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Xanthine oxidoreductase and xanthine oxidase in human cornea

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Summary. Xanthine oxidoreductase (xanthine dehydrogenase + xanthine oxidase) is a complex enzyme that catalyzes the oxidation of hypoxanthine to xanthine, subsequently producing uric acid. The enzyme complex exists in separate but interconvertible forms, xanthine dehydrogenase and xanthine oxidase, which generate reactive oxygen species (ROS), a well known causative factor in ischemia/reperfusion injury and also in some other pathological states and diseases. Because the enzymes had not been localized in human corneas until now, the aim of this study was to detect xanthine oxidoreductase and xanthine oxidase in the corneas of normal post-mortem human eyes using histochemical and immunohistochemical methods. Xanthine oxidoreductase activity was demonstrated by the tetrazolium salt reduction method and xanthine oxidase activity was detected by methods based on cerium ion capture of hydrogen peroxide. For immunohistochemical studies, we used rabbit antibovine xanthine oxidase antibody, rabbit antihuman xanthine oxidase antibody and monoclonal mouse antihuman xanthine oxidase/xanthine dehydrogenase/aldehyde oxidase antibody. The results show that the enzymes are present in the corneal epithelium and endothelium. The activity of xanthine oxidoreductase is higher than that of xanthine oxidase, as clearly seen in the epithelium. Further studies are necessary to elucidate the role of these enzymes in the diseased human cornea. Based on the findings obtained in this study (xanthine oxidoreductase/xanthine oxidase activities are present in normal human corneas), we hypothesize that during various pathological states, xanthine oxidase-generated ROS might be involved in oxidative eye injury.

Key words: Human cornea, Xanthine oxidoreductase in situ

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Introduction

Xanthine oxidoreductase exists in two functionally distinct forms. Under normal conditions, the larger part of the enzyme occurs as an NAD(+)-dependent dehydrogenase form that produces NADH and urate; the smaller part occurs as an oxygen-dependent oxidase form that produces oxygen radicals and /or hydrogen peroxide and urate (e.g. Frederiks and Bosch, 1995). Based on biochemical studies of Kooij (1994), it can be assumed that under physiological conditions, extracellular xanthine oxidoreductase is present exclusively in the xanthine oxidase form, whereas intracellularly, only 10-20% of xanthine oxidoreductase exists in the xanthine oxidase form and 80-90% in the xanthine dehydrogenase form.

The physiological role of xanthine oxidase is still rather unclear. Some authors have proposed a bactericidal function for the enzyme (Jarasch et al., 1981; Tubaro et al., 1980a,b; Van den Munckhof, 1996), whereas others ascribe an antioxidant function to the enzyme based on the production of urate (Becker, 1993; Kooij, 1994). Gossrau et al. (1990) investigated histochemically the activity of xanthine oxidase in the cutaneous epithelia and hypothesized that this enzyme, together with superoxide dismutase (an enzyme scavenging superoxide radicals and producing hydrogen peroxide during the dismutation reaction of a superoxide free radical), might play a protective role against bacteria in the skin. Xanthine oxidase was described in the rabbit cornea (Čejková and Lojda, 1996; Čejková et al., 2001) and superoxide dismutase in the rabbit (Bhuhyan and Bhuyan, 1978; Čejková et al., 1998, 2000) as well as human cornea (Behndig et al., 1998). As discussed previously (Čejková et al., 2001) both enzymes might have an antimicrobial role at the anterior eye surface - similar to that hypothesized by Gossrau et al. (1990) in the skin.

Under various (patho)physiological conditions, xanthine dehydrogenase can be transformed to xanthine oxidase. Among the many enzymatic systems that are capable of producing reactive oxygen species, xanthine

oxidase plays an important role. The involvement of this enzyme in ischemia-reperfusion injury has been extensively studied and discussed (e.g. Frederiks and Bosch, 1995; Saugstad, 1996; Nishino et al., 1997; Pritsos, 2000). In the eye, Kuriyama et al. (2001) proposed that oxygen free radicals might play an important role in retinal ischemic injury, particularly in the early phase. Čejková et al. (1998) hypothesized that the rabbit cornea might be injured by a very similar mechanism due to long lasting corneal hypoxia (after prolonged continuous soft contact lens wear) and reoxygenation of the cornea (after contact lens removal). The damaging effect of ROS-generating oxidases on the rabbit cornea was also demonstrated after the irradiation with UVB rays (Čejková et al., 2001) and after excimer laser keratectomy (Hayashi et al., 1997; Kasetsuwan et al., 1999). Besides experimental studies, the involvement of enzymatic systems that generate ROS in ocular pathologies is very well known. Marak et al. (1990) studied the role of ROS-generating xanthine oxidase in acute uveitis. Serry and Petrillo (1984) and Mittag (1984) discussed the role of ROS in ocular inflammation. Mendelsohn et al. (1998) measured levels of uric acid and xanthine in aqueous humor samples of patients with retinoblastoma, melanoma, Coat's disease, adult cataract, and congenital cataract. The results showed that high levels of uric acid and xanthine present in the aqueous humor of patients with malignancy were consistent with the destructive nature of these conditions.

To examine the possible participation of xanthine oxidase in a number of specific pathological ocular conditions, we decided to detect xanthine oxidase (and also xanthine oxidoreductase) in the human cornea, where until now these enzymes have not been localized. The technique using cerium ions was used for the demonstration of xanthine oxidase activity (Gossrau et al., 1989, 1990; Nakos and Gossrau, 1994), and the method utilizing tetrazolium ions for the localization of xanthine oxidoreductase activity (the dehydrogenase and oxidase forms of the enzyme) (Kooij et al., 1991; Frederiks and Bosch, 1995). Both methods were employed in our previous studies examining xanthine oxidoreductase and xanthine oxidase activity in the normal and experimentally injured rabbit cornea (Čejková and Lojda, 1996; Čejková et al., 1998, 2001). The histochemical studies were completed by immunohistochemical investigations. For this purpose we used rabbit antihuman xanthine oxidase antibody, rabbit antibovine xanthine oxidase antibody and monoclonal mouse antihuman xanthine oxidase/aldehyde oxidase AB-2 antibody.

Material and methods

The enzymes were detected in the corneas of post-mortem human eyes (20-50 years old), 4 to 16 hours after death. Twelve corneas were evaluated, each cornea both histochemically and immunohistochemically. The majority of corneas (8) (central corneal region) that were employed for our investigation were normal human corneas from fresh globes employed for corneolimbal allotransplantation. The remaining corneas (4) used for research purposes were corneas with decreased endothelial cell density. The corneas were excised and quenched in light petroleum chilled with an acetone-dry ice mixture. Sections were cut in a cryostat and transferred to glass slides.

Histochemical examination

Cryostat sections were fixed for 1 min in 0.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2 at 4 °C, and the sections were processed by cerium-DAB-CO-H_2O_2 methods for xanthine oxidase activity (substrate: hypoxanthine) as described by Gossrau et al. (1989, 1990) and Nakos and Gossrau (1994). Incubation was carried out in media consisting of 0.1M Tris-HCl buffer, pH 7.6, 0.1M NaN_3, 5 mM $CeCl_3$, and 5-10 mM substrate. Incubation varied from 15 to 30 min at 37 ºC. After rinsing the sections with several changes of distilled water, visualization of the reaction products was performed in a medium consisting of 0.05M acetate buffer, pH 5.5, 1.4 mM 3,3-diaminobenzidine, 0.1M NaN_3 and 40mM $CoCl_2$ at 37 °C for 25 min. After rinsing in tap water the sections were mounted in glycerol-gelatine.

Control reactions for xanthine oxidase were performed by incubation in either cerium-or substrate-free media. The specificity of the enzyme reactions was checked by using media to which 100 mM allopurinol was added as an inhibitor of xanthine oxidase (Gossrau et al., 1989, 1990).

Xanthine oxidoreductase activity was demonstrated on unfixed cryostat sections according to Kooij et al. (1991) with some minor modifications as described by Frederiks and Bosch (1995) using an incubation medium containing 18% (w/v) PVA, 100 mM phosphate buffer, pH 8.0, 0.5 mM hypoxanthine, 0.45 mM methoxy-phenazine methosulphate (mPMS) and 5 mM tetranitro BT. Control reactions were done in the absence of hypoxanthine. The specificity of the reaction was tested by the addition of allopurinol to the incubation medium.

Immunohistochemical examination

Immunohistochemistry was carried out on acetone-fixed cryostat sections. Antibodies used included rabbit antibovine xanthine oxidase (Chemicon, Temecula, CA, USA), rabbit antihuman xanthine oxidase (Biogenesis, Poole, UK) and monoclonal mouse antihuman xanthine oxidase/aldehyde oxidase AB-2 (LabVision, Fremont, CA, USA). The binding of primary antibodies was demonstrated by the UltraVision detection system (antimouse and antirabbit) (LabVision, Fremont, CA, USA) following the instructions of the manufacturer. The UltraVision Detection systems contain a biotinylated secondary antibody that reacts with the

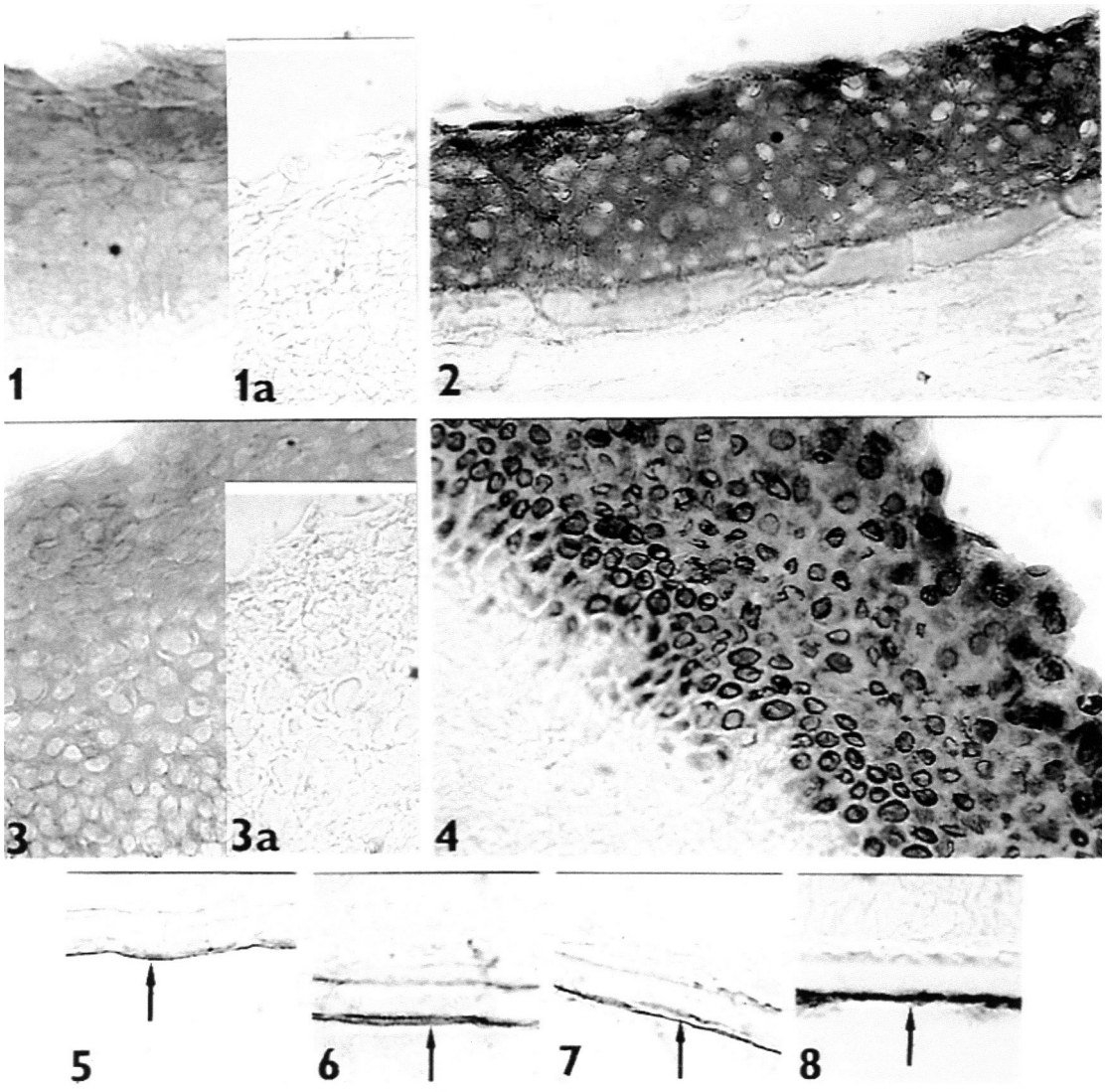
primary antibody, enzyme-labeled streptavidin, and substrate-chromogen. Sections in which the antibodies were omitted served as controls.

Chemicals

All unspecified chemicals were obtained from Aldrich (Steinheim, Germany), Boehringer (Mannheim, Germany), Sigma (Munich, Germany) or Serva (Heidelberg, Germany) and were all of analytical, high or the highest purity grade.

Results

Xanthine oxidase and xanthine oxidoreductase were present in the epithelium and endothelium of all corneas studied. The xanthine oxidase activity detected histochemically was present at lower activity levels in



Figs. 1, 2, 5 and 6. Histochemical demonstration of xanthine oxidase and xanthine oxidoreductase in the corneal epithelium and endothelium. The activity of xanthine oxidase in the epithelium **(Fig.1)** and endothelium (Fig.5) is lower when compared to xanthine oxidoreductase activity **(Fig.2)** – xanthine oxidoreductase in the epithelium, **Fig.6** – xanthine oxidoreductase in the endothelium). x 160

Figs. 3, 4, 7 and 8. Immunohistochemical demonstration of xanthine oxidase using rabbit anti-human xanthine oxidase antibody and immunohistochemistry of xanthine oxidoreductase/ aldehyde oxidase with mouse monoclonal antihuman xanthine oxidase/xanthine dehydrogenase/ aldehyde oxidase antibody. The xanthine oxidase positivity in the corneal epithelium **(Fig. 3)** and endothelium **(Fig. 7)** is less pronounced than that of xanthine oxidoreductase/aldehyd e oxidase **(Fig.4** – corneal epithelium, **Fig.8** – corneal endothelium). x 160

Fig.1a. Control section for the histochemistry of xanthine oxidase (sections were treated in the incubation media without substrate) did not show staining. x 160

Fig. 3a. Control section for the immunohistochemistry of xanthine oxidase (in the procedure the primary antibody was omitted) did not show staining. (No counterstaining). x 160. Comparing the microscopic pattern in Fig.2 with **Fig. 4,** in Fig.2 a diffuse cytoplasmic labeling is seen in the corneal epithelium, whereas in **Fig. 4a** perinuclear labeling pattern can be observed. It must be mentioned that the primary antibody used in Fig. 4 recognizes, besides xanthine oxidoreductase, also aldehyde oxidase. This is in contrast to Fig. 2, where only xanthine oxidoreductase is detected using the histochemical method.

the corneal epithelium (Fig. 1) and corneal endothelium (Fig. 5) as compared to the xanthine oxidoreductase activity (Fig. 2 - xanthine oxidoreductase activity in the corneal epithelium, Fig. 6 - xanthine oxidoreductase activity in the corneal endothelium). Very similar results were obtained using immunohistochemistry: The xanthine oxidase positivity was less pronounced in the corneal epithelium (Fig. 3) and endothelium (Fig. 7) than xanthine oxidoreductase (Fig.4 - corneal epithelium, Fig. 8 - corneal endothelium).

Comparing the histochemical and immunohistochemical patterns of xanthine oxidoreductase in the corneal epithelium, the histochemical demonstration showed diffuse cytoplasmic labeling (Fig. 2), whereas the immunohistochemical demonstration showed perinuclear labeling (Fig. 4). As discussed below, the primary antibody used for immunohistochemistry (monoclonal mouse antihuman xanthine oxidase/aldehyde oxidase antiobody AB-2, LabVision) localized, besides xanthine oxidoreductase, also aldehyde oxidase. This was in contrast to the histochemical studies in which only xanthine oxidoreductase was demonstrated.

The results obtained with both antibodies employed for xanthine oxidase localization were similar. In Fig. 3 and Fig.7 xanthine oxidase was detected with polyclonal rabbit antihuman xanthine oxidase antibody (Biogenesis).

Control sections for histochemistry treated in the incubation media without substrates or in the incubation media with substrates to which allopurinol was added, did not show staining. Also control sections for immunohistochemistry (the primary antibodies were omittted) did not show staining. (Fig.1a: control section for histochemistry of xanthine oxidase; Fig.3a: control section for immunohistochemistry of xanthine oxidase. No counterstaining).

Discussion

Xanthine oxidoreductase is involved in the degradation of adenosine triphosphate to urate, converting hypoxanthine via xanthine to uric acid. It exists in two different forms; an NAD^{+-} reducing form, xanthine dehydrogenase (xanthine: NAD^+, EC 1.1.1.204), and an oxygen-reducing form, xanthine oxidase (xanthine: O_2, EC 1.2.3.2), which generates reactive oxygen species (ROS) (superoxide anions and hydrogen peroxide). Xanthine oxidase has been established in many different tissues, particularly the heart and intestine, as an important source of ROS in ischemia-reperfusion injury (reviewed e.g. by Nishino et al., 1997; Pritsos, 2000). The presence of xanthine oxidase activity in the normal cornea was described by Čejková and Lojda (1996). Under experimental conditions, the role of xanthine oxidase was studied in the rabbit cornea repeatedly irradited with UVB rays (Čejková et al., 2001). ROS produced by xanthine oxidase contributed to the oxidative damage of irradiated corneas. Čejková et al. (1998) found that xanthine oxidase-generated ROS might be involved in the corneal damage related to prolonged contact lens wear, long-lasting corneal hypoxia and quick reoxygenation of the cornea after contact lens removal. It was suggested that xanthine oxidoreductase was released from the corneal epithelium into tears, where it converted to xanthine oxidase causing an additional damage to the cornea by ROS. Fox and van Kuijk (1998) detected xanthine oxidase in the retina of the normal human eye and suggested that this enzyme might be a source of oxidative damage in the retina following ischemia-reperfusion injury. The involvement of ROS in retinal ischemia-reperfusion injury was confirmed by Kuriyama et al. (2001). Cekic et al. (1999) studied xanthine oxidase acting as the source of ROS in the lenses of alloxan-induced diabetic and control rats. Its activity was increased, suggesting an increased oxidative stress to the lens. Hayashi et al. (1997) found after excimer laser therapy in rabbits, lipid peroxidation in the superficial corneal stroma from oxygen free radicals generated by the infiltrating polymorphonuclear cells. (Xanthine oxidase is present in polymorphonuclear cells; see Robinson et al., 1978; Tubaro et al., 1980b; Čejková et al., 2001 for details). Similar findings after excimer laser therapy were obtained by Kasetsuwan et al. (1999). Topical ascorbic acid application decreased oxygen radical damage of the cornea. Jain et al. (1995) described the beneficial effect of antioxidants (dimethyl sulfoxide and superoxide dismutase) on the minimizing free radical-mediated cellular injury after excimer keratectomy in rabbits.

The role of ROS in the eye has also been well documented by experiments using the (hypo)-xanthine/xanthine oxidase system applied on the cornea or injected into the anterior chamber. These studies showed that ROS generated by xanthine oxidase evoked oxidative injury to the cornea (e.g. Hull et al., 1984; Hayden et al., 1990; Yuen et al., 1994; Zeng et al., 1998) and lens (e.g. Varma et al., 1986; Varma and Devamanoharan, 1995; Varma and Morris, 1998).

In contrast to these experiments, until now, xanthine oxidoreductase and xanthine oxidase have not been studied in the human cornea. This is the first communication in this field. The histochemical and immunohistochemical results show that the enzymes are present in the corneal epithelium and endothelium. Xanthine oxidoreductase is more pronounced than xanthine oxidase, as clearly seen in the epithelium.

In the histochemical demonstration of xanthine oxidoreductase (the corneal epithelium), we observed a diffuse cytoplasmic pattern. Very similar pattern was also observed in the histochemical as well as immunohistochemical demonstration of xanthine oxidase. However, using monoclonal mouse antihuman xanthine oxidase/aldehyde oxidase AB-2 antibody (LabVision), we found a perinuclear labeling pattern. Because the antibody mentioned is specific against human xanthine oxidoreductase/aldehyde oxidase (tested

by the manufacturer) and perinuclear labeling is seen only using this antibody, we suggest that the difference is due to the aldehyde oxidase participation. (The AB-2 antibody recognizes aldehyde oxidase in addition to both forms of xanthine oxidoreductase). For xanthine oxidase and aldehyde oxidase, great similarities in biochemical properties, structure and localization have been described (Turner et al., 1995; Moriwaki et al., 1996, 1997; Terao et al., 2000); however, differences in activities, localization and also pathophysiological roles are also known (Beedham et al., 1987; Moriwaki et al., 1997, 1998, 2001).

In physiological conditions the amount of xanthine oxidase is low (only 10-20% of total xanthine oxidoreductase). (Although our present study is not quantitative, the results show that xanthine oxidase activity is less pronounced than xanthine oxidoreductase activity). However, during various diseases and pathological states (such as reperfusion after ischemia) xanthine dehydrogenase might be converted to ROS-generating xanthine oxidase. This has been described in various tissues (such as heart, intestine, kidney, liver, rabbit cornea) (for references see Kooij et al., 1994; Čejková et al., 2001, for review see e.g. Kooij, 1994; Saugstad, 1996; Pritsos, 2000).

In conclusion, previous studies revealed that xanthine oxidase-generated ROS might be involved in pathological states of various tissues, including the rabbit cornea. Halliwel (1991) pointed out that under normal conditions, antioxidants are balanced with the formation of ROS at a level at which these compounds can play their physiological roles without any toxic effects. However, under various pathological states an imbalance appears, which is an indication for oxidative tissue injury. One important factor leading to the oxidant/antioxidant imbalance is the higher amount of ROS.

Further studies are necessary to elucidate the possible participation of xanthine oxidase in corneal pathologies. Based on the results of this paper (xanthine oxidoreductase and xanthine oxidase activities are present in the corneas of normal human eyes), we hypothesize that xanthine oxidase might be involved in oxidative eye injury.

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