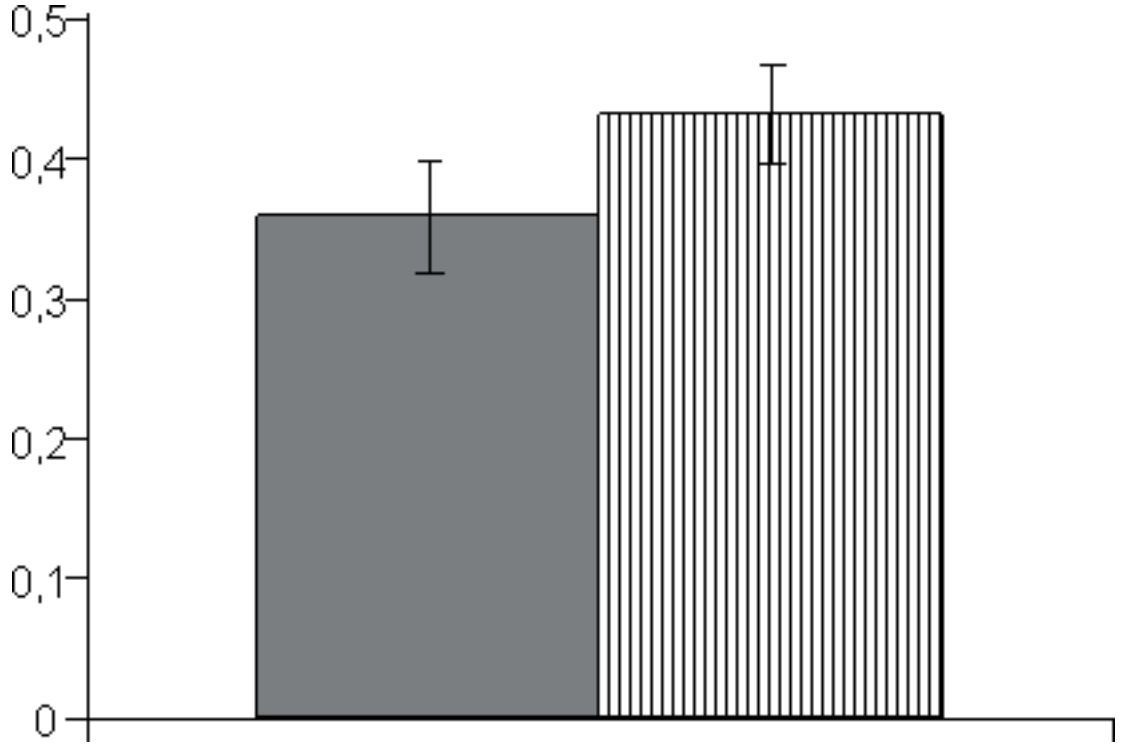


Fig. 1

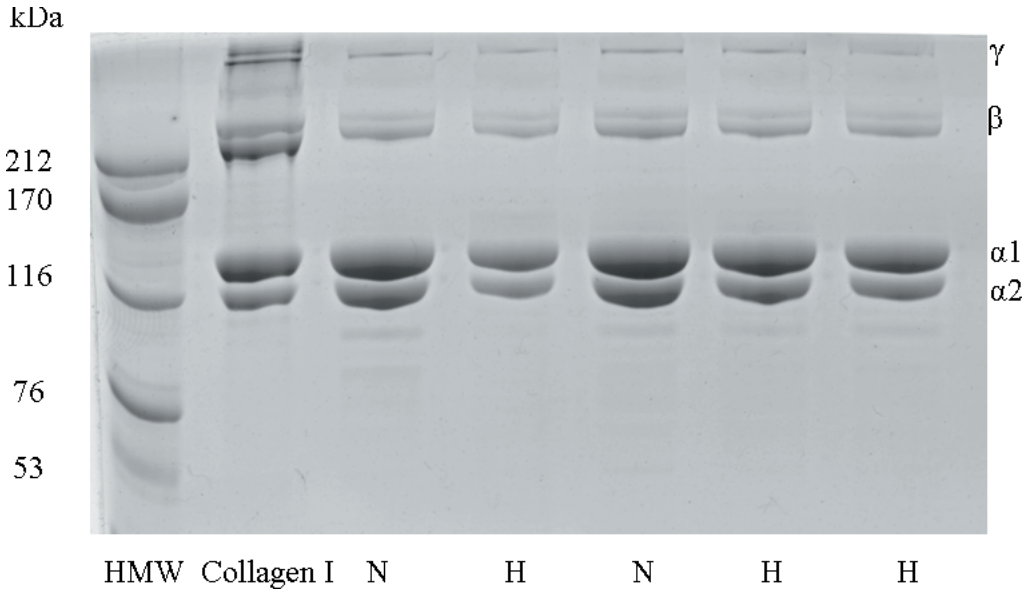
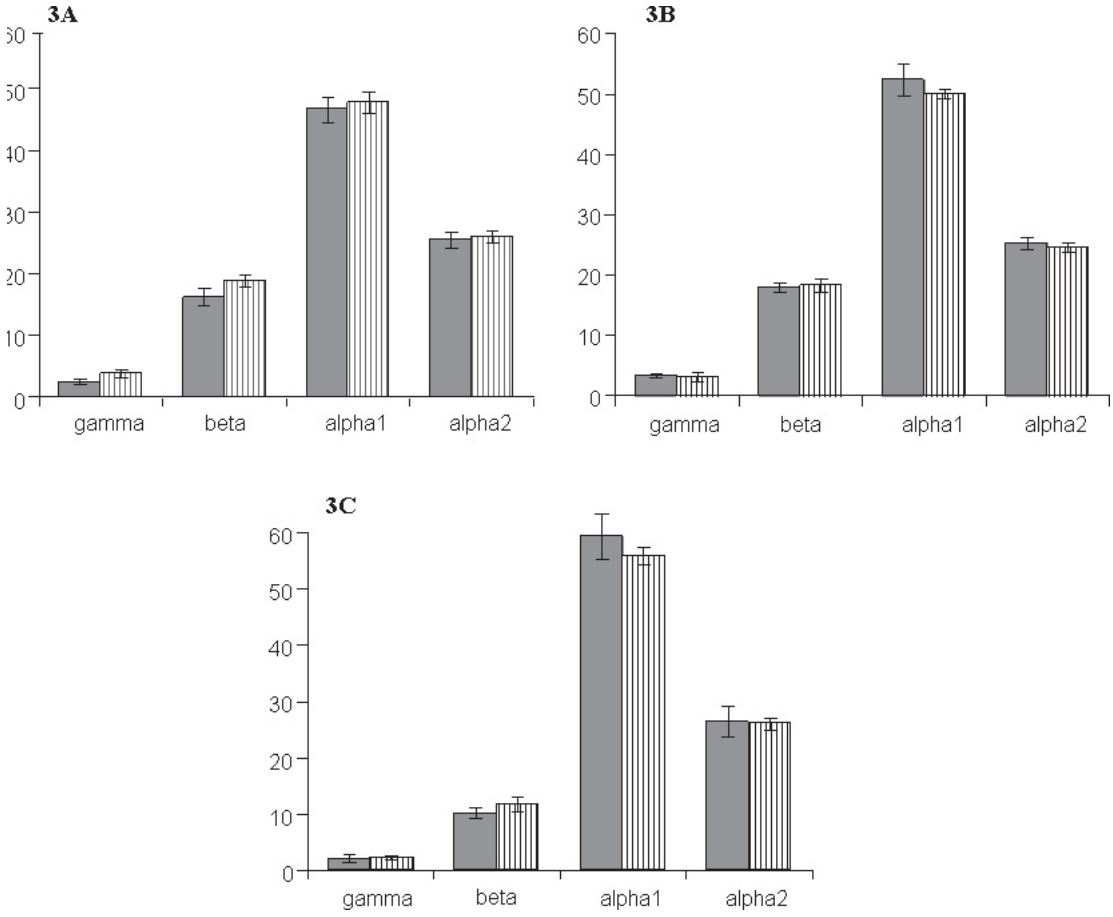


Fig. 2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beta
alpha1
alpha2

Fig. 3

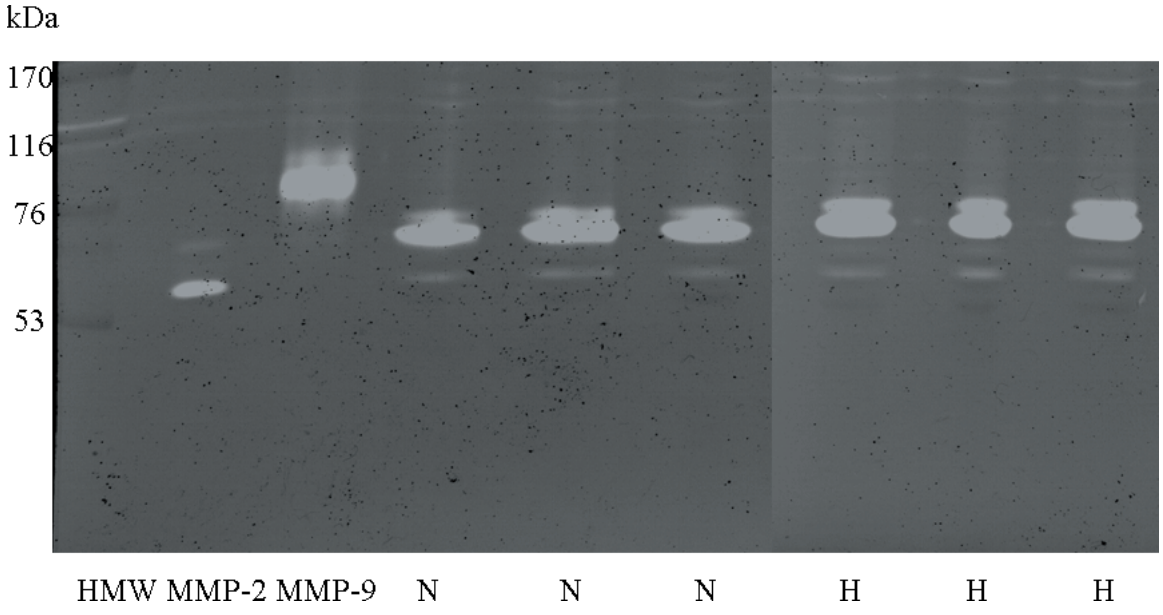

Fig. 4

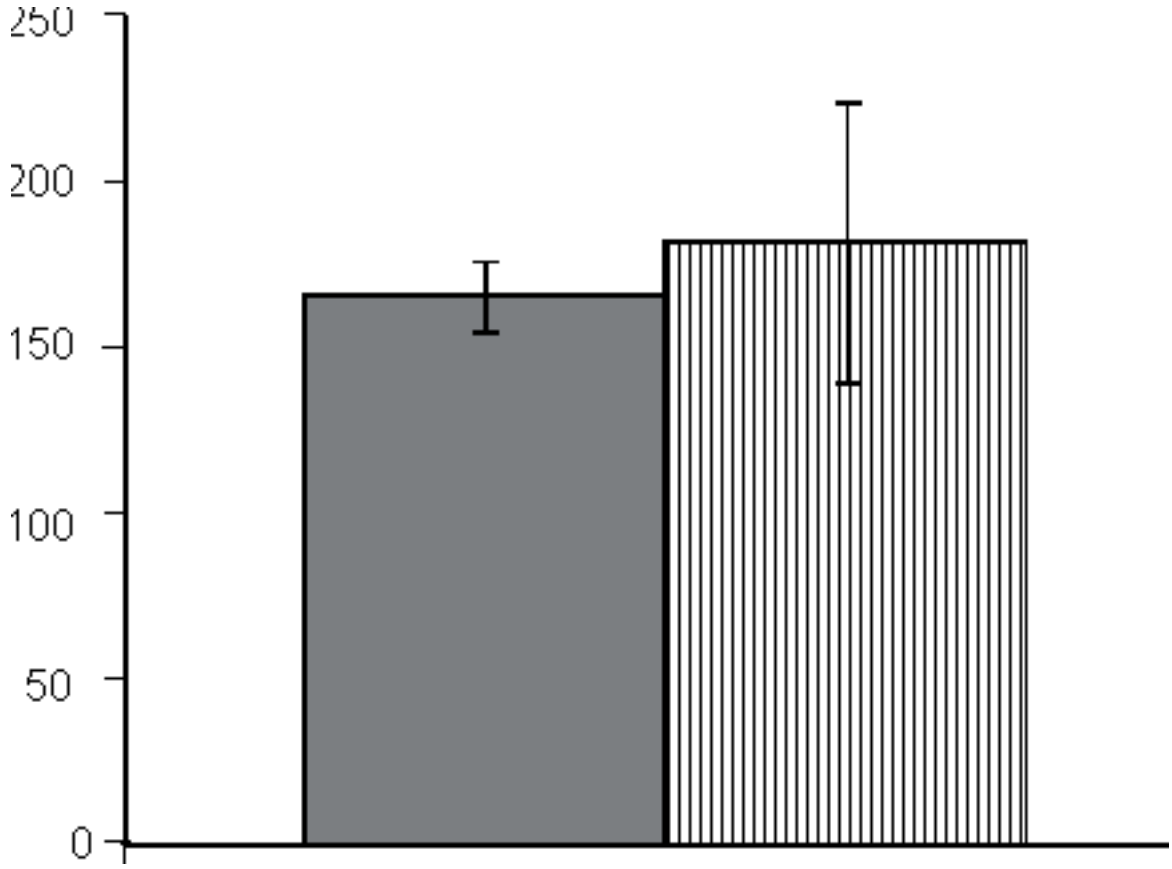

Fig. 5