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Time dependency of metabolic changes in rat lens after in vivo UVB irradiation analysed by HR-MAS ^{1}H NMR spectroscopy

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Abstract

The lens ability to protect against, and repair ultraviolet radiation (UVR) induced damages, is of crucial importance to avoid cataract development. The influence of UVR-induced damage and repair processes on the lens metabolites are not fully understood. Observation of short- and long-term changes in light scattering and the metabolic profile of pigmented rat lenses after threshold UVR exposure might serve to better understand the protective mechanisms in the lens. By using high resolution magic angle spinning (HR-MAS) ^{1}H NMR spectroscopy it was possible to investigate the metabolites of intact rat lenses.

Brown-Norway rats were exposed to 15 kJ m^{-2} UVB irradiation. One eye was exposed and the contralateral served as control. The rats were sacrificed 5, 25, 125, and 625 hr post-exposure and the lenses were removed. The degree of cataract was quantified by measurement of lens forward light scattering. Thereafter, proton NMR spectra from intact lenses were obtained and relative changes in metabolite concentrations were determined.

The light scattering in the lens peaked at 25 hr post-exposure and decreased thereafter. The lowest level of light scattering was measured 625 hr after exposure. No significant changes in concentration were observed for the metabolites 5 and 25 hr post-exposure except the total amount of adenosine tri- and diphosphate (ATP/ADP) that showed a significant decrease already 5 hr after exposure. At 125 hr the lens concentrations of lactate, succinate, phospho-choline, taurine, betaine, myo-inositol, and ATP/ADP showed a significant decrease ($p<0.05$). Phenylalanine was the only metabolite that revealed a significant increase 125 hr post-exposure. At 625 hr most of the metabolic changes seemed to normalise back to control levels. However, the concentration of betaine and phospho-choline were still showing a significant decrease 625 hr after UVB irradiation.

The impact of UVB irradiation on the metabolic profile did not follow the same time dependency as the development of cataract. While the light scattering peaked at 25 hr post-exposure, significant changes in the endogenous metabolites were observed after 125 hr. Both the metabolic changes and the light scattering seemed to average back to normal within a month after exposure. Significant decrease in osmolytes like taurine, myo-inositol and betaine indicated osmotic stress and loss of homeostasis. This study also demonstrated that HR-MAS ^{1}H NMR spectroscopy provides high quality spectra of intact lenses. These spectra contain a variety of information that might contribute to a better understanding of the metabolic response to drugs or endogenous stimuli like UVB irradiation.

Keywords: cataract; UVB; HR-MAS; NMR; metabolites; lens; osmolytes; spectroscopy

1. Introduction

The cataractogenic effect of ultraviolet radiation (UVR) has been known since the beginning of the 20th century (Widmark, 1901). In addition, several epidemiological studies have implicated UVR as one of the environmental factors in human cataract formation (Zigman et al., 1979; Taylor et al., 1988; Cruickshanks et al., 1992; West et al., 1998). Considerable effort in clarifying the biochemical

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mechanisms in the lens tissue caused by UVR exposure has been undertaken, in hopes to avoid or delay the progression of lens opacification.

UVR damage in the lens is complex. Absorbed UVR photons excite lens molecules and create free radicals which increase the oxidative stress on the lens (Spector, 1995; Rose et al., 1998). This induces changes varying from modulated DNA synthesis (Andley et al., 1996), loss of ion homeostasis (Hightower et al., 1999) and accumulation of chromophores (Truscott et al., 1994; Dillon et al., 1999), to crystalline aggregation (Andley and Clark, 1989) and membrane damage (Kochevar, 1990; Hightower et al., 1994a). In the end, UV irradiation might lead to epithelial cell death (Li and Spector, 1996; Michael et al., 1998a). Despite the impressive research effort the metabolic changes involved in these processes are by no means clear.

Severity of the lens damage is dependent on the UVR dose and it is assumed that UVR doses above a certain threshold level cause permanent damages to the lens (Pitts et al., 1977). Previous studies of lens damage after in vivo exposure to threshold doses of UVB irradiation (280–315 nm) found that the spatial organisation of lens fibres was largely reversible 8 weeks after exposure (Michael et al., 2000). However, studies on morphological events alone are not enough to understand the biochemical mechanisms of the repairing processes. Ayala et al. (2000) have suggested that opacities might develop due to an imbalance between damage and repair mechanisms in the lens. Results from our laboratory indicated that time dependent changes in the water-soluble metabolites might differ from the light scattering changes in the lens caused by UVB irradiation (Risa et al., 2004). Investigation of the metabolic profile of the lens tissue under normal and cataractous conditions might contribute to our understanding of how the metabolism is influenced by UVB irradiation.

The aim of this study was to investigate the time dependency of metabolic changes after UVB irradiation by using nuclear magnetic resonance (NMR) spectroscopy.

During the last two decades NMR spectroscopy has proven to be a valuable tool for screening the metabolic profile in both tissue extracts and intact tissues. Thus, new bridges have been constructed between biochemistry and conventional histopathology (Lindon et al., 2003). Phosphorus-31 and proton NMR are the most common nuclei used in biological investigations. Several studies using ^{31}P or ^{1}H NMR spectroscopy have been performed on lens extracts (Meneses et al., 1990; Greiner et al., 1994; Midelfart et al., 1996; Risa et al., 2002).

Until recently only ^{31}P NMR spectroscopy has been found useful studying the metabolic profile of intact lens tissue (Greiner et al., 1981; Kopp et al., 1981). However, new approaches by using high resolution magic angle spinning (HR-MAS) NMR spectroscopy have made it possible to obtain high quality ^{1}H NMR spectra of intact rat lens tissue (Risa et al., 2004). The principle of this method is that line broadening effects from dipolar couplings and chemical shift anisotropy are averaged to zero by rapid spinning of the sample (typically ~4–8 kHz) at an angle of 54.7° relative to the static magnetic field (the magic angle). This non-destructive technique omits the time consuming extraction procedures that require relatively large amounts of tissue and might change the chemical composition of the samples. In comparison to ^{31}P NMR spectroscopy, HR-MAS ^{1}H NMR spectroscopy is more sensitive and can detect many more metabolites due to the natural abundance of proton in almost all metabolites.

In this study HR-MAS ^{1}H NMR spectroscopy was used to elucidate short- and long-term changes in the metabolic profile of pigmented rat lenses after moderate UVB irradiation. This might serve to a better understanding of the repairing mechanisms in the lens after UVR exposure.

2. Material and methods

2.1. Animal experiments

Forty-seven 6-week-old Norwegian Brown rats were anesthetised with 45 mg kg^{-1} ketamine (Ketalar, Parke-Davis, Sweden) and 10 mg kg^{-1} xylazine (Rompun Vet., Bayer AB Sweden) intraperitoneally. Before irradiation pupils were dilated bilaterally with 1% tropicamide (Mydriacyl, Alcon Sverige AB, Sweden). After another 10 min, the eyes were unilaterally exposed to 15 kJ m^{-2} UVB radiation, peak wavelength 302.6 nm, for 15 min. For more detailed description see Michael et al. (1996). The rats were sacrificed by CO_2 asphyxiation 5, 25, 125 and 625 hr after UVB exposure. Both eyes were enucleated and the lenses were dissected free from remnants of ciliary body, zonular fibres and vitreous. The isolated lenses were then put in room tempered Balanced Salt Solution (BSS, Alcon), photographed, and lens forward light scattering was quantified by the technique described previously (Söderberg et al., 1990). The samples were finally frozen and stored at −80°C before NMR spectroscopy. All animals were kept and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. NMR spectroscopy

HR-MAS ^{1}H NMR spectroscopy was performed on a BRUKER Avance DRX600 spectrometer (14.1 T, Bruker BioSpin GmbH, Germany) operating at 600 MHz for protons. The spectra were recorded at 4°C using a 4 mm HR-MAS ^{1}H/^{13}C probe.

The temperature was calibrated using glucose as an internal thermometer (Nicholls and Mortishire-Smith, 2001). While still frozen, the lens sample was immersed in D_2O, containing 0.625 mM sodium-3′-trimethylsilylpropionate-2,2,3,3-d_4 (TSP), in a zirconium 4 mm diameter HR-MAS rotor (92 μl). TSP was used as an internal shift

reference substance (0 ppm). Samples were spun at 5000 Hz.

Proton spectra were obtained using a one-dimensional T_2-filtered sequence [90°-(τ-180°-τ)$_n$-acquisition] (Carr–Purcell–Meiboom–Gill spin-echo pulse sequence, CPMG) (Meiboom and Gill, 1958) to suppress signals from lipids and macromolecules. The inter pulse delay τ was 1 msec and the number of loops n was 72 (giving an effective echo time of $2n\tau = 144$ msec). Spectral region was 12 ppm with 32K data points. Acquisition time was 2.28 sec and water presaturation was done with a single rectangular selective pulse during a repetition delay of 3.1 sec. Number of scans was 512. Zero-filling to 64K was used and exponential line broadening of 1.0 Hz. For peak identification purposes, two-dimensional spectra such as gradient selected homonuclear shift correlated (gs-COSY) and J-resolved spectra were acquired, all under MAS conditions.

Analysis of the spectra was done with special software for analysis of complex mixtures (AMIX, Bruker BioSpin). The spectral region 0.8–10 ppm was reduced to a resolution of 0.001 ppm per point ('bucket' width). By performing a data reduction on the NMR spectra it was possible to summarise and compare sums of buckets over the exact same peak regions in each spectrum. The water peak between 4.7 and 6.3 ppm, and the region between 2.6 and 3.3 ppm, were eliminated from the data reduction. The latter region was excluded due to peak overlap that made it impossible to extract quantitative information on the metabolite concentrations. Peak areas were measured using the noise region (0.3–0.55 ppm) as an internal standard. The identity of samples during analysis was unknown to the spectroscopist.

2.3. Data analysis

Metabolite concentrations in the exposed groups were calculated relative to the levels in the control groups after normalisation (integrated NMR peak per sample wet weight). The level of significance was set to 5%, and mean values were expressed with 95% confidence intervals. ANOVA was used to analyse the time dependency on the concentration change of the metabolites.

3. Results

A significant increase ($p < 0.05$) in light scattering was observed for all UVB exposed lenses. The lens light scattering peaked at 25 hr post-exposure and decreased thereafter (Fig. 1). The lowest level of light scattering was measured 625 hr after exposure. The severity and distribution of the cataract were similar among the exposed lenses at each time point, with mainly anterior subcapsular cataract and equatorial cataract.

The HR-MAS ^{1}H NMR spectra obtained from intact rat lenses were of high resolution quality comparable to those

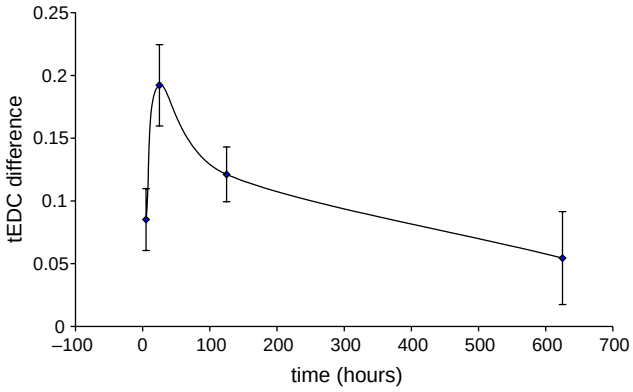

Fig. 1. Difference in intensity of forward light scattering between exposed and non-exposed rat lenses 5 ($n = 12$), 25 ($n = 12$), 125 ($n = 12$) and 625 ($n = 11$) hr after UVB irradiation. The UVRB dose was 15 kJ m^{-2}. The bars represent 95% confidence intervals for the paired-sample mean differences. tEDC represents the transformed equivalent diazepam concentration. (Michael et al., 1998b)

obtained in previous experiments with intact lens tissues (Risa et al., 2004) and tissue extracts (Risa et al., 2002). A representative reduced NMR spectrum (bucket width 0.001 ppm) of a control lens with peak assignments of more than 25 different metabolites is presented in Fig. 2. Due to overlap and insufficient signal-to-noise ratio, some of the assigned metabolites could not be quantified. Fourteen different metabolites were found suitable for quantitative analysis. These were lactate, taurine, myo-inositol, betaine, phospho-choline (p-choline), reduced glutathione (GSH), succinate, glycine, glutamate, tyrosine, valine, alanine, phenylalanine, and the total amount of adenosine tri- and diphosphate (ATP/ADP). The measured peak regions for the respective metabolites are illustrated as dark areas in Fig. 2.

Mean values of the relative concentration differences of the metabolites between exposed and non-exposed lenses were calculated at 5, 25, 125, and 625 hr post-exposure. The results are given in Fig. 3. As shown in this figure no significant changes in concentration were observed for any metabolite at 5 and 25 hr post-exposure with exception of ATP/ADP showing a significant decrease already 5 hr after exposure. At 125 hr, the lens concentrations of lactate, succinate, p-choline, taurine, betaine, myo-inositol, and ATP/ADP showed a significant decrease ($p < 0.05$). The same tendency was observed for glycine and GSH but without reaching a significant level. Valine, alanine and phenylalanine peaked at 125 hr, with phenylalanine increasing significantly. Tyrosine concentration was slightly increased at all post-exposure observation points ($p > 0.05$). The concentration of glutamate had an indicative peak at 25 hr post-exposure, but the following changes were not significantly different from controls for any of the observed time-points after UVB irradiation. At 625 hr most of the metabolic changes seemed to normalise back to near-control values. However, the concentrations of betaine

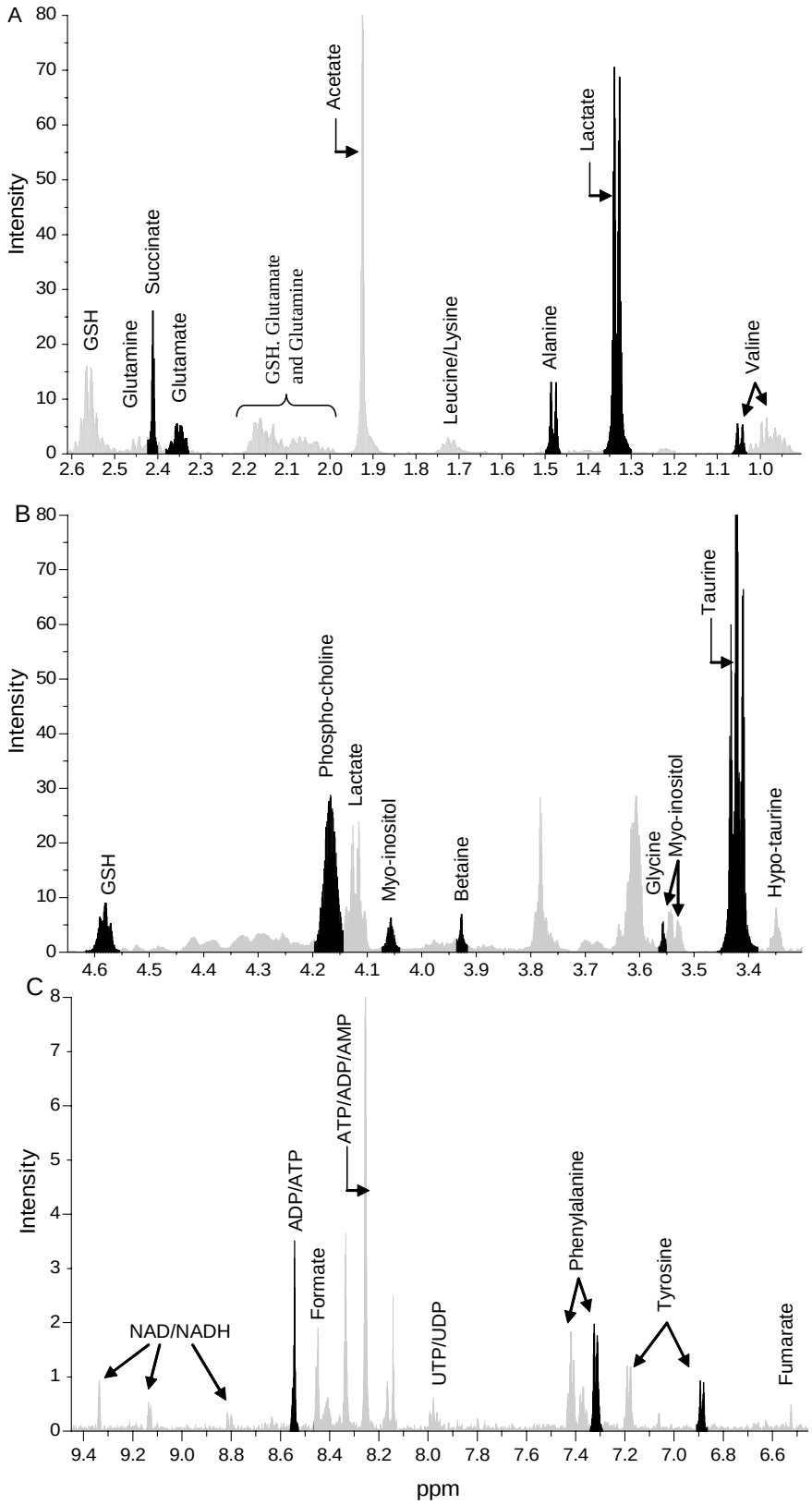

Fig. 2. A representative HR-MAS ^{1}H NMR spin-echo spectrum of an intact rat lens (control), reduced by the software AMIX, Bruker BioSpin to a resolution of 0.001 ppm per point ('bucket' width). The dark regions represent the integrated area of the respective metabolites analysed. (A) High field, (B) middle field and (C) low field region of the obtained spectrum. The ppm values were assigned using TSP as reference substance at 0 ppm. Assignments: GSH, reduced glutathione; NAD/NADH, nicotine adenine dinucleotide; ATP/ADP/AMP, adenosine tri-/di-/monophosphate; UTP/UDP, uridine tri-/diphosphate.

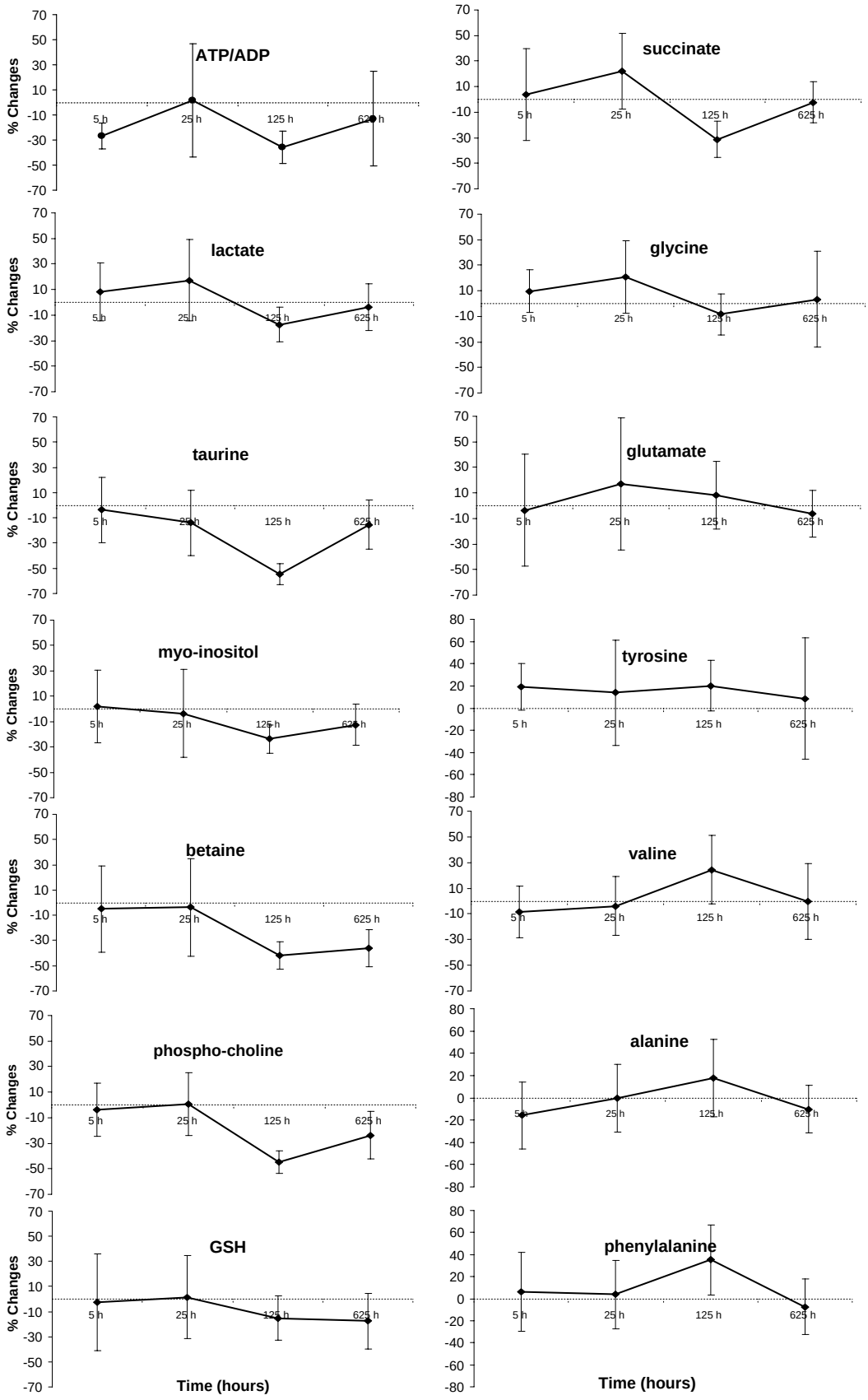

Fig. 3. Relative differences in metabolite concentrations between exposed and non-exposed rat lenses 5 ($n=6$), 25 ($n=6$), 125 ($n=7$) and 625 ($n=6$) hr after UVB irradiation. Data were calculated as (exposed lens − control lens)/control lens. The bars represent 95% confidence intervals for the mean differences.

and p-choline were still showing a significant decrease 625 hr after UVB irradiation.

Statistical analysis (ANOVA) did not reveal any significant time–response relationship for the examined metabolites, except for taurine ($p=0.001$), betaine ($p=0.024$) and p-choline ($p=0.001$).

4. Discussion

As shown recently in our laboratory, HR-MAS ^{1}H NMR spectroscopy has a great potential in analysing the metabolic profile of intact lens tissues (Risa et al., 2004). The detailed information on the metabolic composition was comparable to that acquired by NMR spectroscopy of lens tissue extracts (Midelfart et al., 1996; Risa et al., 2002). In the present study broad resonances from macromolecules and lipids with low mobility and hence short transverse relaxation time (T_2) were suppressed by using a one-dimensional T_2-filtered pulse sequence with an effective spin-echo delay of 144 msec. This allowed an enhancement in relative signal intensity of smaller molecules and better baseline separation of the peaks. However, as explained by Risa et al. (2004) it was difficult to use conventional quantification methods with TSP as internal standard. By analysing the samples under identical conditions and assuming that each metabolite had the same average T_2 in all samples, this problem was avoided by measuring peak intensities relative to a selected noise region and correcting for the lens wet weight. Relative changes could then be extracted.

The metabolic profile of the pigmented rat lens in this study was very similar to previous HR-MAS studies of albino Sprague Dawley rats of the same age (Risa et al., 2004). The same metabolites dominated the one-dimensional proton spectra, and the same cross-peaks were revealed in the assignment work of the two-dimensional spectra. However, a comparative study of the metabolic profile of lenses from pigmented and albino rat eyes were not performed.

The observed increase in light scattering from the lens after UVB irradiation was transient, showing a maximum at 25 hr post-exposure. The decrease in light scattering between 25 and 625 hr latency showed that 15 kJ m^{-2} is below or close-to-threshold dose for the pigmented rat lens (Pitts et al., 1977; Michael et al., 1996). There were not observed any significant light scattering or metabolic effect of the UVB irradiation in the contralateral non-exposed eyes.

Comparing the light scattering differences and changes of the metabolic profile in the lens, the impact of the UVB irradiation on the metabolic processes seemed to be delayed. This is because the major changes in the metabolite concentration were observed first at 125 hr post-exposure. Similar to the normalisation of light scattering, the concentrations of most of the metabolites seemed to converge back to the normal level after 625 hr.

In earlier studies, different time intervals for repeated threshold doses of UVB irradiation have shown that the most severe cataract development occurred in a group that was allowed a 72 hr interval between two exposures. In contrast to that, the damage was the same whether the second exposure was repeated immediately, 6 or 24 hr after. The lowest intensity of light scattering was detected in the 30 days interval group (Ayala et al., 2000). It was suggested that photoproduct formation, different repair mechanisms and apoptosis might make the lens cells more sensitive to a second UVB exposure. In the present work, the peak changes in the endogenous metabolite concentrations at 125 hr post-exposure supports the observations that the lens is vulnerable to additional UVB attacks at certain time intervals. The normalisation of the metabolic changes after 625 hr (26 days) indicates, however, that the biochemical repair in the lens can occur within a month after irradiation. Consequently, when repeated UVB exposures are separated by 30 days, the final damage of the lens might be less than with shorter time intervals.

Both taurine and myo-inositol are among the organic osmolytes that have been previously identified in the lens (Miller et al., 2000; Cammarata et al., 2002) and their significant decrease 125 hr post-exposure might be explained by changes in epithelial membrane permeability, osmotic stress and loss of homeostasis (Hightower et al., 1994b). Michael et al. (1998a) have observed that threshold UVB irradiation induced apoptosis in the lens epithelial cells leading to loss of metabolically competent cells and disturbance of the water balance in the lens. Taurine is released in association with cell shrinkage and water extrusion during apoptosis in neurons (Morán et al., 2000). The observed decrease in taurine and myo-inositol indicates a possible relationship between apoptosis and extrusion of organic osmolytes in rat lens after UVB irradiation. Also the level of betaine, known as a dominating osmolyte in placenta and renal medulla tissue (Miller et al., 2000), fell significantly 125 hr post-exposure. So far, no osmolytic activity has been reported for betaine in the lens (Miller et al., 2000). In fact, the lenticular role of betaine is not exactly known (Rao et al., 1998). Some osmolytes, especially methylamines like betaine, may have stabilising effects on macromolecules (Yancey et al., 1982). This effect is of crucial importance to the lens fibre cells which have limited capacity of damage repair (Spector, 1995).

The phospholipid precursor p-choline is one of the most abundant metabolites in the rat lens and an important metabolite in cell membrane metabolism (Kopp et al., 1981). Studies have shown that cataractogenic osmotic and oxidative stress caused an increased efflux of lenticular organic phosphate compounds, including p-choline (Desouky et al., 1992; Jernigan et al., 1993). Investigation of apoptotic cell death in lymphoblasts has revealed a decrease in p-choline during the cytoskeletal architectural destruction, and suggested that small molecules like p-choline may not be replenished as the cell prepares to die (Blankenberg et al., 1997). In agreement with earlier observations on osmotic stressed rat lenses (Jernigan et al.,

1993), present study revealed a time dependent reduction in lenticular p-choline after UVB irradiation.

The delayed time response of the observed concentration changes indicates that initial changes in the lens epithelium (Li and Spector, 1996; Shui et al., 2000) might induce additional biochemical changes in the bulk of the lens at a later stage. Indeed, it has been suggested that many of the damages associated with cataract such as Na/K-ATPase inhibition, drop in GSH concentration, loss of ATP, changes in water balance and lens protein modifications are all potential results of early changes in the epithelial cells and might occur in a post-insult period 1–12 days later (Hightower and McCready, 1992; Li et al., 1995). Histological analysis of albino rat lenses showed that severe damages in underlying fibre cells did not appear until 7 days after threshold UVB exposure (Michael et al., 1998a). In present study threshold UVB doses was found to cause significant post-insult disturbances as late as 125 hr after irradiation, starting thereafter the repair process with normalisation of the metabolism.

It has been suggested that polymerization of crystalline cleavage products cause the formation of water-insoluble polypeptides in the lens (Srivastava, 1988; Baruch et al., 2001). Therefore, proteases that further degrade the protein fragments into smaller peptides and amino acids might provide an important secondary defence against aggregation and cataract development (Taylor and Davies, 1987; Chaerkady and Sharma, 2004). The significant increase of phenylalanine and the increasing tendency of valine and alanine ($p > 0{\cdot}05$) might be due to induction of site specific hydrolysis of multicatalytic proteases.

GSH has an important role in protecting protein thiol-groups from oxidative damage and preventing cross-linking of soluble crystallines (Reddy, 1990). GSH concentration has showed a rapid depletion in lens epithelium and more slowly in the underlying lens fibres after UVB irradiation (Hightower and McCready, 1992). However, the lens epithelial cells have a remarkable ability to re-establish GSH to a normal level (Spector, 1995). The present findings of unchanged GSH level in the post-irradiation period from 5 to 625 hr indicated that the lens maintained a vital GSH metabolism.

Normal GSH turnover in the lens requires high amount of energy (Reddy, 1990), which might be one of the reasons for the significant reduction in ATP and ADP level post-irradiation. In general, repair processes result in increased energy demands and as shown previously photochemical stress induces a decrease in the ATP level (Thomas et al., 1993; Spector, 1995).

The ATP production in the lens continues even after severe epithelial damage (Spector, 1995). This is because the bulk of the lens relies on anaerobic glycolysis, represented throughout the lens, with lactate as the end product catalysed by lactate dehydrogenase (LDH). Löfgren and Söderberg (2001) have reported that UVB irradiation inhibits the activity of LDH in the lens, and this might lead to energy depletion. The significant decrease in both lactate and ATP/ADP observed at 125 hr post-exposure supports these observations.

Potential local variations in lens nucleus, cortex and epithelium were not addressed in the present study. HR-MAS ^{1}H NMR spectroscopy has the potential to investigate the metabolic profile of very small lenticular sections. Thus, in further studies it might be possible to investigate separately local metabolic changes in the anterior, nuclear and posterior sections of the rat lens.

In summary, this study showed that the UVB irradiation impact on the metabolic profile of rat lens did not follow the same time relationship as the development of light scattering. While the light scattering peaked at 25 hr post-exposure, most significant changes in the endogenous metabolites were observed after 125 hr. Both the metabolic changes and the light scattering seemed to average back to normal within a month after exposure. Most of the observed changes were concentration decrease of several water-soluble metabolites, similar to earlier observations in albino lenses (Risa et al., 2004). Significant decrease in osmolytes like taurine, myo-inositol and betaine indicated a loss of homeostasis and osmotic stress. Our laboratory is the first to report a biological response of betaine metabolism in the lens. This study also demonstrates that HR-MAS ^{1}H NMR spectroscopy provided high quality spectra of intact lens tissue and a large number of water-soluble metabolites could be directly investigated. The results might contribute to a better understanding of the metabolic response to external pathophysiological stimuli like UVB irradiation.

Acknowledgements

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