PROTEOMICS

# Supporting Information for Proteomics

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**A retinal proteomics-based study identifies αA-crystallin as a sex steroid-regulated protein**

## Supplementary Materials and Methods

### Animals

Adult male Wistar rats (weighing approximately 200-250 g) were purchased from Harlan Italy and allowed to acclimate in our animal facility for 1 week before use. They were housed in an air-conditioned room (22 ± 2°C, relative humidity 50 ± 10%) with a 12-h light/dark cycle and had free access to tap water and rat chow (TRM, Harlan Italy). They were divided into four groups: a control group with no injection and three injected groups receiving an intravitreal injection of vehicle or dihydrotestosterone (DHT) or 17β-estradiol (E2). All experiments were conducted in compliance with protocols approved by the local animal use and care authorities.

### Intraocular injection

Intravitreal injection was performed as previously described [25]. Rats were anesthetized using ketamine (85 mg/kg) and xylazine (14 mg/kg); anesthetic drops (0.5% proparacaine hydrochloride) were applied topically to each eye before injections. Each rat received either vehicle (5 μl/eye) or DHT (30 fmol/5 μl/eye) or E2 (1.5 pmol/5μl/eye) in both eyes. Stock solutions of steroids were dissolved in ethanol (final concentration ≤ 0.0003% v/v) and prepared at their final concentration in PBS before injections. Five microliters of test sample were injected using a 10 μl Hamilton syringe adapted with a 25 gauge glass microneedle. The needle tip was inserted into the superior hemisphere of the eye at 45° angle through the sclera into vitreous body. Care was taken not to damage the lens. Following intravitreal injections, the needle was held in place for one min and withdrawn slowly. Animals with retinal bleeding or lens injury from the injection procedure were excluded from the study. Three days after the injection, rats were euthanized, eyes rapidly enucleated and anterior segments including cornea, iris and lens were removed under a stereo microscope (Leica MS5). Then the retina was separated from sclera and washed three times with PBS (pH 7.4) to remove any vitreous humor adhered to the retina. Structural integrity of the retina was assessed by histology. Then each retina was placed in an Eppendorf tube containing ice-cold extraction buffer as described below.

**Two-dimensional electrophoresis**

Proteins were extracted in sample buffer containing 7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1- propanesulfonate, 1% tritonX100, 40mM Tris-HCl pH 8.8, 10mM dithiothreitol, 1.2% Destreak, 0.5% IPG (immobilized pH gradient) buffer (pH 3–11, GE Healthcare), 1% phenylmethylsulfonyl fluoride, 25 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 0.1 μM okadaic acid, and 1 μl/ml protease inhibitor mix (100X solution; GE Healthcare). After centrifugation at 10.000x g, protein concentrations were measured by 2D Quant protein assay (GE Healthcare); all samples were adjusted for equal protein concentration and stored in aliquots at -80°C. In first set of experiments, six different retinal protein samples for each group were independently subjected to the first dimension of isoelectric focusing (IEF) using pH 3 to 11 non-linear gradient strips (GE Healthcare), and to the second dimension separation on 12% SDS-polyacrylamide gels. This allowed to screen retinal protein changes within and among groups. In order to analyze and identify protein spots within the range of p*I* 5.3-6.5 and 10-200 kDa, four-five retinal preparations from each group with similar profiles were pooled. Thirty micrograms (for analytic gels) and one milligram of proteins (for preparative gels and mass spectrometry analysis) from each pool of the four experimental groups were loaded onto18-cm, pH 5.3-6.5 IPG dry strips (GE Healthcare). Rehydratation loading of sample proteins was carried out at 20°C in two steps: a passive step for 2h followed by a step at 30V for 18h. Then strips were subjected to IEF with IPGphorII (Amersham Biosciences) and the following voltage/time profile was used: 0 V for 2 h, 30 V for 6 h and 30 V for 12 h at 20°C, followed by 200 V for 6 h, 1250 V for 2 h, 3500 V for 1.5 h, 5500 V for 3 h and 9500 V for 3 h at 15°C. Post-IEF strips were either used immediately or stored at -80°C. For the second dimension of SDS-PAGE, post-IEF strips were placed in equilibration buffer-1 (50 mM Tris-HCl, pH 8.8, 6.0 M urea, 2.0% SDS, 30% glycerol, 1%DTT) for 15 min and then in equilibration buffer-2 (50 mM Tris-HCl, pH 8.8, 6.0 M urea, 2.0% SDS, 30% glycerol, 2.5% iodoacetamide) for a further 15 min. The equilibrated strips were loaded onto SDS-containing 12% polyacrilamide, and SDS-PAGE was performed at 3 mA/gel for 4h followed by 15 mA/gel for 15 h by using Ettan Dalt six electrophoresis Unit (Amersham Biosciences).

**Protein staining and 2-D gel image analysis**

Gels were stained with silver nitrate and we applied the conditions previously described [26] in order to partly encompass nonlinearly range of protein level changes and carry out a proper quantitative image analysis among groups. Consequently, staining of all gels of control and treated samples were developed at the same time and for a brief period to avoid saturation. We occasionally detected spot saturation that was not included in our analysis. The gels were scanned (Image Scanner II; Amersham Biosciences) and analyzed with Image master 2-D software (Amersham Biosciences) for spot detection, quantification and matching. The analyses were performed as follows: first, spots were compared by considering the distance of X axis direction as the distance of IEF direction and the distance of Y axis direction as that of distance of SDS-PAGE direction; second, the intensity of each spot was evaluated as the percentage of volume, corresponding to pixel intensity integrated over the area of each spot and divided by the sum over all spots in the gel; third, the intensity of each spot was corrected by subtracting a background intensity; and finally, the mean intensity value of each spot of all three gels was calculated. Only protein spot changes present in all the three replicate gels of each group and greater than 2-fold in magnitude in the treated groups when matched to the control group were considered real variations and subjected to MALDI-TOF-MS analysis.

**Protein spot identification by MALDI-TOF Mass Spectrometry**

Silver stained spots from 2-D gels of DHT- and E2-injected samples were excised, destained and acetonitrile dehydratated. They were successively rehydratated and digested overnight at 37°C with trypsin (Promega). From each protein digested, 0.75 µl was spotted onto the MALDI target and allowed to dry. Then 0.75 µl of matrix solution [alpha-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid] was applied to the dried sample and re-dried. The peptide mixtures were analyzed by peptide mass fingerprinting using an Ettan MALDI-TOF/Pro mass spectrometer (Matrix-assisted-laser-desorption-ionization) (Amersham Biosciences),

following the manufacturer's recommended protocol. After tryptic peptide mass acquisition, mass fingerprinting searching was carried out in Swiss-Prot/TREMBL and NCBInr databases using Mascot (Matrix Science Ltd., London, UK, http://www.matrixscience.com) online available software. A mass tolerance of 100 ppm was allowed and the number of accepted missed cleavage sites was set to one. Alkylation of cysteine by carbamidomethylation was assumed as a fixed modification, while oxidation of methionine was considered as a possible modification. The criteria used to accept identifications included the extent of sequence coverage, number of matched peptides and probabilistic score. Peptide sequencing by tandem mass spectrometry (MS/MS) analysis was performed to confirm the sequence of some peptides when the PMF was not clear by using a nanospary/LCQ DECA IT mass spectrometer (Thermo, West Palm Beach, FL). Database searching was carried out using TurboSEQUEST (Thermo) and MASCOT MS/MS ion search software (http://www.matrixscience.com).

**Western Immunoblot analysis**

Western immunoblot analysis was performed to confirm the changes in αA-crystallin expression as detected by silver staining. Total retinal proteins were prepared as for 2D gel analysis and forty micrograms of proteins were first separated by 12% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 3% nonfat dry milk in TBS-T (0.1%) for 2 h and incubated overnight at 4°C with the anti-αA-crystallin monoclonal antibody (1:500; Novus Biologicals). The immunocomplex was detected with a horseradish peroxidase-conjugated anti-mouse IgG (1:1000; Amersham Pharmacia Biotech.) using enhanced chemiluminescence reagent (Super Signal West Pico, Pierce). To confirm equal loading, the membrane was reprobed with the monoclonal anti-βactin antibody (1:5000; Sigma- ImmunoChemicals), and immunocomplex was visualized as described above. Densitometry from scanned images of non-saturated immunoblots was performed using the image analysis system (Chemi-Doc, Quantity-One analysis, Bio-Rad). The pixel intensity of the bands was set as 100%, the individual band was then calculated as percentage of total signal.

**Statistical analysis**

Analysis of 2-DE gels at pH range 5.3-6.5 was based on group variations in the intensity of individual spots shown in three independent gels from a pool of 4-5 different retinal preparations for each experimental group. For immunoblots three independent experiments were analyzed. The means (± SEM) and statistical tests are reported. Analysis was evaluated by the Student's *t*-test and the Mann–Whitney test. Additionally, results from the 2-DE gels were analyzed using One-way analysis of variance (ANOVA) followed by the Tukey–Kramer and Bonferroni tests for multiple comparisons; in some cases the Newman–Keuls method was also used. Differences were considered significant at $p < 0.05$.

## Supplementary Figure

**Supplementary Figure 1**: Two-dimensional separation of retinal proteins in pH range 3 to 11. Silver-stained gels of retinal preparations from adult rats un-injected or injected intravitreally with vehicle, DHT or E2 are shown. These gels are representative of six different retina samples for each group. Within each group 2-DE gel patterns were similar to each other, and almost 90% matching on spot position and quantity was achieved in four-five samples out of the six examined. Among groups, many protein spots were differentially expressed in the p*I* regions of pH 5-9 of the retinal proteome maps of rats treated with DHT or E2 when compared to those of vehicle-treated and untreated rats. Notably, retinal protein expression changes were also seen in vehicle-treated rats with respect to un-injected rats, albeit to a significantly lesser extent. The pI gradient of the first dimension electrophoresis is shown on the top of the gels (*x* axis) and the migration of molecular mass (MW) markers for SDS-PAGE in the second dimension is shown on the side of the gels (*y* axis).

## Supplementary Figure 1.