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Absence of glutaredoxin1 increases lens susceptibility to oxidative stress induced by UVR-B

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ABSTRACT

We investigated if the absence of glutaredoxin1, a critical protein thiol repair enzyme, increases lens susceptibility to oxidative stress caused by *in vivo* exposure to ultraviolet radiation type B (UVR-B). $Glrx^{-/-}$ mice and $Glrx^{+/+}$ mice were unilaterally exposed *in vivo* to UVR-B for 15 min. Groups of 12 animals each received 4.3, 8.7, and 14.5 kJ/m^2 respectively. 48 h post UVR-B exposure, the induced cataract was quantified as forward lens light scattering. Cataract morphology was documented with darkfield illumination photography. Glutathione (GSH/GSSG) content was analyzed in $Glrx^{-/-}$ and $Glrx^{+/+}$ lenses. UVR-B exposure induced anterior sub-capsular cataract (ASC) in $Glrx^{-/-}$ and $Glrx^{+/+}$ mice. In $Glrx^{-/-}$ lenses the opacities extended further towards the lens equator than in wild type animals ($Glrx^{+/+}$). Lens light scattering in $Glrx^{-/-}$ mice was increased in all dose groups compared to lenses with normal glutaredoxin1 function. The difference was more pronounced with increasing exposure dose. Lens sensitivity for UVR-B induced damage was significantly higher in $Glrx^{-/-}$ lenses compared to $Glrx^{+/+}$ lenses. The *Glrx* gene provides a 44% increase of protection against close to threshold UVR-B induced oxidative stress compared to the absence of the *Glrx* gene. In conclusion, the absence of glutaredoxin1 increases lens susceptibility to UVR-B induced oxidative stress in the mouse.

1. Introduction

The lens depends on a balanced redox state for maintaining its transparency (Lou, 2003). Oxidative stress induced by reactive oxygen species (ROS) has long been implicated in the pathogenesis of age-related cataract (Augusteyn, 1981; Spector, 1995; Delcourt et al., 2000; Truscott, 2003, 2005). In an environment rich in oxidants the formation of protein thiol-mixed disulfides (PSSG) through protein S-thiolation can trigger a cascade of events leading to cataract. Starting with protein or enzyme deactivation, changes in protein conformation and the disruption of membranes by lipid peroxidation may lead to protein-crosslinking and the formation of high-molecular weight aggregates in the lens (Lou et al., 1990; Raghavachari et al., 2001; Chylack, 2004). These structural alterations then can cause increased lens light scattering through localized alterations of the refractive index.

To prevent thiol oxidation and to keep the intrinsic redox system in a reduced state, the lens has several antioxidative defence mechanisms and is equipped with an enzyme system that specifically reduces PSSG before further chemical alterations can occur. This dethiolation process is mediated through the redox-regulating glutathione-dependant enzyme glutaredoxin1 (gene symbol: *Glrx*), also known as thioltransferase, which is ubiquitously present in the cytosol of microbial, plant and animal tissues and was first discovered in the lens in 1996 (Raghavachari and Lou, 1996; Sagemark et al., 2007). Glutaredoxin1 function is illustrated in Fig. 1.

Recently, the role of glutaredoxin1 dysfunction was confirmed in the pathogenesis of diabetic retinopathy, Alzheimer's disease, and obstructive lung disease (Akterin et al., 2006; Shelton et al., 2007; Peltoniemi et al., 2006). Furthermore, it was shown that *Glrx* gene expression and function is up regulated two-fold under oxidative stress conditions when other antioxidative enzyme systems such as glutathione reductase or glutathione peroxidase

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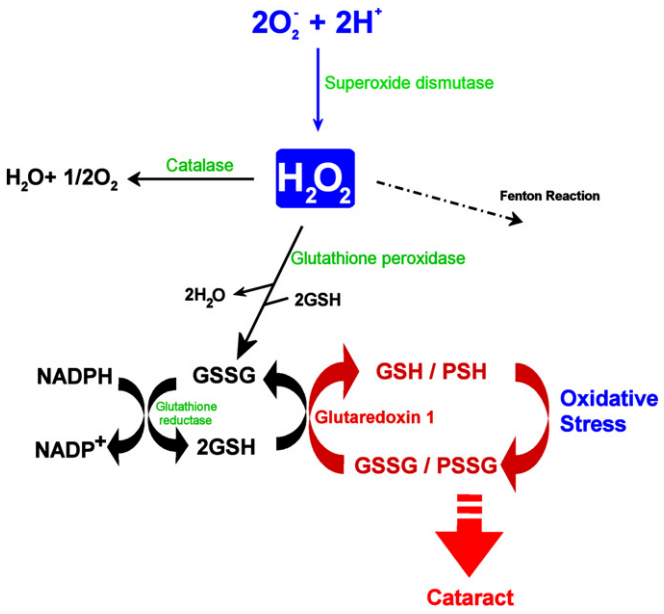

Fig. 1. Antioxidative defence systems and glutaredoxin1 (Thioltransferase, TTase) function in the lens: H_2O_2 generated by the dismutation of superoxide anion is degraded by several pathways including catalase, glutathione peroxidase, and the Fenton reaction. A decreased SH/S–S ratio by oxidation can be reversed by glutathione reductase or glutaredoxin1, the latter specifically reduces protein thiol-mixed disulfides. These mechanisms protect the lens from oxidative damage (Superoxide dismutase).

are already inactivated by ROS damage (Raghavachari et al., 2001). This increased activity and expression of glutaredoxin1 under sustained oxidative stress is held to be essential for cell protection since the dethiolating enzyme can repair oxidatively damaged key enzymes and proteins (Xing and Lou, 2003).

However, the role of glutaredoxin1 and the corresponding gene (*Glrx*) in UVR-B induced cataractogenesis is still unclear. Cataract is the leading cause of blindness worldwide and it has been long established that exposure to UVR-B induces cataract in humans and animals (Jose and Pitts, 1985; Taylor et al., 1988; Cruickshanks et al., 1992; Dillon et al., 1999). UVR-B together with ionizing radiation is the main source of exogenously generated ROS in the lens (Moon et al., 2005).

We investigate for the first time if the absence of glutaredoxin1 sensitizes the lens to oxidative damage from exposure to UVR-B. Recently, we have characterized the C57BL/6J mouse as an *in vivo* model for UVR-B induced cataract (Meyer et al., 2005, 2007) and defined the cataract threshold estimate ($MTD_{2.3:16}$) to 2.9 kJ/m^2 (Meyer et al., 2008). Since glutaredoxin1 is believed to act as a dethiolating antioxidant that repairs and regenerates oxidatively damaged proteins even when primary antioxidative defence systems fail to protect the lens against oxidative stress, (Quiao et al., 2001), it is of high interest to explore whether the inactivation of glutaredoxin1 sensitizes the lens to oxidative insults in an *in vivo* UVR-B cataract model.

2. Materials and methods

2.1. Animals

The generation of *Glrx* knockout mice, in which exons 1 and 2 of the mouse *Glrx* gene are deleted, has been described previously (Ho et al., 2007) The $Glrx^{-/-}$ and $Glrx^{+/+}$ mice used in the current study were bred on the same 129SV and C57BL/6 hybrid background. All animals were maintained and treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Ethical approval was obtained from the Northern Stockholm Animal Experiments Ethics Committee Protocol Number N32/05 and N70/06. Knockout and wild type mice were genotyped with PCR assays at St. Erik's Eye Hospital in Stockholm.

2.2. UVR-B exposure

UVR-B in the 300 nm wavelength region (UVR 300 nm) was generated with a high-pressure mercury arc lamp. The emerging radiation was collimated, passed through a water filter and focused on the entrance slit of a double monochromator (Michael et al., 2000). The radiation output of the UVR-source is shown in Fig. 2.

UVR intensity was measured as irradiance in the corneal plane with a thermopile (model 7101; Oriel, Stratford CT) calibrated to a US National Institute of Standard traceable source. Intensity was adjusted by varying the height of the entrance slit of the first monochromator.

2.3. Experimental procedure

Ten minutes preceding UVR-B exposure, the animal was anaesthetized with a mixture of 40 mg/kg ketamine and 5 mg/kg xylazine injected s.c. Five minutes after the injection, 1% tropicamide was instilled into both eyes to induce mydriasis. Prior to exposure, all animals were checked with a slit lamp to exclude pre-existing cataract. One eye of each mouse was exposed *in vivo* to UVR-B for 15 min based on previous studies (Ayala et al., 2000; Dong et al., 2005). The contralateral, non-exposed eye was lubricated and shielded during exposure and kept as a control. After exposure, lubricating ointment was instilled into both eyes.

Each animal was kept for a latency period of 48 h from the start of UVR exposure, based on data on cataract evolution after *in vivo* exposure to UVR-B (Meyer et al., 2005). The eyes were enucleated and the lenses were extracted microsurgically using an operation microscope. Remnants of the ciliary body were removed from the lens equator keeping the lens in balanced salt solution (BSS, Alcon, Sweden). A few lenses were damaged during the dissection and therefore excluded from data analysis.

2.4. Quantification of intensity of lens forward lens light scattering

Experimentally induced cataract was quantified as intensity of forward lens light scattering, measured with a light dissemination meter (LDM) (Söderberg et al., 1990). The LDM uses the principle of darkfield illumination. Light transilluminates a transparent object (e.g. a mouse lens) at 45° against the horizontal plane. At this angle, the light cannot enter the objective aperture. If the object scatters

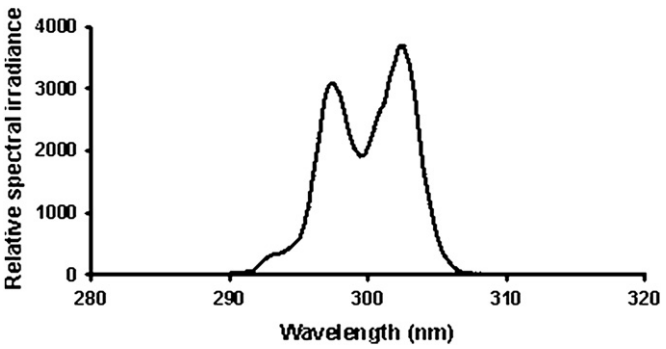

Fig. 2. Spectral irradiance of UVR in the exposure plane.

light in the forward direction, a defined fraction of light reaches the objective and is measured by a photodiode. An opaque lipid emulsion of Diazepam (Stesolid Novum®, Dumex-Alphapharma, Denmark) was used as the scattering standard. Light scattering was expressed as transformed equivalent Diazepam concentration (tEDC) (Söderberg et al., 1990).

2.5. Cataract morphology

Immediately after lens light scattering measurements, cataract morphology was documented in darkfield illumination with a microscope photography system.

2.6. Measurement of GSH and GSSG

Whole lenses were homogenized with lysis buffer and centrifuged; supernatant was used for the estimation of GSH and GSSG based on the method of Ellman (Ellman, 1959).

2.7. Experimental design

The experimental design is given in Fig. 3.

The experiment was designed to allow for analysis of a total of 30 female *Glrx*$^{-/-}$ mice and 30 age-matched female wild type mice (*Glrx*$^{+/+}$) divided into three dose groups ($n = 10$) each, receiving 4.3, 8.7, or 14.5 kJ/m^2 UVR-B. Based on previous experiments dose levels were chosen as 1,5× (4.3 kJ/m^2), 3× (8.7 kJ/m^2), and 5× (14.5 kJ/m^2) equivalents of the estimated cataract threshold dose or Maximum Tolerable Dose (MTD) in the mouse (Meyer et al., 2008). To allow for potential losses during the experiment, two additional animals were included in each dose group. Thus, in total 72 mice were exposed.

2.8. Statistical parameters

Considering the sample size, the significance level was set to 0.05 and the confidence coefficient to 0.95. The data were analyzed with Microsoft Office Excel 2003, SP3.

3. Results

3.1. Cataract morphology

Following exposure to UVR-B, lenses of both *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$ mice developed anterior sub-capsular opacities (Fig. 4a). In *Glrx*$^{-/-}$ mice, the epithelial damage extended circularly further towards the equator two days after exposure, and thus the cataractous area was larger in *Glrx*$^{-/-}$ mice than in *Glrx*$^{+/+}$ mice (Fig. 4b). Furthermore, the sub-capsular opacity in *Glrx*$^{-/-}$ mice was not as clearly demarcated from transparent lens tissue as in mice with normal glutaredoxin1 function (Fig. 4, left).

Animals	UVR-B exposure dose	Latency	Lens light scattering
30 Glrx$^{+/+}$	4.3 kJ/m^2 8.7 kJ/m^2 (n=10) 14.5 kJ/m^2	48 h	Exposed Measurement Control
30 Glrx$^{-/-}$	4.3 kJ/m^2 8.7 kJ/m^2 (n=10) 14.5 kJ/m^2	48 h	Exposed Measurement Control

Fig. 3. Experimental design.

3.2. Lens light scattering at above threshold UVR-B dose

Lenses with dissection damage were excluded from light scattering measurements. Table 1 provides the actual sample sizes for each subgroup.

Lens light scattering in exposed lenses of *Glrx*$^{-/-}$ mice was higher in all three dose groups as compared to age-matched wild type animals (*Glrx*$^{+/+}$; Fig. 5).

To analyze the impact of lack of the *Glrx* gene on lens sensitivity, k, to *in vivo* exposure to UVR-B, the differences of light scattering between exposed and contralateral non-exposed lens, I_d, as a function of dose, H, were fitted separately for the *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$ to a linear-quadratic model, considering an experimental error, ϵ (Equation (1))

$$I_d = kH^2 + \varepsilon \quad (1)$$

This was based on the previous finding that difference of light scattering between exposed and contralateral not exposed lens increases sigmoidally in a double exponential fashion for which the lower interval has been shown to be well approximated to a second order polynomial omitting the first order term (Söderberg et al., 2003).

The best fit dose-response functions for *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$ are given in Fig. 6:

A 95% confidence interval for the sensitivity, k, was estimated for *Glrx*$^{-/-}$ to $3.94 \pm 1.47 \times 10^{-3}$ tEDC/MTD$_{2.3;16}$ and for *Glrx*$^{+/+}$ to $2.21 \pm 0.88 \times 10^{-3}$ (25° of freedom). The sensitivities for *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$, were found significantly different as tested by an approximate t-test (Test statistic = 2.06, $t_{46;0.975} = 2.01$). The approximate t-test was chosen since the variances for the two sensitivities were found significantly different with an F-test.

3.3. GSH/GSSG content

No significant difference in GSH/GSSG concentration was found between exposed and contralateral lenses in *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$ mice (data not shown).

4. Discussion

We investigated cataract development 48 h post unilateral *in vivo* exposure to above threshold doses UVR-B in glutaredoxin1 deficient mice (*Glrx*$^{-/-}$) and age-matched wild type mice (*Glrx*$^{+/+}$). This is the first study that investigates the impact of glutaredoxin1 deficiency in UVR-B induced cataractogenesis.

We aimed to further explore the *in vivo* glutaredoxin1 function and the impact of glutaredoxin1 deficiency in cataract development since it has been shown in previous work that glutaredoxin1 is characterized by a remarkable resistance to oxidative damage (Quiao et al., 2001; Xing and Lou, 2002). Therefore we anticipated that the absence of glutaredoxin1 could impair the lens protective system enough to observe cataractogenetic effects even at around threshold dose UVR-B. This hypothesis was confirmed by our observations in glutaredoxin1 deficient mouse lenses.

4.1. Cataract morphology

The *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$ mice, all with the same hybrid 129SV and C57BL/6 background developed only anterior sub-capsular opacities (Fig. 4) and not any other types of lens opacifications. This contrasts to the UVR cataract pattern in wild type C57BL/6 mice, where around 10% of the animals developed nuclear or cortical cataract, with the remaining 90% developing anterior sub-capsular cataract (Meyer et al., 2005, 2008) and implicates that the genetic

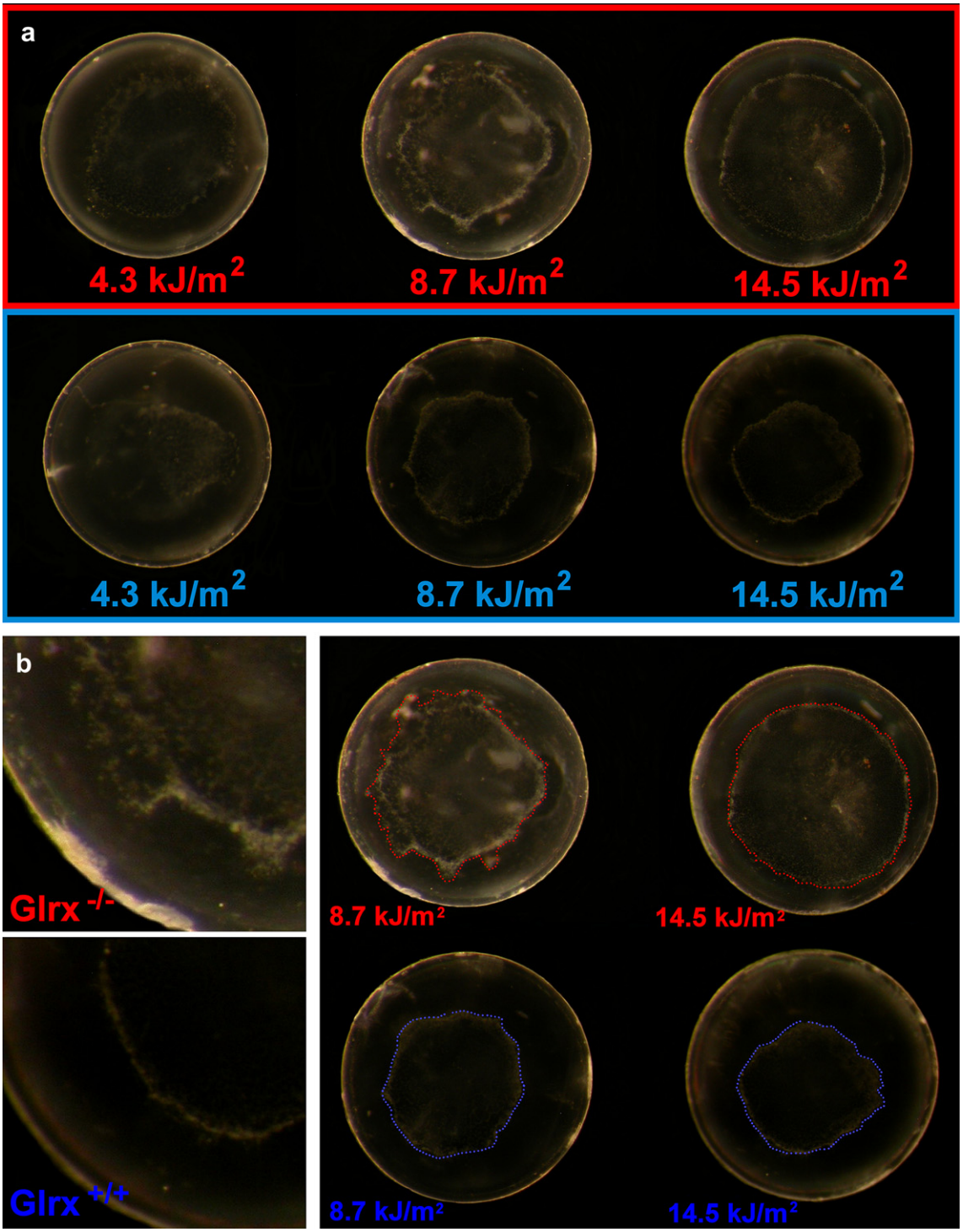

Fig. 4. (a) Anterior sub-capsular cataract in *Glrx*$^{-/-}$ (upper row, red) and *Glrx*$^{+/+}$ (lower row, blue) mouse lens 48 h after UVR exposure to 4.3, 8.7, or 14.5 kJ/m^2 for 15 min. View is from anterior. (b) Representative measurements of cataract area in *Glrx*$^{-/-}$ (upper row, red) and *Glrx*$^{+/+}$ (lower row, blue) mice following UVR exposure. The area covered by the anterior sub-capsular cataract in *Glrx*$^{-/-}$ mice lenses is larger than that in observed in *Glrx*$^{+/+}$ mice at identical post-exposure interval and UVR-dose applied. Furthermore the margin line of cataract repair is less demarcated in *Glrx*$^{-/-}$ lenses compared to *Glrx*$^{+/+}$ lenses (left, higher magnification).

background has an impact on the lens response to UV irradiation. The strictly anterior sub-capsular cataract in the Glrx$^{+/+}$ as well as in *Glrx*$^{-/-}$ lenses indicates that the lens epithelium is the main route of UVR lens damage. However, the morphology of the anterior sub-capsular opacities (Fig. 4) shows remarkable differences between *Glrx*$^{-/-}$ and *Glrx*$^{+/+}$ animals. We know from previous work that UVR-B induced anterior sub-capsular cataract is repaired in mouse lenses starting from the equator towards the anterior lens sutures (Meyer, data unpublished). In the current study, we observed that the sub-capsular damage extended further towards the equator and was less demarcated from clear lens areas in *Glrx*$^{-/-}$ animals compared to *Glrx*$^{+/+}$ animals 48 h after UVR-B exposure (Fig. 4). glutaredoxin1 was recently implicated to play a role in protein regeneration and reactivation of key glycolytic

Table 1
Number of lenses measured for light scattering.

Dose	Genotype	Exposed	Contralateral	Difference
		n	*n*	*n*
1.5	G−/−	9	10	9
3	G−/−	12	11	11
5	G−/−	11	11	10
			Sum	30
1.5	G+/+	9	9	9
3	G+/+	10	8	8
5	G+/+	9	9	9
			Sum	26

enzymes (Wu et al., 1998; Yoshitake et al., 1994). It is therefore not surprising that the absence of glutaredoxin1 delays the reparative migration of the sub-capsular opacity observed here.

4.2. Cataract quantified as forward lens light scattering

The stronger lens light scattering in the $Glrx^{-/-}$ mice (Fig. 5) implicates that the absence of glutaredoxin1 increases the lens susceptibility to oxidative stress induced by UVR-B exposure. Since more than 70% of the protein thiols in cataractous lenses are present as disulfides, compared to less than 10% in normal lenses (Harding, 1970; Reddy and Han, 1976; Lou et al., 1989), the observed increased lens light scattering in $Glrx^{-/-}$ lenses could be due to PSSG accumulation. This assumption is supported by recent findings from Löfgren et al. who demonstrated decreased levels of glutathione and protein thiols and increased levels of PSSG in $Glrx^{-/-}$ lens epithelial cells (Löfgren et al., 2008). The observation that the sensitivity of $Glrx^{-/-}$ lenses is higher than the sensitivity of $Glrx^{+/+}$ lenses (Fig. 6) suggests that the yield for biologically expressed UVR-B induced oxidative stress is higher without the *Glrx* gene.

4.3. Derivation of Grx1 protection

Furthermore, based on our results we were able to derive the relative *protection*, ρ, against biological damage caused by *in vivo* UVR-B exposure that is provided by the *Glrx* gene in the lens (Appendix). This *protection* was estimated to 44%. The uncertainty in the estimates of the sensitivities for $Glrx^{+/+}$ and $Glrx^{-/-}$ as indicated by the confidence intervals (Fig. 5) is mainly caused by the fact that close to threshold responses are close to noise level. This uncertainty is then transferred to the estimate of the protection factor. On the other hand, it would be much less relevant to establish the physiological *protection* of the *Glrx* gene at high UVR-B dose levels where the damage may be high enough to exceed saturation of *Glrx* gene protection. The estimates of the sensitivities could be increased with larger sample sizes.

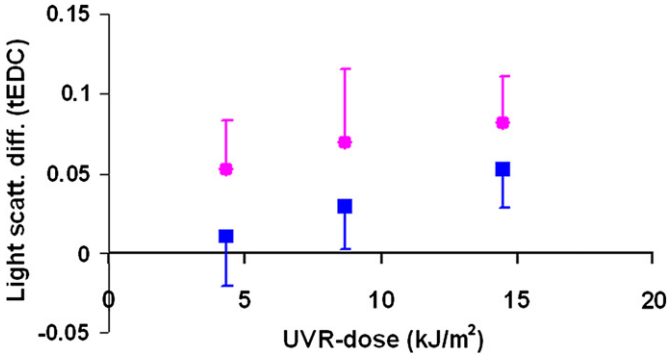

Fig. 5. Mean light scattering difference in $Glrx^{-/-}$ (circles) and $Glrx^{+/+}$ (squares) mouse lenses after 4.3, 8.7, or 14.5 kJ/m^2 UVR-B. Post-exposure interval is 48 h. Bar is 95% confidence interval for the mean.

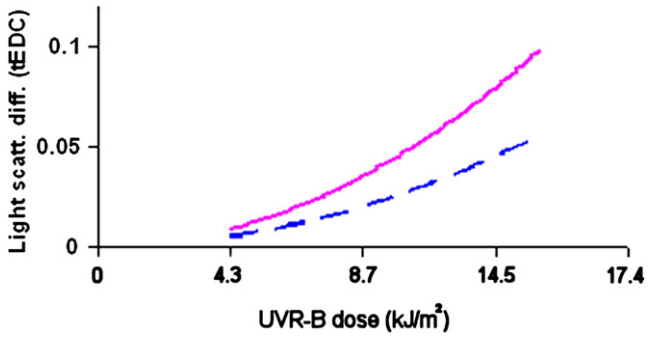

Fig. 6. Dose-response function for $Glrx^{-/-}$ (continuous line) and $Glrx^{+/+}$ (interrupted line) mouse lenses, 48 h post in *in vivo* UVR-B exposure. Response is given as paired difference in light scattering difference between exposed and contralateral lens.

Excessive oxidation and consequently ROS overload increases the risk for inactivation of antioxidative enzyme systems (Xing and Lou, 2002). Ho and coworkers found that $Glrx^{-/-}$ mice were not more susceptible to acute oxidative insults in models of heart and lung injury induced by ischemia/reperfusion and hyperoxia, respectively (Ho et al., 2007). No changes in the activities of and/or expression of compensatory enzymes such as catalase, glutathione peroxidase, copper-zinc- and manganese dismutase were determined in heart and lungs of glutaredoxin1 deficient mice compared to wild type mice. They concluded that generalized S-thiolation of cytosolic proteins is not a major cause of tissue damage in the ischemia/reperfusion model. However, they do not exclude the physiological role of glutaredoxin1 in other models of oxidant-mediated tissue damage such as in UVR-B induced cataractogenesis.

4.4. GSH/GSSG content

The non-significant effect of UVR-B on whole lens GSH/GSSG content is probably due to the limitation of the oxidative insult to the anterior cortical region. It is known that UVR 300 nm is attenuated very close to the anterior lens surface (Löfgren and Söderberg, 2001) which correlates well with the anterior sub-capsular changes seen in the UVR-B exposed $Glrx^{+/+}$ and $Glrx^{-/-}$ lenses. Since we analyzed homogenates of whole lenses, the GSH/GSSG levels in the unharmed nucleus and posterior cortex might have masked changes in the anterior sub-capsular region. Based on recent findings by Löfgren et al. GSH levels should be expected to be lower in $Glrx^{-/-}$ lens (Löfgren et al., 2008) than in $Glrx^{+/+}$ lenses.

The impact of glutaredoxin1 deficiency on the sensitivity of the lens to *in vivo* UVR-B exposure reported here is to our knowledge the first evidence that the absence of an antioxidative enzyme in the lens exacerbates *in vivo* UVR-B cataract development. The work of Wolf and coworkers supports the relevance of ROS detoxifying enzymes such as glutathione peroxidase in the progression and severity of age-related cataract in different mouse models (Wolf et al., 2005). We would like to stress though, that the effect of glutaredoxin1 deficiency on *in vivo* cataractogenesis does not eliminate or question the importance of specific non-mutated genes whose alleles do not code for anti-oxidants and that have been shown to influence age-related cataract in mice and humans (Hammond, 2000; Hammond et al., 2001). Rather the interrelation of both or further predispositions might influence cataract incidence.

5. Summary

We were able to demonstrate that deficiency of the *Glrx* gene increases the lenticular sensitivity to *in vivo* UVR-B exposure. We were further able to quantify the protection associated with the presence of the *Glrx* gene against *in vivo* UVR-B induced light scattering in the lens. These findings demonstrate that glutaredoxin1 plays a key role in the protection of lens proteins from acute oxidative stress induced by UVR-B in the mouse. Our findings implicate that glutaredoxin1 deficiency may have an impact on a long-term low dose repeated daily exposure of the lens to UVR-B. Thus glutaredoxin1 polymorphisms may cause a varying degree of lens sensitivity to age-related cataract associated oxidative stress.

Acknowledgements

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Appendix

Derivation of Glrx protection

If the dose-response function for light scattering induced by *in vivo* exposure to UVR-B (Fig. 6) is analyzed, it is found that an arbitrary dose increase, ΔH, induces a larger damage increase in $Glrx^{-/-}$ lenses, $\Delta D_{Glrx-/-}$, than the corresponding damage increase in $Glrx^{+/+}$ lenses, $\Delta D_{Glrx+/+}$. (Fig. 7).

The *protection*, ρ, of the *Glrx* gene in the lens against biological damage caused by *in vivo* UVR-B exposure can be expressed as the difference between damage increase without the gene, $\Delta D_{Glrx-/-}$, and damage increase with the gene, $\Delta D_{Glrx+/+}$, related to the damage increase without the gene, for an arbitrary dose increase, ΔH (Equation (2))

$$\rho = \frac{\Delta D_{Glrx-/-} - \Delta D_{Glrx+/+}}{\Delta D_{Glrx-/-}} \tag{2}$$

Applying algebra to Fig. 7, it is seen that the *protection* can alternatively be expressed as the ratio of the difference between the sensitivity for $Glrx^{-/-}$, $k_{Glrx-/-}$, and the sensitivity for *Glrx*+/+, $k_{Glrx+/+}$, related to the sensitivity for $Glrx^{-/-}$ (Equation (3)).

$$\rho = \frac{k_{Glrx-/-} - k_{Glrx+/+}}{k_{Glrx-/-}} \tag{3}$$

Applying Equation (1), the *protection* of the *Glrx* gene for *in vivo* close to threshold UVR damage in the lens is estimated to 44% from the sensitivities derived by best fits to the model in Equation (1).

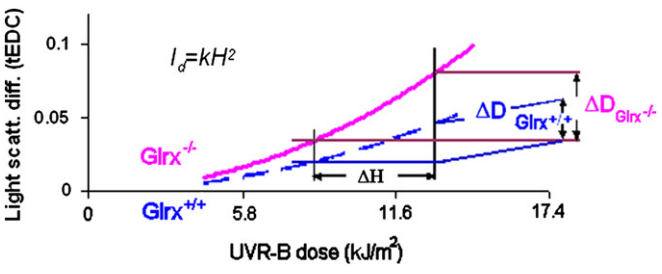

Fig. 7. Derivation of *Glrx protection*. Dose-response function for $Glrx^{-/-}$ (continuous line) and $Glrx^{+/+}$ (interrupted line) mouse lenses. Response is given as paired difference in light scattering between exposed and contralateral lens. The model, $I_d = kH^2$, represent increase of damage, $\Delta D_{Glrx-/-}$ and $\Delta D_{Glrx+/+}$ caused by the arbitrary dose increase ΔH in $Glrx^{-/-}$ and $Glrx^{+/+}$ lenses, respectively.

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