Metabolic Changes in Rat Lens after In Vivo Exposure to Ultraviolet Irradiation: Measurements by High Resolution MAS ^{1}H NMR Spectroscopy

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Purpose. In the present study, high-resolution magic angle spinning proton nuclear magnetic resonance (HR-MAS ^{1}H NMR) spectroscopy was used to investigate changes in the metabolic profile of intact rat lenses after UVB irradiation of the eyes.

Methods. Three groups of Sprague-Dawley rats were exposed to UVB radiation at 2.5, 5.0, and 7.5 kJ/m^2. One eye was exposed, and the contralateral eye served as the control. One week after exposure, the lenses were removed and forward light-scattering was quantified. Thereafter, proton NMR spectra from the intact lenses were obtained. Relative changes in metabolite concentrations were determined.

Results. The lenses in all three groups showed significant increases in light-scattering after UVB irradiation. The high-quality HR-MAS ^{1}H NMR spectra permitted more than 30 different metabolites to be identified. UVB irradiation caused a significant decrease ($P < 0.05$) in concentrations of taurine, hypotaurine, tyrosine, phenylalanine, valine, *myo*-inositol, phosphocholine, betaine, succinate, and glutathione at all three UV doses. For glycine, glutamate, and lactate, significant decreases in concentration were observed at the two lowest UVR-B doses. The total amount of adenosine tri- and diphosphate and (ATP, ADP) decreased significantly and that of adenosine monophosphate AMP increased significantly at the two highest doses. Alanine was the only amino acid that increased after UVB irradiation. None of these metabolites exhibited a significant UVB dose-dependent relationship.

Conclusions. This study demonstrates for the first time the potential of HR-MAS ^{1}H NMR spectroscopy as an analytical tool for use on intact lenses. Near-threshold UVR-B doses led to a generally significant decrease in water-soluble metabolites 1 week after exposure. The lack of dose-dependent changes in the metabolites indicates that repair processes during the first week after UVB irradiation overcome the immediate metabolic disturbances. (*Invest Ophthalmol Vis Sci.* 2004;45:1916–1921) DOI:10.1167/iovs.03-1292

Ultraviolet radiation (UVR) damages the lens by several mechanisms, leading to formation of protein cross-linking, damage to the membrane transport system, and changes in cellular DNA. These alterations have a major impact on metabolic pathways in the lens.

Generation of reactive oxygen species ($^1O_2$, $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$) is a well-documented route for UVR damage.[1,2] Increased levels of oxidants disturb the fine balance between generation of free radicals and their elimination by the oxidant scavengers. This in turn may lead to further alterations of the biological processes in the lens. As shown in many studies, significant changes in enzyme activities and metabolic concentrations have been detected after UVR exposure.[3–6] Disruption of epithelial membrane proteins after UVR exposure induces unbalanced ion concentrations, which causes swelling and osmotic stress.[7] The lens epithelial cells also show swollen mitochondria, subcapsular vacuoles, and nuclear fragmentation after UV irradiation.[8] The wavelength range shown to be most harmful to the lens is 300 to 305 nm,[9,10] in the middle of the UVR-B range.

Despite the many experimental studies of UVR and cataract development, the metabolic changes involved in this process are by no means clear. Knowledge of this may contribute to our understanding of the mechanisms of UVR on lenticular opacity formation.

The purpose of this study was to investigate changes in the metabolic profile of rat lenses after UVR exposure using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Previous studies on rats[11] documented a dose–response relationship between UVR exposure and subsequent lens opacities. It has been shown that forward light-scattering in the rat lens increases exponentially with increased UVR dose between 0.1 and 14 kJ/m^2.[12] At present, the protection criteria for UVR-induced cataract formation are based on threshold levels. In the rabbit and rat, a threshold dose of 5.0 kJ/m^2 for UVR-B–induced cataract has been determined.[9,12] The assumption is that UVR doses above threshold give more intense reaction than those below threshold and cause permanent damage to the lens. Long-term exposure to low-dose UVR, however, is considered a risk factor in humans. For comparison it has been calculated that, with the sun in zenith, an outdoor worker would receive an ocular UVR-B dose of 3.0 kJ/m^2 during 75 hours.[12] The main focus in this study has been directed toward the effect of close-to-threshold UVR-B doses on the lens metabolism 1 week after the exposure.

Our laboratory has found ^{1}H NMR spectroscopy to be very useful in the monitoring of metabolic profiles in tissue extracts from the anterior eye segment.[13,14] Extensive biochemical sample preparations with tissue extraction require relatively large amounts of biological tissue, are time consuming, and may change the chemical composition of the samples.

Thus, to avoid the extraction procedure, application of high-resolution magic angle spinning (HR-MAS) ^{1}H NMR spectroscopy was considered a suitable, nondestructive technique. According to the results reported in other studies[15–19] this technique has the potential to produce high-resolution spectra from small unprocessed tissue samples and intact cells. Because of its qualities HR-MAS ^{1}H NMR spectroscopy has shown an increasing impact in screening the multiparametric meta-

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bolic response of living systems to pathophysiological stimuli or genetic modification, and it enables new bridges to be constructed between tissue biochemistry and conventional histopathology.[20]

In this study HR-MAS ^{1}H NMR spectroscopy has been applied to monitor metabolic changes in the rat lens induced by exposure to different UVR-B doses.

Materials and Methods

UVB Exposure

Six-week-old female outbred albino Sprague-Dawley rats (n = 31, weight approximately 150 g) were anesthetized with 11 mg/kg xylazine (Rompun Veterinary; Bayer Pharma AB, Göteborg, Sweden) and 80 mg/kg ketamine (Ketalar; Parke-Davis Scandinavia AB, Solna, Sweden) intraperitoneally. Before irradiation 1 drop of tropicamide (Mydriacyl 0.5%, Alcon Sverige AB, Stockholm, Sweden) was instilled in both eyes to dilate the pupils. The animals were divided into three groups. In each group, one eye of each animal was exposed to UV radiation. The doses were, 2.5 (n = 9), 5.0 (n = 11), and 7.5 (n = 8) kJ/m^2, measured in the corneal plane (peak wavelength 302.6 nm, 6.4 nm full-width at half maximum, and exposure time 15 minutes). The UVR-B source was a 300-W high-pressure mercury lamp (Oriel, Stratford, CT) equipped with water filter, double monochromator (set to λ_{MAX} 300 nm, 10 nm full width at half maximum) and collimating optics. The irradiance was measured with a thermopile calibrated by the Swedish National Bureau of Standards. The contralateral eye served as a nonexposed control. All animals were kept and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Lens Forward Light-Scattering

One week after exposure the rats were killed by CO_2 asphyxiation, the eyes enucleated, and the lenses dissected from remnants of ciliary body and zonular fibers. The isolated lenses were then put in room-tempered physiologic saline solution (Balanced Salt Solution, BSS) and photographed, and the lens forward light-scattering was measured with a light-dissemination meter.[12] The scattering standard was a lipid emulsion of diazepam (Diazemuls; KabiVitrum, Stockholm, Sweden), and the unit was therefore expressed as transformed equivalent diazepam concentration ($_t$EDC). Finally, the wet weight of the lenses was determined, and the samples were frozen and stored at −80°C before NMR spectroscopy.

HR-MAS ^{1}H NMR Spectroscopy

HR-MAS ^{1}H NMR spectroscopy was performed on a spectrometer (Avance DRX600, 14.1-T; Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 600 MHz for protons. The spectra were recorded at room temperature with a 4-mm HR-MAS ^{1}H/^{13}C probe. The lenses were placed in a Zirconia 4-mm diameter rotor (92 μL) and deuterium oxide (D_2O) containing 0.625 mM sodium-3′-trimethylsilylpropionate-2,2,3,3-d$_4$ (TSP) was added. Spinning rate was 8 kHz. A Carr-Purcell-Meiboom-Gill spin-echo pulse sequence [90° - (τ - 180° - τ)$_n$ - acquisition] was used as a T_2 filter to attenuate resonances from lipids and macromolecules.[21] The interpulse delay (τ) was 1 ms, and the value of n was 72 ($2n\tau$ = 144 ms). Acquisition time was 2.0 seconds and repetition delay 5.0 seconds. The number of scans was 512. Water suppression was achieved with a presaturation selective pulse. Exponential line broadening of 1.0 Hz was used. Chemical shifts were measured relative to TSP at 0 ppm. Analysis of the spectra was performed with special software for analysis of complex mixtures (AMIX; Bruker BioSpin GmbH). Peak areas were measured, using the noise region (0.4–0.7 ppm high field, 9.6–9.8 ppm low field) as an internal standard, and the peak areas were related to the lens wet weight. The identity of samples during analysis was unknown to the spectroscopist.

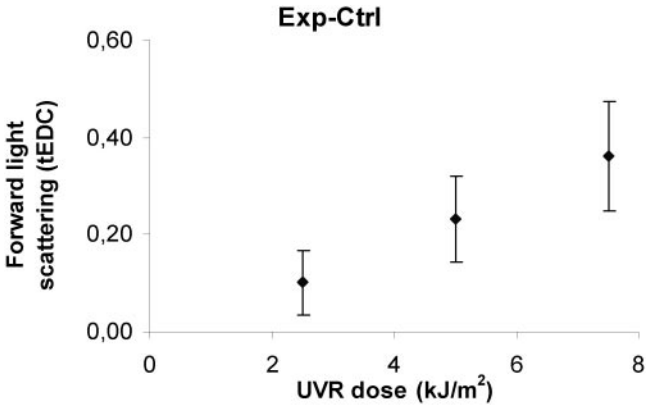

Figure 1. Difference in intensity of forward light-scattering between exposed and nonexposed rat lenses 1 week after a UV dose of 2.5 (n = 8), 5.0 (n = 11), or 7.5 (n = 8) kJ/m^2. The bars represent 95% confidence intervals for the paired-sample mean differences. $_t$EDC represents the transformed equivalent diazepam concentration.[12]

Data Analysis

For each animal, the metabolite concentrations in the exposed eye were compared with the concentrations in the lateral unexposed eye, and relative changes were calculated. Statistical analysis of the calculated changes were performed with significant level set to 5%, and mean values were expressed with 95% confidence intervals. One-way ANOVA was used to analyze the UVR-B dose dependency on the concentration change of the metabolites. Significant results ($P < 0.05$) were further tested by the Bonferroni multiple comparison test to identify the differing group.

Results

One week after UVR-B exposure of 2.5, 5.0, or 7.5 kJ/m^2, all three groups of exposed lenses showed a significant increase in light-scattering compared with nonexposed lenses (Fig. 1). The mean forward light-scattering increased with increasing UVR dose.

The HR-MAS ^{1}H NMR spectra obtained from intact rat lenses were of high-resolution quality comparable to those obtained from lens tissue extracts in previous experiments.[13,14] Assignment of peaks was based on two-dimensional ^{1}H-^{1}H correlation spectroscopy (COSY) and two-dimensional J-resolved ^{1}H NMR spectroscopy, combined with reported data from the literature.[13,19,22,23] It was possible to identify more than 30 different metabolites in the spectra. A representative HR-MAS ^{1}H NMR spectrum of rat lens tissue with assignments of metabolites is shown in Figure 2.

In some regions of the spectra, several metabolites overlapped each other. Thus, only a selected group of the metabolites could be used for quantitative analysis. For some other metabolites such as tryptophan (7.73 ppm) and fumarate (6.51 ppm), the signal-to-noise ratio in the spectra was too low to extract reliable information.

Eight different amino acids and derivatives were analyzed separately. Differences in mean relative concentrations of the amino acids determined in the exposed and nonexposed lenses are given in Figure 3. As shown in this figure, with the exception of alanine, the concentrations of all amino acids showed a decrease after exposure to UVB light. For taurine, hypotaurine, tyrosine, phenylalanine, and valine this decrease was significant for all three UVR-B doses ($P < 0.05$). For glycine and glutamate, the change in concentration was only significant for 2.5 and 5.0 kJ/m^2. Alanine was the only amino acid that increased in concentration after UVB irradiation.

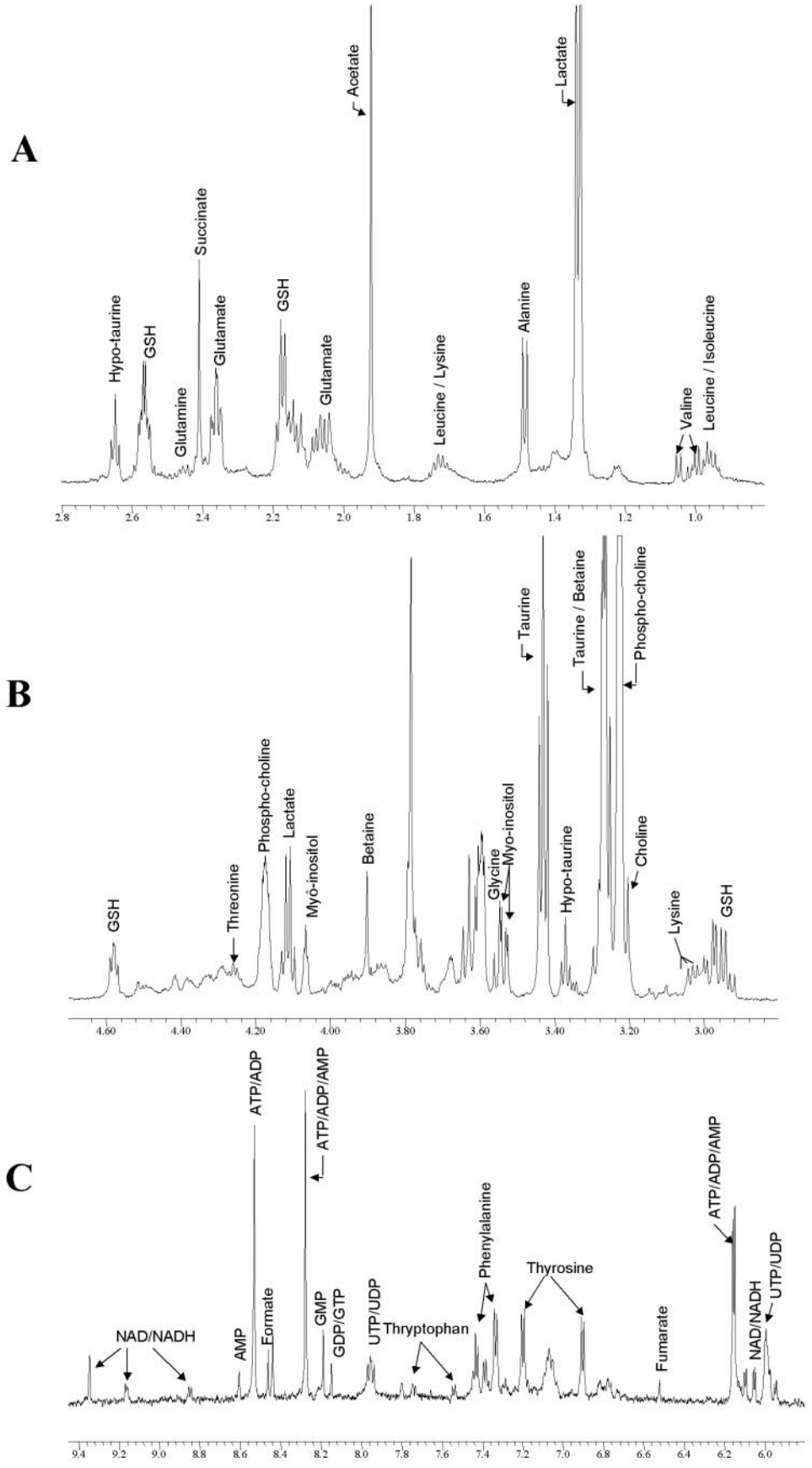

FIGURE 2. Representative metabolic profile of a 600-MHz HR-MAS ^{1}H NMR spectrum from an intact rat lens (control). (**A**) High-field, (**B**) middle-field, and (**C**) low-field region of the obtained spectrum. The units were assigned with TSP used as the reference substance at 0 ppm. GSH, reduced glutathione; NAD/NADH, nicotine adenine dinucleotide; GTP, GDP, GMP, guanosine tri-, di-, monophosphate, respectively; UTP, UDP, uridine tri- and diphosphate, respectively.

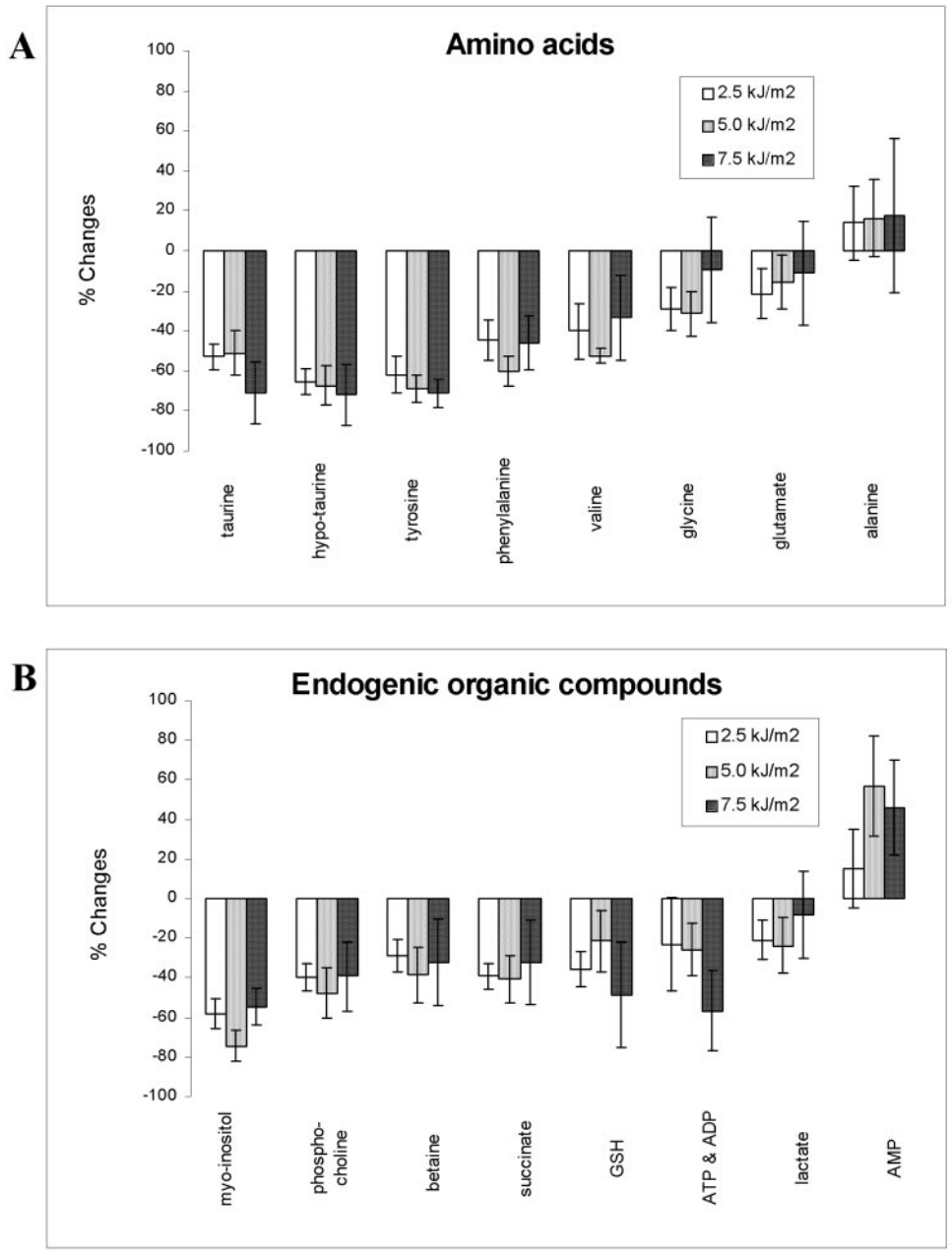

FIGURE 3. Relative differences in metabolite concentrations between exposed and nonexposed contralateral rat lenses 1 week after a UVB dose of 2.5 ($n = 7$), 5.0 ($n = 8$), and 7.5 ($n = 6$) kJ/m^2, respectively. Data were calculated as (exposed lens − control lens)/control lens. (**A**) Relative changes of detectable amino acids in the NMR spectra. (**B**) Relative changes of other quantifiable metabolites visible in the NMR spectra. The bars represent 95% confidence intervals for the mean differences.

In addition to the amino acids, eight other organic metabolites were analyzed. As shown in Figure 3, the concentration of *myo*-inositol, phosphocholine, betaine, succinate, and reduced glutathione (GSH) decreased significantly. *Myo*-inositol showed the most pronounced reduction in concentration (70%–80%) compared with unexposed lenses.

It was not possible to separate adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the spectra; thus, they had to be measured together at 8.54 ppm. The results showed a significant decrease in ATP and ADP at the two highest UVR doses. Adenosine monophosphate (AMP) was measured as one separate peak at 8.60 ppm, and was, like alanine, the only metabolite increasing in concentration after UVR exposure. The increase of AMP was significant, however, only at the two higher UVR doses. Only a small nonsignificant decrease in the lactate level was observed.

Statistical analysis (ANOVA) did not reveal any significant dose–response relationship for the examined metabolites except for *myo*-inositol, showing a significant increase in the concentration difference for UVR doses from 2.5 to 5.0 kJ/m^2 ($P = 0.029$), and a significant decrease in concentration difference for UVR doses from 5.0 to 7.5 kJ/m^2 ($P = 0.01$).

DISCUSSION

To our knowledge, this is the first time HR-MAS ^{1}H NMR spectroscopy is used to investigate the metabolic profile directly in the lens. An advantage of this method is avoidance of time-consuming extraction processes, which may change the metabolic composition of the samples. The obtained spectra revealed a multitude of sharp NMR peaks providing information on the identity and concentrations of many metabolites in the lens. In this way, the method has the potential to reveal biochemical changes in the lens occurring as a result of diseases, toxic effects, trauma, or other factors.

For quantification purposes, it was difficult to use conventional methods with TSP as an internal standard.[24] First, the volume of standard solution added to each lens in the MAS rotor varied because of variations in the individual lens vol-

umes. Second, TSP possesses an aliphatic short chain that can bind to serum albumin.[25] Thus, binding to similar proteins in the lens tissue may occur.

Measuring peak intensities versus noise ratios was found to be a more suitable method to avoid these problems.[26] When samples were processed under identical conditions, this method was found to be reliable for relative quantitative assessments. However, some of the metabolites identified in the spectra could not be precisely quantified due to overlap with other compounds.

The dose–response relationship between UVB irradiation and subsequent lens opacities was quantified in this study (Fig. 1) and was found to be in agreement with the results presented in previous studies by Söderberg et al.[11] However, the observed UVB effect on the lens transparency does not seem to evoke a similar dose–response relationship in the metabolite concentrations (Fig. 3). It is possible that the influence of UVB irradiation on the metabolic state of the lens is more profound immediately after the exposure and then starts to average during the first week of the repairing processes. As shown in other studies,[27,28] the intensity of light-scattering in the lens increases exponentially during the first week after UVB exposure. Thereafter, only small or no changes are observed during the next week and up to 32 weeks after exposure.

Threshold exposure to UVB irradiation induces apoptosis in the lens epithelium.[29] Seven days after exposure, the dead cells are replaced and leave extracellular spaces and superficial fibers in the epithelium and outer lens cortex.[30] This process may lead to disturbance of the water balance in the lens. For near-threshold doses, a decrease in water content has been reported and found to be the same at the 3-, 5-, and 8-kJ/m^2 UVR-B dosages 1 week after irradiation.[31] Our findings revealed a general decrease in water-soluble metabolites without the observed dose–response relationship for similar UVR doses. This may indicate a leakage of water-soluble metabolites from the lens, due to membrane damage caused by UVB irradiation.

Experiments performed by Hightower et al.[32] have shown that UVR damages the lens epithelium and induces changes in membrane permeability and loss of ion homeostasis, which further leads to osmotic stress. As shown in an earlier study, near-threshold UVR doses (3–8 kJ/m^2) lead to a decrease in water content in the lens, whereas higher UV doses lead to swelling.[31] A general decrease in the lens water content may affect osmolytes the most. In the lens, particularly taurine and *myo*-inositol are involved in the osmotic regulation.[33,34] This may explain why these two metabolites and also hypotaurine, a taurine precursor, are among the substances with the tendency to decrease the most after UVB irradiation.

An interesting observation made in this study is the increase of the alanine concentration observed after UVB irradiation at all three doses. In some studies, elevation of alanine concentrations has been reported, as in plasma of patients with presenile cataract[35] and in the aqueous humor of cataractous Wistar rats.[36] However, this is, to our knowledge, the first time an increase of alanine concentration in a cataractous lens has been reported. van Heyningen[37,38] has shown that alanine is one of the nonprotein oxidation products of tryptophan present in the human lens. Moreover, Ansari et al.[39] report that in vitro UV irradiation of phenylalanine in the presence of hydrogen peroxide, forms appreciable amounts of alanine. In this study, a simultaneous decrease of phenylalanine accompanied by an increase of alanine concentration has been observed, which may support this explanation.

Glutathione (GSH) is present in relatively high concentrations in the lens and is involved in protection from oxidative damage,[40] amino acid, and cation transport across the cell membrane.[41,42] After UVB irradiation, GSH has been found to decrease rapidly in the epithelium and more slowly in the underlying lens fibers.[6] In this study, the whole lens was analyzed and a significant decrease in GSH in the range of 20% to 50% was observed after the UVB irradiation. Earlier work observed that, under oxidative stress, the GSH level in the lens was stabilizing 15% to 20% below the normal concentration.[40] No increase in oxidized glutathione (GSSG) was observed in the present work. In fact, GSSG was not detectable in any group, perhaps because the oxidation process forms mainly protein-linked GSSG, which was not possible to detect by the technique used in this study.

Löfgren and Söderberg[4] have reported that after UVB irradiation lenticular lactate dehydrogenase (LDH) activity decreases only in the anterior, not in the posterior, part of the lens. Further studies have shown an intralenticular accumulation of lactate concentrations 6 hours after exposure.[43] In the present study, the whole lens was analyzed, and thus a possible decrease of lactate concentration in the anterior part of the lens could have been masked by the accumulation of lactate in the lens cortex.

Regarding energy metabolism, a reduction in the high-energy phosphates ATP and ADP, combined with an increase in the low-energy phosphate AMP, was observed as a consequence of UVB irradiation. High-energy phosphates are highly abundant in the lens epithelium. Damage of epithelial cells results in increased energy demands, because of the repair processes in the lens after UVB irradiation. A similar response in energy metabolism in the lens has been reported for other experimental stressors such as calcium deprivation,[44] steroid treatment,[45,46] and galactose-induced cataract.[47]

In summary, this study revealed significant changes in the metabolic profile of intact rat lenses 1 week after UVB irradiation. No clear dose–response relationship in metabolic changes was observed in the 2.5- to 7.5-kJ/m^2 UVR-B dose range. This may indicate that the repair processes occurring during the first week after UVB irradiation are sufficiently effective to overcome the immediate metabolic disturbances.

In general, the significant decrease in water-soluble metabolites observed in this study, may indicate a metabolite efflux from the lens induced by UVB-irradiation. Osmolytes such as taurine, hypotaurine, and *myo*-inositol are among the metabolites with the largest concentration decrease. That some metabolites decreased more than others implicates their different role in the protection of the lens against UVB irradiation and illustrates the complexity of this process and the time-dependent changes in the metabolic profile after UVB irradiation. Especially interesting was the tendency of alanine concentrations to increase. This, to our knowledge, is the first time such a trend has been observed in the lens.

The present study has also shown the potential of using HR-MAS ^{1}H NMR spectroscopy as an analytical tool on intact lenses. The NMR spectra provided information on the identity and concentrations of many metabolites in the lens, contributing thereby to a more complete picture of the influence of UVR irradiation on the lens.

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