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RAPID COMMUNICATION

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

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Sex steroids influence the structural and functional organization of ocular tissues, promote survival in several pathological conditions including retinal neurodegeneration and have a prominent role in age-related eye diseases as well as neurodegenerative diseases. However, their underlying mechanisms are still elusive. We explored proteomic profiling of rat retinas following intravitreal injection of the bioactive 17β-estradiol or androgen dihydrotestosterone. Using narrow range 2-DE gels and MALDI-TOF-MS analysis, we identified three sex steroid-regulated proteins: the galectin-related-inter-fiber (GRIFIN) which is a galectin family member protein of unknown function, the fatty acid-binding protein epidermal-5 (FABP5) protein responsible for the fatty acid uptake and transport and the small heat shock αA-crystallin (CRYAA) protein involved in preventing aggregation of denatured or unfolded proteins. Changes in the expression of these proteins revealed a predominant estrogenic effect and the multiple CRYAA protein species reflected posttranslational modifications. Sex steroid-mediated modifications of CRYAA were confirmed by Western blotting analysis. This study provides new target proteins for sex steroids with a potential link to age-related diseases associated with proteotoxic stress.

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Sex steroids, here especially referring to estrogens and androgens, have an important impact on the anatomy, physiology and pathophysiology of the eye [1]. Gender dissimilarities are reported in the structural and functional organization of ocular tissues and there is compelling evidence that fluctuations in the levels of sex steroids during life affect vision. Studies especially highlight their critical role in the pathogenesis of age-related ocular diseases as well as neurodegenerative diseases, which share the common pathological mechanism of protein aggregation and are collectively termed protein misfolding or conformational diseases [2]. For instance, estrogen depletion in postmenopausal women is associated with higher risk of developing age-related macular degeneration (AMD) as well as Alzheimer's disease, and risks seem to be relieved among postmenopausal hormone users [3–5]. Androgen effects, so far mainly characterized at the anterior segment of the eye, are associated with dry eye syndromes [6].

Human and mammalian ocular tissues possess estrogen receptor subtypes (ERα and ERβ), androgen receptors (ARs) and enzymes of synthesis and metabolism of their ligands [1, 7]. This outlines that sex steroids may act on the eye through paracrine as well as local, intracrine mechanisms and by exerting genomic and nongenomic actions. Our own investigations set the adult male rat retina as an androgenic

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Abbreviations: AMD, age-related macular degeneration; **ARs,** androgen receptors; **CRYAA,** αA-crystallin; **DHT,** dihydrotestosterone; **E2,** 17β-estradiol; **ERα,** estrogen receptor subtype alpha; **ERβ,** estrogen receptor subtype beta; **FABP5,** fatty acid binding protein epidermal-5; **GRIFIN,** galectin-related-inter-fiber

and estrogenic tissue where synthesis and function of testosterone and its transformed product estradiol may rely on the control of aromatase and presence of ERα, ERβ and ARs [7, 8]. Moreover, there is evidence of neuroprotection by estradiol and the androgen precursor dehydroepiandrosterone in the retina as well as brain, occurring for instance in excitotoxicity or ischemia-reperfusion injury and, quite interestingly, independently of sex [7, 9, 10].

However, the functional pathways through which sex steroids exert their effects are only partly understood and the broad range of their effects makes it plausible that many target molecules are still unknown. Here, we used the intravitreal injection technique, which is a mainstay of ophthalmic practice [11], to perform the first survey of the rat retinal proteome following the administration of 17β-estradiol (E2) or the biological active testosterone metabolite dihydrotestosterone (DHT).

We first generated 2-DE gels by using wide-range pH 3–11 IPG (see ''Materials and methods'' in Supporting Information). After three days of a single intravitreal dose of DHT or E2, the retina proteome maps exhibited several reliable spot variations in the p*I* regions of pH 5–9 when compared to the maps of vehicle- and un-injected rat retina samples, and the same occurred in at least six independent gels of six different retinal preparations for each experimental group (Supporting Information Fig. 1). Then, the clustering of variations packed in the region of pH 5.3–6.5 was considered for our further investigation. Proteins were resolved within the range of p*I* 5.3–6.5 for improving the resolution of spot profiles, and the identity of spots showing ≥two-fold changes in intensity in at least triplicate gels of treated samples was analyzed through MALDI-TOF-MS as described in Supporting Information. In narrow-range 2-DE gels, we identified five to nine spots in treated samples (Fig. 1A and B). Three spots were increased by vehicle or steroids treatments (spots 2, 8 and 13) and suggested that the intraocular injection-related trauma could have affected the retinal proteome; the other spots were fully expressed by DHT or E2 treatments and indicated specificity of sex steroid effects (Fig. 1B and C). The peptide mass fingerprinting analysis revealed that two spots corresponded to the galectin-related-inter-fiber protein (GRIFIN), one to fatty acid-binding protein epidermal-5 (FABP5) and the others were isoforms of the αA-crystallin (CRYAA) protein (Table 1).

GRIFIN is a galectin family member protein of unknown function and so far found exclusively in differentiated lens fiber cells [12]. Three GRIFIN mRNA species have been isolated but the existence of splice variants is uncertain. Our results originally display GRIFIN expression in rat retinal

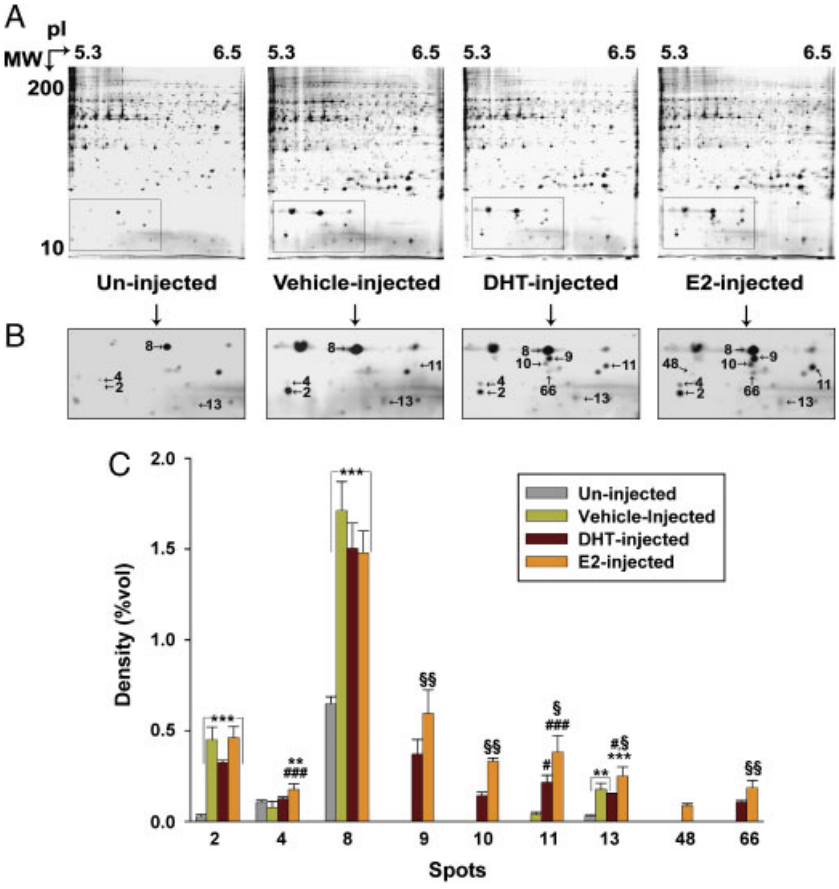

Figure 1. (A) Representative 2-DE images of retinal proteins of rats un-injected or injected intravitreally with (5 μL/eye), DHT (30 pmol/5 μL/eye) or E2 (1.5 pmol/5 μL/eye) for three days. The 2-DE analyses were performed using narrow 5.3–6.5 pH IPG strips in IEF and 12% T SDS-PAGE in the second dimension, and silver staining detection (see ''Materials and methods'' in Supporting Information). The p*I* gradient of the first dimension is given on the *x*-axis, and the migration of molecular mass (MW) markers for the second dimension on the *y*-axis. (B) Magnification of the boxes in (A). Numbers refer to spots that showed ≥two-fold changes in intensity or were uniquely expressed, and correspond to the proteins identified by MS in Table 1. (C) Densitometry of protein spots performed on triplicate gels for each group (mean ± SEM) showing significant differences: (*) versus un-injected group, (♯) versus vehicle-injected group and (§) versus DHT-injected group. ♯, § $p<0.05$; **, ♯♯, §§ $p<0.01$; ***, ♯♯♯ $p<0.001$.

Table 1. Protein identification by MS

Spot	Protein	Accession no.[a)]	Theoretical MW (kDa)/p*I*	Experimental MW (kDa)/p*I*	MASCOT search results		
					Matched/searched peptides	Coverage %	Score
2	GRIFIN	NP-476535	15.8/5.39	14.9/5.36	4/4	34	84
4	GRIFIN	NP-476535	15.8/5.39	15.5/5.36	4/4	34	84
8	αA-crystallin	NP-036666	19.9/5.81	18.4/5.44	10/26	46	127
9	αA-crystallin	NP-036666	19.9/5.81	17.6/5.44	10/24	46	116
10	αA-crystallin	NP-036666	19.9/5.81	17.1/5.47	5/9	20	75
11	αA-crystallin	NP-036666	19.9/5.81	16.8/5.66	6/10	30	92
13	FABP5	NP-665885	15.3/6.73	14.3/5.47	4/4	32	76
48	αA-crystallin	NP-036666	19.9/5.81	16.2/5.37	4/5	18	69
66	αA-crystallin	NP-036666	19.9/5.81	16.4/5.44	5/6	23	88

a) Accession no. from GenBank, NCBI, Bethesda, MD; www.ncbi.nlm.nih.gov/GenBank

2-DE maps that resolved into spots 2 and 4 differing in molecular weights; spot 2 was increased after either vehicle or steroid treatments as a stress response to intraocular injection, whereas spot 4 was specifically increased by E2 treatment.

FABP5 is a small cytoplasmic protein responsible for the fatty acid uptake and transport. In the retina, it is upregulated during development and regeneration [13] or after stimuli leading to oxidative stress, and acts by scavenging reactive lipids [14], but it is virtually absent in adult animals [13]. Our results accordingly demonstrate FABP5 resolving as single spot 13 at a very low level in the control adult retina and increasing by vehicle treatment as a possible adaptive defensive response to the intraocular injection-induced stress. However, E2-mediated specific increase in the protein expression was also detected.

CRYAA belongs to the α-crystallin family and is a small heat shock protein with a chaperone-like activity to prevent aggregation of denatured or unfolded proteins, but also with a function in remodeling the cytoskeleton, inhibiting apoptosis and enhancing cellular resistance to stress [15]. It is essential for the optical and refractive properties of lens and implicated in cataract. In the retina, CRYAA protein changes are seen in AMD patients, experimental glaucoma and diabetic retinopathy, and during aging [16–18]. Like FABP5, CRYAA may promote survival and axonal regeneration of retinal ganglion cells after optic nerve injury [18]. The recent role of α-crystallins in preventing amyloid fibril formation suggests an important implication of these chaperones in protein misfolding diseases, such as Alzheimer's, type II diabetes, AMD and cataract [19]. The possibility, however, that crystallins aggregate and form fibrils with still undefined roles has lately been discussed. We found that CRYAA was the most abundant protein within the p*I* range of 5.3–6.5. Following DHT or E2 treatments, CRYAA resolved into multiple spots (spots 9–11, 48 and 66), varying in both the molecular weight and p*I* in comparison to its origin (spot 8) and likely reflecting proteolytic processing and/or changes in intrinsic charge as a consequence of posttranslational modifications (Fig. 1B and C; Table 1). The increased intensity of CRYAA spot 8 after vehicle treatment and similarly after steroids treatments may underline a possible defensive mechanism to injection-induced stress, as suggested for FABP5. Steroid-induced changes, primarily influencing CRYAA posttranslational modifications, appear more confident with steroid actions at posttranslational rather than transcription level of the protein. The biological significance of CRYAA isoforms is unknown and whether posttranslational modifications minimize the protein functions is still controversial [20].

This study first demonstrates the regulation of CRYAA, GRIFIN and FABP5 proteins in the adult male retina after intravitreal injection of sex steroids. Although these proteins are highly expressed in lens, their changes are expected to be specific to the retinal tissue since possible lens contamination was accurately avoided and results were reproducible in different, individual retinal preparations as described in Supporting Information. Interestingly, CRYAA changes often co-occur with GRIFIN or FABP5 changes in proteomic studies [14, 16, 21, 22], GRIFIN may directly interact with CRYAA [12], and they were here upregulated after intravitreal steroid administration. Considering the current ophthalmic practice of intravitreal injections for the treatment of vitreoretinal diseases [11], it is also intriguing that these proteins with a proposed role in promoting survival and resistance to cellular stress were upregulated after the vehicle injection. Moreover, a single intraocular injection of E2 and DHT at physiological concentrations acting at their respective receptors was used in our investigation without any apparent adverse effect. Likewise, loss of the eye transparency, normally occurring by intraocular glucocorticoids, was not found by Kosano and Nishigori [23] after intraocular methyltestosterone, estradiol or ethinylestradiol administration.

In general, E2 treatment was found to be more effective than DHT treatment. This was especially confirmed by Western blotting analysis of CRYAA carried out to validate

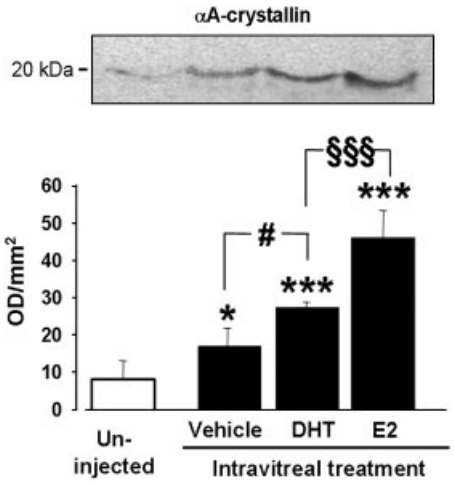

Figure 2. Western blot analysis of retinal extracts confirming increased expression of CRYAA after intravitreal treatment with DHT and even higher with E2. CRYAA protein levels were normalized by β-actin. Data represent mean ± SEM ($n = 3$). (*) versus un-injected group, (♯) versus vehicle group and (§) versus DHT group. *, ♯ $p < 0.05$; ***, §§§ $p < 0.001$.

changes in spot densities estimated through silver staining (see ''Materials and methods'' in Supporting Information) (Fig. 2). The predominant estrogenic effect in protein changes might result from the largest estrogen receptors distribution in the retina, if compared to the ARs presence, seemingly most abundant in other parts of the eye [1, 7, 8]. Alternatively, DHT might promote estrogenic-mediated effects by its conversion into 5α-androstan-3β,17β-diol that is known to act as an ERβ agonist.

Estrogens and androgens regulate the transcription and posttranscription expression of several molecules with a great impact on the flow of information into proteome. A novel finding reports E2 regulation of the brain mitochondrial proteome [24]. Our study provides the first survey of retinal proteins regulated by E2 and DHT. The functional meaning of the steroid-induced regulation of the three proteins identified has to be investigated, as well as many other proteins seen to be affected in our initial large-scale analysis remain to be characterized. Amyloidosis is a clue in protein misfolding diseases where steroids and cholesterol acting as a lipid membrane compound and precursor of cerebral steroids may have important roles [25]. It is noteworthy that CRYAA, estrogens and androgens have antioxidant and antiapoptotic properties and reduce amyloid β-peptide accumulation and toxicity [5, 15, 24, 26]; moreover, age-related ocular and neurodegenerative conformational diseases are associated with crystallin changes [19] and sex steroid depletion [1]. Although some clinical studies disclose benefits among hormone therapy users, the therapeutic value of estrogens and androgens is far to be definitive and studies are needed. Our findings on sex steroid-mediated regulation of CRYAA together with GRIFIN and FABP5 give evidence for novel mechanism(s) of sex steroids and a potential link to age-related disorders associated with proteotoxic stress.

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