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Reactive oxygen species (ROS)-generating oxidases in the normal rabbit cornea and their involvement in the corneal damage evoked by UVB rays

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Summary. The corneas of albino rabbits were irradiated (5 min exposure once a day) with UVB rays (312 nm) for 4 days (shorter procedure) or 8 days (longer procedure). The eyes were examined microbiologically and only the corneas of sterile eyes or eyes with non-pathogenic microbes were employed. Histochemically, the activities of reactive oxygen species (ROS)-generating oxidases (xanthine oxidase, D-amino acid oxidase and α-hydroxy acid oxidase) were examined in cryostat sections of the whole corneas. Biochemically, the activity of xanthine oxidoreductase/xanthine oxidase was investigated in the scraped corneal epithelium. UVB rays significantly changed enzyme activities in the corneas. In comparison to the normal cornea, where of ROS-generating oxidases only xanthine oxidase showed significant activity in the corneal epithelium and endothelium, D-amino acid oxidase was very low and α-hydroxy acid oxidase could not be detected at all, in the cornea repeatedly irradiated with UVB rays, increased activities of xanthine oxidase and D-amino acid oxidase were observed in all corneal layers. Only after the longer procedure the xanthine oxidase and D-amino acid oxidase activities were decreased in the thinned epithelium in parallel with its morphological disturbances. Further results show that the xanthine oxidase/xanthine oxidoreductase ratio increased in the epithelium together with the repeated irradiation with UVB rays. This might suggest that xanthine dehydrogenase is converted to xanthine oxidase. However, in comparison to the normal corneal epithelium, the total amount of xanthine oxidoredutase was decreased in the irradiated epithelium. It is presumed that xanthine oxidoreductase might be released extracellularly (into tears) or the enzyme molecules were denatured due to UVB rays (particulary after the longer procedure). Comparative histochemical and biochemical findings suggest that reactive oxygen species-generating oxidases (xanthine oxidase, D-amino acid oxidase) contribute to the corneal damage evoked by UVB rays.

Key words: ROS-generating oxidases, cornea, UVB rays

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Introduction

The cornea is exposed to reactive oxygen species (ROS) generated from environmental pollutants, i.e. ozone, nitric oxide, sulphur dioxide, smoke (including cigarette smoke) (Chace et al., 1991). Recently, the health risk associated with ozone depletion will principally be those due to increased ultraviolet (UVB) radiation in the environment, i.e., increased damage to the eyes, the immune system and the skin. Chronic eye conditions likely to increase with the ozone depletion include cataract, squamous cell carcinoma, ocular melanoma, and a variety of corneal and conjunctival effects (reviewed by Longstreth et al., 1998). In the skin, exposure to UV radiation appears to be the most important environmental risk factor for basal and squamous cell carcinomas and cutaneous melanoma (Marks and Fürstenberger, 1985; de Gruijl, 2000). A 10% ozone reduction increases non-melanoma skin cancer by 26% and cataract (the loss of intraocular lens transparency) by 6-8% (Ambach and Blumthaler, 1993).

One of the causes of ocular as well as cutaneous damage induced by UV irradiation is the generation of reactive oxygen species (ROS) (Riley, 1988). ROS (superoxide and hydroxyl radicals, hydrogen peroxide, singlet oxygen) are danger to biological systems. ROS might cause cellular damage by reacting with lipids, proteins and DNA (Chace et al., 1991; Kehrer, 1993). Many pathologies have been attributed to the action of

ROS and one of the dominant theory of aging contends that senescent changes are a consequence of the accumulated action of ROS (Emerit, 1992). According to Ogura et al. (1991) ROS are important mediators of lipid peroxidation in the epidermis exposed to UV light. ROS are suggested to play a major role in a number of specific pathological conditions of intraocular tissues, such as cataract formation and retinal degeneration (Mittag, 1984).

Normally, the cornea contains low molecular weight antioxidants (such as ascorbic acid, glutathione and alpha-tocopherol) as well as high molecular weight antioxidants (such as catalase, superoxide dismutase, glutathione peroxidase and reductase) protecting the cornea against the oxidative damage (e.g. Bhuyan and Bhuyan, 1977, 1978; Redmond et al., 1984; Atalla et al., 1987, 1988, 1990; Čejková and Lojda 1994, 1996; Behndig et al., 1998). Corneal epithelium has the physiological capacity to absorb UV rays (below 310 nm wavelength), it acts as a UVB-filter. Aqueous humor, containing ascorbic acid, the proteins and some aminoacids (tyrosine, phenylalanine, cysteine, tryptophane) is also responsible for UVB absorption so that only small amount of this radiation reaches the intraocular lens (Ringvold, 1998). The lens acts as filter of light between 300-400 nm from reaching the retina (Eaton, 1994-95). Experimental studies show that UVB rays (and ROS generated by them) cause morphologic disturbances in the cornea, particularly in the epithelium. A single dose of irradiation of the rat corneal epithelium blocked the cell proliferation (Haaskjold et al., 1993). Higher doses of UVB rays resulted in a considerable reduction of epithelial thickness (Koliopoulos and Margaritis, 1979). Cullen (1980) (using slit-lamp biomicroscopy, light and electron microscopy) described a loss of superficial corneal epithelial layers and selective UV induced autolysis of the wing cells. Čejková and Lojda (1996) found that irradiation of the rabbit cornea with UVB rays led to the decrease of Na^+-K^+-dependent adenosintriphosphatase first in the corneal epithelium and later in the corneal endothelium which was accompanied by the increased corneal hydration and changes of corneal transparency. The Na^+-K^+-dependent adenosintriphosphatase is the major agent of transcorneal sodium and chloride transport, and, hence, a major control of corneal hydration (Redmond et al., 1984).

For the maintenance of normal corneal hydration and transparency the corneal endothelium plays the key role. Under physiological conditions, the corneal endothelium is exposed to the oxidants of the aqueous humor. Hydrogen peroxide is present in the normal rabbit aqueous humor at a concentration of 20 mM (Ringvold, 1980). The corneal endothelium containing antioxidants (with the most importance glutathion and catalase) (Bhuyan and Bhuyan, 1984; Redmond et al., 1984) is able to adequately defend against a physiological level of hydrogen peroxide; however, it is very susceptible to the damage from increased level of hydrogen peroxide (Riley and Giblin, 1983; Riley et al., 1987; Hull and Green, 1989; Ren and Wilson, 1994).

The danger of ROS to the corneal epithelium and endothelium was well documented with experiments using xanthine oxidase/(hypo)xanthine system generating ROS. Yuen et al. (1994) described the damage of cultured rabbit corneal epithelial cells by ROS. Several antioxidants had the cytoprotective effect against oxidative injury to rabbit corneal epithelial cells. Hayden et al. (1990) observed that ROS lyse epithelial cells of the human and rat cornea. Partial protection from lysis was afforded by superoxide dismutase and complete protection by catalase. Hull et al. (1984) described the damage to the corneal endothelium by ROS which caused the anatomic disruption of endothelial cells with the interference in the function of endothelial water movement and resultant swelling of the corneal stroma. Hull and Green (1989) examined the effect of antioxidant enzymes on the endotelial damage evoked by ROS which could be blocked with catalase removing hydrogen peroxide from the system (not with superoxide dismutase which removes superoxide from the system). According to these authors this indicates that hydrogen peroxide is more toxic to the endothelium than superoxide anion. Zeng et al. (1998) found that the corneal endothelial cell cultures prepared from New Zealand white rabbits were damaged by ROS. Morine hydrate highly reduced this damage. Zeng et al. (1995) prevented the cultured rabbit corneal endothelial cell damage from ROS by low-molecular weight antioxidants (Vitamin E, alpha-tocopherol and ascorbate). Rootman et al. (1994) with their criteria for cell necrosis (cytoplasmic shrinkage, dissolution of plasma membranes and presence of "haloes" around the cells on phase contrast microscopy, confirmed by transmission electron microscopy) found that purogallin may be useful in preventing endothelial cell damage preserved for corneal transplantation.

The xanthine oxidase/(hypo)xanthine system for ROS generation was employed also in a row of experiments with the intraocular lenses with the aim to better understand the damage of the lens by ROS as well as to elucidate whether these disturbances might be reduced by antioxidants. Varma and Devamanoharan (1995) and Varma and Morris (1998) reduced the lens damage with pyruvate; Varma et al. (1986) by ascorbic acid. Hull et al. (1985) demonstrated that ROS increased iris vascular permeability and also blood-aqueous barrier permeability.

Results of above mentioned papers clearly show that UVB rays have the damaging effect on corneal structure and function. The experiments with the xanthine oxidase/(hypo)xanthine system show that ROS generated by xanthine oxidase are danger to individual corneal layers. In contrast to these information, little is known about oxidases-generating ROS in situ. Such enzymes were described in the epithelia of intestine and skin by Gossrau et al. (1990). In the cornea, only xanthine oxidase (and xanthine oxidoreductase) were

described (Čejková and Lojda, 1996; Čejková et al., 1998). Until now, other oxidases-producing ROS (such as D-amino acid oxidase, α-hydro acid oxidase) have not been studied. Therefore, in this study, we decided to fill this gap. Activities of xanthine oxidase, D-amino acid oxidase and α-hydroacid oxidase were examined histochemically both in the normal rabbit cornea and in the cornea irradiated with UVB rays. With the aim to better understand the role of xanthine oxidase in the irradiated epithelium, we determined this enzyme also biochemically in the scraped corneal epithelium.

To study the xanthine oxidase and some other oxidases-generating ROS in the cornea irradiated with UVB rays seemed to us to be very important because these enzymes generating ROS (when activated) might contribute to the development of oxidant/antioxidant imbalance in the cornea. We found previously that UVB rays decreased activities of antioxidant enzymes (catalase and glutathione peroxidase) in the corneal epithelium (Čejková et al., 2000). The relationship between depletion of antioxidants and cataract formation was well documented (Spector and Garner, 1981; Giblin et al., 1982; Fecondo and Augusteyn, 1983; Ohrloff and Hockwin, 1984; Babu et al., 1995). The existence of ROS and the damage they can do to living systems has been known for some time (e.g. Pryor, 1970) but only recently it has been recognized that they would exist in great toxic profusion if not continuosly counteracted and removed by specific defense mechanisms (Riley, 1988; Kelly, 1993).

Materials and methods

Adult New Zealand white rabbits (body weight 2.5-3.0 kg) were used in our experiments. The investigation was conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were anesthetized by i.m. injection of Rometar (Xylazinum hydrochloricum, 2%, Spofa, 0.2ml/1kg body weight) and Narkamon (Ketaminum hydrochloricum, 5%, Spofa, 1ml/1kg body weight). The open eyes were irradiated with a UVB lamp (Illkirch Cedex, France; 312 nm wavelength, 6W) from a distance of 0.03m. Only corneas were irradiated; the rest of the eye surface was protected from UV rays. The animals were irradiated for 5 minutes with UVB rays once daily, for 4 days (shorter procedure) or 8 days (longer procedure). Every day a microbial examination was performed on all eyes, and only sterile eyes or eyes with non-pathogenic microbes were used. After finishing the experiments, the animals were sacrificed under thiopental anesthesia. For the histochemical examination, each group contained 12 corneas. For the biochemical examination, scraped corneal epithelium was employed (the corneal epithelium was carefully scraped off - limbus to limbus - with a Graefe´s knife). For the biochemical as well as histochemical investigations, normal corneas served as controls.

Histochemical examination

The anterior eye segments were dissected out and quenched in light petroleum chilled with an acetone-dry ice mixture. Sections were cut in a cryostat and transferred to glass slides. 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 (substrate: hypoxanthine), D-amino acid oxidase (substrate: D-proline) and α-hydroxy acid oxidase (substrate: L-mandelic acid) 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 °C. 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 ROS-producing oxidases were performed by incubation in either cerium- or substrate-free media or complete media free of oxygen (by bubbling with nitrogen for 10 min). The specificity of the enzyme reactions was investigated by using media to which 1-100 mM allopurinol, Kojic acid and 2-ketobutyrate were added as inhibitors of xanthine oxidase, D-amino acid oxidase and α-hydroxy acid oxidase, respectively (Gossrau et al., 1990; Robinson et al., 1990).

Morphological disturbances of corneas were examined by hematoxylin-eosin staining.

Biochemical investigation

Total xanthine oxidoreductase (D and O forms together) and xanthine oxidase (O-form) were measured spectrofluorometrically (Perkin Elmer LS-50B fluorometer, excitation at 340 nm, emission at 390 nm) in the same sample using the method of Beckman et al. (1989) modified by Hermes-Lima and Storey (1995) based on the xanthine oxidoreductase-catalyzed conversion of pterin to isoxanthopterine.

Samples of the epithelium were obtained by pooling epithelial cells from 3-4 corneas (after the irradiation with UVB rays) and 1-2 corneas (controls). The epithelium homogenate was centrifuged at 5 °C and 25000 g for 25 minutes. 100 ml of supernatant was added to 2 ml of 0.1 mM potassium phosphate (pH 7.6) buffer containing 0.5 mM EDTA at 25 °C. The reaction was started by 20 μl of 2 mM pterine, and the xanthine oxidase activity was recorded. After 3 minutes, 20 μl of 1 mM methylene blue was added as the ultimate electron receiver, and total xanthine oxidoreductase activity was recorded for the next 3 min. The reaction was stopped by 50 μl of 4 mM allopurinol. To calibrate the assay, 20 μl

of 0.02 mM isoxanthopterine was added, and the increase of fluorescence was recorded. Perkin Elmer software and MS Excel were used for kinetics evaluations. In a control experiment, several crystals of phenylmethylsulfonyl fluoride were added to a stock of water to test the proteolytic conversion of xanthine oxidoreductase during sample preparation, but we did not observe any effect of this protease inhibitor on the results under the described conditions.

Chemicals

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

Results

Histochemical examination

The specifity of the oxidase reactions (histochemical procedures) was proven by control experiments. In cryostat sections the xanthine oxidase activity was inhibited only by allopurinol and not by other inhibitors tested. Similar results were obtained with appropriate inhibitors for the inhibition of D-amino acid oxidase and α-hydroxy acid oxidase. The lack of a final reaction product after incubation in the presence of catalase or in the absence of cerium ions indicated the production of H_2O_2.

Normal cornea (cryostat sections of the whole corneas)

In the central parts of the normal cornea, the activity of xanthine oxidase was present in all layers of the corneal epithelium (Fig. 1c, E-epithelium). In the peripheral region of the corneal epithelium, the xanthine oxidase activity was less pronounced in basal cell layers. In the corneal endothelium, xanthine oxidase activity was present and did not reveal regional differences (Fig. 1i, arrow points to the endothelium). The actitivity of D-amino acid oxidase was low in all layers of the corneal epithelium (without any differences between the central and peripheral parts of the cornea) (Fig. 1d, E-epithelium), and some activity was present also in the endothelium (Fig. 1j, arrow points to the endothelium); α-hydroxy acid oxidase activity was absent both in the corneal epithelium and endothelium.

Shorter irradiation procedure with UVB rays (cryostat sections of the whole corneas)

In the corneal epithelium the activity of xanthine oxidase was high (Fig. 1a, E-epithelium). D-amino acid oxidase activity appeared higher in the superficial layers of the corneal epithelium (Fig. 1b, thick arrows point to the increased activity in the epithelium, E). Individual inflammatory cells (mainly polymorphonuclear leukocytes) were present in the corneal stroma; they were active for xanthine oxidase (Fig. 1a, arrows) and D-amino acid oxidase (Fig. 1b, thin arrows). Activities of xanthine oxidase (Fig. 1f) and D-amino acid oxidase (Fig. 1g) were more pronounced in the corneal endothelium (arrows) (compare with enzyme activities in the normal corneal endothelium, Fig. 1i,j, arrows point to the endothelium).

Longer irradiation procedure with UVB rays (cryostat sections of the whole corneas)

In eyes repeatedly irradiated with UVB rays (for 8 days), decreased xanthine oxidase and D-amino acid oxidase (Fig. 1e) activities were found in the damaged corneal epithelium (E). (The damage to the corneal epithelium was revealed by hematoxylin-eosin staining). The epithelium became thinner; the anterior portion of the corneal epithelium was lost. The number of enzymatically active inflammatory cells - D-amino acid oxidase (Fig. 1e, arrows point to inflammatory cells), xanthine oxidase - was increased in the corneal stroma (as compared to the shorter irradiation procedure, Fig. 1b); the activities of xanthine oxidase and D-amino acid oxidase in the corneal endothelium (arrows) were more pronounced (Fig. 1h,ch; arrows point to the endothelium). Moreover, some enzymatically active inflammatory cells present in the anterior chamber were attracted to the endothelium. (Compare with the shorter procedure, Fig. 1f,g).

Biochemical investigation (scraped corneal epithelium)

UVB rays (shorter procedure) significantly inhibited xanthine oxidoreductase. After the longer procedure with UVB rays also the xanthine oxidase activity was significantly decreased in the scraped corneal epithelium (ANOVA, $p>0.01$). UVB irradiation induced an increase in the xanthine oxidase/xanthine oxidoreductase ratio (Fig. 2).

Discussion

Which functions can be attributed to the described ROS-generating oxidase activities in the normal and experimentally injured cornea? Until now, details have not been precisely elucidated. The oxidase activities have not been systematically studied in the cornea. This is the first investigation in this field.

D-amino acid oxidase (D-amino acid: O_2 oxidoreductase, EC 1.4.3.3) has been localized in cells of various organs at varying levels of activity (van den Munckohof, 1996). D'Aniello et al. (1993) hypothetized that the main function of this enzyme would be the degradation of D-amino acids from exogenous sources, such as food or bacteria, and detoxification of endogenous D-amino acids that may be accumulated during aging (Robinson et al., 1990).

Xanthine oxidoreductase is involved in the degradation of adenosintriphosphate to urate by converting hypoxanthine via xanthine into uric acid. This enzyme exists in two forms: Xanthine oxidase (xanthine: O_2, EC 1.2.3.2), an oxygen-reducing form generating superoxide anions and hydrogen peroxide, and xanthine dehydrogenase (xanthine: NAD^+, EC 1.1.1.204), an NAD-reducing form. Kooij et al. (1992)

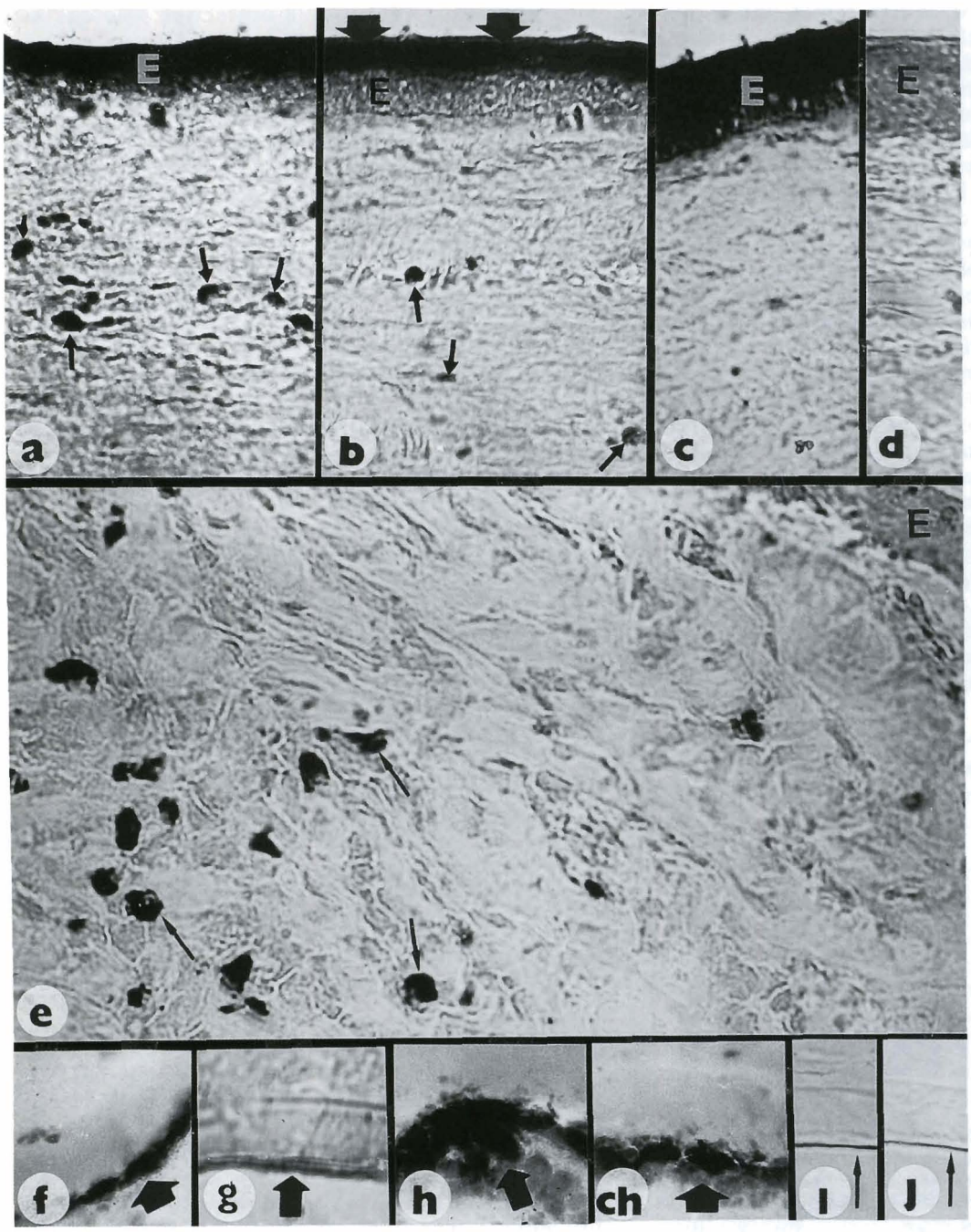

Fig. 1. Histochemical identification of xanthine oxidase and D-amino acid oxidase in the central region of the normal cornea and the cornea irradiated with the shorter or longer UVB ray procedure (a-j). **a.** Xanthine oxidase. The activity is high in the corneal epithelium (E) of the cornea irradiated with UVB rays (shorter procedure). Some inflammatory cells (arrows) present in the corneal stroma are also active for xanthine oxidase. **b.** D-amino acid oxidase activity. In the cornea irradiated with the shorter procedure, the activity is increased in the superficial layers of the epithelium (thick arrows) E: epithelium. Thin arrows point to the inflammatory cells that are active for D-amino acid oxidase. **c.** Xanthine oxidase. In the normal cornea the activity is highly pronounced in the epithelium (E). **d.** D-amino acid oxidase. The enzyme activity is very low in the epithelium (E) of the normal cornea. **e.** D-amino acid oxidase. In the cornea irradiated with UVB rays (longer procedure) the activity is greatly decreased in the thinned corneal epithelium (E) as compared with the shorter procedure (b). **f.** Xanthine oxidase. After irradiation with UVB rays (shorter procedure) the enzyme activity is increased in the endothelium (arrow). Compare with the normal corneal endothelium (i) where the activity is much less pronounced. **g.** D-amino acid oxidase. In the cornea irradiated with UVB rays (shorter procedure), the enzyme activity is increased in the endothelium (arrow) as compared to the normal corneal endothelium (j). **h.** Xanthine oxidase. After the longer procedure with UVB rays the xanthine oxidase activity is much more pronounced in the corneal endothelium (arrow). Some inflammatory cells present in the anterior chamber are also active and attracted to the endothelium. Compare with the shorter procedure (f). **ch.** D-amino acid oxidase. After the longer procedure with UVB rays the enzyme activity is more pronounced in the endothelium (arrow) as compared to the shorter procedure (g). **i.** Xanthine oxidase. In the normal corneal endothelium (arrow) the enzyme is present with significant activity. **j.** D-amino acid oxidase. Some enzyme activity is present in the normal corneal endothelium (arrow). a-d, x 140; b-j, x 160

and Frederiks and Bosch (1996) suggested that the main function of this enzyme is not production of superoxide radicals and/or hydrogen peroxide but rather the metabolism of xanthine to uric acid, which can act as a potent antioxidant. This presupposition is based on the finding of enzyme activity in epithelial as well as endothelial cells of various organs that are subject to relatively high oxidant stress; therefore, the authors postulate that in these cells xanthine oxidoreductase is involved in the antioxidant enzyme defense system. Under various (patho)physiological conditions, xanthine dehydrogenase can be converted into xanthine oxidase (e.g. Granger et al., 1986) reversibly via oxidation of sulphydryl groups and irreversibely by proteolysis (Kooij et al., 1992). Kooij et al. (1994) found biochemically that conversion of xanthine dehydrogenase into xanthine oxidase occurs exclusively in vitro or extracellularly and that in vivo the amount of xanthine oxidase is only 10-20% of total xanthine oxidoreductase. In this study (scraped corneal epithelium) we found approximately 48% of xanthine oxidoreductase in the oxygen reducing form; however, it is not presumed that this ratio exists in vivo. We cannot exclude enzyme conversion ex vivo during preparation of the organ and tissue extract despite the laboratory conditions used for the inhibition of xanthine oxidoreductase conversion to xanthine oxidase by atmospheric oxygen. The difficulties with the biochemical determination of enzyme activities, including the relevance of the in vivo fixation of the xanthine oxidase/xanthine dehydrogenase ratio, is discussed by Haberland et al. (1989).

The physiological role of xanthine oxidase is still rather unclear (Kooij et al., 1994), some authors have proposed a bactericidal function for the enzyme (Tubaro et al., 1980a,b; Jarasch et al., 1981; Van den Munckhof, 1996; Gossrau et al., 1990). Gossrau et al. (1990) described 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 as well as superoxide dismutase are present in the corneal epithelium (Bhuyan and Bhuyan, 1978; Behndig et al., 1998; Čejková et al., 1998, 2000). Thus, under physiological conditions, both enzymes might have an antimicrobial role at the anterior eye surface - similarly to that hypothesized by Gossrau et al. (1990) in the skin.

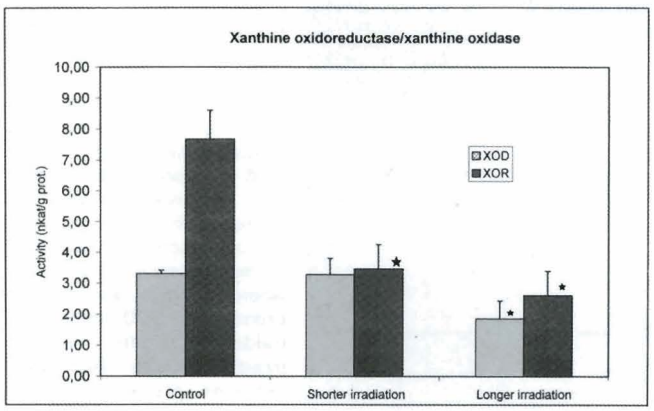

Fig. 2. Biochemical evaluation of the xanthine oxidoreductase/xanthine oxidase activities (scraped corneal epithelium) after the shorter and longer irradiation procedures with UVB rays. The xanthine oxidoreductase activity is significantly decreased already after the shorter irradiation procedure. The longer irradiation procedure causes the more profound decrease of the xanthine oxidoreductase activity and evokes the significant decrease of the xanthine oxidase activity. The xanthine oxidase/xanthine oxidoreductase ratio is increased in the corneal epithelium irradiated with UVB rays. *: $p<0.01$ (Bonferroni multiple comparison test - ANOVA); mean±SD of 8 samples.

On the other hand, according to Behndig et al. (1998) and Čejková et al. (2000), the high levels of superoxide dismutase in the anterior eye surface tissues and fluids might be related to the risk of photochemical production of superoxide in this location – tears (Crouch et al., 1988; Behndig et al., 1998), corneal epithelium, stroma, corneal endothelium (Behndig et al., 1998); conjunctiva (Modis et al., 1998). Superoxide dismutase catalyzes the superoxide radical dismutation to hydrogen peroxide that is cleaved under physiological conditions by other antioxidant enzymes, mainly catalase and glutathione peroxidase.

Under pathological conditions, the role of xanthine oxidase in hypoxia-reoxygenation injury has often been proposed (e.g. Kooij et al., 1990, 1994) and critically reviewed (e.g. Saugstad, 1996). Kooij et al. (1994) described in liver that the conversion of xanthine dehydrogenase into xanthine oxidase might occur exclusively in vitro or extracellularly. In contrast, Cote et al. (1996) found in rat lung that exposure to hypoxia produced a significant increase in lung tissue xanthine oxidase activity and an increase in the ratio of xanthine oxidase to xanthine dehydrogenase.

In this connection, the role of xanthine oxidase in the additional corneal damage evoked by prolonged wearing of hydrophilic contact lenses, long-lasting corneal hypoxia and quick reoxygenation of the cornea after contact lens removal was described by Čejková et al. (1998). It was suggested that xanthine oxidoreductase was released from the corneal epithelium into tears, converted to xanthine oxidase and caused additional damage to the superficial layers of the corneal epithelium by ROS.

The damaging effect of oxidases-generating ROS to the cornea is suggested also in this paper in relation to the repeated irradiation of the cornea with UVB rays. UVB rays evoke a gradual increase of xanthine oxidase and D-amino acid oxidase activities in all corneal layers (in the corneal epithelium only after the shorter procedure). The increase of D-amino acid oxidase activity in the epithelium shortly irradiated with UVB rays is located in superficial layers. This is very well seen because the activity of this enzyme is low in the normal corneal epithelium. In contrast, xanthine oxidase

activity is high already in the normal corneal epithelium and therefore the increase of activity cannot be precisely evaluated. After the longer irradiation procedure xanthine oxidase and D-amino acid oxidase are decreased in the thinned corneal epithelium which goes parallel with its morphological disturbances. Mainly D-amino acid oxidase reveals the profound decrease in activity because the anterior portion of the corneal epithelium is lost (area where the enzyme activity was increased). The reduction of the epithelial thickness after the prolonged repeated irradiation of the cornea with UVB rays due to the loss of superficial epithelial layers was described also by Koliopoulos and Margaritis (1979) in the ultrastructural study, by Cullen (1980) using slit-lamp biomicroscopy, light and electron microscopy and by Ringvold (1983) in scanning microscopic study. Cullen (1980) described that the biomicroscopically observed granules in the epithelium were the clinical manifestation of the secondary lysosomes revealed by light and electron microscopy. According to this author UV breaks down primary lysosome membranes to release hydrolytic enzymes which in turn results in destruction of the corneal epithelium. In this connection, it is necessary to mention that after UVB irradiation of the rabbit cornea, increased activities of lysosomal hydrolases were detected histochemically in the anterior portion of the corneal epithelium that correlated with increased levels of enzymes in tears (Čejková, 1998, 1999).

Wickert et al. (1999) determined the type of cell death (apoptosis versus necrosis) after UVB rays in the corneal and lens epithelium of the rat. Regarding the type of cell death, after UVB exposure, morphological signs of apoptosis and TUNEL positive cells were visible in the epithelium of rat cornea. UVB irradiated lens epithelial cells exhibited features typical of necrosis. Opacificication of the lens appeared to follow the death of lens epithelial cells.

The biochemical results of this paper show that the xanthine oxidase proportion of total xanthine oxidoreductase increases with repeated irradiation with UVB rays. These results might indicate the conversion of xanthine dehydrogenase to xanthine oxidase. Very similar results were obtained by Srivastava and Kale (1999) in the liver after irradiation of albino mice with gamma rays. Because the ratio of the activity of xanthine dehydrogenase to that of xanthine oxidase decreased with the irradiation procedure, these authors suggested that xanthine dehydrogenase might be converted into xanthine oxidase. However, in the above-mentioned study the total activity of xanthine oxidoreductase (xanthine dehydrogenase + xanthine oxidase) remained constant at all radiation doses. In contrast, we found a significant decrease of xanthine oxidoreductase in the scraped corneal epithelium after the irradiation procedures with UVB rays. The release of xanthine oxidoreductase from the corneal epithelium extracellularly into tears is presumed. However, we suggest that after the longer irradiation procedure some enzyme molecules are denaturated.

Further results show that enzymatically active inflammatory cells (for xanthine oxidase, D-amino acid oxidase) were present in the corneal stroma and their amount was increasing along the irradiation. The relationship among xanthine - generated ROS, neutrophil infiltration and tissue damage in the intestine was demonstrated by Grisham et al. (1986). The localization of D-amino acid oxidase on the cell surface of human leukocytes was described by Robinson et al. (1978) and the presence of xanthine oxidase in mouse leukocytes by Tubaro et al. (1980b). The findings of ROS-generating oxidases on the cell surface of leukocytes supported the concept that the plasma membrane is involved in peroxide formation. Tubaro et al. (1976, 1980a-c) found increased xanthine oxidase activities in the liver and polymorphonuclear leukocytes of mice after various pathological conditions, mainly during infectious processes. The authors suggest that in polymorpho-nuclear leukocytes, xanthine oxidase plays a role in the killing mechanism. Because in our studies only sterile eyes or eyes with nonpathogenic microbes were used, we cannot discuss the influence of infection on the xanthine oxidase activity in leukocytes. However, as documented in this study, following UVB irradiation inflammatory cells were present in the corneal stroma and these cells bore activities of xanthine oxidase and D-amino acid oxidase. It is suggested that the presence of polymorphonuclear leukocytes in the cornea irradiated with UVB rays is due to UVB-induced inflammation, similarly to that described by Hawk et al. (1988) in the skin irradiated with UVB rays. Moreover, Riley and Elgebaly (1990) described the release of a chemotactic factor following UVB irradiation in experiments with isolated rabbit corneas, providing a mechanism for the recruitment of neutrophils.

Comparing our results in the cornea with results of some other authors in the skin it follows that there are great similarities in relation to UVB rays, ROS generated by them and tissue damage – in spite of well known differences between the corneal and skin structure (and function). The cornea is transparent and avascular; the enzymes involved in ROS generation or cleavage are located mainly in the epithelium and endothelium (less in the corneal stroma). In the skin various cell types participate in oxidant/antioxidant mechanisms, including fibroblasts, keratinocytes and vascular endothelium (e.g. Shindo et al., 1991; Kooij et al., 1992; Deliconstantinos et al., 1996; Sasaki et al., 1997; Masaki et al., 1998); melanocytes have an important role for the skin pigmentation and photoprotection (Cario-Andre et al., 1999).

The cornea and the skin both are directly open to environmental noxes, radiation and ROS generated by them (reviewed by Longstreth et al., 1998). They contain low molecular weigt antioxidants (such as ascorbic acid, glutathione and alpha-tocopherol) as well as high molecular weight antioxidants (such as catalase, superoxide dismutase and the enzymes of the glutathione

redox cycle – glutathione peroxidase and reductase) that protect tissues against the oxidative injury. (References for corneal antioxidants see above, references for the skin antioxidants, e.g. Shindo et al., 1993, 1994; Tyrrel, 1995). In the skin antioxidants are located both in the epidermis and dermis, however, the amount of them is greater in epidermis (Shindo et al., 1993).

Ocular or cutanous disturbances appear when antioxidants are reduced. There is evidence to suggest that antioxidant enzymes (glutathione peroxidase, superoxide dismutase) decrease with the increasing degree of cataract (Spector and Garner, 1981; Giblin et al., 1982; Fecondo and Augusteyn, 1983; Ohrloff and Hockwin, 1984). Babu et al. (1995) described that UVB rays (and ROS generated by them) decrease antioxidant-defence system in aqueous humor and lens followed by the increase in H_2O_2 leading to the formation of cataract. Čejková and Lojda (1996) and Čejková et al. (2000) found that UVB rays gradually decrease catalase and glutathione peroxidase (less superoxide dismutase) in the corneal epithelium and endothelium. The depletion of antioxidants went parallel with the decrease of Na^+-K^+-dependent adenosintriphosphatase a γ-glutamyl transferase - enzymes associated with the transport of ions of water and metabolites through the cornea (reviewed by Čejková, 1998). Swelling of the cornea appeared. In this connection, Hightower and McCready (1992) described that irradiation of the intraocular lens (anterior irradiation of the cultured lenses) resulted in reduced Na^+-K^+-dependent adenosintriphosphatase activity in the lens epithelium. In the skin, Hasegawa et al. (1992) and Iizawa et al. (1994) described that UVB rays caused the decrease in catalase, superoxide dismutase and glutathione peroxidase which went along with the increased lipid peroxidation. Podda et al. (1998) found that the higher doses of UVB rays caused the decrease in antioxidants (ascorbate, alpha-tocopherol, ubiquinol) associated with the detectable oxidative damage. Pence and Naylor (1990) found that already a single exposure to UVB irradiation in the skin of hairless mouse leads to significant decreases of skin antioxidants (superoxide dismutase and catalase). Shindo et al. (1993) examined that UVB rays caused the reduction in antioxidants (mainly catalase and ascorbate) in the skin, however, the reduction was much more severe in epidermis than in dermis.

In contrast to the decrease of antioxidants, including antioxidant enzymes, pro-oxidative enzymes (such as xanthine oxidase, D-amino acid oxidase) are increased in the cornea irradiated with UVB rays. This was shown in this paper. In the skin irradiated with UVB rays, the activation of xanthine oxidase was also observed, however, it was dose dependent. No induction of xanthine dehydrogenase or xanthine oxidase were evident in the skin of mouse after the single UVB irradion (Pence and Naylor, 1990) although significant hyperplasia was evident. However, as demonstrated by Deliconsttantinos et al. (1996), after longer irradiation of the skin with UVB rays xanthine oxidase and NO synthase of human keratinocytes were activated to release NO, superoxide and peroxynitrite which might be involved in the pathogenesis of sunburn erythema and inflammation. According to these authors UVB rays act as a potent stimulator of NO synthase and xanthine oxidase themselves, as well as in their neighboring endothelial and smooth muscle cells. The xanthine oxidase and NO synthase activation due to UVB rays might be a major part of the integrated response leading to erythema production and the inflammatory response.

We can conclude that under normal conditions, antioxidants in the cornea (and also other tissues, including the skin) are balanced with the formation of ROS at the level at which these compounds can play their physiological role without any toxic effects (Halliwel, 1991). Under the influence of UVB rays, an imbalance appears. ROS generated in tissues by UVB rays and from other sources, such as cellular infiltrations or oxidase reaction (xanthine oxidase, D-amino acid oxidase) evoke the decrease in antioxidants. This is an indication to oxidative injury.

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