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Irradiation of the rabbit cornea with UVB rays stimulates the expression of nitric oxide synthases-generated nitric oxide and the formation of cytotoxic nitrogen-related oxidants

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Summary. Until now, the role of nitric oxide (NO) in cornea irradiated with UVB rays remains unknown. Therefore, we investigated nitric oxide synthase isomers (NOS), enzymes that generate NO, nitrotyrosine (NT), a cytotoxic byproduct of NO, and malondialdehyde (MDA), a byproduct of lipid peroxidation, in rabbit corneas repeatedly irradiated with UVB rays (312 nm, 1x daily for 6 days, the dose per day 1.01 J/cm^2) using immunohistochemical methods. The biochemical measurement of nitrite and nitrate has been used for the indirect investigation of NO concentration in the aqueous humor. Results show that in contrast to normal corneas, where of the NOS isomers only endothelial nitric oxide synthase (NOS3) was expressed in a significant amount (in the epithelium and endothelium), in irradiated corneas all NOS isomers (also brain nitric oxide synthase, NOS1, and inducible nitric oxide synthase, NOS2) as well as an indirect measure of ONOO-formation and MDA were gradually expressed, first in the epithelium, the endothelium and the keratocytes beneath the epithelium and finally in the cells of all corneal layers and the inflammatory cells that invaded the corneal stroma. This was accompanied by an elevated concentration of NO in the aqueous humor. In conclusion, repeated irradiation with UVB rays evoked the stimulation of NO production, peroxynitrite formation (demonstrated by NT residues) and lipid peroxidation (evaluated by MDA staining).

Key words: UVB rays, Reactive nitrogen species, Cornea

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Introduction

The cornea is directly exposed to UV light, and due to the thinned ozone layer, to more pronounced penetration not only of UV rays of longer wavelength (UVA rays) but also UV light of shorter wavelength (UVB rays) known to generate reactive oxygen species (superoxide and hydroxyl radicals, hydrogen peroxide, singlet oxygen) (e.g. Riley, 1988; Meseley and Mackie, 1997; Young, 1997; Wenk et al., 2001) that are dangerous to biological systems, causing cellular damage by reacting with lipids, proteins and DNA (Kehrer, 1993). Although a number of antioxidants are present in the eye (low-molecular weight antioxidants, e.g. ascorbic acid, glutathione and alpha-tocopherol, as well as high molecular weight antioxidants, such as catalase, superoxide dismutase and glutathione peroxidase that protect ocular tissues against oxidative injury, the cornea may be damaged by oxidative stress when the production of damaging reactive oxygen species overwhelms the antioxidants. This occurs in the cornea after repeated irradiation with UVB rays (Čejková, et al., 2000, 2001, 2004). The antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) gradually decreased during irradiation, whereas the pro-oxidant enzymes (xanthine oxidoreductase/xanthine oxidase) remained at normal levels or even increased.

Very similar results were desribed by Lodovici et al. (2003) in in vitro experiments with rabbit corneal-derived cells. Superoxide dismutase decreased in UVB irradiated cells, whereas xanthine oxidase activity increased. This resulted in the insufficient cleavage of reactive oxygen species and contributed to the development of oxidative corneal injury.

Surprisingly, the potential role of nitric oxide (NO) species in oxidative eye damage related to UVB rays has

not been investigated. This is in contrast to the skin, where the effect of UVB rays on NO production has been studied and the involvement of nitride oxide in the development of skin disturbances well documented. Rhodes et al. (2001) described that prostaglandin E2 and nitric oxide were the mediators of ultraviolet-induced erythema. They also showed that the erythemal response was accompanied by the prolonged synthesis of both mediators and that the synthesis of nitric oxide was induced by lower doses of ultraviolet B compared with that of prostaglandin E_2. Deliconstantinos et al. (1997) and Horikoshi et al. (2000) showed that L-NAME (N-nitro-L-arginine methylester hydrochloride), an inhibitor of nitric oxide synthases, delayed both skin erythema and pigmentation. Romero-Graillet et al. (1997) pointed out that normal human keratinocytes secreted nitric oxide in response to UV rays and demonstrated that keratinocyte NOS3 was involved in human skin pigmentation (UV-induced melanogenesis). Deliconstantinos et al. (1996 a,b) described the induction of NOS3 and xanthine oxidase following exposure to UVB rays leading to the release of nitric oxide and peroxynitrite formation. Moreover, these authors investigated whether this light-induced response could be involved in the pathogenesis of sunburn erythema and inflammation. The studies indicated that UVB radiation could act as a potent stimulator of nitric oxide synthase-generated NO and xanthine oxidase-generated reactive oxygen species in human endothelial cells and that the cytotoxic effects of nitric oxide and peroxynitrite might be involved in skin erythema and the inflammatory process.

The results on xanthine oxidase in irradiated skin correspond with our previous findings in the rabbit cornea irradiated with UVB rays. UVB rays stimulated xanthine oxidase activity in the corneal epithelium and endothelium (Čejková and Lojda, 1996; Čejková et al., 2001). Also, Lodovici et al. (2003) described an increase in xanthine oxidase activity in rabbit corneal-derived cells after UVB irradiation. However, the potential role of NO in the development of oxidative corneal injury after UVB irradiation has not been examined. Therefore, we decided to study this problem to fill this gap. Rabbit corneas were repeatedly irradiated with UVB rays, and the expression of NOS isomers, NT and MDA was evaluated immunohistochemically. The amount of NO in the aqueous humor, measured indirectly by nitrite and nitrate concentrations, was evaluated biochemically.

Materials and methods

Animals and experimental injuries

Adult New Zealand white rabbits (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 an i.m. injection of Rometar (Xylazinum hydrochloricum, 2%, Spofa, 0.2ml/1kg body weight) and Narkamon (Ketaminum hydrochloricum, 5%, Spofa, 1 ml/kg body weight). The open eyes were irradiated (both eyes of each rabbit) with a UVB lamp (Illkirch Cedex, France; 312 nm wavelength, 6W) from a distance of 0.03m for 5 minutes once daily (a dose per day of 1.01 J/cm^2). Only the corneas were irradiated; the rest of the eye surface was protected from UV rays. The animals were irradiated for 3 days (the first group, 5 rabbits) or for 6 days (the second group, 12 rabbits). 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 left without any treatment for 24 hours and afterwards they were sacrificed under thiopental anesthesia (Thiopental, Spofa). For the immunohistochemical examination, each group contained 10 corneas. Normal corneas (10) served as controls. For the biochemical examination, 7 rabbits from the second group were employed. Normal eyes of 7 rabbits served as controls.

Immunohistochemical 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. Unfixed cryostat sections were fixed in acetone at 4 °C for 5 min.

For the immunohistochemical localization of NOS and NT, the following primary antibodies were used: monoclonal mouse anti-human NOS3 antibody, monoclonal mouse anti-human NOS1 antibody and monoclonal mouse anti-human NOS2 antibody (BD Biosciences, San Jose, CA, USA), monoclonal mouse anti-nitrotyrosine (Abcam, Cambridge, UK). Subsequently, an anti-mouse HRP/DAB Ultravision Detection System (Lab Vision, Fremont, CA, USA) was employed as recommended by the manufacturer: hydrogen peroxide block (20 min), ultra V block (5 min), primary antibody incubation (60 min), biotinylated goat anti-mouse antibody incubation (10 min), and streptavidin peroxidase incubation (10 min). Visualization was performed using a freshly prepared DAB substrate/chromogen solution. Some sections were counterstained with Mayer's hematoxylin (Sigma). Negative controls included the omission of the primary antibody. To confirm the specifity of the staining for nitrotyrosine, the antibody was incubated with 10 mM nitrotyrosine (Kooy et al., 1997).

Measurement of nitric oxide levels in the aqueous humor

Immediately after killing the animals, aqueous humor samples were collected by anterior puncture from the eyes of each animal. Nitric oxide was determined using a spectrophotometric assay based on the Griess reaction. Nitrate was first converted to nitrite by nitrate reductase (see Tracey et al., 1995 for details). The absorbance at 540 nm was recorded using a microplate

spectrophotometer (Tecan, Grödig, Austria). A nitrite calibration curve was used to convert absorbance to µM nitrite.

Analysis of data

Student's t-test test for unpaired data and the graphical presentation of results were performed using the Prism program (GraphPad Software, San Diego, CA, USA).

Results

Immunohistochemical stainings for NOS, NT and MDA in normal corneas and corneas irradiated with UVB rays for 3 days or 6 days

Fig.1. In the normal cornea, the epithelium (arrow, a)

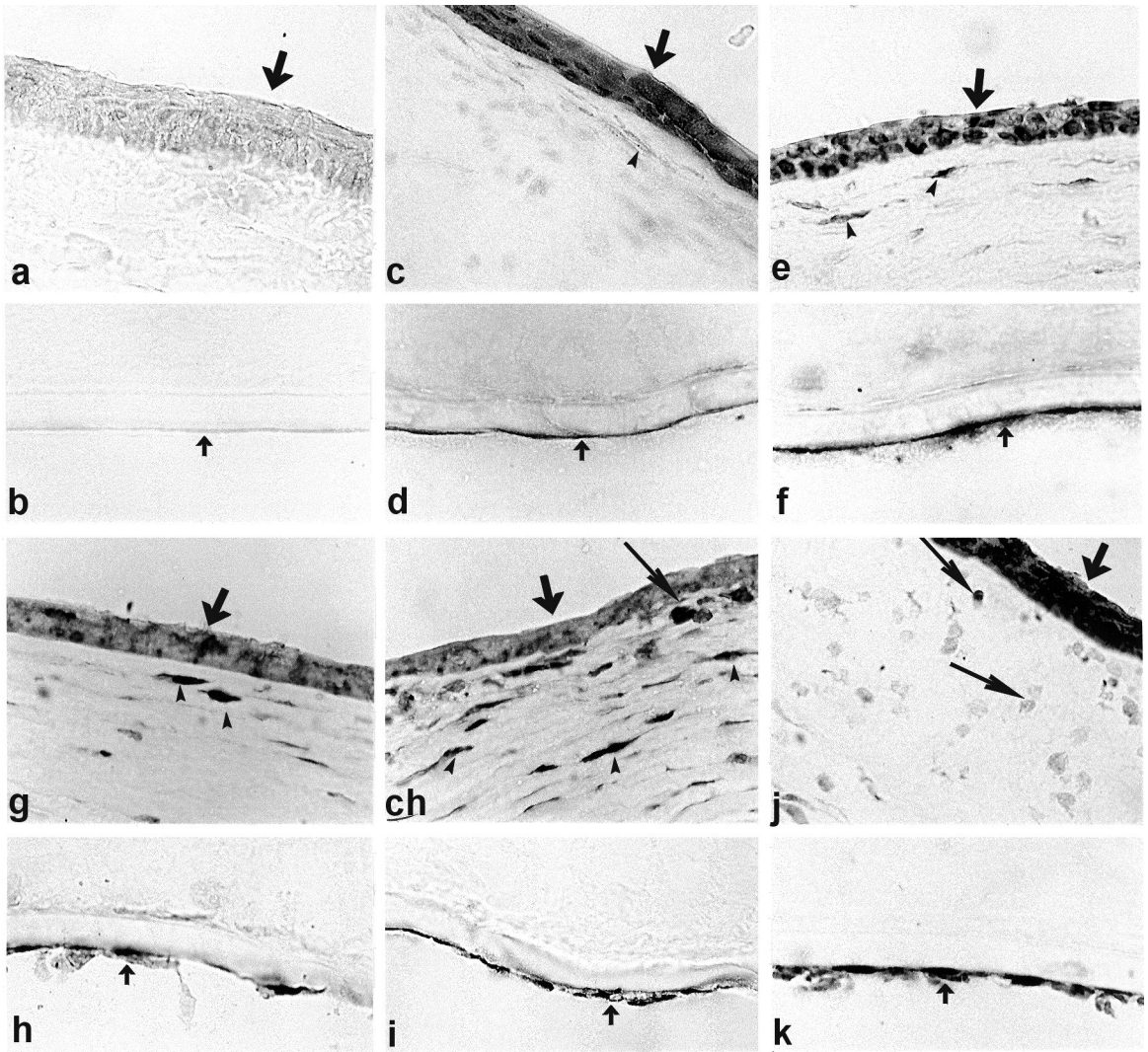

Fig. 1. Imunohistochemical staining for NOS3 in the normal rabbit cornea and staining for NOS3, NOS1, NOS2, NT and MDA in corneas irradiated with UVB rays for 3 days. **a, b.** In the normal cornea, significant NOS3 staining is present in the epithelium (arrow, a) and slight staining in the endothelium (arrow, b). **c, d.** A cornea irradiated with UVB rays for 3 days. NOS3 staining is strong in the epithelium (arrow, c) as well as the endothelium (arrow, d) and weak in keratocytes beneath the epithelium (arowhead, c). **e, f.** In a cornea irradiated with UVB rays for 3 days, distinct NOS1 staining appears in the epithelium (arrow, e) and endothelium (arrow, f). Keratocytes in the superficial portion of the corneal stroma also show staining for NOS1 (arrowhead, e). **g, h.** A cornea irradiated with UVB rays for 3 days. NOS2 staining is apparent in the thinned corneal epithelium (arrow, g), keratocytes (arrowheads, g) beneath the epithelium and in the endothelium (arrow, h). **ch, i.** A cornea irradiated with UVB rays for 3 days. NT is expressed in the thinned epithelium (arrow, ch) and endothelium (arrow, i). Keratocytes (arrowheads, 1ch) in the corneal stroma strongly express NT as do the scarce polymorphonuclear leukocytes (large arrow). **j, k.** In a cornea repeatedly irradiated with UVB rays for 3 days, MDA is highly expressed in the epithelium (arrow, j) and endothelium (arrow, k). The large arrow points to polymorphonuclear leukocytes in the corneal stroma (j), which also show MDA positivity. x 120

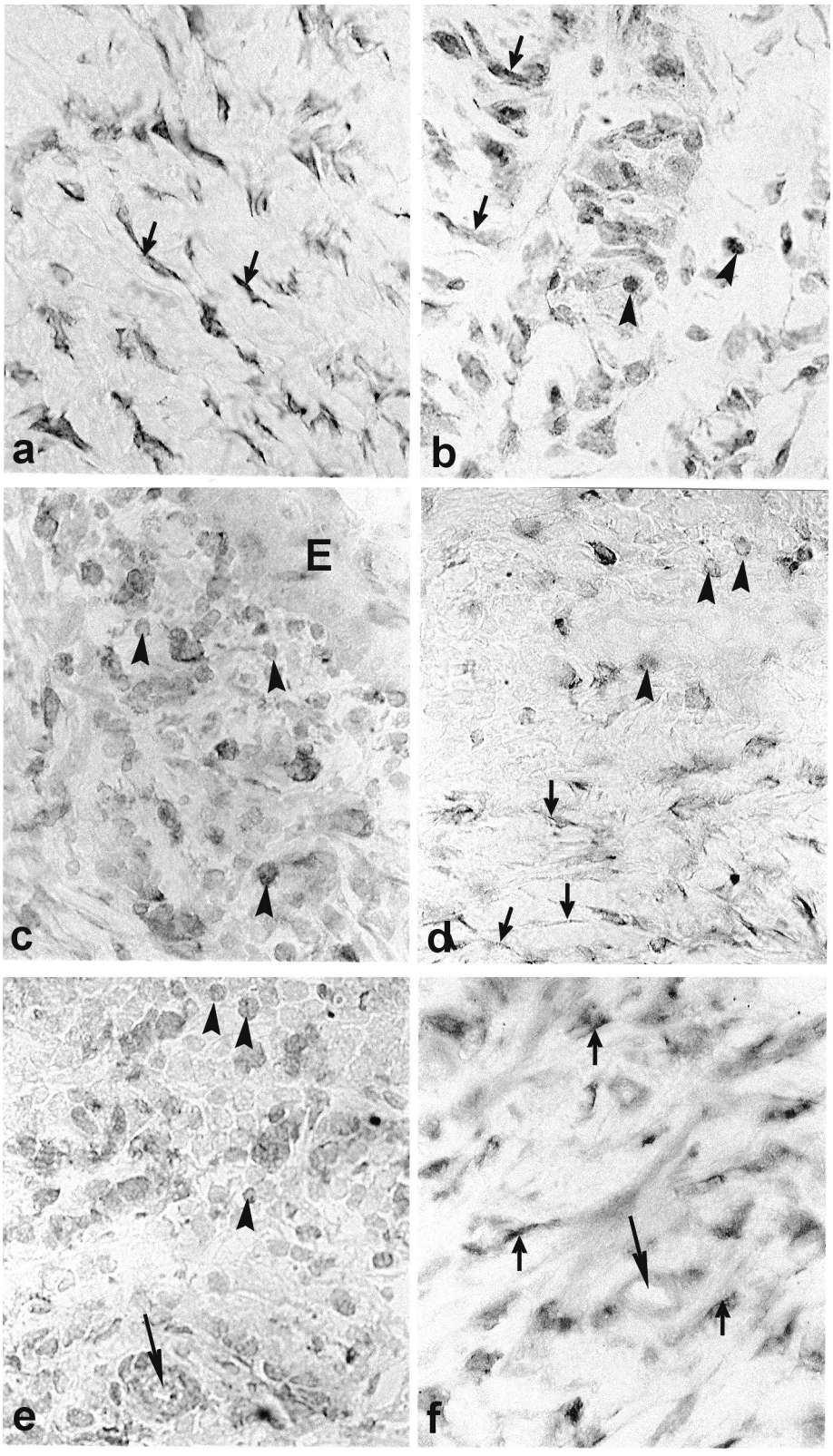

Fig. 2. Immunohistochemical staining for NOS3, NOS1, NOS2, NT and MDA in cornea irradiated with UVB rays for 6 days. **a.** Strong NOS3 staining in keratocytes of the corneal stroma (arrows). **b.** NOS1 staining is present in keratocytes (arrows) and also polymorphonuclear leukocytes (arrowheads) present in small number in the middle part of the corneal stroma. **c.** NOS2 staining can be seen in numerous polymorphonuclear leukocytes (arrowheads) in the upper portion of the corneal stroma at the limbus. The remaining part of the epithelium (E) shows slight NOS2 staining. **d.** NT staining is apparent in keratocytes (arrows) as well as in polymorphonuclear leukocytes (arrowheads), which are present in smaller number in the middle portion of the corneal stroma. **e.** MDA staining in polymorphonuclear leukocytes (arrowheads) present in large number in the upper portion of the corneal stroma. The large arrow points to the site of corneal vascularization. **f.** MDA staining in keratocytes in the deep part of the corneal stroma where polymorphonuclear leukocytes are nearly absent. The stroma is vascularized (large arrow). x 120

and endothelium (arrow, b) showed some staining for NOS3, whereas staining for NOS1, NT and MDA was essentially absent (not shown). On day 3 of repeated irradiation, NOS3 was expressed strongly in the thinned epithelium (arrow, c), slightly in the keratocytes beneath the epithelium (arrowhead, c) and moderately in the endothelium (arrow, d). On the same day of irradiation (day 3), NOS1 staining appeared in the epithelium (arrow, e), in keratocytes in the upper part of the corneal stroma (arrowheads, e) and in the endothelium (arrow, f). NT staining (g, h) and NOS2 staining (ch, i) showed a very similar picture, however, in the case of NOS2 rare polymorphonuclear leukocytes were also stained in the corneal stroma (large arrow, ch). Already on day 3 of UVB irradiation, MDA was strongly expressed in the thinned epithelium (arrow, j) and also in the endothelium (arrow, k). In the corneal stroma some polymorphonuclear leukocytes showed slight staining for MDA (large arrows, j).

Fig. 2. On day 6 of irradiation, the corneas were nearly without epithelium. In the endothelium, pronounced stainings for NOS isomers, NT and MDA was observed (not shown). In the corneal stroma distinct staining for NOS3 (arrows, a) and NOS1 (arrows, b) was seen in keratocytes. Some polymorphonuclear leukocytes in the corneal stroma showed staining for NOS1 (arrowheads, b). NOS2 was expressed in numerous polymorphonuclear leukocytes in the upper part of the corneal stroma (arrowheads, c). The remaining epithelium (E) at the limbus showed slight NOS2 staining. NT was expressed in keratocytes (arrows, d) and inflammatory cells (arrowheads, d), which were present in low number in the middle part of the corneal stroma. MDA was expressed in inflammatory cells (arrowheads, e) present in large amount in the upper portion of the corneal stroma as well as in keratocytes (arrows, f) in the deep part of the corneal stroma, where polymorphonuclear leukocytes were nearly absent. The large arrows point to the sites of corneal vascularization (e, f).

In controls in which the primary antibody was omitted, the samples were negative. In controls for the specificity of nitrotirosine staining, highly reduced staining was observed when the primary antibody was incubated with an excess of nitrotyrosine.

Biochemical determination of NO in the aqueous humor

The level of NO in the aqueous humor, measured by nitrate and nitrite concentration, was significantly increased ($t/22/=2.59$, $P<0.05$) in eyes irradiated with UVB rays (20.7±2.8 mM) as compared to the levels of NO in the aqueous humor of normal eyes (13.5±0.9 mM) (means ± S.E.M.) (Fig. 3). (Although 14 UVB-treated eyes and 14 controls were taken in our experiment, finally, 11 aqueous humor samples of UVB-treated eyes and 13 aqueous humor samples of normal eyes were examined; the rest of samples was lost due to the unsuccessful aqueous humor aspiration).

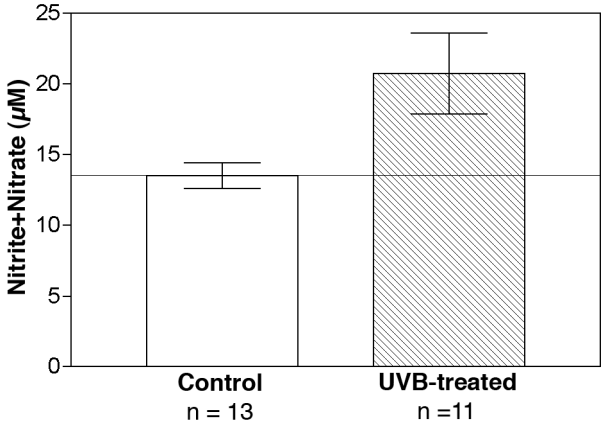

Fig. 3. Biochemical determination of NO in the aqueous humor (measured by nitrate and nitrite concentration) in eyes repeatedly irradiated with UVB rays for 6 days as compared to normal eyes. Concentrations of nitrite and nitrate in the aqueous humor of irradiated eyes (UVB-treated) significantly increased ($t/_{22}/=2.59$, $P<0.05$) as compared to normal eyes (control).

Discussion

In contrast to the well documented involvement of reactive oxygen species in oxidative eye damage related to UVB rays, the potential role of reactive nitrogen species has not been investigated. This is the first study dealing with this problem. The results show that reactive nitrogen species are highly involved. UVB rays evoke the gradual expression of all NOS isomers (NOS1, NOS2, NOS3) and also NT and MDA, first in the corneal epithelium, endothelium and the keratocytes beneath the epithelium. At the end of irradiation, the source of NO, NT and MDA were cells of all corneal layers and also polymorphonuclear leukocytes that invaded the corneal stroma.

Nitric oxide, produced by the oxidative deamination of L-arginine by NOS, is an important mediator of homeostatic processes in the eye, such as the regulation of aqueous humor dynamics, retinal neurotransmission and phototransduction (Becquet et al., 1997; Schneemann et al., 2002). In the cornea, nitric oxide spontaneously produced in the corneal endothelium is highly involved in the maintainance of corneal thickness (Yanagiya et al., 1997). However, changes in nitric oxide generation or action have been found to contribute to various pathological states, such as ocular inflammatory or degenerative diseases (Wang and Hakanson, 1995; Becquet et al., 1997; Allen et al., 1998; Koss, 1999; Chiou, 2001; Chiou et al., 2001; Park et al., 2001; Buddi et al., 2002; Kim et al., 2002; Tsai et al., 2002). Moreover, Ko et al. (2000) described the role of nitric oxide in experimental allergic conjunctivitis and Jeng et al. (2002) and Meisler et al. (2004) the damaging effect of increased NO production in corneas waiting for

transplantation in storage media. Kao et al., 2002 pointed out that increased levels of NO might be a risk factor in cataract formation. Evidence of oxidative ocular damage from the cytotoxic byproduct peroxynitrite, generated by the rapid reaction of NO with the superoxide, has been demonstrated in endotoxin - induced uveitis (Allen et al., 1996, 1998). Wu et al. (1997) described that peroxynitrite caused oxidative damage of the retina through lipid peroxidation of photoreceptors in experimental autoimmune uveitis. Lipid peroxidation is an important biological consequence of oxidative damage of cell membranes and the formation of cytotoxic aldehydes. These aldehydes can result in changed enzyme activities, the inhibition of DNA/RNA protein synthesis and other damaging events (Esterbauer et al., 1991). Increased levels of MDA, the toxic aldehyde byproduct of lipid peroxidation, were described in the cornea after the intrastromal injection of liposaccharide (Qian and Wu, 1998). Buddi et al. (2002) first described the presence of MDA in human diseased corneas, and Park and Tseng (2000), investigating the role of acute inflammation in keratocyte death after photorefractive keratectomy, described distinct immunohistochemical staining for MDA in the corneal stroma.

Immunohistochemical studies allow one to demonstrate the cellular source of NO in situ. In agreement with other authors (Yanagiya et al., 1997; Buddi et al., 2002; Kim et al., 2002), we found that NOS3 was expressed to some extent in the normal cornea only in the epithelium and endothelium and that NOS2 and NOS1 staining were essentially absent. However, according to our findings as well as other studies on various ocular diseases or experimental injuries (Buddi et al. 2002; Kim et al. 2002; Park and Tseng, 2000), NOS isomers were continually expressed in cells of all corneal layers. Also, NT and MDA staining appeared in corneal cells and in inflammatory cells (mainly polymorphonuclear leukocytes). Of the NOS isomers, NOS1 and mainly NOS2 have been found to be present in inflammatory cells.

Comparing our results in corneas irradiated with UVB rays with the above-mentioned results on UVB-irradiated skin, where the influence of UVB rays on nitric oxide generation was well documented (Deliconstantinos et al., 1996a,b, 1997; Romero-Grailett et al., 1997; Horikoshi et al., 2000; Rhodes et al., 2001) also in the cornea we can show that as in the skin, UVB rays stimulated NOS, resulting in elevated nitric oxide production. Besides corneal cells, polymorphonuclear leukocytes strongly contributed to NOS generation. It is known that UVB rays generate reactive oxygen species, including superoxide. Moreover, UVB rays evoke in the eye as well as in the skin an increase in xanthine oxidase activity (Čejková and Lojda, 1996; Deliconstantinos et al., 1996 a,b; Čejková et al., 2001; Lodovici et al., 2003). Xanthine oxidase is an enzyme known to generate reactive oxygen species, including superoxide. Nitric oxide reacts rapidly with superoxide to form the potent oxidant peroxynitrite. We detected peroxynitrite formation in the cornea by the demonstration of nitrotyrosine residues, which are considered a specific marker for peroxynitrite and nitrogen-related oxidants (Kooy et al.,1995; Cross et al., 1997; Saleh et al., 1997). MDA, one of the most abundant carbonyl products of lipid peroxidation (Marnett, 2002), we also found in corneas irradiated with UVB rays, suggesting profound oxidative corneal damage.

NOS expression in corneas irradiated with UVB rays was accompanied by a significant increase in NO concentration in the aqueous humor, measured by nitrate/nitrite levels (breakdown products of NO). Although the cornea absorbs a large proportion of damaging UVB rays (Schive et al., 1984; Ringvold 1998), increased levels of NO in the aqueous humor may as well result from the inflammatory response secondary to the (rather profound) corneal injury in this model.

In conclusion, reactive nitrogen species are highly involved in oxidative injury of the cornea related to UVB irradiation.

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