Transcriptional regulation of RACK1 and modulation of its expression: Role of steroid hormones and significance in health and aging

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"Io e mia Mamma abbiamo raggiunto un'intesa:
siamo d'accordo che non andremo mai d'accordo"

Alla mia Mamma

Preface

The experiments I carried out during my PhD have been all related to the study of RACK1 (Receptor for the Activated C Kinase 1) protein and in particular on its implication in immune system functionality and breast cancer progression.

RACK1 is a 36 kDa highly conserved intracellular protein member of the WD (tryptophan-aspartate)-repeat protein family. It is homologous (42%) to the β subunit of G-protein (Gβ), the best characterized WD repeat protein, and contains a seven-bladed β-propeller structure that serves as a docking site for interacting proteins. Indeed, although all WD family members show different functions, they share a common role as scaffolding protein complexes, serving as hubs for multiple signal transduction pathways associated with diverse biological functions [1–3].

RACK1 protein is encoded by rack1 gene (previously know as GNB2L1, guanine nucleotide binding protein β polypeptide 2-like 1), mapped to chromosome 5q35.3 [4]. RACK1 was originally cloned from a chicken liver cDNA library and human B-lymphoblastoid cell line [5]. Several years later Mochly-Rosen's group cloned the protein from a rat brain cDNA library screen designed to isolate anchor proteins that bound PKC (Protein Kinase C) in the presence of its activators, diacylglycerol, calcium, and phosphatidylserine [6-8]. Hence the name Receptor for the Activated C Kinase 1 [9,10].

Although originally found to act as a shuttling protein for activated PKCβII, the extensive investigation of the relationship between RACK1 and its binding partners has led to the realization that RACK1 interacts with numerous proteins, either directly or as a part of a larger complex in distinct cellular compartments [1,2].

RACK1 main functions as a scaffold protein are 1) shuttling its binding proteins to their sites of action in order to facilitate the cross-talk between different signaling pathways and 2) induce conformational changes in its binding partners in order to enhance their stability, modify their activity or change their interaction with specific molecules [2,11]. The some of RACK1 signaling partners include Mitogen Activated Protein Kinase (MAPK), Jun-N-terminal Kinase (JNK), and cAMP specific phosphodiesterase PDE4D5, as well as Src kinase and integrins [3,12-14]. Other signal transduction partners identified are the tyrosine kinase oncoprotein SRC [15-17], the protein serine phosphatase PP2A [18,19] and the focal adhesion kinase FAK [19,20]. As a partner for multiple signaling cascades, RACK1 can interact with the cytoplasmic tail of several receptors including the Insulin-like Growth Factor Receptor I (IGF-IR), the NMDA receptor, the β-integrin receptor, the common beta-chain of the IL-5/IL-3/GM-CSF receptor, type I interferon receptor, several ion channels, as well as the androgen receptor [1–3]. The biological processes supported by these interactions include cell growth and survival, transcription, translation, apoptosis and cell mobility

[21,22]. The specific role of RACK1 in these aspects is still controversial and appears to be cell and contest-dependent [3,23]. Interestingly, RACK1 is also a ribosomal protein present at the small ribosomal unit next to the mRNA exit channel where it can influence ribosomal translational activity and selectivity [24,25]. Finally some recent studies show that RACK1 can also participate in the miRNA pathways [26].

Despite the plethora of RACK1 signaling partners mentioned above many others still remain uncharacterized and the difficulty in their identification is further increased by RACK1 miscellaneous intracellular compartmentalization (nucleus, cytosol, ribosomes, cytoskeleton) in different cell lines. However, the large number of RACK1 already known interactions undoubtedly identifies a complex network of signaling elements that characterize the physiological and pathological role of RACK1. Since its discovery, in fact, RACK1 has been associated to many diseases and its alterations have held responsible for cognitive disorders, heart failure, pulmonary arterial hypertension, renal failure, muscle atrophy, immunological alterations, dysfunctional sperm production and many types of cancer [21,27]. An increased RACK1 expression has also been observed in the frontal cortex of patiens affected by bipolarism, while a decreased in RACK1 expression is reported in brains of Down's syndrome and Alzheimer's disease patients [2]. It becomes clear that correlation between RACK1 protein levels and conditions related to physiological and pathological changes are of particular significance.

Although much is known about RACK1 protein localization, interactions and related functions, the mechanisms regulating its expression are rather unexplored. The considerations made above underline that a deeper understanding of RACK1 transcriptional regulation is of pivotal interest for all biological pathways involving RACK1 scaffolding and signaling functions.

In this regard, I can start introducing the study of RACK1 hormonal regulation I carried out within the immune and the immunosenescence context during the first part of my Phd.

The term immunosenescence refers to the substantial reduction of the immune system functionality our body is subject as we age [28,29]. The immunological functions decline is due to the progressive impairment of both innate and adaptative immunity and results in immune cells phenotype and functionality alteration [30,31,32]. Impairment of lymphoid organs and immune cells maturation, activation and functional differentiation, with the consequent altered release of cytokines, molecules that are essential for an efficient reaction to pathogens [33]. Thymus atrophy represents the most striking anatomical and histological event [34-36], it reaches its peak around the age of 50 and it leads to the reduction of lymphocytes B and T precursors and of their ability to differentiate. Indeed, T lymphocytes distribution (naïve, helper, suppressor, cytotoxic and memory T) resulted alterated, with a consequent imbalance between pro- and anti-inflammatory cytokines production, which in turn facilitate the onset of autoimmune disorders. NK (Natural Killer) cells number increases

but their cytotoxic result lower, thus reducing protection against pathogens and tumors [37]. The number of B lymphocytes remains unchanged, but antibodies production is lower than young people and anti-idiotypic and auto-antibodies prevail [38,39]. Macrophages and monocytes show a decreased functionality as well [40], IL-2 [41], IL-3, IL-6 and TNF-α level are lower [42,43]. Immune functions impairment is not only due to the inadequate cytokines secretion but also to defects in intracellular cytokines-induced transduction signalling pathways. In this regard, evidences showed alterations in PKC, a kinase involved in immune cells activation, proliferation, differentiation and survival signalling pathaways [44]. PKC failing signal trasduction has to be link to the reduction of RACK1 protein level that occurs in elderly [45,46]. RACK1 in fact is responsible for PKC anchoring, active conformation stabilization and translocation of the kinase to its sprcific substrates in order to phosphorylate them and activate defined pathways [46]. Therefore PKC defective signalling transduction can be ascribed to RACK1 lower expression and to PKC instability that comes with it, resulting in a significant impairment of the immunological response [47,48].

In order to explain RACK1 age-related decrease expression, we have to consider another important age-associated physiological aspect we still didn't discuss: endocrinosenescence. The endocrine system in fact plays an important role in modulating immune response and, as the immune system, its functions are affected by the passing of the years.

Human life is characterized by continuous changes in hormonal levels and it is well established that the aging process is accompanied by a natural multiple hormonal dysregulation. The levels of hormones like testosterone, progesterone, estrogen, aldosterone, and dehydroepiandrosterone decrease compared to younger age and some target tissues become less sensitive to their control [49]. In general, age-related hormonal changes are characterized by an imbalance between catabolic and anabolic hormones, where the former remain stable and the latter decrease.

Within my laboratory we focused on the imbalance existing between two specific hormones: the anabolic dehydroepiandrosterone (DHEA) and the catabolic cortisol and we studied their role on RACK1 modulation.

Cortisol and DHEA are both biosynthesized under the control of the hypothalamic-pituitary-adrenal (HPA) axis by the adrenal gland, but in different zones of the adrenal cortex. Cortisol is synthesized in the *zona fasciculata*, whereas DHEA in the *zona reticularis*. Aging brings to a differential degeneration of these two zones with the latter being the most affected and resulting in a steady reduction of the synthesis of DHEA after puberty and toward aging [50]. Therefore the increased ratio cortisol : DHEA observed in the elderly is mainly derived as a result of a significant reduction in the levels of DHEA [50–52].

DHEA and its sulfated form (DHEAS) represent the most abundant circulating adrenal steroids in humans [53]. The non-sulfated form is further metabolized into androstenedione,

testosterone and estrogens in brain, bones, breast and ovaries [54]. DHEA is described as the 'elixir of youth' for its anti-ageing properties, anti-obesity, anti-diabetic and immune-promoting effects [55]. Its mechanism of action and physiological implications are not fully understood and the possibility to be converted to either androgens or estrogens further extends its spectrum of action.

Cortisol is the most abundant endogenous glucocorticoid circulating in our body and it is essential to maintain homeostasis. Under basal conditions it is released under the control of HPA axis following a circadian rhythm [56]. Cortisol acts in different body districts and is involved in many physiological processes like blood sugar raising, hepatic gluconeogenesis increase, glucagon release stymulation, collagen and bone matrix synthesis decrease, proteins catabolism, fatty acids release. Moreover it inhibits pro-inflammatory cytokines (INFγ, TNFα) production in favour of the anti-inflammatory ones (IL-4, IL-10) leading to lymphod cells apoptosis and prostaglandin, leukotriene and tromboxanes synthesis reduction. In stress conditions (physical, emotional, immunological stressors) cortisol levels arise and its immunesuppressive and anti-inflammatory effects occur.

Similarly to the others glucocorticoids (GC), cortisol exerts its functions binding to its cytoplasmatic receptor (Glucocorticoid Receptor, GR) and translocating into the nucleus, where it acts as a transcription factor regulating about 15% of genome expression.

Obviously the wide range of genes regulated by GR includes also rack1 gene.

The first bioinformatic analysis able to describe rack1 gene promoter was carried out in 1999 and it focused on a 800 bp sequence within porcine rack1 gene 5'-flanking region. This analysis allowed to identify several putative transcriptional binding sites including a serum responsive element (SRE), a AP1 (Activator Protein 1) binding site, at least two E2F (Elongation Factor 2) binding sites, a SP1 (Specific Factor 1) binding site, a c-myc binding site, a YY1 binding site, a NF1 (Nuclear Factor 1) binding site, a (NF-kB)/c-rel (Nuclear Factor-kB) binding site and a EF1 (delta-crystallin/E2-box Factor 1) [57].

The same analysis was perfomed also on the murine rack1 gene promoter leading to the identification of putative binding site for HINF-A (Histone Nuclear Factor 4), F2F (Footprint 2 Factor), IRF2 (Interferon Regulatory Factor 2), RIPE3B (Rat Insulin Promoter Element 3B), URTF (Urokinase Transcription Factor), SP1, NF1 and NF-kB, whose binding to rack1 gene promoter was particurarly investigated. Apparently in fact, PKC can induce NF-kB activation through the up-regulation of a ikB (Inhibitor of kappa B) kinases group which phosphorylates ikB releasing Nf-kB and enabling it to translocate into the nucleus regulating genes expression [58,59]. According to this mechanism PKC would be able to regulate RACK1 expression through Nf-kB. In addition, Nf-kB mediation would explain the mechanism by which LPS (Lipopolysaccharide) and PMA (phorbol 12-myristate 13-acetate)

can induce RACK1 expression [57,60], demonstrating its fundamental role in the regulation of the protein [58].

Thus far, the only analysis of human rack1 gene available is the one conducted by Del Vecchio in 2009 within our laboratory [60]. Similar to those found in porcine and mouse genes, four c-Rel binding sites were identified. Oct-1 (Octamer binding protein 1), Elk-1 (E-26-Like Protein) and Pax-4 (Paired box gene 4) transcription binding sites were also founded and a consensus sequence for GR binding was detected at nucleotide -186 [60].

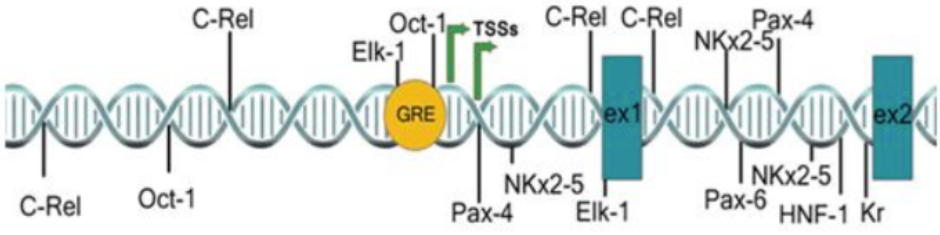

Fig. 1 - Human rack1 gene bioinformatic analysis

This sequence appeared to be similar to the consensus for a negative glucocorticoid responsive element (nGRE). Since cortisol exerts its functions binding to glucocorticoid receptor, this finding was particularly relevant in our context. Indeed, we observed that thanks to the presence of nGRE sequence, physiological concentrations (0.1-0.5 μM) of cortisol can inhibit RACK1 expression in THP-1 cells (human monocytic cell line), inducing an impairment of PKC signal transduction and a defective cytokines production in response to inflammatory events. We demonstrated that cortisol acts as a rack1 gene promoter repressor in a dose-dependent manner, reducing RACK1 mRNA expression and protein level. Conversely, as assessed by LPS-induced TNF-α release *in vitro*, prolonged DHEA (10-100 nM) exposures are able to counteract cortisol immunosuppressive effects [61]. Furthermore, in 2002 Corsini et al. demonstrated that *in vivo* DHEA administration restored RACK1 levels and immune functions, indicating that this hormone acts as a positive regulator of RACK1 expression, exerting anti-glucocorticoid effects [62].

However, the counteracting effect of DHEA against cortisol was still not fully understood. Therefore, in *Chapter 2* we use THP-1 cells (human monocytic leukemia cell line) to demonstrate that DHEA interferes with the splicing of glucocorticoid mRNA inducing the formation of the β isoform of the receptor (GRβ) through the over-expression of the serine/arginine (SR)-rich splicing factor 9 (SRSF9), whereas cortisol induces the formation of isoform α through the over-expression of the splicing protein SRSF3, where SRSF9 and

SRSF3 are two GR pre-mRNA splicing central proteins.

The human GR gene (NR3C1), in fact, is composed of 9 exons and the alternative splicing in exon 9 generates two homologous receptor isoforms termed GRα and GRβ [63,64]. GRα, glucocorticoid classic receptor, mediates most of the know glucocorticoids functions; after binding to its agonist ligand, GRα undergoes conformational changes, dissociates from heat shock proteins, homo-dimerizes, translocates into the nucleus, interacts with GRE sequences and modulates the transcription of its target genes. GRβ, instead, shares the same N-terminal domain and DNA-binding domain with GRα, but it has a unique ligand-binding domain. As result of this, GRβ does not bind GCs, thus it lacks transcriptional activity [65]. There is evidence that GRβ acts as a dominant negative on GRα [66,67], antagonizing GRα functions by the formation of GRβ/GRα heterodimers.

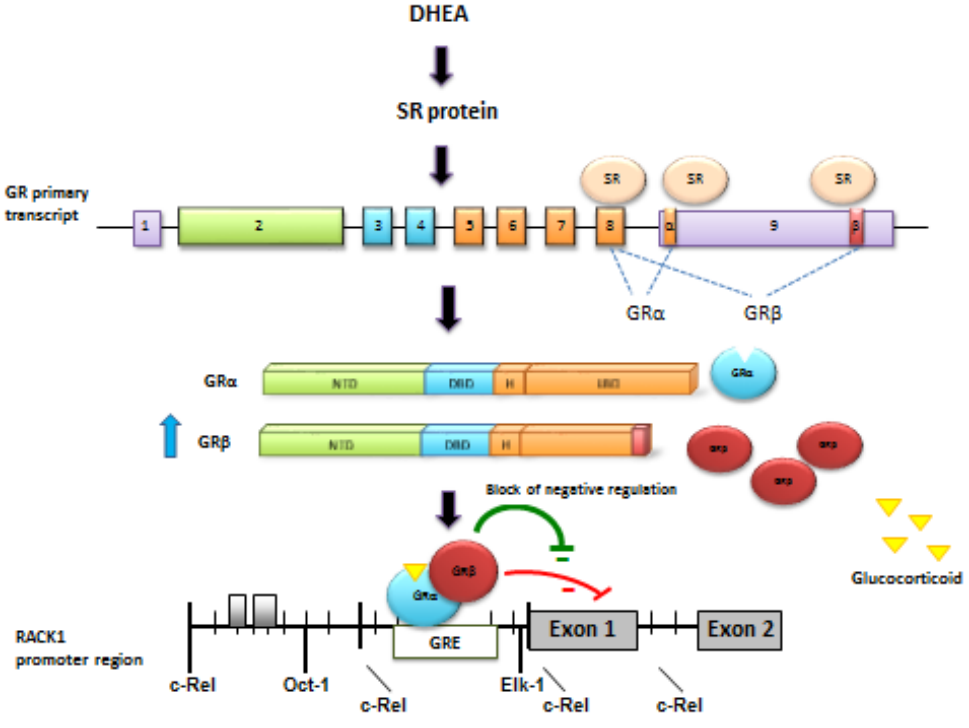

Fig. 2 - DHEA induction of GRβ production and its anti-glucocorticoid effect.

In this paper we also demonstrated that physiological concentrations of DHEA are able to increase total GR mRNA levels and that this effect is completely prevented by the pre-treatment with the androgen receptor (AR) antagonist flutamide. This result can be consistent with DHEA metabolization into active androgens and with AR capability in binding GRE

sequence. Indeed it is known that AR and GR can interact at the transcriptional level forming heterodimers at a common DNA site, usually termed as a canonical androgen/glucocorticoid response element (ARE/GRE) [68], with AR selective receptor binding achieved through relaxed *cis*-element stringency rather than a distinct and strict ARE sequence [69]. Furthermore, AR can bind other DNA sequences as a monomer, or in alternative dimer conformation, or even as a heterodimer with transcription factors that do not belong to the nuclear receptors superfamily [70]. Hence, differential interactions among factors, rather than their stringent specificity, can confer precise promoter and cell context-dependent hormonal response[71]. In the context of rack1 gene promoter we identified an Oct-1 binding site close to GRE sequence, we speculate that this transcription factor may regulate RACK1 expression in association with AR and the non-canonical GRE sequence.

Functional consequences of this regulatory pattern have come to light also in *Chapter 3*.

The demonstration of the existence of a complex hormonal balance between steroid hormones in the control of RACK1 expression and immune activation, in fact, suggested that RACK1 might also be targeted by endocrine disrupting chemicals (EDCs). As a proof of concept in we investigated the effect of the doping agent nandrolone, an AR agonist, and of p,p'DDT (dichlorodiphenyltrichloroethane) and p,p'DDE (dichlorodiphenyldichloroethylene), respectively a weak and a strong AR antagonist, on RACK1 expression and innate immune response. Compounds affinity for both AR and GR was estimated and it resulted higher for AR, suggesting these compounds exert their activity through AR binding. THP-1 treatment confirmed androgens positve role on RACK1 expression and innate immune functions since nandrolone was able to increase RACK1 mRNA expression and protein level as well as LPS-induced TNF-α and IL-8 release, while the opposite effect was observed for p,p'DDT and p,p'DDE.

Thus far I discussed of RACK1 modulation only within the immune context but, as mentioned above, literature data suggested its potential role also in the development and spread of cancerous cells. Aging is a key risk factor for degenerative immunological changes but it has also a pivotal role in the development and progression of some forms of cancer and there is evidence that RACK1 expression and function are linked to these pathological changes.

RACK1 seems to be implicated in cancer resistance to cell death, angiogenesis induction, sustaining of proliferative signal, invasion and metastasis. RACK1 expression has been shown to be dysregulated in some cancers like pulmonary adenocarcinoma, hepatocellular carcinoma and metastatic melanoma [2,23,72,73]; it can promote *in vitro* and *in vivo* breast and prostate cancer cell proliferation, invasion and metastasis [74,75]; it has also been suggested as a prognostic marker. In breast cancer, elevated expression of RACK1 seems to be associated with poor clinical outcome [76].

In *Chapter 5* we demonstrated cortisol-driven RACK1 over-expression in triple negative breast cancer (TNBC) cells and its implication in cell proliferation and migration.

TNBC cells lack the expression of estrogen (ER), progesterone (PR) and human epidermal growth factor 2 (HER2) receptor, however about 25% of invasive TNBCs are GR-positve. Our experiments were performed using MDA-MB-231, a highly aggressive, invasive and poorly differentiated TNBC cell line characterized by a significant basal increase of GR expression (GRα in particular), an ideal model to investigate GR-positive TNBCs [77-79]. In these cells the majority of GR target genes were aberrantly expressed and associated with unfavourable clinical outcomes.

Also RACK1 expression resulted aberrant, with elevated level of protein even in basal conditions [78-80].

As previously done with THP-1 we demonstrated that, also in this new cell context, cortisol could direct GR splicing to the formation of the α isoform by a mechanism involving SRSF3 up-regulation and that GRα regulated RACK1 expression binding to the non-canonical GRE sequence present within its promoter. Differently from THP-1, however, MDA-MB-231 cortisol treatment brought to an increase and not a decrease of RACK1 mRNA expression and protein level, confirming once more that RACK1 modulation and function are closely reletad to the cell context.

Considering the established role of RACK1 in cancer cell migration and invasion [81] we decided to evaluate cortisol-induced RACK1 impact on cell motility. MDA-MB-231, in fact, is a cell line commonly used in identification genes and pathways that are potential mediators of metastasis to brain, bones and lungs [82,86]. Subsequently we also demonstrated that RACK1 increased expression had an impact on cell proliferation and migration. Firstly we investigated cortisol-induced RACK1 subcellular localization and we found it significantly higher in the cytoskeleton comportment, which is consistent with a possible effect on cell migration; then we performed a scratch wound healing assay. After 24 hours of cortisol treatment 80% of the wound area was helded whereas only 60% was covered in untreated control cells. We also demonstrated that cortisol-induce cell migration could be prevented by the treatment with the GR antagonist mifepristone.

Taking into account that there is no specific targeted therapy for TNBC patients and that resistance to standard cytotoxic chemotherapies is growing, our results are particularly relevant in a clinical context. GR antagonism, in fact, was demonstrated to sensitize cells to chemotherapy-induced cytotoxicity in TNBC [78,79] and a phase I clinical trail conducted in advanced breast cancer patients suggests that GR could be a useful biomarker and a promising target in TNBCs [87,88].

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Transcriptional regulation of RACK1 and modulation of its expression: Role of steroid hormones and significance in health and aging

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Transcriptional regulation of RACK1 and modulation of its expression: Role of steroid hormones and significance in health and aging

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ABSTRACT

The Receptor for Activated C Kinase 1 (RACK1) is a scaffold protein for different kinases and membrane receptors. RACK1 can shuttle proteins to their sites of action, facilitate cross-talk among distinct signaling pathways or recruit other signaling proteins into the complexes. Therefore, it is a key mediator of various pathways and is involved in various biological events including development, immune response, brain activity and cancer. Because of its importance, it is of extreme significance to understand the transcriptional mechanisms governing its expression. The identification of regulatory elements in the promoter of RACK1 shed some light on its transcriptional modulation in physiological and pathological context. Literature data support the existence of a complex hormonal balance, between glucocorticoids and androgens, in the control of RACK1 expression due to specific and complex interactions on the RACK1 promoter. These and other informations suggest that a better understanding of RACK1 transcriptional regulation is essential to unravel its role. Furthermore, the modulation of its expression in physiological or pathological conditions may be of interest in different context, such as aging and cancer.

1. Introduction

The Receptor for Activated C Kinase 1 (RACK1) is a member of the tryptophan-aspartate repeat (WD-repeat) family of proteins and is homologous to the β subunit of G-proteins (Gβ). Family members share a common role as scaffolding protein complexes, often with multiple and competing partners, thereby serving as hubs for diverse signal transduction pathways associated with many biological functions [1–3]. RACK1 was originally cloned from a chicken liver cDNA library and human B- lymphoblastoid cell line (BLCL) [4] and was then isolated from a rat brain cDNA library as a partner of specific protein kinase C (PKC) isoform [5–8]. Although originally found to act as a shuttling protein for activated PKCβII, RACK1 was also demonstrated to bind to other signaling partners including the MAP kinase (MAPK), Jun N-terminal kinase (JNK) [9] and the cAMP-specific phosphodiesterase PDE4D5 [10–13]. Other signal transduction partners identified are the tyrosine kinase oncoprotein SRC [14–16], the protein serine phosphatase PP2A [17,18] and the focal adhesion kinase FAK [19,20]. As a partner for multiple signaling cascades, RACK1 can interact with the cytoplasmic tail of several receptors including the Insulin-like Growth Factor Receptor I (IGF-IR), the NMDA receptor, the β-integrin receptor, the common beta-chain of the IL-5/IL-3/GM-CSF receptor, type I interferon receptor, several ion channels, as well as the androgen receptor (reviewed in [1–3]).

RACK1 is also a ribosomal protein that is present at the small ribosomal unit next to the mRNA exit channel and can influence ribosomal translational activity and selectivity [21,22].

Finally some recent studies show that RACK1 can also participate in the miRNA pathways. There is evidence that RACK1 is involved in the recruitment of miRNAs into miRISC [23], and may facilitate interaction between component of RISC and the translational machinery.

It is clear from this brief outlook that there are multiple binding partners for RACK1 although several of these remain uncharacterized and may not bind directly to RACK1, some of these proteins may be constitutively bound while some require specific signals for binding to RACK1 and other possible partners within the complex. The overall role of RACK1 spans from shuttling signaling proteins to their sites of action to facilitating the cross-talk between signaling pathways and the specific function of the protein RACK1 within these complexes may vary. [see ref. 2]. This undoubtedly identifies a complex network of signaling elements that characterize the physiological and pathological role of RACK1. In fact, the correlation between RACK1 protein levels and conditions related to physiological and pathological changes are of particular significance.

Aging is a key risk factor for degenerative changes for example in the immune and the nervous system. Aging is also a risk factor in the development and progression of some forms of cancer. Evidence is substantial concerning the fact that RACK1 expression and function are linked to these pathological changes.

Concerning aging of the immune system, also known as immunosenescence, the evidence suggest that some age-related defective response of immune cells can be ascribed to an altered PKCβII signaling which depends on a reduced target translocation of the kinase [24,25]. The major contributor to this event is the reduced expression of RACK1 with consequences that include response to influenza vaccination [26], cell proliferation and cytokine production [24–27]. The same defective PKC signaling was also investigated in the brain of aging animals and it was demonstrated to be a key factor in the impairment in memory processes [28,29].

RACK1 has also been implicated in the development and maintenance of some hallmarks of cancer, which include resistance to cell death, induction of angiogenesis, sustaining proliferative signal, invasion and metastasis. RACK1 expression has been shown to be dysregulated in some cancers like pulmonary adenocarcinoma, hepatocellular carcinoma and metastatic melanoma [2,30–32]. Furthermore RACK1 can promote cell proliferation, invasion and metastasis in vitro and in vivo in breast and prostate cancer [33,34]. Finally RACK1 has been suggested to be a prognostic marker. Elevated expression of this protein seems to be associated with poor clinical outcome in breast cancer [35]. High expression of the protein correlates with a poor clinical out- come in early stage non small cell lung cancer (NSCLC) because of a polymorphism in the promoter region of the gene [36]

All these considerations underline that RACK1 participates in numerous cellular functions exerting multiple and different roles. It is clear that a deeper understanding of RACK1 transcriptional regulation is of pivotal interest for all biological pathways involving RACK1 scaffolding and signaling functions.

2. Rack1 structure and promoter element

Although much is known about RACK1 protein localization, interactions and related functions, the mechanisms regulating its expression are rather unexplored. The porcine rack1 gene and its organization was the first to be described in the literature [37]. A 4.5-kb fragment of DNA was obtained by PCR amplification of porcine genomic DNA; the clone contained the putative 5′-regulatory sequence of porcine rack1 gene spanning a length of 850-bp. Several putative transcriptional factor binding sites were identified within this 5′-flanking region. There are, at least, two E2F binding sites, an AP1 binding site, a SP1 binding site, a c-myc binding site, a serum response element (SRE), a YY1 binding site, a NF1 binding site and a NF-κB/c-rel binding site. In porcine ST cells, it was observed that RACK1 protein was transiently induced by serum growth factors. Moreover, RACK1 expression was stimulated by phorbol esters through the mediation of the AP1 binding site on the promoter. These results, taken together, suggest that the 5′-upstream YY1 binding site, SRE and AP1 site can be associated with the regulation of porcine rack1 gene expression [37].

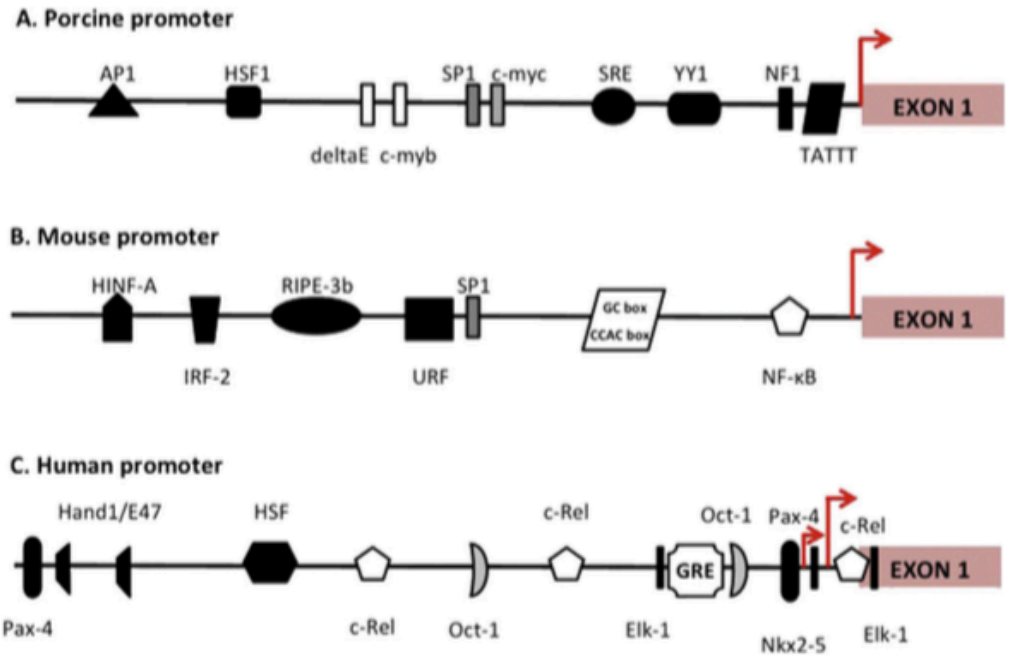

Fig. 1. Bioinformatic analysis of the promoter region of mammalian genes encoding RACK1. (A) Porcine promoter - [37] Map of the 5′-flanking region within 800 bp upstream of the putative promoter region of porcine GNB2L1 gene, potential binding sites for the transcriptional factors are shown. (B) Mouse promoter - [38] Functional analysis of the 5′-flanking within 1.5 kb upstream of the putative promoter region of mouse Rack1 gene, potential binding sites for the transcriptional factors are shown. (C) Human promoter - [39] Bioinformatic analysis of the 5′-flanking region within 7 kb upstream of the putative promoter region of the human RACK1 gene identified two major transcription sites which are indicated with arrows. Several putative cis-acting elements are shown; in particular, the putative unique GRE (Glucocorticoid Response Element) is detected at the nucleotidic position –186 with the sequence AGAACACCCTCCGGAAGCACA.

Subsequent studies characterized the mouse rack1 coding gene structure, identified within a 14-kb region of mouse genomic DNA and found to contain 8 exons and 7 introns. Several putative cis-acting elements in 1.5-kb of the proximal 5′-flanking sequence were recognized, including a CCAC box and sites for histone nuclear factor-A, footprint II factor, interferon regulatory factor-2, rat insulin promoter element 3b, urokinase transcription factor, SP1, and NF-κB transcription factors. Moreover, primer extension analysis identified two distinct transcription start sites at 265 and 285 nucleotides upstream of the translation initiation site. Although no TATA box was predicted in proximity to either transcription start site, two GC box/SP1 sites and four CCAC-binding protein boxes were found within the 150-bp upstream region [38]. This indicates that RACK1 transcripts are driven by a TATA-less, GC-rich promoter region. Further functional characterization of the RACK1 gene promoter suggested that the NF-κB binding site is an important positive regulatory element. Gel mobility shift

assay confirmed that NF-κB interacts with the RACK1 promoter in a region that is critical for gene expression and that seems to be essential for the role of RACK1 in nerve growth factor (NGF)-mediated cell survival [38].

The promoter of the human rack1-encoding gene, previously described in DNA databases as guanine nucleotide binding protein, beta polypeptide 2-like 1 (GNB2L1), was studied by cloning a 2-kb region 5′ of the rack1 human gene to observe possible differences from the above reported species. The human gene has 8 exons and 7 introns, spanning approximately 7-kb. Rack1 is mapped to chromosome 5q35.3, in close proximity to the telomere of chromosome 5 [39].

Similarly, to what observed in the mouse gene, the human rack1 gene has two major transcription start sites: G at 4702 nt (+1, 395 nt 5′ of coding ATG) and C at 4769 nt (+68, 328 nt 5′ of the coding ATG). The shorter and predominant transcription start site (+68) was in a non-canonical oligo pyrimidine sequence (TCATCCCT) and the alternative longer transcription start site (+1) contained in a sequence which does not match characteristic motifs believed to be peculiar of riboproteins coding transcripts [40]. A parallel bioinformatic analysis revealed several putative cis-acting elements. Binding sites for transcription factors belonging to a smooth muscle/cardiomyocyte specific-family were recognized. Indeed, Hand1/E47, which is implicated in cardiomyocytes differentiation, the smooth muscle specific factors Elk- 1 and Nkx2–5, which are cardiac specific homeobox were identified; moreover, myogenin/NF1 factor can also be considered of this group, even if it is more in general involved in muscle growth and differentiation. Another interesting result, similar to that obtained for the mouse and porcine genes, was the finding of four c-Rel binding sites, a member of NF-κB transcription factor family; in addition, Oct-1 and Elk-1 sites were also identified. Then three Pax-4 binding sites were found and interestingly, one of these was contained in the 3′ of the first mapped transcription start; moreover Nkx2–5, another binding element, was found in the 3′ of the second transcription start site. Finally, a consensus sequence for the binding of GR (Glucococorticoid Receptor) was detected at the nucleotide −186 (+1 is the first TSS) [39] [Fig. 1].

3. Functional analysis of RACK1 promoter region

Functional analysis of the promoter region was performed by constructing deletion fragments and cloning them into a firefly luciferase- reporter vector [41]. These constructs were used in two different cellular models to investigate their transcriptional modulation in cells of neural (human neuroblastoma SH-SY5Y cells) and immune (human monocytic THP-1 cells) origin.

To better understand the regulation of the mapped promoter region, two well-known stimuli were used: lipopolysaccharide (LPS) is an immune stimulus, and phorbol 12-myristate 13-

acetate (PMA) which is a direct activator of the PKC pathway and was previously reported to induce RACK1 protein expression [37]. Both molecules are directly or indirectly linked to cellular signals directed to the nucleus by means of the NF-κB pathway.

Consistently with the direct nature of the stimulus, treatment of both THP-1 and SH-SY5Y cells transfected with the full length clone Δ1, resulted in significant increase of luciferase activity [39]. On the other hand a differential response was obtained when using LPS which induced a significant increase in promoter activity in THP-1 cells transfected with the full length Δ1 construct while in SH-SY5Y cells, known to possess a cytosolic non-functional TLR4 receptor for LPS [42] there was no significant response [39].

3.1. cRel sites and their significance in cognitive functions and neurodegeneration

Fragmentation of the Δ1 construct and subsequent functional analysis suggested that one interesting feature shared by the porcine, mouse and human gene was the possibility to respond to stimuli connected with the NF-κB pathway. In particular, considering the mouse gene, it was demonstrated that the effect of NGF on PC12 cells survival was mediated by the activation of NF-κB and resulted in maintenance of RACK1 levels and cell survival in serum-free medium. On the other hand, inhibition of NF-κB activity blocked cell survival and reduced RACK1 expression [38].

The NF-κB transcription factor has been demonstrated to have a role in various context, particularly in the field of neuronal plasticity and survival [43,44]. NF-κB is activated by several cytokines and neurotrophic factors in response to various cell stressors, and it is known to be activated in neurons and glial cells in acute and chronic neurodegenerative conditions. Some evidence suggest that both RACK1 [45] and NF-κB [46] are reduced in the brain of patients affected by Alzheimer's disease (AD). AD is one of the most common neurodegenerative disorder characterized by the deposition in the brain of fibrillar aggregates of a peptide named beta-amyloid (Aβ), derived from proteolytic processing of a precursor called amyloid precursor protein (APP). APP is an integral membrane protein with a complex proteolytic metabolism that can be simplified in a so called “amyloidogenic” pathway, based on the activities known as β and γ secretases, which generate Aβ and an alternative “non amyloidogenic” pathway, based on the action of α secretase cleaving APP inside the Aβ region [47]. This step generates sAPPα, a soluble APP fragment secreted in conditioned medium of cultured cells, human plasma and the cerebrospinal fluid. APP processing by α-secretase occurs via a constitutive pathway as well as by receptor mediated activation of multiple signal transduction pathways among which protein kinase C (PKC) is a major player [48,49].

Several studies have suggested that sAPPα is involved in physiological processes such as neuroprotection, synaptic plasticity, neurite outgrowth and synaptogenesis [50] and the role in neuroprotection may be associated with effects on several signaling pathways related to cell

survival. Among them, the PI3K/Akt pathway has been shown to be activated by sAPPα in models in vitro and in vivo [50]. Moreover there is also evidence of the involvement of the NF-ϰB pathway down- stream of PI3K/Akt [43,51].

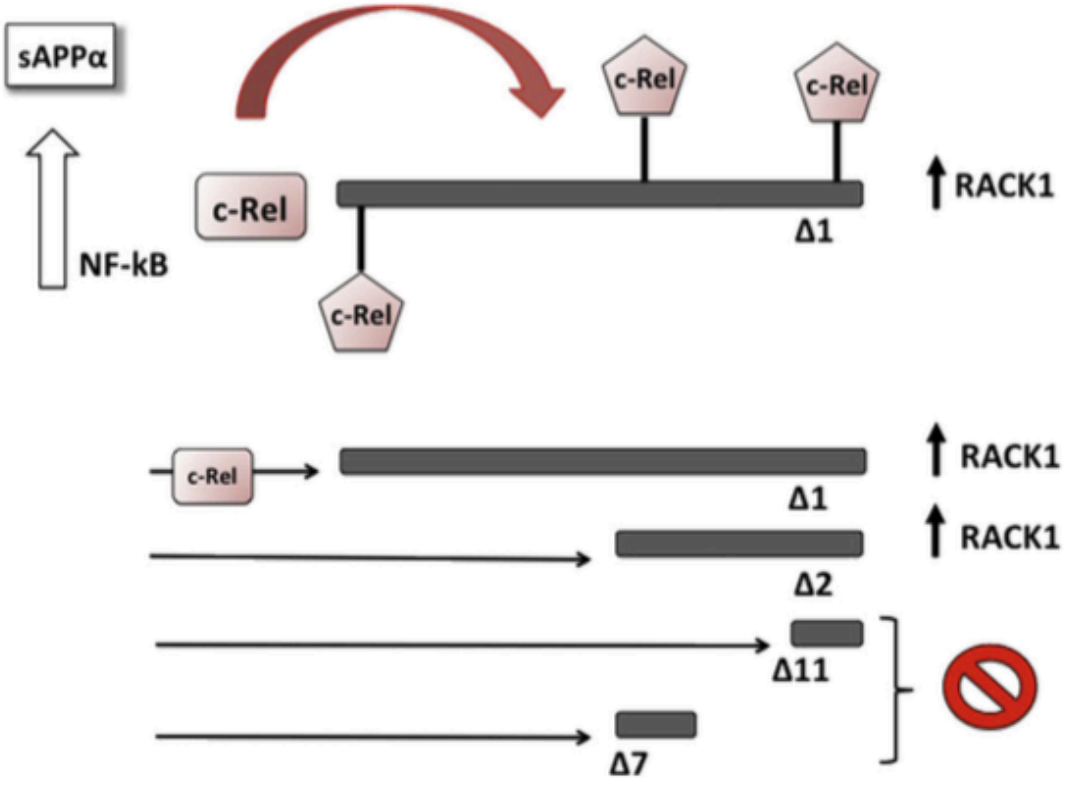

Fig. 2. sAPPα promotes RACK1 promoter activation. In nervous system, sAPPα is able to activate PI3K/Akt and NF-ϰB pathways promoting nuclear translocation of c-Rel. sAPPα-induced c- Rel translocation was investigated through the construction of four RACK1 promoter deletion mutant on a luciferase reporter. A 2 kb region 5′ of the rack1 human gene containing all three c-Rel sites was clone (Δ1). Deletion mutants Δ2 excluding the distal c-Rel cis sites, Δ7 containing only the proximal cis site and Δ11 containing only the intermediate c-Rel site were generated. Exposures to sAPPα of SH-SY5Y cells transfected with the Δ1 and Δ2 constructs resulted in a significant increase of luciferase activity whereas the transfections with Δ7 and Δ11 constructs did not elicit a luciferase response. These data highlighted that sAPPα treatment was able to activate the c-Rel sites on the RACK1 promoter and that both proximal cis sites (located in both Δ1 and Δ2) were necessary for its activation.

In this regard, it was demonstrated that, in SH-SY5Y cells, sAPPα can modulate the expression of RACK1 and the signaling activity of PKCβII through the activation of the PI3K/Akt and NF-ϰB pathways [52]. In fact in vitro experiments sAPPα was able to promote nuclear translocation of c-Rel. Since three consensus c-Rel responsive elements were mapped

on the RACK1 promoter region, it was investigated whether the c-Rel translocation induced by sAPPα was able to influence RACK1 promoter activity. For this purpose, four different luciferase constructs were designed in order to include all three c-Rel sites or alternative combination of them and verify their response to sAPPα (see Fig. 2 and data in ref. 52). Exposures to sAPPα of cells transfected with the Δ1 and Δ2 constructs resulted in a significant increase of rack1 luciferase activity whereas SH-SY5Y cells transfected with Δ7 and Δ11 constructs did not elicit a luciferase response. These data highlighted that sAPPα treatment was able to activate the c-Rel sites on the RACK1 promoter and that both cis sites, located in Δ1 and Δ2 constructs, were necessary for its activation. Activation of the promoter resulted also in increased expression of RACK1 protein as well as increased translocation and activity of PKCβII thus suggesting that the effect of sAPPα on RACK1 also induced an effect on PKCβII signaling. The specific involvement of the PI3K/Akt cascade and NF-ϰB were confirmed by appropriate control with pathway inhibitors. Blocking the activation of the Akt/PI3K with wortmannin and blocking the activation and translocation of cRel prevented all effects on the RACK1 promoter constructs and on RACK1 protein expression, thus strongly supporting a direct link between sAPPα and the effects on rack1 gene expression through the activity of the transcription factor NF-ϰB [52].

These observations are relevant in the context of AD. In fact, AD patients have reduced levels of sAPPα in the cerebrospinal fluid and reduced constitutive and regulated release of sAPPα has been demonstrated in cells from AD patients [for a review see ref. 49]. Moreover, there is strong evidence that PKC signaling pathways are causally involved in memory and cognition and, therefore may be associated also to molecular events in the neurodegenerative pathophysiology of dementia such as AD. Age related loss of PKC dependent signaling were described in several species. Loss of PKC in the membrane fraction of hippocampal neurons was demonstrated in rabbits and rats where the loss of PKC was not dependent from overall loss of the kinase protein. However, a dramatic decrease of the PKC anchoring protein RACK1 suggested that the latter was the major contributor to the dysregulation of the PKC system in the aging animals [for a review see ref. 53]. It was also shown that RACK1 levels were significantly decreased in AD brains in both membrane and cytosolic fractions when compared to age and post mortem interval matched control cases. This change was not associated with modified levels of PKCβII, suggesting a specific impairment of the mechanism of anchoring of this PKC isoform [45] pointing to a major role of RACK1 expression in cognitive degeneration and other age related conditions [53,54].

3.2. *The GRE site and the interaction of DHEA and glucocorticoids*

One significant observation on the bioinformatic analysis of the RACK1 promoter was the presence of a GRE (Glucocorticoid Response Element) consensus sequence (5′-AGAACACCCTCCGGAAGCACA-3′) identified at the nucleotide position – 186/– 165 relative to the transcription start site [39]. Many mechanistic studies have shown that GR transactivation requires the presence of (+) GREs, which allosterically mediate GR binding, recruitment of coactivators and transcription. These elements contain two inverted repeat AGAACA sequences separated by three nucleotides, with G and C bases critical for GR

binding. The three-nucleotide spacing between half-sites is strictly required to preserve dimerization potential of GR on the element. In contrast, a newly discovered negative glucocorticoid response element (nGRE) mediates DNA-dependent transrepression by the GR across the genome and has a major role in immunosuppressive therapy. This nGRE consensus sequence, CTCC(N)0-2GGAGA, differs dramatically from activating sequences. The spacing required in the nGRE is variable, ranging from 0 to 2 nucleotides. To unravel the mechanism of nGRE-mediated transrepression by the GR, Hudson and colleagues studied the interaction between GR and nGRE in the thymic stromal lymphopoietin (TSLP) promoter [55]. They demonstrated that two GR monomers bind nGREs in a reverted repeat orientation with strong negative cooperativity. When combined, the unique GR conformation and negative cooperativity ensure the presence of monomeric GR at nGREs. This interaction mechanism represents a new mode of GR-DNA binding and a new paradigm for GR-mediated transrepression [55]. The site identified on the RACK1 promoter appears to be similar to the consensus for a negative GRE.

The preliminary functional analysis of the RACK1 reporter constructs demonstrated that in SH-SY5Y cells 1 μM of cortisol for 24 h was able to repress the activity of the promoter constructs. By using two different constructs one full length and a very short one without the GRE sequence it was shown that cortisol inhibition of RACK1 driven luciferase expression was dependent on the presence of the GRE element [39]. A more detailed analysis of the role of the GRE element in the RACK1 promoter was conducted in a second cellular model. Human monocytic THP-1 cells were transiently transfected with three luciferase reporter constructs Δ1, Δ6 and a new construct Δ9 which includes only the GRE sequence and its immediate surroundings. Cells were incubated in the presence of cortisol at the concentration of 0.1 μM or 0.5 μM; these concentrations were chosen as representative of the most common range of total plasma concentration of cortisol in humans. Δ1 luciferase activity was strongly reduced by both cortisol concentrations. Similar results were obtained in cells transfected with the Δ9 construct which contained only the GCs responsive element. Conversely, cortisol treatment did not show evidence of inhibition of luciferase activity in cells transfected with the Δ6 reporter construct because of GRE sequence absence [56]. These data showed that when promoter constructs bearing the putative GCs responsive element were challenged with cortisol the effect of treatment was an inhibition of luciferase activity. In line with this evidence, cortisol treatment exerted its effect also at RACK1 mRNA and protein levels. Overall these data suggest that cortisol at physiological concentrations can inhibit the expression of RACK1 protein via inhibition of the activity of its gene promoter [56]. On the other hand, mifepristone, a potent GR antagonist, completely prevent the effect of cortisol [57]. Further evidence suggest that other corticosteroids such as betamethasone, budesonide, methylprednisolone, prednisone and prednisolone can target RACK1. All these corticosteroids, acting at the transcriptional level, are able to modulate RACK1 expression, supporting the notion that this protein is an important target of corticosteroid-induced anti-inflammatory effects [57] (Fig. 3).

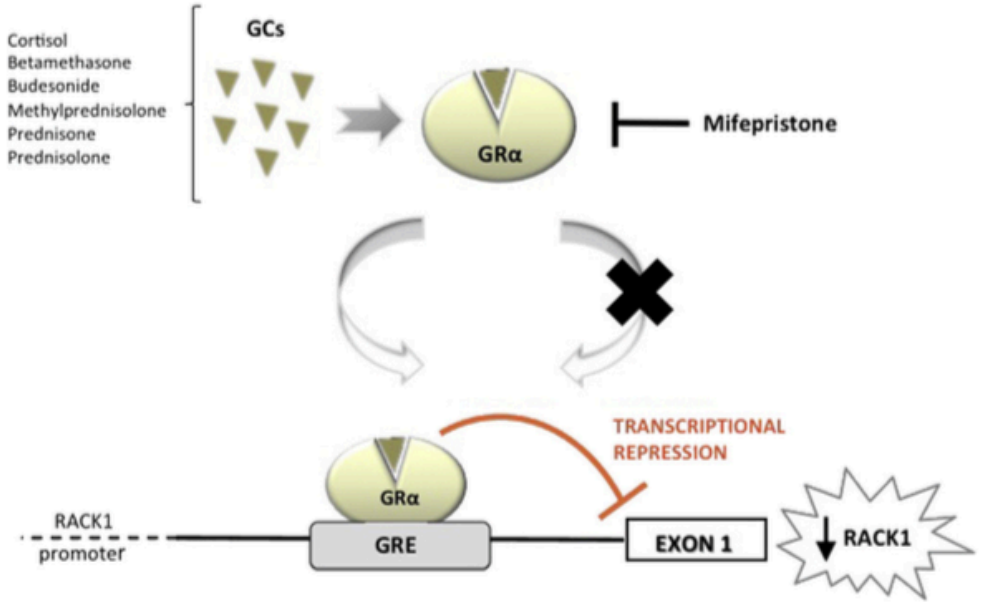

Fig. 3. Schematic representation of the interaction between GRα and GRE sequence located on RACK1 promoter. Glucocorticoids (GCs) bind GRα (Glucocorticoid Receptor α) thus interacting with the GRE (Glucocorticoid Response Element) sequence on the RACK1 promoter. This binding determines a down-regulation of RACK1 expression. Treatment with GR antagonist mifepristone is able to prevent the inhibitory effect of GCs on the expression of RACK1 [56,57].

The investigation of the transcriptional regulation of rack1 began with the observation that some age related deficits of the immune system are correlated to a reduced expression of RACK1 [24,25]. Further investigation into the mechanisms led to the observation that reduced secretion of dehydroepiandrosterone (DHEA) during aging was associated to the decrease of RACK1 expression and PKC-dependent functions and that in vitro and in vivo administration of DHEA was able to restore RACK1 expression to normal levels in cells from aged animals and humans [25,27,54].

It is well established that the aging process is accompanied by hormonal changes characterized by an imbalance between catabolic and anabolic hormones and in the elderly, the imbalance between cortisol and DHEA may affect immune functions [58,59]. Cortisol and DHEA are both produced by the adrenal gland however in different zones of the adrenal cortex. Cortisol is synthesized in the zona fasciculata, whereas DHEA in the zona reticularis. Aging brings to a differential degeneration of these two zones with the latter being the most affected and resulting in a steady reduction of the synthesis of DHEA after puberty and toward aging [58]. The common observation is that the increased ratio cortisol:DHEA in the

elderly is derived mainly as a result of a significant reduction in the levels of DHEA [58–60].

On the other hand it is a consolidated evidence that DHEA exerts anti-glucocorticoid properties in the regulation of many processes involved in the immune response and the evidence concerning the effect on RACK1 is consistent with this antagonistic paradigm.

The activities of DHEA have not yet been unequivocally correlated with a classical nuclear steroid receptor interaction and some evidence has been provided for the presence of a specific G protein coupled plasma membrane receptor [61].

The effect of cortisol on RACK1 expression is clearly transcriptional and the experimental evidence suggest that also the effect of DHEA on RACK1 is transcriptional in nature. However, besides the hypotheses concerning a cell surface receptor there is no clear indication of the interaction of DHEA with a nuclear receptor with canonical transcriptional activity.

The reporter constructs bearing the full length and other fragments of the RACK1 promoter were transfected in the two cellular models used by Del Vecchio et al. [39] and challenged with DHEA; however, no direct effect was observed in the experimental paradigm chosen.

It is therefore very important to understand, in the context of immunosenescence, the molecular mechanism through which cortisol and DHEA regulate RACK1 gene expression.

Once established that the effect of DHEA on RACK1 expression cannot be explained by a direct interaction on the promoter region cloned [56], and established that the contrasting effect on cortisol cannot be explained with simple pharmacological antagonism, a number of different indirect mechanisms of action can be accounted for the activity of DHEA. In this context, it was recently demonstrated that DHEA modulates the effect of cortisol on RACK1 expression via interference with the splicing of the glucocorticoid receptor [62].

The human GR gene (NR3C1) is expressed in several isoforms and the most representative are generated by the alternative splicing in exon 9 and are termed GRα and GRβ [63]. GRα mediates most of the known glucocorticoid actions, while the GRβ isoform is expressed in most tissues but lacks the ligand-binding domain. The specific functions of GRβ had not been completely elucidated but it is known to have a dominant negative effect on GRα [64]. DHEA treatment induced an increase of GRβ secondary to the induction of expression of a member of the Serine/Arginine rich proteins (SRp) family. These proteins play many significant roles in the regulation of RNA processing and gene expression, spanning from transcription to translation [65] and particularly pre-mRNA splicing [66]. Emerging data establish an association between high levels of the GRβ isoform and altered SR protein expression [67,68,69]. The upregulation afforded by DHEA influenced the effect of cortisol on RACK1 expression suggesting that DHEA may have an indirect transcriptional effect on the RACK1 promoter via induction of the alternative splicing of the GR [62].

3.3. Effect of androgens on the RACK1 promoter

Another possible mechanism through which DHEA can modulate cortisol activity on RACK1 expression could be dependent on the transformation of DHEA into active androgen steroids. In THP-1 cells, it was found that DHEA is converted to metabolites 3α-diol and 3β-diol as well as testosterone and dihydrotestosterone (DHT). The use of the drug finasteride, an inhibitor of the enzyme 5α -reductase, prevents the conversion of testosterone to DHT as well as the conversion of 4- androstene-3,17-dione to 5α-androstanedione which is then metabolized by 17β-hydroxysteroid oxidoreductase to DHT. Hence, the effect of DHEA on RACK1 expression can be prevented, implicating DHT as an effector androgen [70]. Previous indications already suggested that the effect of DHEA on RACK1 expression in cells from aging animals and humans could be recapitulated also by testosterone [27]. More specific indication that the androgen receptor (AR) is involved in the activity of DHEA came from data demonstrating that the effect of DHEA on RACK1 expression could be completely prevented by using flutamide as an AR antagonist. Similarly, blocking the expression of the receptor by the use of siRNA resulted in no response to DHEA in terms of RACK1 expression. The effect of blocking the receptor had also an influence on the modulation of GR splicing since blockade of the AR using flutamide prevented the induction of GRβ following DHEA treatment [70].

All together, these data clearly indicate that the modulation of the AR is a key step in the mechanism supporting DHEA-induced RACK1 expression and these indications grant further detailed analysis of the RACK1 promoter region which is limited to bioinformatic informations and need a more specific functional verification.

It is known that the AR and GR can interact at the transcriptional level and that this interaction is correlated with their ability to form heterodimers at a common DNA site, in vitro and in vivo. Indeed, identical P boxes of GR, AR, mineralocorticoid receptor (MR) and progesterone receptor (PR) allow them to bind to similar hormone response elements (HRE). In transient transactivation assays, AR, GR, MR, and PR are all capable of activating reporter genes by recognizing a similar palindromic response element that comprises an inverted repeat of the 5′- AGAACA-3′ hexamer with a 3-nt spacer, usually termed as a canonical androgen/glucocorticoid response element (ARE/GRE) [71].

Analysis of the sequences specific for the wild-type AR identified a response element with a well-conserved 5′-hexamer (5′-AGAACA-3′) but a less stringent sequence requirement for the 3′-hexamer [72–74]. Moreover, alternative AREs with different spacer-lengths or different hexamer-orientations have been proposed [75,76]. Such difference between GR and AR could well be due to the stronger dimerization function in the AR-DBD which allows the AR to dimerize on sequences that are more divergent or to AR-binding to response elements that are organized as direct repeats (AGAACA [0–8 n] AGAACA) instead of the canonical inverted repeats [76,77]. Collectively, AR selective receptor binding in vivo is achieved

through relaxed cis-element stringency rather than a distinct and strict ARE sequence [74].

In case of the AR, it is still possible that the AR can bind other DNA sequences as a monomer, or in alternative dimer conformations, or even as a heterodimer with transcription factors that do not belong to the NR superfamily [78,79]. The latter may include interactions with AP-1, NFI, and Oct-1 or with transcriptional coactivators that do not themselves bind DNA such as SRC-1, GRIP1, and TIF2. These interactions sum to regulate the steroid response in a promoter- and cell context-dependent manner. In this regard, the interaction of Oct-1 with AR versus GR in vivo and in vitro was tested. Oct-1 co-immunoprecipitated from cell lysates with both AR and GR, but in qualitatively different manners. First, in contrast to GR, AR interacts well with Oct-1 only when both factors are bound to DNA. Second, binding to the DNA induces selective changes in the conformation of the ligand-binding domain of AR, but not GR, and leads to increased interaction with Oct-1. Finally, the coactivator SRC-1 interacts more efficiently with the AR-Oct-1 complex when both transcription factors are bound to DNA. These results suggest that DNA-dependent protein-protein interactions, functionally amplified by enhanced coactivator recruitment, may promote receptor-selective activation. Hence, differential interactions among factors, rather than their stringent specificity, can confer precise hormonal response [80].

In the context of the RACK1 promoter we identified an Oct-1 binding site close to GRE sequence. We can speculate that this transcription factor may regulate RACK1 expression in association with AR; in fact the non-canonical GRE sequence described may be also a cis-regulatory target of AR as it consist of direct repeats 5′-AGAACAccctccggaAGCACA-3′ including the already mentioned well-conserved 5′-hexamer (5′- AGAACA-3′) (Fig. 4).

Functional consequences of these AR regulatory elements on the RACK1 promoter can be postulated in the context of cancer and in particular those cancers that are most sensitive to hormonal regulation.

AR remains an important therapeutic target in the treatment of metastatic prostate cancer (PC), and steroidal or nonsteroidal AR inhibitors commonly known as antiandrogens, such as cyproterone acetate, flutamide, nilutamide, and bicalutamide have been used for several decades for this purpose [71]. Literature data report that Oct-1 coordinates genome-wide AR signaling for prostate cancer growth. It was also observed that treatment with Oct-1 polyamides suppressed castration-resistant tumor growth and specifically repressed global Oct-1 chromatin association and androgen signaling in prostate cancer cells. Hence, targeting Oct-1 binding could be a novel therapeutic strategy for AR-activated castration-resistant prostate cancer [81]. In this context, it is also interesting to underline that RACK1 promotes PC cell proliferation, invasion and metastasis in vitro and in vivo by PTEN downregulation and Ki67 upregulation [33].

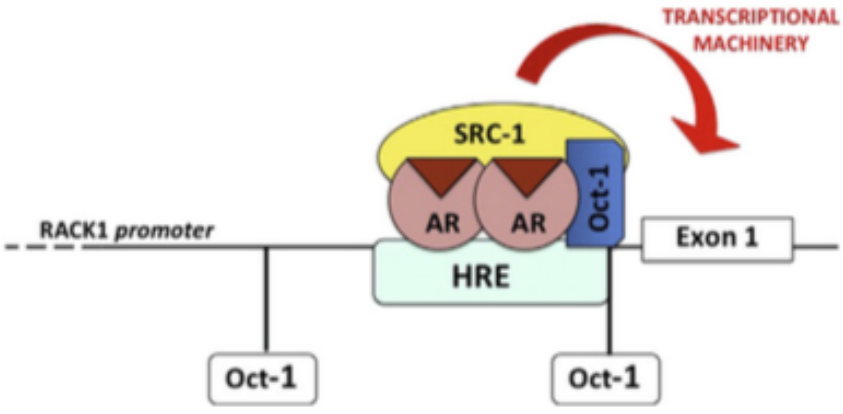

Fig. 4. Model for RACK1 regulation in prostate cancer cells. AR and Oct-1 binding to cognate sites (within the RACK1 promoter) induces conformational changes in both AR and Oct-1 thus favoring their interaction and facilitates recruitment of the co-activator SRC-1 to promote transcription. One SRC-1 molecule is shown for simplicity (see text for details).

Approximately one-half of the AR cistrome overlaps with that of GR in a prostate cancer cell line expressing both AR and GR and in cell lines established from antiandrogen-resistant xenograft tumors. The fact that AR and GR bind to similar response elements has raised questions about their interplay on chromatin binding in vivo. The interplay between AR and GR signaling is of potential clinical importance because about 30% of prostate cancers express GR, and RACK1 basal expression is regulated by increased splicing toward GRβ [62] therefore RACK1 over-expression in prostate cancer cells may also be attributed to this signaling pathway. Indeed, GRβ may interact with numerous transcriptional cofactors and transcriptional factors, lodge into the transcription intermediate complex formed on the promoter region of GRβ responsive genes, and modulate their transcriptional activity [64]. It is interesting to note that, in immune context, DHEA was able to increase RACK1 expression by the up-regulation of GRβ expression. This observation is interesting as in the inflammatory prostate tissue microenvironment it is possible that one of the mechanisms of cancer promotion includes increased metabolism of endogenous DHEA, to either androgens or estrogens, and also increased induction of paracrine factors, including cytokines, chemokines and growth factors, that induce proliferation or inhibit apoptosis [82].

4. Conclusion

Due to the numerous interacting partners, RACK1 appears to be a fulcrum of cellular homeostasis, controlling essential cellular processes such as transcription and translation, cell proliferation and growth as well as cell spreading and cell-cell interactions. The informations collected clearly suggest that the transcriptional control of RACK1 expression may assume a pivotal significance in several physiological and pathological context. The functional analysis of the promoter elements has been conducted only in a limited set of experimental paradigms indicating for example the important role of the NF-κB pathway, which may not be limited to neuroprotection or cell survival but implicate also more complex interactions because this signaling pathway is also central to immune activation as well as in cancer.

Hence, considering the central role of RACK1 and the different contexts in which it is involved, an updated analysis of RACK1 promoter region should be made in order to identify other possible transcription factor sites which could be different or similar in porcine and in mouse promoters. Changes in RACK1 levels are likely to subvert physiological functions and may lead to defective immune surveillance, endocrine effects and some features of cancer cells. Understanding the molecular mechanisms involved in RACK1 expression may offer the possibility to control its expression, which is going to be beneficial in all those physiological and pathological conditions in which the role of RACK1 is central.

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Role of spliceosome proteins in the regulation of glucocorticoid receptor isoforms by cortisol and dehydroepiandrosterone

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Role of spliceosome proteins in the regulation of glucocorticoid receptor isoforms by cortisol and dehydroepiandrosterone

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ABSTRACT

Dehydroepiandrosterone (DHEA) can counteract the activity of cortisol by modulating the glucocorticoid receptor β (GRβ) expression and antagonizing the binding of GRα to the glucocorticoid responsive element (GRE) in RACK1 (Receptor for Activated C Kinase 1) promoter. These observations are important in the context of immunosenescence and can be extended to recognize a complex hormonal balance in the control of GR isoform expression and consequently in the expression of GR responsive genes. To elucidate the mechanism of DHEA on GR alternative splicing, we investigated its possible involvement in the expression of proteins such as the Serine/arginine (SR)-Rich Splicing Factors (SRSF) regulating GR splicing, specifically SRSF9 and SRSF3 also known as SRp30c and SRp20 respectively. We demonstrated that DHEA can induce the up-regulation of GRβ mRNA which is preferentially directed toward the isoform. The effect is due to an increase in expression of the splicing factor SRSF9. On the other hand cortisol up-regulated SRSF3, the splicing factor promoting GRα isoform. We demonstrated that DHEA and cortisol modulate SRSF9 and SRSF3 in a different way and our data suggest that the anti-glucocorticoid effect of DHEA, among other mechanisms, is also exerted by modulating the expression of proteins involved in the splicing of the GR pre-mRNA.

1. Introduction

The aging of the immune system is related to the impairment of some signal transduction systems. In this context, we demonstrated that the reduced expression of the scaffold protein RACK1 (Receptor for Activated C Kinase 1) was related to a defective PCK II (Protein Kinase C II) translocation. As a consequence of this signal transduction impairment, a significant decrease in immune cells functionality was observed [1,2]. The age-associated decline in RACK1 expression and immune functions were restored in vitro and in vivo by dehydroepiandrosterone (DHEA) administration [2,3]. The endocrine system plays an important role in modulating immune functions [4]; in particular, we demonstrated that DHEA and cortisol interaction is relevant for RACK1 expression [5]. DHEA behaved as a positive RACK1 regulator, whereas cortisol acted as a transcriptional repressor for the rack1 gene, previously known as GNB2L1 (guanine nucleotide-binding protein-2-like 1) [5]. Physiological concentrations of cortisol exerted an inhibitory effect on RACK1 expression because of the presence of a glucocorticoid receptor (GR) binding consensus sequence [glucocorticoid-responsive element (GRE) sequence] in RACK1 promoter region [5–7].

It is a consolidated evidence that DHEA exerts anti-glucocorticoid properties in the regulation of many processes including the immune response. However, the counteracting effect of DHEA against cortisol within the context of RACK1 expression is not fully understood. We have recently demonstrated that DHEA may interfere with the splicing of glucocorticoid receptor (GR) mRNA inducing the formation of the β isoform; in detail, DHEA induced a dose related up-regulation of GRβ and GRβ knockdown completely prevented DHEA-induced RACK1 expression and cytokine release [7].

The human GR gene (NR3C1), coding for glucocorticoid receptor (GR), is composed of 9 exons. The alternative splicing in exon 9 generates two homologous receptor isoforms, termed GRα and GRβ [8,9].

GRα, the classic receptor, mediates most of the known glucocorticoid actions. After binding to its agonist ligand, GRα undergoes conformational changes, dissociates from heat shock proteins (HSPs), homo-dimerizes and translocates into the nucleus. The ligand-activated GRα directly interacts with specific DNA sequences (GRE sequences) in the promoter regions of target genes [10]. Similarly to the classic human GRα, the human GRβ isoform is also ubiquitously expressed in most tissues and it contains 742 amino acids and shares the first 727 amino acids from the N-terminus with GRα [11]. GRβ encodes an additional 15 non-homologous amino acids in the C-terminus whereas GRα possesses an additional 50 amino acids forming a 777 amino acid protein. Therefore, GR shares the same N-terminal or immunogenic domain (NTD) and DNA-binding domain (DBD) with GRα , but has a unique ligand-binding domain (LBD). As a result of these differences, GRβ does not bind glucocorticoids and thus it is unable to activate glucocorticoid-responsive gene promoters [10,11]. Indeed, there is evidence that GRβ acts as a dominant negative on GRα [12,13]. As

this regard, we demonstrated that GRβ antagonize the function of GRα by the formation of an inactive GRβ/GRα heterodimer. In the context of RACK1 expression, the presence of this inactive GRβ/GRα complex was demonstrated by an ELISA based transcription factor binding assay. DHEA counteracts the cortisol-induced binding of GRα to the RACK1 promoter region, thus reinforcing the idea that GRβ is a dominant-negative regulator of GRα activity [7].

In order to understand the selective increase in GRβ, it is imporant to consider the alternative splicing machinery represented by the spliceosome and its components. In this regard, it has been reported that different Serine/arginine (SR)-Rich Splicing Factors (SRSF), such as SRSF9 and SRSF3 (also known as SRp30c and SRp20 respectively), could be involved in alternative splicing of GR, thus altering relative levels of GRα and GRβ [14]. In particular, SRSF9 has been predominantly found to direct alternative splicing of GR gene in neutrophils [15].

Recently, we demonstrated that DHEA treatment induced an over-expression of SRSF9 [7] thus suggesting that modulation of the spliceosome proteins involved in GR mRNA splicing may represent a relevant mechanism in the regulation of glucocorticoid activities.

The purpose of this work was to investigate whether DHEA and cortisol treatment influence the alternative splicing of GR pre-mRNA by modulating specific SR proteins. In particular, we focused our attention on SRSF9 and SRSF3, two central proteins for GR pre-mRNA splicing, in order to elucidate the mechanism of action of DHEA and cortisol on GR functions.

2. Materials and Methods

2.1. Chemicals

DHEA, cortisol and flutamide were obtained from Sigma Aldrich (St. Louis, MO, USA). They were dissolved in DMSO at concentration of 1 mM and 10 mM and frozen (–20 ° C) in stock aliquots. Stock aliquots were diluted at a final concentration in culture media at the time of use (final concentration of DMSO in culture medium <0.1%). Cell culture media and all supplements were from Sigma Aldrich. Mouse anti-human RACK1 monoclonal antibody (610177) and mouse monoclonal anti-actin (612656) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal anti-SRp30c (sc-134036) and mouse monoclonal anti-tubulin were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) Mouse monoclonal anti-SRP20 (MABE 116, Millipore) was purchased from Sigma Aldrich. Rabbit polyclonal anti-GR (H-300) (sc-8992) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA) while mouse monoclonal anti-GR (ab130227) were acquired from Abcam (Cambridge, UK). Host specific peroxidase conjugated IgG secondary antibody (31460) was purchased from Thermo Scientific (Waltham, MA, USA). All reagents were purchased at the highest purity available. Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA).

2.2. Cells and treatments

THP-1 human promyelocytic cells (ECACC, Salisbury, UK) were maintained at 37 ° C in 5% CO_2 and cultured in RPMI 1640 medium supplemented with 10% heated-inactivated FBS, 2 mM L-glutamine, 0.1 mg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin.

Treatments were performed using 1.5×10^6 cells seeded in 6 well dishes. In line with our previous results, cells were treated for 6 h with 0.1 M cortisol [5] whereas DHEA treatment was performed for 18 h at the concentration of 0.1 M [7]. To demonstrate the role of androgen receptor (AR), 50 M flutamide was used [16]. Other specific details of times and concentrations are given in figure legends.

2.3. RT-PCR and real-time PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. QuantiTect reversion transcription kit from Qiagen was used for cDNA synthesis following manufacturer's instructions. RT-PCR was performed using GRα and GRβ primers, which were custom, designed and synthesized by Primm (Milan, Italy) and the nucleotide primer sequences are found in Table 1.

Table 1

Name	Forward primer sequence (5′ to 3′)	Forward primer sequence (3′ to 5′)	Ref.
GRα	CTAAGGACGGTCTGAAGAGC	GCCAAGTCTTGGCCCTCTAT	[17]
GRβ	CTTCCAGAACCATGGTAGCC	TACGAAACTCCACCCAAAGG	[7]

Amplification was obtained by polymerase chain reaction (PCR) using GoTaq® G2 DNA polymerase (Promega, Madison, WI). RpL6, SRSF9 (SRp30c), SRSF3 (SRp20) and NR3C1 (GR) primers were provided by Qiagen.

Real-time PCR was performed by using QuantiTect Syber Green PCR kit from Qiagen. PCRs were performed in duplicate and according to the standard protocol suggested by the manufacturer. The RpL6 (ribosomal protein L6) RNA transcription was used as endogenous reference [18] and the quantification of the transcripts was performed by using the $2^{-\Delta\Delta CT}$ method [19].

2.4. Western blot analysis

The expression of GRα, GRβ, SRSF9, SRSF3 and RACK1 in cell homogenates was assessed by Western blot analysis. Briefly, cells were treated and then collected, washed once with PBS, centrifuged and lysed in 100 L of homogenization buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitor mix). The protein content was measured using the Bradford method (Bio-Rad Protein Assay). Western blotting samples were prepared mixing the cell lysate with sample buffer (125 mM Tris-HCl pH 6, 8.4% SDS, 20% glycerol, 6% -mercaptoethanol, 0.1% bromophenol) and denaturing at 95 ° C for 5 min. Equivalent amounts of extracted protein (20 g) were electrophoresed into an appropriate % SDS-PAGE under reducing conditions. The proteins were then transferred to a PVDF membrane (Amersham, Little Chalfont, UK) which was blocked in 5% w/v BSA, 1X TBS, 0.1% Tween-20 for 1h with gentle shaking. The proteins were visualized using primary antibodies diluted in 5% w/v BSA, 1X TBS, 0.1% Tween-20 for RACK1 (1:1000), GRα (1:1000), GRβ (1:1000), SRSF3 (1:500), SRSF9 (1:1000), β-actin (1:2000) and α-tubulin (1:1000). In all experiments, immuno-reactivity was measured using host specific secondary IgG peroxidase conjugated antibodies (1:5000 diluted) and developed using enhanced chemiluminescence reagent (Pierce, Thermo Scientific, Rockford, IL, USA).

2.5. Small interference RNA (siRNA)

To evaluate the role of SRSF3 and SRSF9 in GRα and GRβ expression respectively, silencing experiments were conducted. The effect of inducing RNA interference on SRSF3 and SRSF9 were assessed using commercially available reagents from Life Technologies and Santa Cruz Biotechnology respectively [20]. siRNA transfection was performed with Lipofectamine® 2000 following manufacturer's instructions. Forty-eight hours after transfection cells were treated as previously described.

2.6. Statistical analysis

Following acquisition of the Western blot or RT-PCR images, the optical density of the bands was calculated and analyzed with Scion Image program for digital image processing (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD). The relative densities of the bands were expressed as arbitrary units and normalized to data obtained from control sample run under the same conditions. All experiments were performed at least three times, with representative results shown. Data are expressed as mean ± standard error (SE). Statistical analysis was performed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA). The data were analyzed by analysis of variance (ANOVA) followed by an appropriate post hoc comparison test as indicated in figure legend. Effects were designed significant if $p \leq 0.05$.

3. Results

3.1. Effect of cortiso land DHEA on total GR expression

We previously demonstrated that DHEA can interfere with the action of glucocorticoids counteracting the inhibitory effect of cortisol on RACK1 expression [5,7].

To better understand this effect, we analyzed the role of cortisol and DHEA on total GR expression in the human promyelocytic cell line THP-1 (Fig. 1A). Cells were treated with physiologically relevant concentrations of DHEA and cortisol and the figure shows that while cortisol does not affect the overall levels of the GR, the effect of DHEA results in a significant increase of total GR mRNA levels.

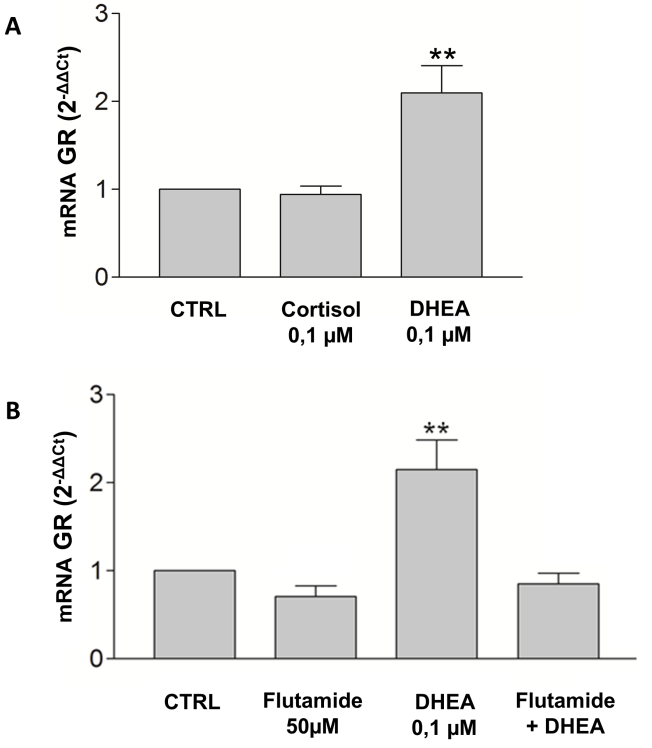

Fig. 1. Effect of cortisol and DHEA on total GR mRNA expression and the role of DHEA as androgenic agonist. (A) THP-1 cells were treated for 6 h with 0.1 M cortisol or 0.1 M DHEA. The effect on mRNA levels was evaluated by real-time PCR in three independent experiments using RpL6 as an endogenous reference. (B) The anti-androgen flutamide prevents DHEA-induced GR mRNA expression. THP-1 cells were treated with 50 M flutamide (1 h), DHEA 0.1 M (18 h) or 50 M flutamide for 1 h followed by 0.1 M DHEA for 18 h. The effect on GR mRNA levels was evaluated by Real-time PCR in three independent experiments using RpL6 as an endogenous reference. Each value in the graphs represents the mean ± SE of three independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with **p < 0.01 versus CTRL (control; vehicle-treated cells).

We have also recently demonstrated that the conversion of DHEA to active androgens is a key event in DHEA-induced expression and monocyte activation [16]. When cells were pre-treated with flutamide (50 M) and subsequently with DHEA the androgen antagonist completely prevented the effect of DHEA on the expression of GR mRNA (Fig. 1B). This result can be consistent with recent data that identified an androgen responsive element 160K base pairs upstream of the GR gene [21].

3.2. Effect of cortisol and DHEA on GRα and GRβ expression

We have recently demonstrated that cells treated with DHEA and then stimulated with cortisol showed a significant increase in the GRβ /GRα ratio, suggesting a possible involvement of DHEA in GR alternative splicing [7]. Hence, we decided to analyze the role of cortisol and DHEA on the expression of GR and isoforms.

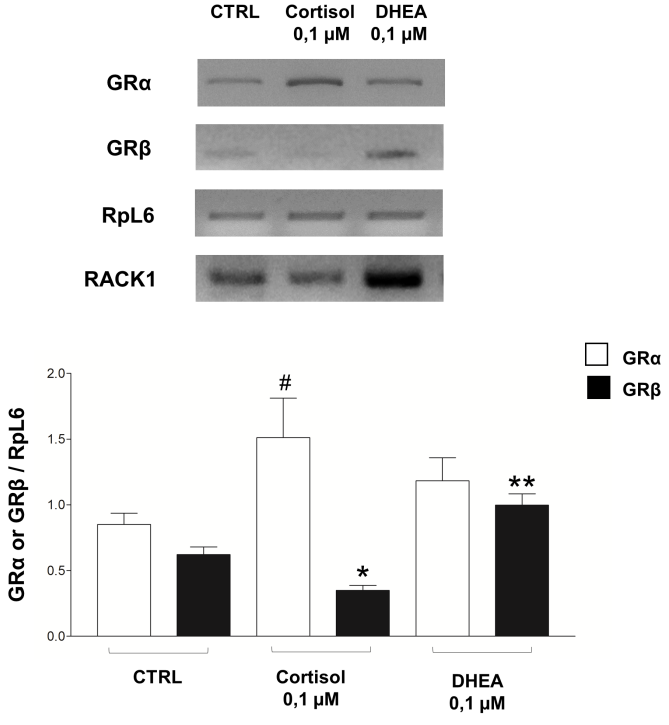

Fig.2. Effect of cortisol and DHEA on GR alternative splicing. THP-1 cells were incubated in a medium containing 0.1 M cortisol (6h) or 0.1 M DHEA (18h). RNA splicing variants, GR and GR were detected by RT-PCR and analyzed on 3% agarose gel. RpL6 expression was detected to normalize the samples while RACK1 expres- sion was used as a positive control. The image is a representative RT-PCR. Each value in the graph represents at least the mean ± SE of three independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $^{\#}$ $p < 0.05$ versus GR CTRL and $^{*}p < 0.05$, $^{**}p < 0.01$ versus GR CTRL (control; vehicle-treated cells).

For these purpose, treatment times corresponded to our previous published experimental scheme which was demonstrated to be optimal to investigate DHEA and cortisol actions [5,7]. THP-1 cells were treated for 18 h with 0.1 M DHEA or for 6 h with cortisol and subsequently analyzed by RT-PCR for the expression levels of the two receptor isoforms. As shown in Fig. 2, cortisol treatment induced a significant increase of GRα mRNA at the expense of GRα, which resulted significantly reduced compared to control cells. This result was also confirmed by real-time PCR (data not shown). Conversely DHEA treatment induced an up-regulation of GRβ maintaining GRα mRNA levels similar to control cells as we previously demonstrated by real time PCR [7]. As a positive control of the effect of DHEA and cortisol we analyzed the mRNA levels of RACK1 (Fig. 2) and, as expected, DHEA induces the expression of RACK1 whereas cortisol inhibits its expression [5].

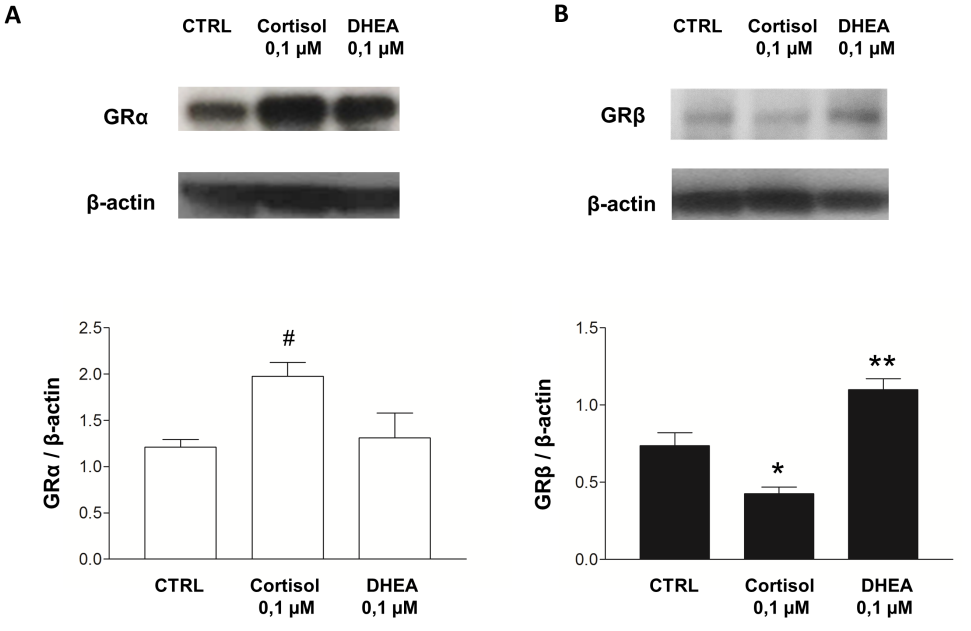

Fig. 3. Effect of cortisol and DHEA on GR (A) and GR (B) protein expression. THP-1 cells were incubated in a medium containing 0.1 M cortisol (6 h) or 0.1 M DHEA (18 h). β-actin expression was detected to normalize the samples. The images are a representative Western blot result respectively for GR (A) and GR (B). Each value in the graph represents the mean ± SE of three independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with (A) $^{\#}$ $p < 0.05$ versus GR CTRL and (B) *$p < 0.05$, **$p < 0.01$ versus GR CTRL (control; vehicle-treated cells).

Concerning the protein expression, cortisol induced a significant up-regulation of GRα (Fig. 3A) with a consequent and significant decline of GRβ protein levels compared to control

cells (Fig. 3B). On the other hand, as previously demonstrated, DHEA induced a significant increase of GRβ (Fig. 3B) expression without affecting GRα protein levels (Fig. 3A). These results suggest that cortisol and DHEA have a different effect on GR mRNA expression. Cortisol only affects GR pre-mRNA splicing without affecting total expression levels, whereas DHEA shows an increase in total GR expression and also induces a splicing mechanism directed toward the β isoform.

3.3. Characterization of the effects of cortisol and DHEA on GR splicing factors

Data suggests that cortisol and DHEA can influence the expression of GR by modulating splicing factors [7]. In particular, SRSF9 has been found to direct the alternative splicing of GR gene in neutrophils [15,20]. In our experimental model, we had preliminary evidence that DHEA induced an overexpression of SRSF9 supporting the modulation of spliceosome proteins in GR splicing. To investigate the effect of cortisol and to confirm the effect of DHEA on SRSF9, its expression was evaluated both at the mRNA and protein levels. As shown in Fig. 4A, DHEA induced SRSF9 mRNA expression at 6 h, whereas at 18 h its effect was remarkable at protein level (Fig. 4B). This difference in time of DHEA treatment necessary to obtain a significant effect may reflect a different turnover of the molecule involved (mRNA versus protein). We demonstrated that SRSF9 over-expression induced by DHEA depended on its up-regulation at transcriptional level. We also observed that cortisol treatment promoted a significant down-regulation of SRSF9 mRNA expression (Fig. 4A), thus causing a significant reduction in SRSF9 protein levels (Fig. 4B), which can support the decrease in GRβ protein (Fig. 3B).

Similarly to SRSF9 and taking into account that cortisol shifted GR splicing toward the α isoform, we investigated whether cortisol can modulate SRSF3 expression, a splicing factor which was involved in GR splicing [14]. As anticipated, cortisol induced a significant up-regulation of SRSF3 at mRNA and protein level, whereas DHEA had not effect (Fig. 4C and D).

3.3.1. *Effect of SRSF3 silencing on GR alternative splicing*

To confirm the role of SRSF3 in cortisol-induced GRα splicing, its expression was silenced using a specific small-interfering (si) RNA. After forty-eight hours of silencing, SRSF3 protein levels were significantly suppressed compared to control cells (data not shown); SRSF3 knockdown completely blocked the effect of cortisol (data not shown) with a significant effect on GRα expression; indeed, in the presence of SRSF3 siRNA, cortisol failed to stimulate GRα-induced expression at both mRNA (Fig. 5A) and protein levels (Fig. 5B). These results suggest that the effect of cortisol is driven by a modulation of GRα

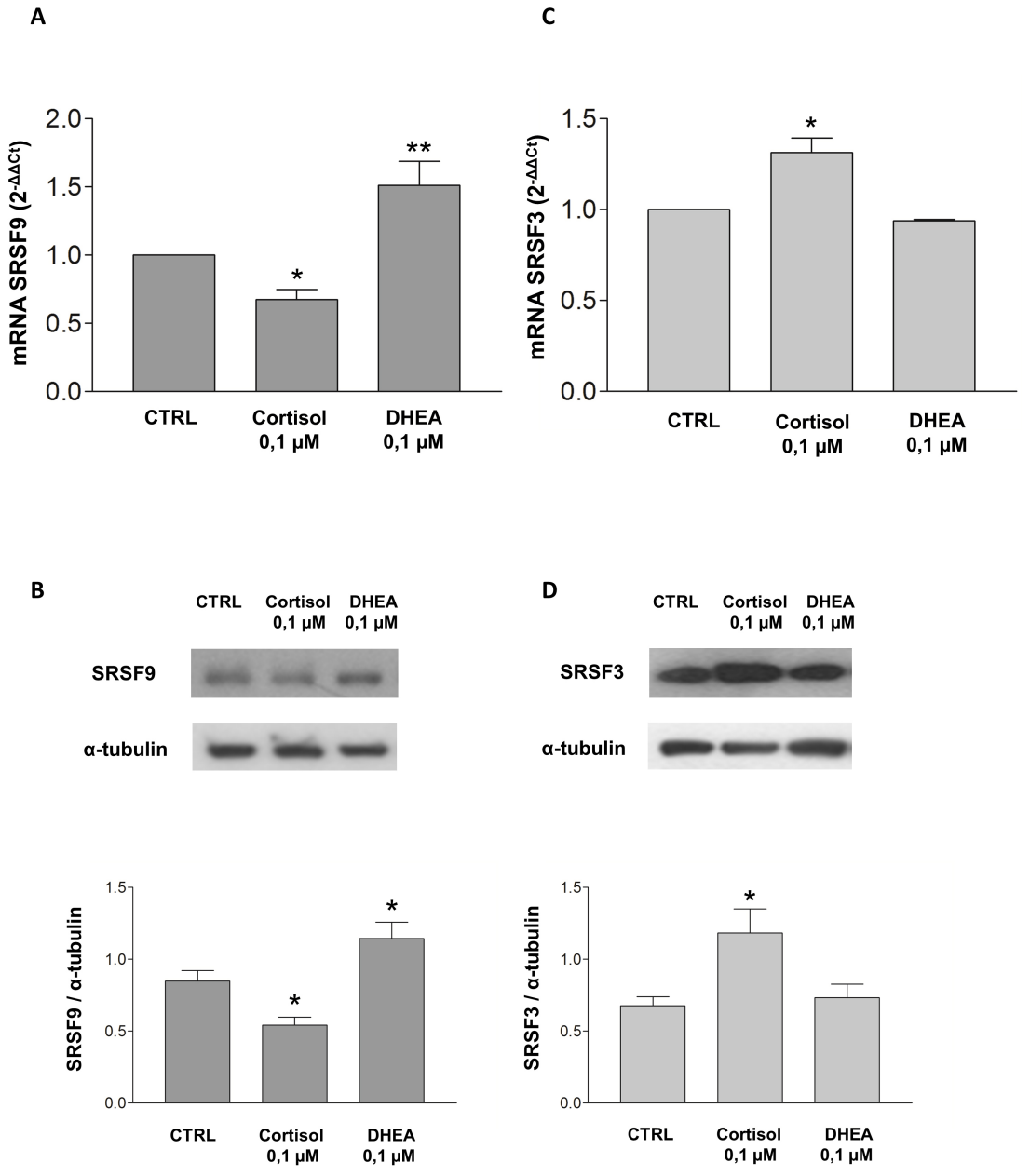

Fig. 4. Effect of cortisol and DHEA on SRSF9 and SRSF3 expression. (A–C) THP-1 cells were treated for 6 h with 0.1 M cortisol or 0.1 M DHEA. The effect on SRSF9 (A) and SRSF3 (C) mRNA levels was evaluated by real-time PCR in three independent experiments using RpL6 as an endogenous reference. Each value in the graph represents the mean ± SE of three independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with (A) *p < 0.05, **p < 0.01 versus SRSF9 CTRL and (C) *p < 0.05 versus SRSF3 CTRL (control; vehicle-treated cells). (B–D) THP-1 cells were incubated in a medium containing 0.1 M cortisol (6 h) or 0.1 M DHEA (18 h). α-tubulin expression was detected to normalize the samples. The images are a representative Western blot result respectively for SRSF9 (B) and SRSF3 (D). Each value in the graph represents the mean ± SE of three independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with (B) *p < 0.05 versus SRSF9 CTRL and (D) *p < 0.05 versus SRSF3 CTRL (control; vehicle-treated cells).

expression and activity. Finally, SRSF3 silencing resulted in the prevention of the cortisol repression action which determined an unaffected RACK1 expression, thus maintaining RACK1 levels comparable to control cells (Fig. 5B).

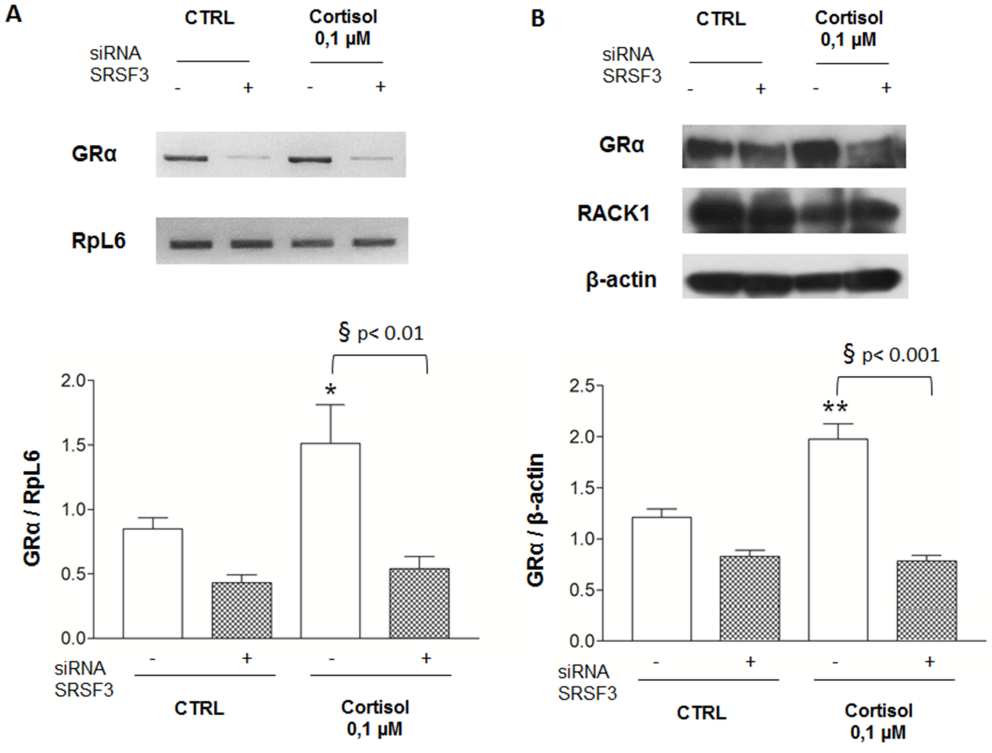

Fig. 5. SRSF3 silencing prevented cortisol induced GR increase. (A) After 48 h of silencing THP-1 cells were incubated for 6 h in a medium containing vehicle or 0.1 M cortisol. GR splicing variant was detected by RT-PCR and analyzed on 3% agarose gel. RpL6 expression was detected to normalize the samples. The image is a representative RT-PCR. Each value in the graph represents at least the mean ± SE of three independent experiments. Statistical analysis was performed with Bonferoni's multiple comparison test with *$p < 0.05$ versus no silenced CTRL; §§ $p < 0.01$ versus SRSF3 silenced cells. (B) After 48 h of silencing THP-1 cells were incubated in a medium containing vehicle or 0.1 M cortisol (6 h). Cellular extracts were examined by Western blotting using a specific antibody against GR and RACK1. β-actin expression was detected to normalize the samples. The image is a representative Western blot result. Each value in the graph represents the mean ± SE of three independent experiments relative to GR . Statistical analysis was performed with Bonferroni's multiple comparison test with **$p < 0.01$, versus no silenced CTRL; §§§ $p < 0.001$ versus SRSF3 silenced cells.

3.3.2. Effect of SRSF9 silencing on GRβ alternative splicing

We then investigated the role of SRSF9 in DHEA-induced GRβ splicing. After silencing, SRSF9 protein levels were significantly suppressed compared to control cells (data not shown). SRSF9 knockdown completely blocked the effect of DHEA on GR expression preventing the increase of GRβ at mRNA (Fig. 6A) and protein expression levels (Fig. 6B). SRSF9 knockdown prevented DHEA- induced RACK1 expression (Fig. 6B) thus reinforcing the idea that GR is a dominant-negative regulator of GRα activity. These results suggest that the effect of DHEA is driven by a modulation of SRSF9, which, in turn, influences GRβ expression and activity.

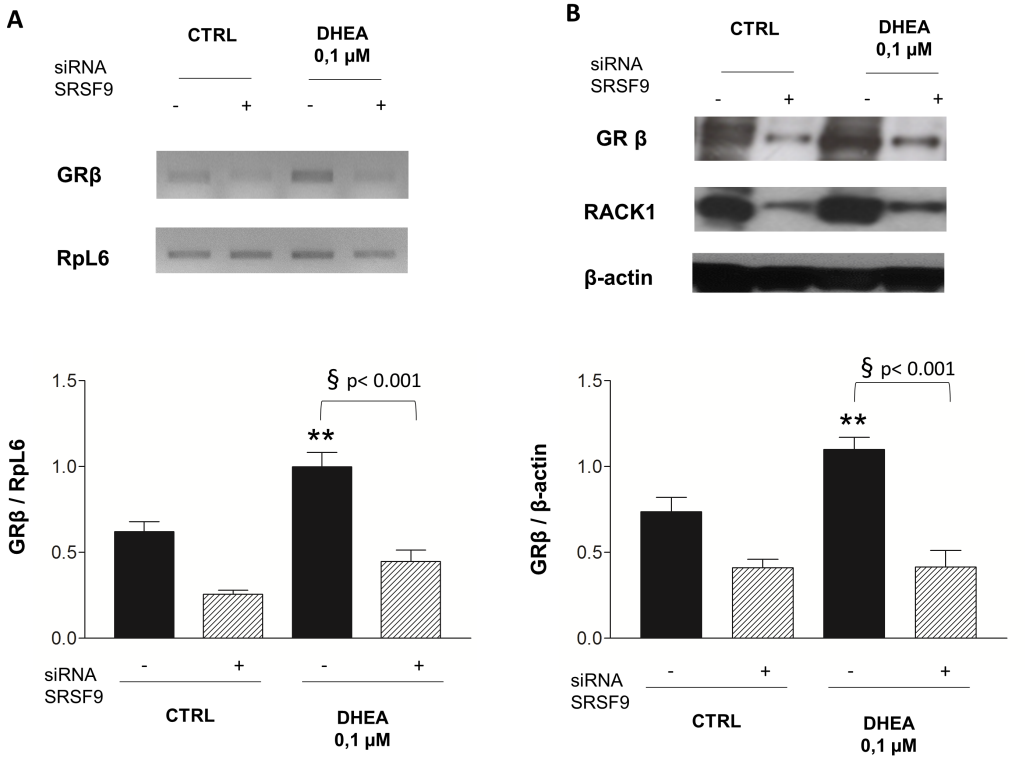

Fig.6. SRSF9 silencing inhibited the effect of DHEA on GR protein expression. (A) After 48h of silencing THP-1cellswereincubatedfor18hinamediumcontainingvehicle or 0.1 M DHEA. GR splicing variant was detected by RT-PCR and analyzed on 3% agarose gel. RpL6 expression was detected to normalize the samples. The image is a representative RT-PCR. Each value in the graph represents at least the mean ± SE of three independent experiments. Statistical analysis was performed with Bonferroni's multiple comparison test with **p < 0.01 versus no silenced CTRL; §§§ p < 0.001 versus SRSF9 silenced cells. (B) After 48 h of silencing THP-1 cells were incubated in a medium containing vehicle or 0.1 M DHEA (18 h). Cellular extracts were examined by Western blotting using a specific antibody against GR and RACK1. -actin expression was detected to normalize the samples. The image is a representative Western blot result. Each value in the graph represents the mean ± SE of three independent experiments relative to GR . Statistical analysis was performed with Bonferroni's multiple comparison test with **p < 0.01 versus no silenced CTRL; §§§ p < 0.001 versus SRSF9 silenced cells.

4. Discussion

Over the past few years, we have focused our attention on the effects that age-related imbalance between DHEA and cortisol have on RACK1 expression and its implication in immune functionality [2,3,5,6,22]. Physiological concentrations of cortisol exert an inhibitory effect on RACK1 expression, whereas treatment with DHEA increases RACK1 expression and can restore the levels of RACK1 protein both in vivo and in vitro [2]. Our previous results demonstrated that DHEA exerted its anti-glucocorticoid effect by modulating GRβ expression and antagonizing the function of GRα binding to GRE sequence in RACK1 promoter [7]. In the current manuscript, we examine more in depth the mechanism of action of DHEA on the alternative splicing of GR. We demonstrated that cortisol specifically exerted a shift in the pattern of expression of GR promoting the α isoform at the expenses of GRβ. Cortisol did not affect total GR mRNA levels; on the contrary, it influenced and controlled the exon inclusion/exclusion in GR mRNA transcript by modulating SRSF3 and SRSF9 expression, two splicing factors involved in GR alternative splicing [14]. According to cortisol effect on GR expression, SRSF3 and SRSF9 were modulated in an oppo- site way. Cortisol up-regulated SRSF3, the GRα promoting splicing factor whereas down-regulated SRSF9, which has a specific role in increasing GRβ alternative splicing. Moreover, SRSF3 silencing resulted in the prevention of cortisol effect on GRα expression which, in turn, prevented the inhibitory effect of cortisol on RACK1 expression levels.

Conversely, we demonstrated that DHEA exerted its action on SRSF9 expression thus promoting GRβ increase in order to antagonize the function of GR and to counteract the effect of cortisol. Our previous data demonstrated that GRβ knock-down completely blocked the effect of DHEA on RACK1 protein expression [7]. In line with these results, we demonstrated that SRSF9 knockdown prevented GRβ expression thus affecting RACK1 expression and also preventing DHEA action. All together, these results suggest that DHEA influences RACK1 expression by modulating SRSF9 protein, which in turn shifts GR alternative splicing in favor of β isoform, counteracting the inhibitory effect of GRα on RACK1 transcription. In this regard, altogether our data suggest that DHEA exposure counteracted cortisol effect on SRSF9 thus indicating a specific role for SRSF9 in increasing GRβ mediated by DHEA treatment.

Physiological actions of DHEA have been attributed to its conversion to either androgens or estrogen. Our recent data indicates the ability of THP-1 cells to rapidly convert DHEA to dihydrotestosterone (DHT) with its downstream metabolites 3α-diol and 3β-diol already appearing after 1 h of exposure. More specific indication that the androgen receptor (AR) is involved in the activity of DHEA came from our data demonstrating that the effect of DHEAon RACK1 expression could be completely prevented by using flutamide, an AR antagonist. In addition, we demonstrated that flutamide also prevented DHEA induced GRβ protein expression [16]. In the current manuscript, we also demonstrated that flutamide treatment prevented DHEA-induced GR mRNA expression. This is in line with recent

evidence demonstrating an androgen response element upstream of the GR gene [21]. Altogether, data clearly indicate that AR is a key step in the anti-glucocorticoid action of DHEA. Hence, our current evidence allows us to suggest a model of DHEA action which elucidate and reconcile data that we have accumulated in the last years [2,7,16]. DHEA conversion to androgens and AR activation resulted in GR mRNA up-regulation which is preferentially shifted to β isoform by DHEA-induced SRSF9 increase expression. The increase in GRβ then counteracts the cortisol-induced binding of GRα to the RACK1 promoter region by forming an inactive GRβ/GRα heterodimer.

Overall, these data supports the existence of a complex hormonal balance in the control of RACK1 expression and monocyte activation, leading the way to novel therapeutic targets for immune modulation.

5. Conclusion

Our data highlight, for the first time, an important role of DHEA and cortisol in GR alternative splicing. We demonstrated that DHEA treatment induced an overexpression of SRSF9, suggesting that modulation of the spliceosome proteins involved in GR mRNA splicing can be a significant mechanism for glucocorticoid activities regulation. We also demonstrated that cortisol was able to shift GR splicing toward α isoform by SRSF3 up-regulation and SRSF9 down-regulation resulting in a significant decrease of GRβ . The latter may also be particularly relevant in the context of glucocorticoid resistance. Tissue-specific alteration of local glucocorticoid sensitivity have been attributed to GRβ activity; GRβ mediated insensitivity to glucocorticoids is associated with dysregulation of immune function in various disorders, including glucocorticoid-resistant asthma, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis, chronic lymphocytic leukemia, and nasal polyps [10,23]. Considering that the physiological activity of cortisol decreases GRβ protein by modulating SRSF9, we consider that it may be interesting to analyze the possible effect on this splicing factor protein operated by other corticosteroids such as betamethasone, budesonide, methylprednisolone, prednisone and prednisolone, opening a new way of pharmacological investigation.

Finally, differences in the levels and/or activities of these spliceosomal proteins and their tissue specificity are still largely an open question. Hence it is a relevant issue that should be exam- ined more in details. This topic is of particular importance when considering the clinical role of glucocorticoids and their receptors in the pathophysiology and treatment of human disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2017.03. 019.

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The scaffold protein RACK1 is a target of endocrine disrupting chemicals (EDCs) with important implication in immunity

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The scaffold protein RACK1 is a target of endocrine disrupting chemicals (EDCs) with important implication in immunity

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ABSTRACT

We recently demonstrated the existence of a complex hormonal balance between steroid hormones in the control of RACK1 (Receptor for Activated C Kinase 1) expression and immune activation, suggesting that this scaffold protein may also be targeted by endocrine disrupting chemicals (EDCs). As a proof of concept, we investigated the effect of the doping agent nandrolone, an androgen receptor (AR) agonist, and of p,p′DDT (dichlorodiphenyl- trichloroethane) and its main metabolite p,p′DDE (dichlorodiphenyldichloroethylene), a weak and strong AR antagonist, respectively, on RACK1 expression and innate immune response. In analogy to endogenous androgens, nandrolone induced a dose-related increase in RACK1 transcriptional activity and protein expression, resulting in increased LPS-induced IL-8 and TNF-α production and proliferation in THP-1 cells. Conversely, p,p′DDT and p,p′ DDE significantly decrease RACK1 expression, LPS-induced cytokine production and CD86 expression; with p,p′ DDE exerting a stronger repressor effect than p,p′DDT, consistent with its stronger AR antagonistic effect. These results indicate that RACK1 could be a relevant target of EDCs, responding in opposite ways to agonist or antag- onist of AR, representing a bridge between the endocrine system and the innate immune system.

1. Introduction

Over the past decades most industrialized countries faced a significant increase in the prevalence of diseases, such as cancer (i.e. breast, lung and prostate cancer), allergy, and autoimmunity (i.e. arthritis, SLE, type I diabetes) that can be linked to immune alterations. Environmental factors, including the extensive presence of endocrine disrupting chemicals (EDCs), are believed to be a major factor responsible for such increased prevalence (Nahta et al., 2015). EDCs derive mainly from industrial and agricultural sources, and include pesticides (e.g. fungicides, insecticides, herbicides), chemicals used in the plastics industry and in consumer products (e.g. PCB, BPA, phthalates). Growing evidence indicates that EDCs can interfere with the immune system in human and wildlife (Kuo et al., 2012).

Many EDCs have been shown to possess immunosuppressive properties, as well as to increase autoimmune reactions and enhance inflammation. It is interesting to note that virtually all compounds in the Group I of the priority list of chemicals developed within the EU-Strategy for Endocrine Disruptors (http://ec.europa.eu/environment/chemicals/endocrine/strategy/substances_en.htm) are also immunotoxic compounds, but in most cases the relationship between endocrine disruption and immunotoxicity must be demonstrated. Evidence is accumulating on the existence of bidirectional interactions among oxidative stress, immune and endocrine systems, and cancer (Kravchenko et al., 2015). This is a relatively new area of research and we cannot currently list the precise chemicals due to an insufficient knowledge.

Regarding the chemicals investigated in the current study and their effects on the immune system, literature data supports immunomodulatory effects for all. In particular, for anabolic steroids animal studies reported both decreased and increased thymic cellularity (Panteleeva et al., 2013; Grönbladh et al., 2013), increased and decreased CD4+ cells (Inamdar Doddamani and Jayamma, 2012); aggravation of septic shock by increasing pro-inflammatory cytokine production (Lin et al., 2011); increased proinflammatory cytokine production (Hughes et al., 1995); increased anticancer activity (Rigberg and Brodsky, 1975). In a study on competitive bodybuilders that self-administered anabolic androgenic steroids compared with a group of bodybuilders not using these drugs, Calabrese et al. (1989) observed no significant differences in T-cell subsets while enhanced B cells proliferative responses, and increased NK activity were present in the anabolic-androgenic steroid- using group. Significantly lower serum immunoglobulin levels, in particular IgA, in the steroid-using group were also demonstrated. The authors concluded that anabolic-androgenic steroid use as practiced by these categories of athletes is a potent modulator of immune responsiveness. In addition, the well-known competitive antagonism of androgens to the glucocorticoid receptors is likely to be relevant for immunomodulation (Browne et al., 1992; Chen et al., 1997). Regarding DDT immunosuppressive effects, reduction in immunoglobulin levels and decreased response to bacterial challenges have been demonstrated in rodents (Banerjee, 1987a,b; Banerjee et al., 1997; Gabliks et al., 1975; Rehana and Rao, 1992). In

humans, Vine et al. (2001) reported in residents living around a dump site a positive association between DDE and IgA, no association between DDE and IgG (IgA was used as measure of mucosal immune responses and IgG as indicator of humoral immune responses), modestly increased total lymphocytes, and lowered mitogen-induced lymphoproliferative activity. Cooper et al. (2004) reported in African-American farmers an inverse association between levels of p,p′DDE and IgG, and no significant association between the concentration of p,p′DDE and IgA. Changes in Ig levels provide evidences of potential immunosuppression, which is consistent with studies indicating an increased incidence of infections in exposed people. Higher levels of prenatal p,p′DDE were associated with an increased incidence of otitis media in Inuit infants (Dewailly et al., 2000). Hermanowicz et al. (1982) found a higher prevalence of infectious diseases in workers who had directly worked with DDT and lindane for 12–30 years compared with controls.

The identification of the regulatory elements in the promoter region of the rack1 gene [also known as guanine nucleotide binding protein, beta polypeptide 2-like 1 (GNB2L1) gene], encoding RACK1 (Receptor for Activated C Kinase 1), shed some light on the transcriptional modulation of RACK1 in physiological and pathological context. Our published data supports the existence of a complex hormonal balance among cortisol, estrogens and androgens in the control of RACK1 expression and immune functionality, suggesting that this scaffold protein may also be the target of the action of EDCs, linking the endocrine with the immune system. We demonstrated that RACK1 gene expression is negatively regulated by glucocorticoids (Del Vecchio et al., 2009; Buoso et al., 2011; Corsini et al., 2014) and estrogen (Viviani et al., 2002), whereas dehydroepiandrosterone (DHEA), through an androgenic mechanism (Corsini et al., 2016), and endogenous androgens have an opposite effect on RACK1 expression and on the regulation of PKC activity involved in immune cells activation (Corsini et al., 2002, 2005; Buoso et al., 2011). These findings led us to hypothesize that RACK1 could be an important target of the EDC and to investigate the effects of EDCs on RACK1 expression. The protein RACK1 has been identified and cloned in the early nineties and so named because of its interaction with Protein Kinase C (PKC) (Ron et al., 1994). Since then our understanding of its functions has increased dramatically and RACK1 is now widely recognized as a multiple target scaffolding protein involved in various biological events, such as development, immune response, neuronal activity, and cancer (reviewed by Adams et al., 2011; Ron et al., 2013; Li and Xie, 2015). Due to its plethora of interaction partners, RACK1 appears therefore to be a fulcrum of cellular homeostasis, controlling essential cellular processes such as transcription and translation, cell proliferation and growth as well as cell spreading and cell-cell interactions. Our working hypothesis is that the modulation of RACK1 could represent the molecular initiating event that can bridge together several adverse effects, including immunotoxicity and steroid endocrine interference linking another pathway to the immune modulatory effect of EDCs.

The purpose of this work was to investigate the ability of EDCs to modulate RACK1 expression. As a proof of concept, we used p,p′DDT, p,p′DDE and nandrolone, a synthetic

anabolic-androgenic steroid. These compounds were chosen as they are known to have opposite effects on the AR: whereas nandrolone is a potent agonist, p,p′DDE is a strong antagonist and p,p′DDT a weak antagonist. As experimental model, the human promyelocytic cell line THP-1 was used. In addition, the human whole blood assay was used to confirm the immunomodulatory effects on LPS-induced cytokine production. Results obtained support our working hypothesis: EDCs can target RACK1, with consequent modulation of innate immune functionality and cell proliferation. This data provides evidence that EDCs exert a role in the control of innate immune modulation, with a specific effect on RACK1 expression, contributing to our understanding of the mechanism of action of EDCs.

2. Materials and methods

2.1. Chemicals

Nandrolone or 19-nortestosterone (Cas No. 434-22-0), p,p′DDT (Cas No. 50-29-3) and p,p′DDE (Cas No. 72-55-9) were obtained from Sigma Aldrich (St Louis, MO, USA). They were dissolved in DMSO at concentration of 50 mM and frozen (−20 °C) in stock aliquots. Stocks were diluted at final concentrations in culture media at the time of use (final concentration of DMSO in culture medium b0.2%). Lipopolysaccharide from Escherichia coli serotype 0127:B8 (LPS) was from Sigma Aldrich. Cell culture media and all supplements were from Sigma Aldrich. Mouse anti-human RACK1 monoclonal antibody (Cat. No. 610177) and mouse monoclonal anti-β-actin (Cat. No. 612656) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Host specific peroxidase conjugated IgG secondary antibody (Cat. No. 31460) was purchased from ThermoScientific (Waltham, MA, USA). Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA). All reagents were purchased at the highest purity available.

2.2. Receptor affinity

Affinity of the tested compounds was measured by an in silico analysis. Crystallographic structures of the ligand binding domains of the human androgen (AR), co-crystallized with 2-chloro-4-{[(1R,2R)-2-hydroxy-2-methylcyclopentyl]amino}-3-methylbenzonitrile, and gluco- corticoid receptors (GR), co-crystallized with mifepristone, were downloaded from the RCSB PDB, respectively codes: 5CJ6 (http://dx. doi.org/10.1021/acs.jmedchem.5b01168) and 1NHZ [10.1074/jbc. M212711200].

The crystallographic structures were prepared through the QuickPrep program of the Molecular Operating Environment (MOE) version 2016.08 (http://www.chemcomp.com), which deletes distant solvent, adds hydrogens, installs tethers, calculates charges and performs initial refinement of the systems. The tested compounds were downloaded from the NCBI Pubchem repository, and organized in a MOE database file. Each compound was docked on both the receptor LBDs through the Dock program of the MOE suite, using default settings, the co-crystallized ligands for the identification of the binding site, and the MMFF94x force field. Five refined poses were generated for each complex and scored according to their approximate binding free energy, computed by using the GBVI/WSA dG empirical scoring function (Naïm et al., 2007). The dissociation constant (K_i) of the complexes have been computed from the GBVI/WSA dG value by the following equation: $\Delta G = -RT\ln K_i$.

2.3. Cells

For experiments, THP-1 cells, obtained from Istituto Zooprofilattico di Brescia (Italy), were diluted to 10^6 cells/ml (or 2.0×10^5/ml for cell proliferation experiments) in RPMI 1640 without phenol red containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, gentamycin 10 μg/ml, 50 μM 2-mercaptoethanol, supplemented with 5% heated-inactivated dialyzed fetal calf serum (culture media) and cul- tured at 37 °C in 5% CO_2 incubator. Cells were treated as reported in the legend to figures or tables.

Blood samples were taken by venous puncture with sodium citrate 0.5 M as anticoagulant. Healthy subjects (n = 4) were selected according to the guidelines of the Italian Health authorities and to the Declaration of Helsinki principles and signed an informed consent (average 40 yr, min 25 max 53). Criteria for exclusion were the use of medication known to affect the immune system, i.e. steroids, or patients suffering from malignancies, inflammations and infections. Regarding the number of donors enrolled, in previous experiments conducted as part of a project sponsored by the Dutch Institute ZonMw (# 63112), we found that three donors were sufficient to characterize in vitro the immunomodulatory potential of chemicals (unpublished data). This is also in line with data published by Langezaal et al. (2002) for the whole blood assay that reported a CV = 20 ± 5% in the response among donors (CV, coefficient of variance). Blood samples were diluted 1:10 in cell culture medium RPMI 1640 without phenol red containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin. Diluted blood samples were treated for 24 h with the selected compounds and then in the presence or absence of LPS at final concentration of 10 ng/ml for 24 h to assess TNF-α and IL-8 production.

2.4. Real-time PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valen- cia, CA, USA) following manufacturer's instructions. For the synthesis of cDNA, 2.0 μg of total RNA was retro-transcribed using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA) following the supplier's instructions. RACK-1 gene expression was evaluated by Real time reverse transcription-polymerase chain reaction (Real time-PCR). For PCR-analysis, Taq-ManTM-PCR technology was used. Primers were purchased from Applied Biosystems. PCRs were per- formed in duplicate according to the standard protocol suggested by the manufacturer. For each PCR reaction, 10 ng of total RNA were used. The 18S ribosomal RNA transcription was used as endogenous ref- erence and the quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Western blot analysis

The expression of RACK1 in cell homogenates was assessed by Western blot analysis. Briefly, cells were treated and then collected, washed once with PBS, centrifuged and lysed in 100 μl of homogenization buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitor mix). The protein content was measured using the Bradford method. Western blotting samples were prepared mixing the cell lysate with sample buffer (125 mM Tris-HCl pH 6, 8.4% SDS, 20% glycerol, 6% β-mercaptoethanol, 0.1% bromophenol) and denaturing at 95 °C for 5 min. Equivalent amounts of extracted protein (10 μg) were electrophoresed into an appropriate 10% SDS-PAGE under reducing conditions. The proteins were then transferred to a PVDF mem- brane (Amersham, Little Chalfont, UK) which was blocked in 5% w/v BSA, 1× TBS, 0.1% Tween-20 for 1 h with gentle shaking. The proteins were visualized using primary antibodies diluted in 5% w/v BSA, 1× TBS, 0.1% Tween-20 for RACK1 (1:1000) and β-actin (1:2000). In all experiments, immuno-reactivity was measured using host specific sec- ondary IgG peroxidase conjugated antibodies (1:5000 diluted) and developed using enhanced chemiluminescence reagent (Pierce, Thermo Scientific, Rockford, IL, USA). Following acquisition of the Western blot, the optical density of the bands was calculated and analyzed with Scion Image program for digital image processing (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD). The relative densities of the bands were expressed as arbitrary units and normalized to data obtained from con- trol sample run under the same conditions.

2.6. Cell viability

Cytotoxicity was assessed by leakage of lactate dehydrogenase (LDH). LDH activity was determined in cell-free supernatants using a commercially available kit (Takara Bio Inc., Japan). Results are expressed as OD.

2.7. Cytokine production

Cytokine production was assessed in cell free supernatants by specific sandwich ELISAs, commercially available (R&D System, Minneapolis, MN, USA). Cell-free supernatants obtained by centrifugation at 1200 rpm for 5 min were stored at −20 °C until measurement. Limits of detection were 15 pg/ml. Results are expressed in pg/ml or as Stimulation Index (SI).

2.8. Flow cytometric analysis of CD86 expression

Briefly, after 24 h of treatment, THP-1 cells were centrifuged, washed once with cold PBS and suspended in PBS supplemented with 1% FCS. 10^5 cells were stained in the dark for 30 min with specific FITC-conjugates antibodies against CD86 (BD Biosciences) or with isotype control antibody at room temperature (BD Biosciences) following supplier's instructions. 1 ml of PBS was then added and cells centrifuged at 1200 rpm for 5 min and suspended in 0.5 ml of PBS supplemented with 1% FCS. The intensity of fluorescence and the percentage of positive cells were analyzed using a FACSCalibur flow cytometer and data were quantified using CellQuest software (Becton Dickinson). 10,000 viable cells were analyzed for mean fluorescence intensity (MFI). MFI of isotype control was subtracted to MFI of CD86 stained cells. All experiments were performed in triplicate.

Changes in CD86 expression are reported as stimulation index (SI) calculated by the following equation:

SI 1⁄4 MFIt=MFIcMFIt stand for chemical-treated cells, whereas MFIc for the untreated ones.

2.9. Plasmid DNA preparation, transient transfections and luciferase assays

Δ1 reporter plasmid construct has been described previously (Del Vecchio et al., 2009) and it was the longest construct available, 2105 nt long, which contained the rack1 gene promoter region between nucleotide − 1744 and + 361. It included the GRE sequence. Plasmids for transfections were purified with the HiSpeed® Plasmid Midi Kit (Qiagen, Valencia, CA). DNA was quantified and assayed for purity using a DUR24 530 UV/Vis Spectrophotometer (Beckman Coulter Inc., Fullerton, CA). Transient transfections were performed in 6-well plates; for each well 8×10^5 cells were seeded in RPMI 1640 medium without phenol red, FBS antibiotics and supplemented with 1% L-glutamine. Transfections were carried out using Lipofectamine 2000 (Invitrogen Carlsbad, CA) following manufacturer's instructions. Each luciferase-reporter construct plasmid DNA was co-transfected with pRL-TK Renilla luciferase expressing vector to measure transfection efficiency (Promega, Madison, WI). During transfection THP-1 cells were incubat- ed at 37 °C in 5% CO_2 and then treated with p,p′DDT and p,p′DDE for the times and at concentrations specified in figure legends. Cells were lysed with Passive Lysis Buffer provide by Dual-Luciferase Reporter Assay System following manufacturer's specifications (Promega, Madison, WI). The luminescence was measured with a 20/20n Luminometer with 10 s of integration (Turner BioSystems, Sunnyvale, CA).

2.10. Statistical analysis

All experiments have been performed at least three times in different moments (independent experiments). For RT-PCR or Western blot analysis, experiments were conducted in single, while for immune assays or promoter activity in triplicate or quadruplicate (replicates). Results reported are either the mean ± SD (standard deviation) of independent experiments, as for RT-PCR or Western blot analysis, or as mean ± SD of 3 replicates, representative of the independent experiments for immune assays. Statistical analysis was performed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA). The data were analyzed by analysis of variance (ANOVA) followed by an appropriate post-hoc comparison test as indicated in figure legend. Effects were designed significant if $p \leq 0.05$. Statistical comparisons in the responses to LPS after treatment with p,p′DDT and p,p′DDE were made to highlight differences that could be related to the different AR antagonistic effect of p,p′DDT (weak) and p,p′DDE (strong), and they are reported in Fig. 3 using the symbol asterisk (*).

3. Results

3.1. Estimated affinity for the androgen and glucocorticoid receptors of the selected compounds

Table 1 contains the top-scoring binding free energy (affinity) values for each complex, and the logP of the tested ligands, computed through the MOE ligand properties program.

Table 1
Estimated affinity for the androgen and glucocorticoid receptors of the selected compounds.

Compounds	LogP	Affinity for AR (kcal/mol)/Dissociation constant	Affinity for GR (kcal/mol)/Dissociation constant
pp′DDE	7.18	$-8.0/1.3 \times 10^{-6}$	$-6.0/3.8 \times 10^{-5}$
pp′DDT	7.05	$-7.3/4.2 \times 10^{-6}$	$-6.4/2.0 \times 10^{-5}$
nandrolone	3.32	$-9.4/1.2 \times 10^{-7}$	$-7.5/3.0 \times 10^{-6}$
testosterone	3.82	$-7.0/7.1 \times 10^{-6}$	$-7.0/7.1 \times 10^{-6}$

The evaluation of the approximate binding free energy and dissociation constants is an approach frequently based on the use of empirical scoring functions, useful for evaluating the affinity of a set of ligands for a specific target/receptor (Eberini et al., 2011). The affinity was estimated for both AR and GR as we have showing that RACK1 expression in under the control of both receptors (reviewed in Buoso et al., 2017). All the tested molecules showed higher affinity values for AR than GR, suggesting that all of them can bind and modulate the activity of AR. Nandrolone showed the highest affinity for both AR and GR. pp′DDE and pp′DDT showed a significantly higher affinity for AR than for GR, suggesting an activity of these compounds directed to the AR ligand binding domain. In detail, pp′DDE showed an approximately 3 fold higher affinity than pp′DDT on the AR.

3.2. Nandrolone increases RACK1 expression and cytokine production

Most of DHEA physiological actions have been attributed to its conversion to either androgens or estrogens (Labrie et al., 2001; Webb et al., 2006). In THP-1 cells, we recently demonstrated that DHEA is rapidly converted to DHT, and using flutamide to block the AR or finasteride to block DHT synthesis or the silencing of AR, the effect of on RACK1 expression could be completely prevented, indicating a role of androgens and AR in RACK1 homeostasis. (Corsini et al., 2016). To confirm that this protein can also be the target of

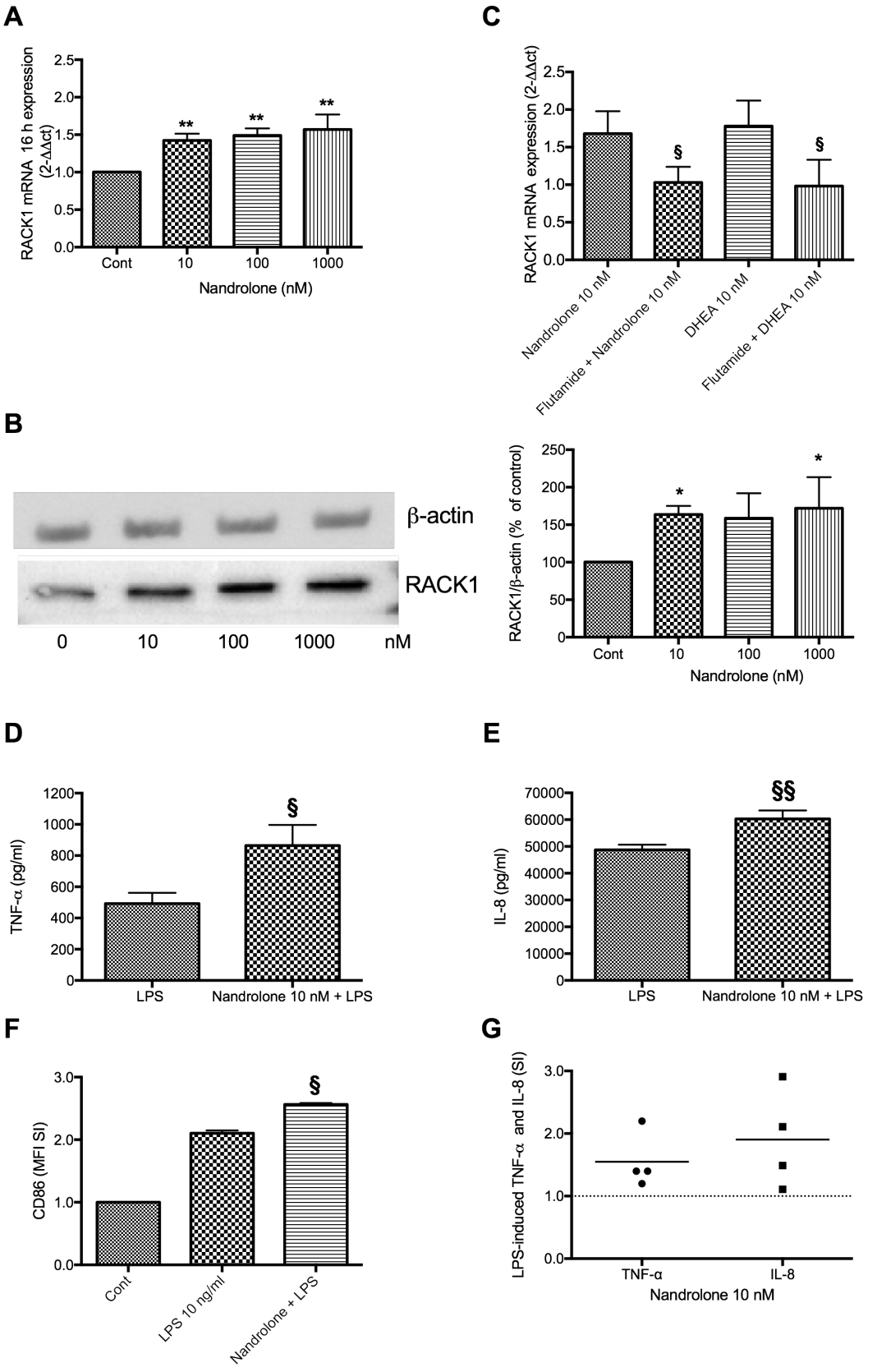
A
RACK1 mRNA 16 h expression (2-ΔΔct)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
Cont
10
100
1000
Nandrolone (nM)
C
RACK1 mRNA expression (2-ΔΔct)
2.5
2.0
1.5
1.0
0.5
0.0
§
§
Nandrolone 10 nM
Flutamide + Nandrolone 10 nM
DHEA 10 nM
Flutamide + DHEA 10 nM
B
β-actin
RACK1
0
10
100
1000
nM
RACK1/β-actin (% of control)
250
200
150
100
50
0
*
*
Cont
10
100
1000
Nandrolone (nM)
D
TNF-α (pg/ml)
1200
1000
800
600
400
200
0
§
LPS
Nandrolone 10 nM + LPS
E
IL-8 (pg/ml)
70000
60000
50000
40000
30000
20000
10000
0
§§
LPS
Nandrolone 10 nM + LPS
F
CD86 (MFI SI)
3.0
2.0
1.0
0.0
§
Cont
LPS 10 ng/ml
Nandrolone + LPS
G
LPS-induced TNF-α and IL-8 (SI)
3.0
2.0
1.0
0.0
TNF-α
IL-8
Nandrolone 10 nM

Fig. 1. Effect of nandrolone on RACK1 expression and response to LPS. A. THP-1 cells were treated for 16 h with increasing concentrations of nandrolone (10–1000 nM) or DMSO as vehicle control (Cont). The effect on mRNA levels was evaluated by real-time PCR using 18S as an endogenous reference. Each value represents the mean ± SD, n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with **p b 0.01 versus control (Cont). B. THP-1 cells were treated for 24 h with increasing concentrations of nandrolone (10–1000 nM) or DMSO as vehicle control (Cont). β-Actin expression was used to normalize RACK1 expression. The image is a representative Western blot. Each value represents the mean ± SD, n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *p b 0.01 versus control (Cont). C. THP-1 cells were treated for 1 h with flutamide (50 μM), and then nandrolone (10 nM) or DHEA (10 nM) was added for 16 h. The effect on mRNA levels was evaluated by real-time PCR using 18S as an endogenous reference. Each value represents the mean ± SD, n = 3 independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with §p b 0.05 versus nandrolone or DHEA treated cells. D, E, F. THP-1 cells were treated with nandrolone 10 nM for 24 h and then LPS (10 ng/ml) was added for 24 h to assess TNF-α release (D), IL-8 release (E), and CD86 expression. Results are expressed as mean ± SD, n = 3 replicates. Data is representative of three independent experiments. Statistical analysis was performed with Student's t-test with §p b 0.05, §§p b 0.01 versus LPS treated cells. G. To confirm the modulatory effect, the whole blood assay was used. Peripheral blood obtained from healthy donors was diluted 1:10, treated with nandrolone (10 nM) for 24 h, and then LPS (10 ng/ml) was added for 24 h to assess TNF-α and IL-8 release. Each dot represents the response of each donor, with mean values reported. Dotted line represents LPS release set at 1.

exogenous substances capable of interfering with steroid hormones, we investigated the response to the doping agent nandrolone, a synthetic anabolic-androgenic steroid derived from testosterone. THP-1 cells were treated for 16 h or 24 h with increasing concentrations of nandrolone (10–1000 nM). These times were chosen from previous experiments as optimal for DHEA-induced mRNA and protein expression, respectively (Buoso et al., 2011). As shown in Fig. 1, nandrolone induced a dose-related and significant increase of RACK1 mRNA expression at 16 h (Fig. 1A) and protein at 24 h (Fig. 1B). To demonstrate the involvement of AR in the observed effect, we investigated the ability of the anti-androgen flutamide to modulate the stimulatory effects of nandrolone. DHEA was used as reference compound. THP-1 cells were treated for 1 h with flutamide (50 μM), then physiologically relevant concentrations of DHEA (10 nM) or nandrolone (10 nM), or DMSO as vehicle control were added. After 16 h of treatment, real time-PCR was used to assess RACK1 mRNA expression. Flutamide completely prevented nandrolone-induced RACK1 mRNA expression with an effect comparable to DHEA (Fig. 1C), confirming AR as a key mechanism in the regulation of RACK1 expression (§p b 0.05 vs nandrolone or DHEA alone).

Next, we investigated if nandrolone-induced RACK1 protein expression could have consequences on LPS-induced cytokine production and CD86 upregulation. Cells were treated in the presence or absence of nandrolone (10 nM) for 24 h, then LPS (10 ng/ml) was added for 24 h to assess TNF-α (Fig. 1D) and IL-8 (Fig. 1E) release, and CD86 expression (Fig. 1F). Nandrolone induced a statistically significant increase in the response to LPS in all parameters investigated (§p b 0.05 and §§p b 0.01 vs LPS treated cells). The MeanFL1 subtracted of isotype control in control cells was 4.9 ± 0.2, 3.8 ± 0.5 in nandrolone alone treated cells and 8.7 ± 0.2 and 9.4 ± 0.1 in LPS and nandrolone + LPS treated cells (§p b 0.05 vs LPS). To confirm the relevance of these results in primary human cells, the whole blood

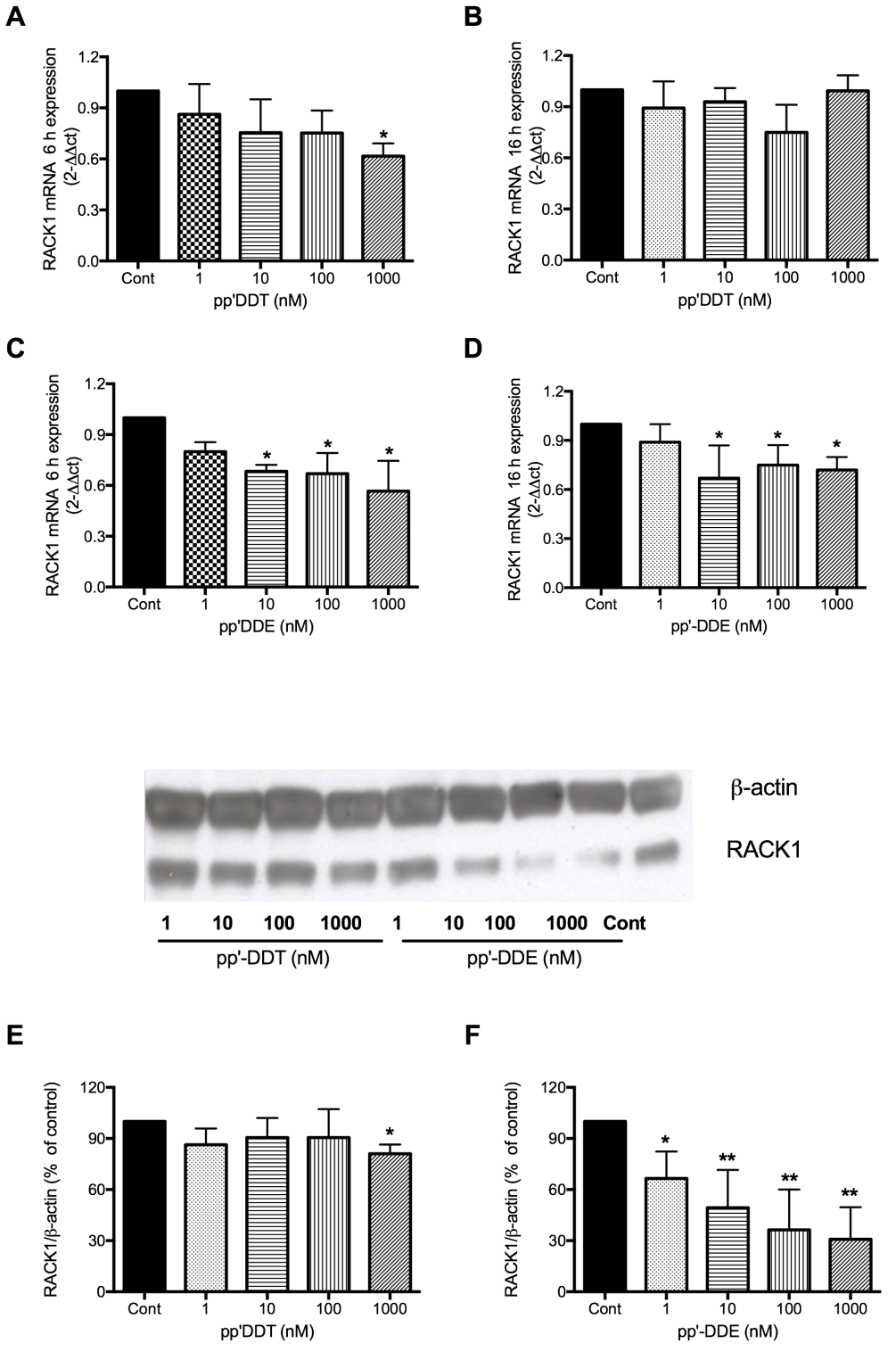
A
RACK1 mRNA 6 h expression (2-ΔΔct)
1.2
0.9
0.6
0.3
0.0
Cont
1
10
100
1000
pp'DDT (nM)
*
B
RACK1 mRNA 16 h expression (2-ΔΔct)
pp'DDT (nM)
C
RACK1 mRNA 6 h expression (2-ΔΔct)
pp'DDE (nM)
*
D
RACK1 mRNA 16 h expression (2-ΔΔct)
pp'-DDE (nM)
β-actin
RACK1
1 10 100 1000 1 10 100 1000 Cont
pp'-DDT (nM)
pp'-DDE (nM)
E
RACK1/β-actin (% of control)
120
90
60
30
0
pp'DDT (nM)
F
**
pp'-DDE (nM)

Fig. 2. Negative effect of p,p′DDTand p,p′DDE on RACK1 expression. A–D. THP-1 cells were treated with increasing concentrations of p,p′DDT (1–1000 nM) for 6 h (A) and 16 h (B) and p,p′ DDE (1–1000 nM) for 6 h (C) and 16 h (D) or DMSO as vehicle control (Cont). The effect on mRNA levels was evaluated by real-time PCR using 18S as an endogenous reference. Each value represents the mean ± SD, n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *p b 0.05 versus control. E–F. THP-1 cells were treated with increasing concentrations of p,p′DDT (1–1000 nM) for 24 h (E) and p,p′DDE (1–1000 nM) for 24 h (F) or DMSO as vehicle control (Cont). β-Actin expression was used to normalize RACK1 expression. The image is a representative Western blot. Each value represents the mean ± SD, n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *p b 0.05 and **p b 0.01 versus control.

assay was used. Diluted whole blood obtained for healthy donors (n = 4) was treated for 24 h with nandrolone (10 nM), then LPS (10 ng/ml) was added for further 24 h. As shown in Fig. 1G, similarly to results obtained in THP-1 cells, LPS-induced TNF-α and IL-8 releases were increased in all donors (dotted line represents LPS responses set at 1). In term of absolute values, in untreated whole blood cells TNF- α release was b15 pg/ml, 89 ± 22 in LPS treated cells, while IL-8 was 244 ± 160 and 7087 ± 1658 in control vs LPS treated cells. CD86 expression was not evaluated in human primary cells. Results obtained confirm our working hypothesis that AR agonists can induce RACK1 expression, enhancing the response to classic innate immunologic stimuli.

3.3. DDT and its metabolite p,p'DDE negatively affect RACK1 expression and immune activation

As a proof of principle that RACK1 can be a target of EDCs, we then investigated if EDCs known to be AR antagonists could modulate RACK1 expression and innate immune response in an opposite way compared to what shown for nandrolone. p,p′DDT and its main metabolite p,p′DDE were used (Kelner et al., 1986; Kitamura et al., 2002), where p,p′DDE is known to be a potent AR antagonist (Kelce et al., 1995). We compared pp′DDT vs pp′DDE in relation to their weak and strong AR antagonistic effects, respectively, with the idea that strong AR antagonist will induced a more pronounced RACK1 inhibition leading to a more pronounced modulation of the innate immune response. Considering that RACK1 repressors, like cortisol (Buoso et al., 2011), act in a short-term compared to DHEA, THP-1 cells were treated also for 6 h with increasing concentrations of p,p′DDT and p,p′DDE (1– 1000 nM) and RACK1 mRNA expression was analyzed by real-time PCR. As shown in Fig. 2A, p,p′DDT was able to reduce significantly RACK1 mRNA expression only at the concentration of 1000 nM, with no effect at 16 h (Fig. 2B). Differently, at 6 h p,p′DDE induced a significant reduction also at the other concentrations tested (Fig. 2C). Moreover, the repression effect of p,p′DDE was detectable also at 16 h (Fig. 2D). Similarly, to the results obtained at the mRNA level, p,p′DDT (Fig. 2E) and p,p′DDE (Fig. 2F) also inhibited the expression of RACK1 at the protein level as assessed by Western blot analysis at 24 h, with p,p ′DDE being more potent. After 24 h of treatment RACK1 protein was decreased by

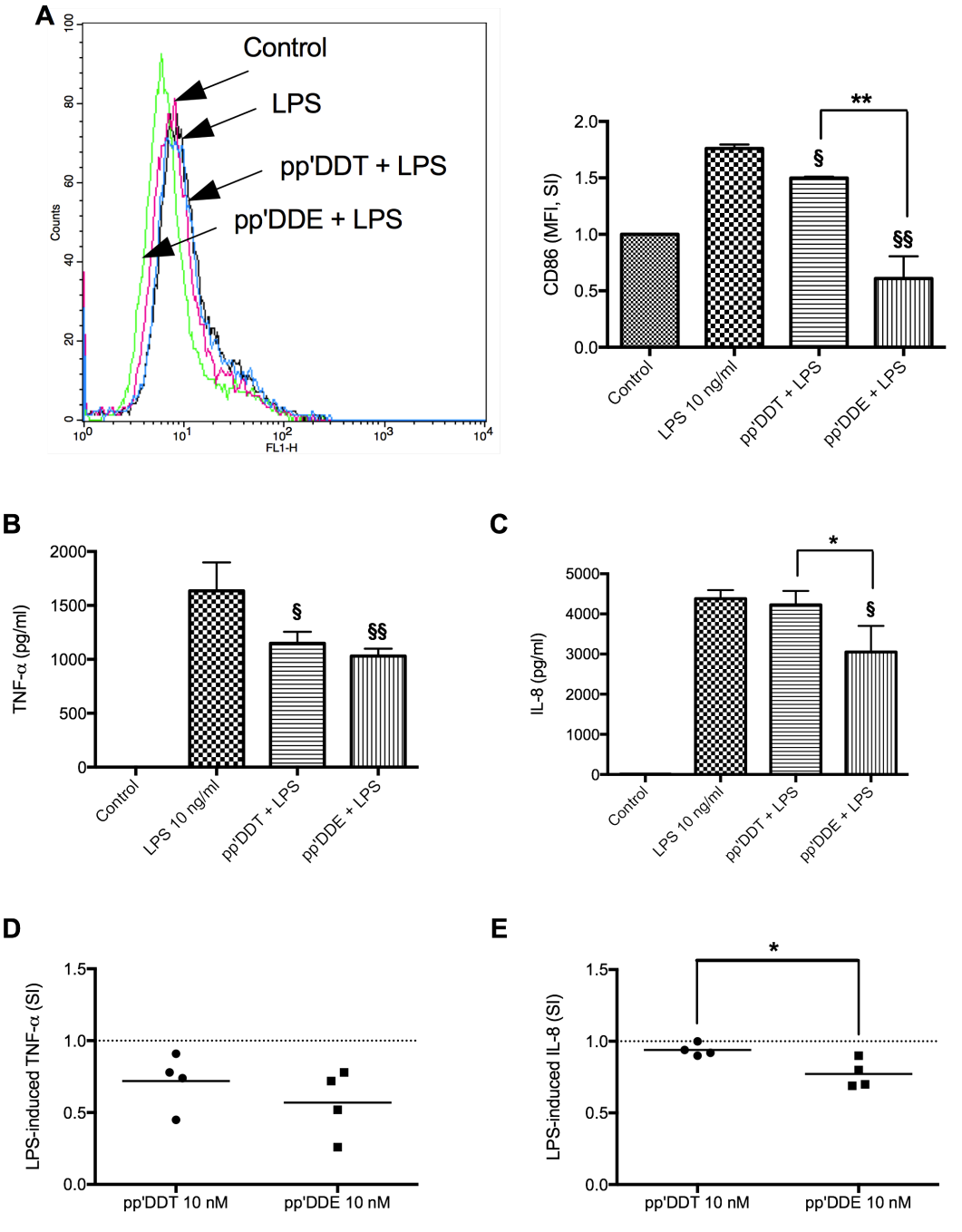
A
Control
LPS
pp'DDT + LPS
pp'DDE + LPS
Counts
FL1-H
CD86 (MFI, SI)
**
§
§§
Control
LPS 10 ng/ml
pp'DDT + LPS
pp'DDE + LPS
B
TNF-α (pg/ml)
C
IL-8 (pg/ml)
*
D
LPS-induced TNF-α (SI)
pp'DDT 10 nM
pp'DDE 10 nM
E
LPS-induced IL-8 (SI)

Fig. 3. Negative effect of p,p′DDT and p,p′DDE on LPS-induced THP-1 activation. THP-1 cells were treated with p,p′DDT (10 nM) and p,p′DDE (10 nM) for 24 h, and then LPS (10 ng/ml) was added for 24 h to assess CD86 expression (A), TNF-α release (B) and IL-8 release (C). Results are expressed as mean ± SD, n = 3 replicates. Data is representative of three independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with §p b 0.05, §§p b 0.01 versus LPS treated cells, and *p b 0.05 and **p b 0.01 versus pp′DDT. To confirm the modulatory effect, the whole blood assay was used. Peripheral blood obtained from healthy donors was diluted 1:10, treated with p,p′DDT (10 nM) and p,p′DDE (10 nM) for 24 h, and then LPS (10 ng/ml) was added for 24 h to assess TNF-α release (D) and IL-8 release (E). Each dot represents the response of each donor, with mean values reported. Dotted line represents LPS release set at 1. Statistical analysis was performed with Student's t-test with *p b 0.05 and **p b 0.01 versus pp′DDT. Comparison in the responses to LPS observed following treatment with p,p′DDT and p,p′DDE were made to highlight differences related to the AR antagonistic effect.

approximately 50–70% by p,p′DDE (Fig. 2F). These results suggest that p,p′DDT and p,p′DDE are able to negatively regulate RACK1 expression with a stronger effect exerted by p,p′DDE, the strongest AR antagonist.

To investigate whether p,p′DDT and p,p′DDE-induced inhibition of RACK1 protein expression could have consequences on monocyte activation, their modulatory effects on LPS-induced cytokine production and CD86 expression were investigated and the effect induced by p,p′ DDT and p,p′DDE compared. THP-1 were treated with p,p′DDT (10 nM) and p,p′DDE (10 nM) for 24 h to modulate RACK1 levels, then LPS (10 ng/ml) was added. CD86 expression and cytokine release were evaluated after 24 h. p,p′DDT and p,p′DDE alone have no effect on basal release of TNF-α and IL-8 release. As shown in Fig. 3A, p,p′ DDT and p,p′DDE significantly decreased LPS-induced CD86 expression as assessed by FACS analysis (§p b 0.05 and §§p b 0.01 vs LPS treated cells), with p,p′DDE being more effective compared to p,p′DDT (*p b 0.05 and **p b 0.01). The MeanFL1 subtracted of isotype control in control cells was 1.4 ± 0.1, 2.6 ± 0.1 in LPS treated cells and 2.2 ± 0.1 and 1.6 ± 0.6 in p,p′DDT + LPS and p,p′DDE + LPS treated cells (§p b 0.05 and §§ p b 0.01 vs LPS; and ** p b 0.01 p,p′DDT vs p,p′DDE). Regarding cytokine production, both LPS-induced TNF-α (Fig. 3B) and IL-8 (Fig. 3C) release were reduced, with p,p′DDE being once again more effective (*p b 0.5 vs LPS-induced IL-8 release). To confirm the relevance of these results in primary human cells, the whole blood assay was used. Diluted whole blood obtained for healthy donors (n = 4) was treated for 24 h with p,p′DDT (10 nM) and p,p′DDE (10 nM) for 24 h, then LPS (10 ng/ml) was added for further 24 h. As shown in Fig. 3 panel D (TNF-α) and panel E (IL-8), results obtained relative to cytokine production overlapped to that observed in THP-1 cells. In term of absolute values, in untreated whole blood cells TNF- α release was b15 pg/ml, 107 ± 44 in LPS treated cells, while IL-8 was 227 ± 42 and 5565 ± 1675 in control vs LPS treated cells. CD86 expression was not evaluated in human primary cells.

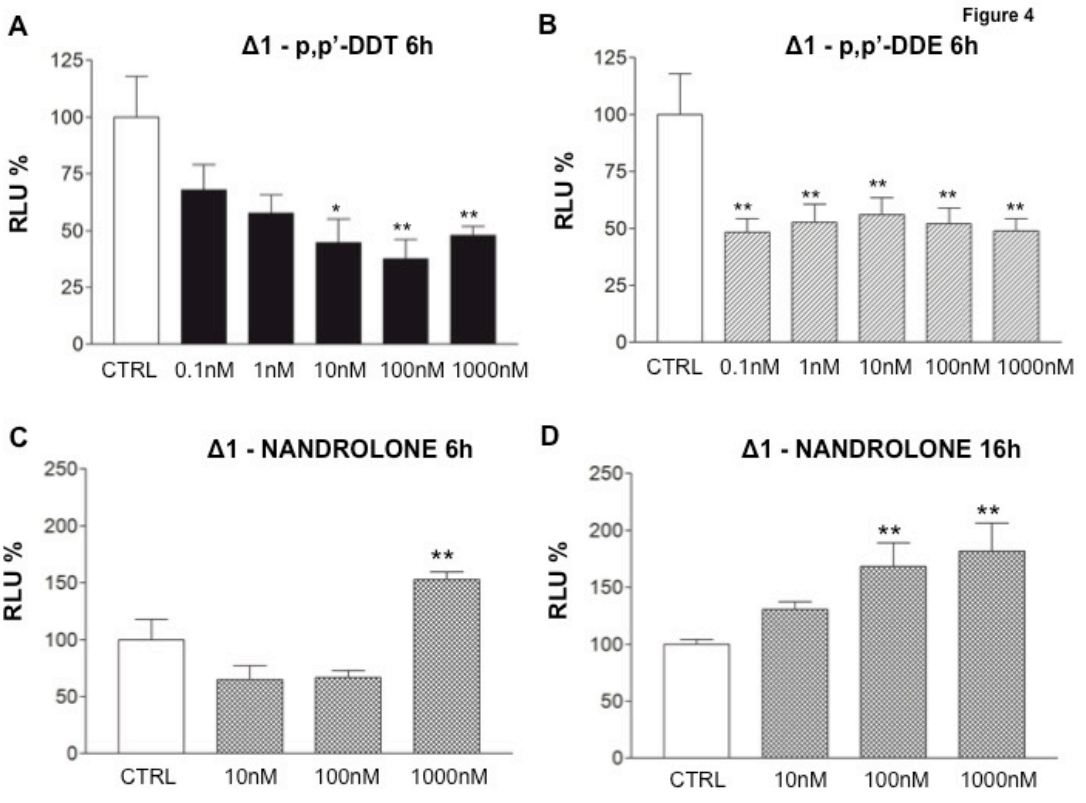

Fig. 4. Modulation of luciferase activity of the GNB2L1 promoter. Δ1 construct (Del Vecchio et al., 2009) was transiently transfected into THP-1 cells; after transfection THP-1 cells were treated for 6 h (A, B) with increasing concentration of p,p′DDT (1–1000 nM) (A) and p,p′DDE (B), or for 6 h (C) and 16 h (D) with increasing concentrations of nandrolone or DMSO as vehicle control (CTRL). Cells were lysed and luciferase activity was measured as described in Materials and Methods section. Luciferase activities are expressed as RLU% respected to non- treated construct (considered as 100%). Results are expressed as mean ± SD, n = 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *p b 0.05, **p b 0.01 vs control (CTRL).

3.4. Effects of the selected compounds on rack1 gene transcription

In view of these results, we considered to investigate the possible effects of the selected compounds on the human rack1 gene promoter, which could be used in future for screening purposes. The Δ1 luciferase reporter deletion mutant, was prepared and used as previously described (Del Vecchio et al., 2009). THP-1 cells were transiently transfected with Δ1 luciferase reporter construct and subsequently incubated for 6 h with increasing concentration of p,p′DDE, p,p′DDT (0.1–1000 nM) and for 6 and 16 h with nandrolone (10–1000 nM). As shown in Fig. 4A, reporter luciferase activity was strongly reduced by p,p′DDT at the 10, 100, 1000 nM concentrations, whereas p,p′DDE exerted a significant decrease in luciferase

activity at all the concentrations tested (Fig. 4B), with no clear dose-response being the activity already significantly reduced at 0.1 nM. Consistent with data presented on RNA and protein expression, nandrolone induced at 16 h a dose-related increase in the luciferase activity (Fig. 4D). The activity at 6 h was induced only at the highest concentration tested.

3.5. Effect of the selected compounds on cell proliferation

In THP-1 cells, it has been elegantly demonstrated by Zhang et al. (2013) that RACK1 has a key role in their proliferation. Therefore, we investigated if nandrolone and p,p′DDE could affect cell proliferation. Cells (2.0×10^5/ml) were starved for 24 h and then 5% final concentration of dialyzed serum was added in the presence or absence of the selected compounds (10 nM) or DMSO as vehicle control for 72 h. As shown in Table 2, an increase in cell numbers was observed following treatment with nandrolone (p b 0.01 vs control cells), while, consistent with the decrease in RACK1 expression, a decrease in cell number was observed in the cells treated with p,p′DDE (p b 0.05 vs control cells). The decrease in cell number following exposure to p,p′DDE was not due to cytotoxicity, as assessed by lactate dehydrogenase leakage that was similar to the one observed in vehicle treated cells (Table 2). This result indicates that targeting RACK1 may also have important implication on the oncogenic growth of cancer cells.

4. Discussion

Our working hypothesis was that EDCs can target RACK1 expression, and that the modulation of RACK1 by EDCs could represent a molecular initiating event that can bridge together several adverse effects concerning steroid endocrine interference and immunotoxicity. As a proof of concept, p,p′DDT, p,p′DDE and nandrolone were used. These compounds were chosen as they are known to have opposite effects on the AR: whereas nandrolone is a potent agonist, p,p′DDE is a strong antagonist and p,p′DDT a weak antagonist. Our in silico approach is useful for measuring the approximate affinity of a set of putative ligands. These values can be expressed either as binding free energies or as complex dissociation constants. In order to better describe the activity of the investigated compounds and the differences between the in vitro potency of p,p′DDE and p,p′DDT, the intrinsic activity (α) of each ligand should also be considered. Time expensive computational strategies for discriminating intrinsic activity of sex hormone LBD ligands have been described (Galli et al., 2014), and could be useful to classify a compound as agonist ($\alpha = 1$) or an antagonist ($\alpha = 0$), but they are not accurate enough for predicting intermediate α values. However, the relatively higher affinity of pp′DDE than of pp′DDT for AR, obtained via molecular docking, supports their literature definition as respectively: "strong" and "weak" AR antagonists.

Results obtained confirm that AR agonist/antagonist can modulate RACK1 expression in opposite way, leading to an enhancement or a decrease in the response to classic innate immunity stimuli and modulation of cell proliferation.

The concentrations used were relevant to human exposure, as for nandrolone following intramuscular injection of either 50 mg 19- nortestosterone-3-(p-hexoxyphenyl)-propionate or 50 mg 19-nortestosterone-decanoate, serum nandrolone increased rapidly to maximal concentrations of 4.6 ± 3.2 and 2.0 ± 1.3 nM (±SD), respec- tively (Belkien et al., 1985). While the levels of p,p′DDT and p,p′DDE in the human population are highly variable, in a study conducted in a relatively highly exposed population of farmers in the United States, the median plasma p,p′DDE concentration was 7.7 μg/l equivalent to 24 nM (range, 0.6–77.4 μg/l) (Cooper et al., 2004), while Barraza- Villarreal et al. (2004) reported in Mexican males mean plasma p,p′ DDE and p,p′DDT levels of 203.5 μg/l equivalent to 640 nM and 67.4 μg/l equivalent to 21 nM, respectively.

The rationale of this study has to be traced back to the observation on the bioinformatic analysis of the rack1 gene promoter of the presence of a negative GRE (Glucocorticoid Response Element) consensus sequence (Del Vecchio et al., 2009; Hudson et al., 2013). In line with this evidence, cortisol treatment inhibits the expression of RACK1 protein via inhibition of the activity of its gene promoter (Buoso et al., 2011). In addition, other corticosteroids such as betamethasone, budesonide, methylprednisolone, prednisone and prednisolone can target RACK1, modulate its expression and support the notion that this

protein is an important target of corticosteroid-induced anti-inflammatory effects (Corsini et al., 2014). On the other hand, it is a consolidated evidence that DHEA exerts anti-glucocorticoid properties in the regulation of many processes involved in the immune response and the evidence concerning the effect on RACK1 is consistent with a complex molecular paradigm (reviewed in Buoso et al., 2017). We demonstrated that DHEA modulates the effect of cortisol on RACK1 expression via interference with the splicing of the glucocorticoid receptor with the induction of the GRβ isoform (Pinto et al., 2015), and via its transformation into active androgen steroids (Corsini et al., 2016).

It is also known that the AR and GR can interact at the transcriptional level and that this interaction is correlated with their ability to form heterodimers at a common DNA site, usually termed as a canonical androgen/glucocorticoid response element (ARE/GRE) (Pihlajamaa et al., 2015), with AR selective receptor binding achieved through relaxed cis-element stringency rather than a distinct and strict ARE sequence (Sahu et al., 2014). Furthermore, AR can bind other DNA sequences as a monomer, or in alternative dimer conformation, or even as a heterodimer with transcription factors that do not belong to the NR superfamily (Hu et al., 2010; Helsen et al., 2012) including interactions with AP-1, NFI, and Oct-1 or with transcriptional coactivators that do not themselves bind DNA such as SRC-1, GRIP1, and TIF2. These interactions sum to regulate the steroid response in a promoter and cell context-dependent manner. DNA-dependent protein-protein interactions, functionally amplified by enhanced coactivator recruitment, may promote receptor-selective activation. Hence, differential interactions among factors, rather than their stringent specificity, can confer precise hormonal response (González et al., 2001). In the context of the rack1 gene promoter we identified an Oct-1 binding site close to GRE sequence, we speculate that this transcription factor may regulate RACK1 expression in association with AR, and the non-canonical GRE sequence described may be also a cis-regulatory target of AR as it consists of direct repeats 5′-AGAACAccctccggaAGCACA-3′ including the well-conserved 5′-hexamer (5′-AGAACA-3′) (Buoso et al., 2017).

Functional consequences and practical use of these regulatory elements in the rack1 gene promoter can be foreseen in the context of EDCs, immunoregulation and cancer cells. RACK1 expression can be used as a target to identify EDCs and immunotoxic compounds, with im- portant implication for cancer progression, as described in the current manuscript. Our results suggest the potential use of RACK1 expression as predictor of EDCs that may interfere with the normal innate immune response. The latest being supported by the fact that, in the last decade, we demonstrated the pivotal role of RACK1, as scaffold protein of PKCβII, in the activation of both innate and acquired immune response in vitro and in vivo. Reduced expression of RACK1 is the major contributor to PKCβII defective activation. The consequence of this signal transduction deficiency is a significant decrease in immune cells functionality including response to influenza vaccination (Corsini et al., 2006), cell proliferation and cytokine production (Corsini et al., 1999, 2002, 2005; Racchi et al., 2006). In addition, Ballek et al. (2016) recently demonstrated that T-cell activation upon TCR

engagement induces a rapid, concomitant, and transient co-redistribution of Lck and RACK1 into the forming immunological synapse, which is required for the subcellular redistribution of Lck. Furthermore, within the immune system, RACK-1 also functions as an adaptor recruiting the transcription factor STAT1 to the IFN receptor complex and is a scaffold protein for the IFN-alpha receptor 2/beta-chain of the receptor, Janus kinase 1, and tyrosine kinase 2 (Usacheva et al., 2003). RACK-1 may also serve as a scaffold protein in other cytokine systems such as IL-2, IL-4, and erythropoietin as well as the signaling pathways of TNF-R55 (Tcherkasowa et al., 2002). Furthermore, considering the central role of RACK1 in cellular homeostasis, chemical-induced changes in RACK1 levels are likely to subvert physiological functions, which go far beyond the immune system, possibly affecting tumor progression as demonstrated for nandrolone and pp′DDE affecting THP-1 cell proliferation in opposite way.

We propose a strategy where as a screening, molecular modelling and docking simulation to assess the affinity for steroid hormones receptors together with the rack1 gene promoter activity should be the initial step, followed by RACK1 mRNA and protein expression to confirm that changes in the promoter activity have an impact on cellular RACK1 level, and finally, the physiological consequences of its modulation, should be investigated for example by evaluating immune functions or cell proliferation to mention some of the possible targets.

Results warrant further analysis of panels of EDCs differently targeting steroid receptors.

Conflict of interest

Authors declare of not having any financial, personal, or association with any of individuals or organizations that have could inappropriately influence the submitted work.

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Review

Role of Hormones in the Regulation of RACK1 Expression as a Signaling Checkpointin Immunosenescence

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Review

Role of Hormones in the Regulation of RACK1 Expression as a Signaling Checkpoint in Immunosenescence

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ABSTRACT

Immunosenescence defines the decline in immune function that occurs with aging. This has been associated, at least in part, with defective cellular signaling via protein kinase C (PKC) signal transduction pathways. Our data suggest reduced PKC activation and consequently reduced response to lipopolysaccharide (LPS) stimulation and cytokine release. The lack of PKC activation seems to be dependent on the reduced expression of the receptor for activated C kinase 1 (RACK1), a scaffolding protein involved in multiple signal transduction cascades. The defective expression of RACK1 may be dependent on age-related alteration of the balance between the adrenal hormones cortisol and dehydroepiandrosterone (DHEA). DHEA levels reduce with aging, while cortisol levels remain substantially unchanged, resulting in an overall increase in the cortisol:DHEA ratio. These hormonal changes are significant in the context of RACK1 expression and signaling function because DHEA administration in vivo and in vitro can restore the levels of RACK1 and the function of the PKC signaling cascade in aged animals and in human cells. In contrast, there is evidence that cortisol can act as a negative transcriptional regulator of RACK1 expression. The *rack1* gene promoter contains a glucocorticoid responsive element that is also involved in androgen signaling. Furthermore DHEA may have an indirect influence on the post-transcriptional regulation of the functions of the glucocorticoid receptor. In this review, we will examine the role of the hormonal regulation of *rack1* gene transcriptional regulation and the consequences on signaling and function in immune cells and immunosenescence.

1. Introduction

One of the most acknowledged consequences of aging is the reduced effectiveness of the immune system, which shows profound and age-dependent changes in the response to immunological challenges. The age-dependent decrease in immunological competence results from the progressive deterioration of both innate and adaptive immune responses [1]. Many factors contribute to this phenomenon, including stem-cell defects, thymic involution, aging of resting immune cells, replicative senescence of clonally expanding cells because of the erosion of telomere ends, defects in antigen-presenting cells, dysfunction in several signal transduction pathways, and dysregulation ofthe cytokine network [2]. Among these, the age-dependent decline of immune functions can be, at least in part, correlated with defective protein kinase C (PKC) signal transduction, which can be ascribed to the reduced expression of the Receptor for Activated C Kinase 1 (RACK1), a scaffold protein for different kinases and membrane receptors [3].

RACK1 binds activated PKCβII in order to stabilize its active conformation [4] and promote its translocation close to specific PKCβII substrates essential for immune cell activation, proliferation, differentiation, and survival [5]. We and others ([6] and refs within) have demonstrated that PKCβII activation plays a key role in the inflammatory response by inducing TNF-α release. An age-associated decrease in the release of TNF-α after lipopolysaccharide (LPS) stimulation was initially observed in alveolar macrophages obtained from aged rats, which produced ~50% less TNF-α than those from young rats [6]. A similar observation was also reported in human monocytes/macrophages, as well as in peripheral blood leukocytes, and was attributed to deficient PKC translocation due to an age-dependent decline in RACK1 expression [7,8]. As a consequence of the signal transduction impairment, a significant decrease in immune function, including the response to influenza vaccination [8], cell proliferation, and cytokine production was observed [6,7,9,10]. Interestingly, the same defective PKC signaling was also observed in the brain of aging animals, and it was demonstrated to be central in the impairment of memory processes [11–13].

The decrease in RACK1 expression is correlated with reduced secretion of dehydroepiandrosterone (DHEA) during aging [7]. Blood levels of DHEA are age-dependent and increase throughout childhood and puberty. After the age of 30, they decrease until reaching a minimum after the age of 80 [14]. This aspect is particularly relevant for the PKC signaling pathway because, in aged animals and in human cells, DHEA administration in vitro and in vivo can restore RACK1 levels, thus re-establishing a dose dependent TNF-α release after LPS stimulation [7,9]. Hence, part of the defective signaling in immune cells can be ascribed to age-related alteration of the hormonal balance.

This finding is particularly significant considering that cortisol levels remain substantially unchanged throughout an individual's life, resulting in an overall increase in the cortisol:DHEA ratio [8]. This increase leads to an imbalance between the actions of these hormones, impairing the ability of DHEA to counter the effect of cortisol [15]. Cortisol acts as a negative regulator of RACK1, while DHEA inhibits cortisol activity, thereby promoting RACK1 expression [16]. The opposing effects of cortisol and DHEA seem to be derived, at least in part, from a complex influence on the post-transcriptional regulation of the glucocorticoid receptor (GR) [17,18].

These considerations underline the importance of exploring the role of RACK1 in the context of immunosenescence and the current knowledge of the mechanisms supporting the role of cortisol and DHEA in the regulation of RACK1 expression.

2. The Critical Involvement of Hormonal Balance Affecting RACK1 Expression

The endocrine system plays an important role in modulating immune function, and it is well established that the aging process is accompanied by hormonal changes characterized by an imbalance between catabolic hormones that remain stable and anabolic hormones such as DHEA that decrease with age [19]. In the elderly, a common observation is an imbalance between cortisol and DHEA, with an increase in the cortisol:DHEA ratio, mainly due to a significant reduction in the levels of DHEA [14,19].

Glucocorticoids have a wide spectrum of biological functions, which include stress resistance, the regulation of gluconeogenesis, cell proliferation, control of inflammation, and immune responses. Particularly relevant is the last property, which allows for their widespread use as therapeutic agents for acute and chronic inflammation, as well as in autoimmune disorders and in the treatment of leukemia and lymphoma [20].

As reviewed by Hazeldine et al. [21], there is significant evidence that DHEA can exert immunomodulatory effects that include the inhibition of glucocorticoid activity. There are some concerns about the applicability of rodents as a useful model, as the site of DHEA production in rodents appears to be localized to tissues such as the brain rather than in the adrenal glands, as is the case in humans. Nevertheless, studies in humans and human derived cells have demonstrated the role of DHEA in regulating human immunity ([7,8], reviewed in [21]). Although the clinical data, derived from the attempted modulation of the immune function with DHEA supplementation, is conflicting, there is still interest in the potential role of this steroid hormone in age-related immunosenescence, provided that its mechanism of action is properly elucidated.

The evidence that DHEA exerts anti-glucocorticoid properties on RACK1 is consistent with an antagonistic paradigm. For example, it has been demonstrated that RACK1 down-regulation caused by physiological cortisol concentrations (0.1 and 0.5 μM) could be counterbalanced by pre-treatment with physiological (10 nM) and pharmacological (100 nM) concentrations of DHEA. The most effective time of pre-incubation was 72 h, although significant effects could be demonstrated also at 16 h. The effect of DHEA was observed on the promoter activity, on the mRNA levels, and at protein level. The interaction between DHEA and cortisol on RACK1 was also tested in the context of a functional immune response, wherein THP-1 cells were treated with LPS in order to induce TNF-α release, and, as expected, pre-treatment with DHEA reduced the inhibitory effect of cortisol on LPS-induced TNF-α release [16].

The effect of cortisol on RACK1 expression is clearly transcriptional, and the experimental evidence suggests that the effect of DHEA on RACK1 is similarly transcriptional in nature. However, there is no clear indication of the interaction of DHEA with a nuclear receptor with canonical transcriptional activity. As the contrasting effect of DHEA on RACK1 expression cannot be explained by a direct interaction on the promoter region or with simple pharmacological antagonism, a number of different indirect mechanisms have been explored (Reviewed in [22]).

3. RACK1 and Its Transcriptional Regulation

3.1. RACK1: A Versatile Hub of Different Signaling Pathways

The human *rack1* gene is mapped to chromosome 5q35.3 in close proximity to the telomere of chromosome 5. The open reading frame of the gene is 1142 bp, and it encodes for a protein with 317 amino acids, registering as a 36 kDa protein on Sodium Docecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) gel [23,24]. RACK1 belongs to the tryptophan-aspartate repeat family (WD-repeat). It is homologous to the β subunit of G-protein (Gβ), the best-characterized WD repeat protein, and contains a seven-bladed β-propeller structure that serves as a docking site for interacting proteins [3].

RACK1 was first identified in a rat brain cDNA library screen designed to isolate anchor proteins that bound PKC in the presence of its activators, diacylglycerol, calcium, and phosphatidylserine [25–27]. The binding of RACK1 to promote signaling via PKC has been characterized for specific isoforms, including PKCβII [5], PKCδ [28], and PKCμ [29]. The extensive investigation of the relationship between RACK1 and its binding partners has led to the realization that RACK1 interacts with numerous proteins (mostly engaged in signaling), either directly or as a part of a larger complex in distinct cellular compartments [3,30]. Some of the signaling partners include Mitogen Activated Protein Kinase (MAPK), Jun-N-terminal

Kinase (JNK), and cAMP specific phosphodiesterase PDE4D5, as well as Src kinase and integrins [22,31–33]. The functions supported by these interactions range from cell growth and survival to cell mobility and suggest a potential role for RACK1 in the development and spread of cancerous cells. The specific role of RACK1 in these aspects is, however, still controversial and appears to be cell, context, and stimulus dependent (see [31,34] for a review).

RACK1 has also been implicated as a ribosomal protein [35,36], suggesting an alternate mechanism via which this protein can alter gene translation and signal transduction. RACK1 is part of the small ribosomal subunit and promotes translation via the recruitment of PKC and the phosphorylation of the eIF6. In some types of cancers, RACK1's function as ribosomal protein can promote the proliferation and survival of neoplastic cells [34]

Although much is known about RACK1 protein localization, interactions, and related functions, the mechanisms regulating its expression remain relatively unexplored.

3.2. *The RACK1 Promoter Element and Its Transcription Factor Binding Sites*

A bioinformatics analysis on the porcine *rack1* gene promoter identified a serum responsive element (SRE) controlling gene expression. In porcine cells, it was observed that RACK1 protein was transiently induced by serum growth factors. Similarly, RACK1 expression was positively stimulated by phorbol esters through the mediation of the AP1 binding site. Moreover, a site for the Nuclear Factor-κB (NF-κB)/c-rel transcription factor was identified [37] and later mapped in a mouse promoter, where it demonstrated a fundamental role in the regulation of RACK1 expression [38].

The promoter of the human *rack1*-encoding gene, previously described in DNA databases as guanine nucleotide binding protein β polypeptide 2-like 1 (GNB2L1), was studied by cloning a 2-kb region 5′ of the *rack1* human gene [20]. Analysis in silico suggested the presence of several binding sites for transcription factors and two major transcription start sites (TSS), similar to what was observed in the mouse gene [20,38]. Binding sites for transcription factors belonging to a smooth muscle/cardiomyocyte specific family were recognized. Consensus binding sites for Hand1/E47, Elk-1, and Nkx2-5, which are cardiac specific homeobox, and myogenin/NF1 factor, which is involved in muscle differentiation and growth, were specifically identified.

Similar to those found in the mouse and porcine genes, four c-Rel binding sites were identified in the human RACK1 promoter [24]. c-Rel is a member of the NF-κB transcription factor family, which has been demonstrated to have a role in neuronal plasticity and survival [39,40]. In SH-SY5Y human neuroblastoma cells, sAPPα, a soluble amyloid precursor protein (APP) fragment secreted in conditioned medium of cultured cells, human plasma, and

the cerebrospinal fluid, could modulate the expression of RACK1 and the signaling activity of PKCβII through the activation of the PI3K/Akt and NF-ϰB pathways. sAPPα treatment induced c-Rel nuclear translocation, favoring its binding to the RACK1 promoter, which correlated with an increase in RACK1 expression [13]. These observations are particularly relevant in the context of Alzheimer's disease (AD), wherein RACK1 levels have been found to be significantly decreased in both membrane and cytosolic fractions obtained from AD brains when compared to age and post mortem matched control cases, suggesting a role for RACK1 expression in cognitive degeneration and age related conditions [41,42].

NF-ϰB involvement in RACK1 regulation was also demonstrated in cells of neural and immune origin using two well-known stimuli; LPS, an immune stimulus, and phorbol 12-myristate 13-acetate (PMA), a direct activator of the PKC pathway, which was previously reported to induce RACK1 protein expression [37]. Both molecules are linked, directly or indirectly, to nuclear cellular signals by means of the NF-ϰB pathway. The treatment of THP-1 (human monocytic cell line) and SH-SY5Y cells with either LPS or PMA resulted in a significant increase in RACK1 expression [24].

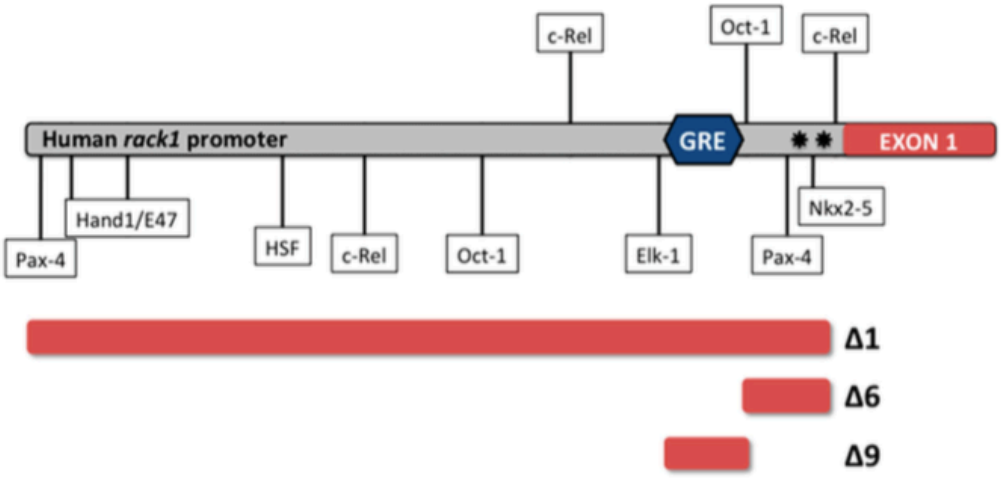

Figure 1. Structural analysis of the human receptor for activated C kinase 1 (RACK1) gene promoter region. Bioinformatic analysis of the 5′-flanking region within 7 kb upstream of the putative promoter region of the human *rack1* gene identified two major transcription sites, which are indicated with stars just before the beginning of Exon 1. Several putative cis-acting elements are shown; in particular, the putative unique GRE (Glucocorticoid Responsive Element) is detected at the nucleotidic position -186 with the sequence AGAACACCCTCCGGAAGCACA. Functional characterization of the GRE site was performed with deletion constructs (Δ1, Δ6, and Δ9), including or excluding the GRE site. More details can be found in the text and in [16,24].

In addition, Oct-1, Elk-1, and Pax-4 transcription factor binding sites were also identified. Finally, a consensus sequence for the binding of GR (Glucocorticoid Receptor), which appears to be similar to the consensus for a negative glucocorticoid responsive element (GRE) or nGRE, was detected at nucleotide −186 (+1 is the first TSS) [24]. nGRE binding is a new mode of sequence recognition for the human GR; two GR monomers bind nGREs in an inverted repeat orientation with strong negative cooperativity, which mediates DNA-dependent transrepression. The ability to repress to the GR at nGRE sites may allow targeted immunosuppressive therapy without the side effects often observed with glucocorticoid treatment [43]. (Figure 1).

3.3. RACK1 and Its Glucocorticoid Regulation

The discovery of the putative nGRE consensus sequence on the *rack1* gene promoter led to an investigation of the role of glucocorticoids in the regulation of RACK1 expression. Preliminary analyses performed in SH-SY5Y cells showed a significant repression of the activity of the *rack1* gene promoter following 24 h of treatment with 1 μM of cortisol [24]. A more detailed analysis of the role of the GRE element in controlling the RACK1 promoter was conducted in THP-1 cells transiently transfected with three luciferase reporter constructs; Δ1, Δ6, and Δ9 [16]. The Δ1 luciferase reporter construct represented the entire 2-kb region 5′ of the human *rack1* gene [24], whereas the construct Δ6 was a promoter fragment only that did not include the GRE sequence; the Δ9 construct included only the GRE sequence. THP-1 cells were transfected with these reporter constructs and treated with two physiological cortisol concentrations, 0.1 μM and 0.5 μM, which were chosen as they are representative of the most common range of the total plasma concentration of cortisol in humans. These studies demonstrated that in the presence of promoter constructs bearing the putative GCs responsive element cortisol induced a significant down-regulation of luciferase activity. In line with this evidence, cortisol was also able to drastically reduce RACK1 expression at both the mRNA and protein level, with a decline of about 70–80% compared to control cells. Additional support for a direct effect of cortisol on the promoter region of RACK1 comes from later observations that the potent GR antagonist mifepristone or RU486 abolished the cortisol-induced inhibition of luciferase activity, preventing RACK1 down-regulation [44], while the GR binding to the GRE sequence was demonstrated by ELISA based transcription factor binding assay [17]. Further evidence suggests that other corticosteroids such as betamethasone, budesonide, methylprednisolone, prednisone, and prednisolone can also target RACK1. The most effective inhibitors of LPS-induced cytokine release, namely budesonide, betamethasone, and methylprednisolone, were also most effective in reducing RACK1 mRNA expression and protein levels, thus confirming a correlation between RACK1 expression and the level of cytokine released in response to LPS. Finally, the importance of RACK1 modulation in the anti-inflammatory effect of cortisol was demonstrated using a

RACK1 pseudosubstrate, which directly activates PKCβ. Cortisol inhibition of LPS-induced cytokine release was prevented when RACK1 pseudosubstrate was added together with LPS [45], suggesting that RACK1 expression is central to the anti-inflammatory effect of cortisol. Synthetic glucocorticoid recapitulated these results [44], supporting the notion that RACK1 protein is an important target of corticosteroid-induced anti-inflammatory effects. RACK1 can therefore be considered a novel transcriptional target of corticosteroid-induced anti-inflammatory effects.

4. DHEA and Cortisol in the Regulation of GR Isoforms

The human gene *NR3C1*, which encodes the GR, is composed of nine exons. Alternative splicing in exon 9 generates two homologous receptor isoforms, termed GRα and GRβ [46,47]. GRα mediates most of the known glucocorticoid actions, while the GRβ isoform is expressed in most tissues but lacks the ligand-binding domain. As a result, GRβ does not bind glucocorticoids and thus is unable to activate glucocorticoid-responsive gene promoters [48–50]. Indeed, there is evidence that GRβ acts as a dominant negative of GRα [50,51]. In the context of RACK1 expression, the presence of the GRβ/GRα inactive complex on a GRE site was demonstrated by transcription factor binding assay. When THP-1 cells were treated for 16–18 h with DHEA (10 and 100 nM) and then stimulated with cortisol (0.1 and 0.5 μM), a significant increase in the GRβ/GRα binding ratio was observed [17]. Hence, DHEA induces the increase of GRβ/GRα complex by GRβ up-regulation and counteracts the cortisol-induced binding of GRα to the RACK1 promoter region, thus reinforcing the idea that GRβ is a dominant-negative regulator of GRα activity [17]. Further investigation into the mechanism of action of DHEA in the context of GR splicing showed that DHEA induced the up-regulation of total GR mRNA, which was preferentially directed toward the β isoform, by increasing expression of the splicing factor SRSF9 (Serine/arginine Rich Splicing Factors 9), also known as SRp20 [18].

As discussed in Section 2, DHEA can modulate RACK1 protein levels via a transcriptional mechanism that does not involve a direct interaction with the promoter region of the *rack1* gene, and hence it can partially act by GRβ modulation. In line with these considerations, it was demonstrated that GRβ knockdown completely prevented DHEA-induced RACK1 expression and the modulation of cytokine release, highlighting that the effect of DHEA is driven by a modulation of GRβ expression and activity [17]. DHEA involvement in GRβ expression was confirmed by SRSF9 silencing; SRSF9 knockdown completely blocked the increase of GRβ induced by DHEA with a consequent prevention of DHEA-induced RACK1 expression [18]. These results suggest that the effect of DHEA is driven by a modulation of SRSF9, which, in turn, influences GRβ expression and activity, thus reinforcing the idea that GRβ is a dominant-negative regulator of GRα activity.

In contrast, cortisol specifically exerted a shift in the pattern of expression of the GR, promoting the α isoform at the expense of GRβ. Hence, cortisol did not affect the total GR mRNA levels, but it influenced and controlled the exon inclusion and exclusion in GR mRNA transcript by modulating, in an opposite way, SRSF3 (also known as SRp30c) and SRSF9 expression, which are two splicing factors involved in GR alternative splicing. Cortisol up-regulated SRSF3, the GRα promoting splicing factor, and down-regulated SRSF9. Moreover, cortisol-induced GRα expression was correlated with RACK1 down-regulation. In fact, SRSF3 silencing prevented the inhibitory effect of cortisol on RACK1 expression levels [18].

These data suggest that cortisol and DHEA can influence the alternative splicing of GR and underline the necessity of a critical balance between these serine/arginine-rich proteins to control the level of exon inclusion/exclusion in the mRNA transcript. Finally, these data also support the idea that the anti-glucocorticoid effect of DHEA, among other mechanisms, is also exerted by the modulation of the expression of proteins involved in the splicing of the GR pre-mRNA.

It is also worthwhile to note that the effect of DHEA on RACK1 expression could be completely prevented by using flutamide, an androgen receptor (AR) antagonist. It was demonstrated that flutamide prevented DHEA induced GRβ protein expression [52] In line with this result, DHEA-induced total GR mRNA expression was also prevented by flutamide treatment [18], according to recent evidence demonstrating an androgen response element upstream of the GR gene.

5. Effect of Androgens in DHEA-Induced RACK1 Expression

The physiological actions of DHEA have been attributed to its conversion to either androgens or estrogens. Recent data indicates that both THP-1 and human peripheral blood mononuclear cells (PBMCs) are able to rapidly convert DHEA to dihydrotestosterone (DHT). Hence, the ability of testosterone, DHT, and androstenedione to induce RACK1 expression and cytokine production was evaluated. As with DHEA, an increase in RACK1 expression and in LPS-induced IL-8 and TNF-α production was observed after treatment with these selected androgens. The role of DHT in DHEA-induced RACK1 expression was also corroborated by the ability of finasteride, a 5α-reductase inhibitor, to completely block the effect of DHEA on RACK1 mRNA expression. The key role of the AR to mediate DHEA-induced RACK1 expression was finally confirmed by silencing experiments [52].

Overall, these data, together with the ability of physiologically relevant concentrations of testosterone and DHT to induce RACK1 expression, support the notion that the metabolic transformation of DHEA to androgens and their binding to the AR are required for DHEA-

induced RACK1 expression and cell activation.

It is important to note that approximately one-half of the AR cistrome overlaps with that of the GR. Indeed, the DNA-binding domain (DBD) of class I steroid receptors, including the AR, GR, progesterone receptor (PR), and mineralocorticoid receptor (MR), is highly conserved. All recognize a response element usually described as a canonical androgen/glucocorticoid response element (ARE/GRE) and are characterized by a well-conserved 5′-hexamer (5′-AGAACA-3′) and a less stringent sequence requirement for the 3′-hexamer [53]. In fact, different spacer-lengths or different hexamer-orientations have been proposed [54,55]. Therefore, the non-canonical GRE sequence described in the *rack1* gene promoter may also be considered as a *cis*-regulatory target of the AR, as it consists of direct repeats of the sequence 5′-AGAACAccctccggaAGCACA-3′.

In this context, and to further support the role of AR in RACK1 expression, recent data suggested a direct involvement of the AR in RACK1 regulation mediated by p,p′DDT (dichlorodiphenyltrichloroethane) and p,p′DDE (dichlorodiphenyldichloroethylene), a weak and a strong AR antagonist, respectively. In THP-1 cells transiently transfected with a luciferase reporter construct of the *rack1* gene promoter and incubated with increasing concentrations of p,p′DDT and p,p′DDE, the reporter luciferase activity was strongly reduced by both endocrine disrupting chemicals (EDC), with p,p′DDE being more potent than p,p′DDT. Moreover, the decrease in RACK1 expression was accompanied by a consequent impairment of IL-8 and TNFα release following LPS stimulation. In contrast, treatment with the AR agonist nandrolone resulted in a dose-related increase in luciferase activity and consequently in RACK1 expression. These findings suggest that RACK1 could be a relevant target of EDCs, responding in an opposing manner to agonists or antagonists of the AR and representing a bridge between the endocrine system and the innate immune system [56].

These last observations should also be considered in the context of RACK1, taking into account that both the AR and GR can interact at the transcriptional level and that this interaction is correlated with their ability to form heterodimers at a common DNA site, both in vitro and in vivo. Moreover, GREs differ in their precise sequence motifs and in the functional GR surfaces required for binding or regulation. In vivo, many genomic regions that contain the GR binding sites consist only of half sites, and these regions are likely responsible for the regulation of a subset of target genes [57]. (Figure 2).

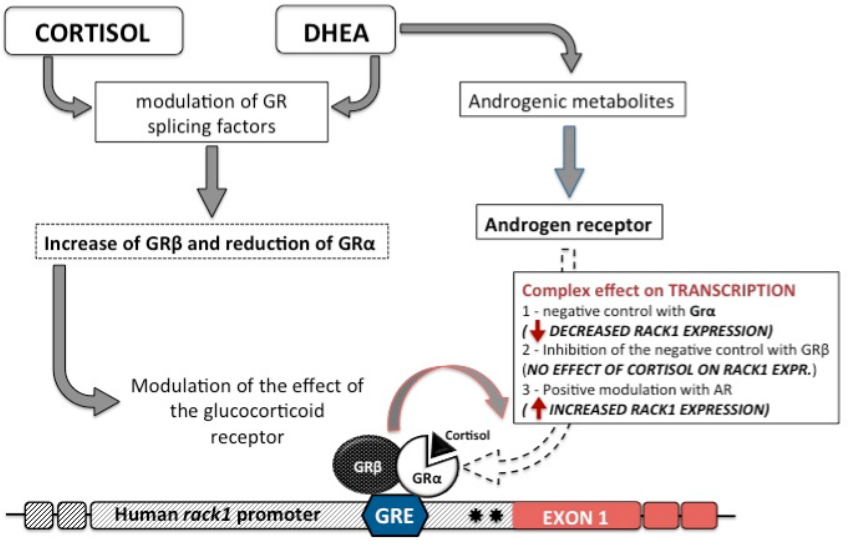

Figure 2. Scheme of the complex hormonal balance in the control of RACK1 expression. Data suggest that cortisol and dehydroepiandrosterone (DHEA) can influence alternative splicing of the GR, controlling the level of exon inclusion/exclusion in the mRNA transcript [17,18], and therefore suggesting that the anti-glucocorticoid effect of DHEA is due, in part, to modulation of the proteins involved in the splicing of the glucocorticoid receptor (GR) pre-mRNA. In addition, the effect expression of DHEA on RACK1 expression is dependent on its transformation into active androgen steroids [52]. Although pharmacological evidence supports the role of the AR, there is not yet direct demonstration of the interaction of the androgen receptor (AR) with the hormone sensitive site on the *rack1* gene promoter; hence the dotted line arrow in the scheme.

6. Conclusions

Taken together, these data support the existence of a complex hormonal balance between hormones in the control of immune modulation, which should be further investigated within the steroid hormones in the control of immune modulation, which should be further investigated within context of immunosenescence and endocrinosenescence. A majority of the data points to a role for he context of immunosenescence and endocrinosenescence. A majority of the data points to a role the cortisol:DHEA ratio in the determination of an appropriate functional response within cells of for the cortisol:DHEA ratio in the determination of an appropriate functional response within cells the immune system during aging. The hormonal imbalance between cortisol and DHEA observed of the immune system during aging. The hormonal imbalance between cortisol and DHEA observed with aging may affect directly the signal transduction cascade involved in the normal functions with aging may affect directly the signal transduction cascade involved in the normal functions of of key players of the innate immune system. It is therefore critical to understand the molecular key

players of the innate immune system. It is therefore critical to understand the molecular mechanism through which cortisol and DHEA regulate RACK1 expression, especially considering the central role of RACK1 in cellular homeostasis. Indeed, changes in RACK1 levels are likely to subvert physiological functions, which go far beyond the immune system, possibly affecting tumor progression as demonstrated by the opposing effects of nandrolone and p,p′DDE on THP-1 cell proliferation [56].

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Abbreviations

RACK1	Receptor for Activated C Kinase1
DHEA	Dehydroepiandrosterone
PKC	Protein Kinase C
AD	Alzheimer 's disease
GRE	Glucocorticoid responsive element
AR	Androgen receptor
GR	Glucocorticoid receptor
EDC	Endocrine disrupting chemicals
LPS	Lipopolysaccharide

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Cortisol-induced SRSF3 expression promotes GR splicing, RACK1 expression and breast cancer cells migration

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Cortisol-induced SRSF3 expression promotes GR splicing, RACK1 expression and breast cancer cells migration

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ABSTRACT

Recent data have demonstrated that triple negative breast cancer (TNBC) with high glucocorticoid receptor (GR) expression are associated to therapy resistance and increased mortality. Given that GR alternative splicing generates mainly GRα, responsible of glucocorticoids action, we investigated its role in the regulation of RACK1 (Receptor for Activated C Kinase 1), a scaffolding protein with a GRE (Glucocorticoid Response Element) site on its promoter and involved in breast cancer cells migration and invasion. We provide the first evidence that GRα transcriptionally regulates RACK1 by a mechanism connected to SRSF3 splicing factor, which promotes GRα, essential for RACK1 transcriptional regulation and consequently for cells migration. We also establish that this mechanism can be positively regulated by cortisol. Hence, our data elucidate RACK1 transcriptional regulation and demonstrate that SRSF3 involvement in cells migration implies its role in controlling different pathways thus highlighting that new players have to be considered in GR-positive TNBC.

1. Introduction

Triple-negative breast cancer (TNBC) lacks effective targeted therapies and consequently cytotoxic chemotherapy offers the only systemic treatment option. Approximately 25% of invasive TNBCs are glucocorticoid receptor (GR)-positive. Previous reports have found a significant association between high tumor GR expression and shortened relapse-free survival (RFS), suggesting that GR-mediated regulation of gene expression contributes to chemotherapy resistance [1–6]. It was found that physiological concentrations of glucocorticoids (GCs) decrease TNBC sensitivity to chemotherapy both in vitro and in vivo [7,8], suggesting that GR activation in TNBCs may contribute to chemotherapy resistance in tumor cells following GR activation by endogenous cortisol. Indeed, it was demonstrated that GR antagonism by mifepristone can counteract these effects of GC activation and increase paclitaxel cytotoxicity both in vitro and in vivo [5]. To identify GR- regulated genes, two chemically distinct GR antagonists, mifepristone and CORT108297 were used to perform global gene expression and GR ChIP-sequencing in MDA-MB-231cells, the TNBC cell line showing the highest GR expression, particularly GRα isoform, which mediates most of the known glucocorticoid actions [1,4,9]. The resulting subset of GR targeted genes were aberrantly expressed in TNBC patients and were associated with unfavorable clinical outcomes thus demonstrating how GR activity signature could be useful for patient stratification [6,4,9]. A deep mRNA sequencing of the transcriptional and post-transcriptional profiling of TNBC, Non-TNBC and HER2-positive breast cancers also allowed elucidating several modulators, including RACK1 [10] which, was involved in proliferation and invasion/metastasis both in vitro and in vivo and suggested as a predictor factor of poor outcome [11–13]. In addition, altered RACK1 expression was also reported in melanoma, non-small cell lung cancer and hepatocellular carcinoma [14–17].

RACK1 is a 36-kDa protein, recognized as a key element in multiple signalling pathways, partnering with proteins such as the oncoprotein Src [18–20], protein phosphatase 2A (PP2A) [21,22] and focal adhesion kinase (FAK) [23,24] thus playing a critical role in cancer cell migration and invasion [25]. Therefore, a deeper understanding of RACK1 transcriptional regulation is becoming of pivotal interest considering that RACK1 could be a GR target gene. Indeed, bioinformatics analysis of RACK1 gene promoter demonstrated the presence of a functional glucocorticoid responsive element (GRE) consensus sequence [26,27]. In THP-1 cells, we conducted a detailed analysis of GRE role in RACK1 promoter, demonstrating that cortisol and synthetic corticosteroids can target RACK1 by acting at transcriptional level [28–31]. Recently we also demonstrated that cortisol treatment in THP-1 cells induced a significant increase of GRα isoform [28]. Our data also demonstrated that cortisol-mediated GR splicing towards the α isoform was related to the up-regulation of SRSF3 (Serine/Arginine-Rich Splicing Factor 3 also known as SRp20), a splicing protein which was previously involved in GR splicing thus demonstrating that cortisol effect is also driven by modulation of GR splicing [32,33].

The purpose of this work was to investigate RACK1 transcriptional regulation in MDA-MB-231, a highly aggressive, invasive and poorly differentiated TNBC cell line characterized by a significant increase of GR expression compared to other similar cell lines [4,34] and consequently used as a model to investigate GR-positive TNBC. In fact, it was reported that MDA-MB-231 cells were used to identify gene expression profiles induced by dexamethasone (Dex), commonly associated with chemotherapy. This analysis demonstrated a striking association between Dex up- and down-regulated genes in MDA-MB-231 cells and in patients with invasive ductal breast carcinoma [9]. The same cell line was also used to study GR antagonism effects by mifepristone treatments [4,35–37] and differentially expressed genes forming GR activity signature in MDA-MB-231 cells were validated in two independent ER- negative BC cohorts [6].

To understand whether and how RACK1 could be a GR target gene in MDA-MB-231, we focused on the role of cortisol in the regulation of RACK1 expression and the consequent effect on cell migration. To elucidate cortisol mechanism of action, we investigated whether it was able to modulate SRSF3 expression and direct GR splicing towards GRα. Our work establishes that RACK1 is a GR target gene through a mechanism involving SRSF3 and can be positively regulated by cortisol. We have demonstrated that SRSF3-induced expression promotes GR splicing toward GRα, which in turn, up-regulated RACK1 determining a significant increase of MDA-MB-231 cells migration which is prevented by RACK1 or SRSF3 silencing. Therefore, our results not only confirm literature data about SRSF3 and RACK1 role in cell migration but also elucidate RACK1 transcriptional regulation and how this mechanism seems to be correlated with cell migration.

2. Materials and methods

2.1. Chemicals

Cortisol (PubChem CID:5754), mifepristone (PubChem CID:55245) and G418 were obtained from Sigma Aldrich (St. Louis, MO, USA). They were dissolved in DMSO at concentration of 1 mM and 10 mM and frozen (−20 °C) in stock aliquots. Stock aliquots were diluted at a final concentration in culture media at the time of use (final concentration of DMSO in culture medium < 0.1%). Cell culture media and all supplements were from Sigma Aldrich. Mouse monoclonal antibodies anti-RACK1 (sc-17754) and anti-GRα (sc-393232), anti-GAPDH (sc-32233) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse monoclonal antibodies anti α-tubulin and anti-SRP20 [SRSF3] (MABE 116) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Host specific peroxidase conjugated IgG secondary antibody (31,460) was purchased from Thermo Scientific (Waltham, MA, USA). All reagents were purchased at the highest purity available. Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA).

2.2. Cell culture and treatments

The TNBC cell line MDA-MB-231(ATCC®) was maintained at 37 °C in 5% CO_2 and cultured in DMEM medium supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 2 mM penicillin/streptomycin. In line with our previous results, cells were treated for 6 h in a medium containing vehicle or 0,1 μM cortisol [32]. To demonstrate the role of glucocorticoid receptor, cells were pretreated for 1 h with 30 μM mifepristone before addition of cortisol and for 7 h with mifepristone alone [37]. Other specific details of times and concentrations are given in figure legends.

2.3. Construction of GR minigene and generation of a stable MDA-MB-231 cell line (GRmini-MDA231)

GR minigene was obtained as described in Ref. [38]. Briefly, human genomic DNA was isolated from peripheral blood using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). The GR genomic region including exon 8, intron H exon 9α, intron J and exon 9β was amplified by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase (F530S -Thermo Fisher Scientific, Rockford, IL, USA) with specific primers located in exon 8 (GR forward including a BamHI site F: 5′-CGGGATCCAGGACGGTCTGAAGAGCCAAGAGCTATTTG-3′) and exon 9β (GR reverse including XhoI site R: 5′–CCGCTCGAGCCCAGAGCTCATCCCATGCTAATTATCCAG -3′) as in- dicated in Ref. [38]. PCR fragment was inserted into pcDNA3 vector (Invitrogen, USA) between BamHI and XhoI sites to generate GR minigene eukaryotic expression vector (pcDNA3-GR). The construct was sequenced by BMR Genomics (Padova, Italy) and subsequently MDA-MB-231

cells were transfected with pcDNA3-GR vector by using Lipofectamine 2000 following manufacturer's instructions. Stable clones obtained by limiting dilution in medium with 1 mg/ml G418 were tested for GR minigene expression by Reverse Transcriptase-PCR (RT-PCR).

2.4. Plasmid DNA preparation, transient transfections and luciferase assays

Plasmids for transfections were purified with the HiSpeed® Plasmid Midi Kit (Qiagen, Valencia, CA). DNA was quantified by QuantusTM Fluorometer (Promega, Madison, WI). Transient transfections for luciferase assay were performed in 6 multi well culture plates; for each well $2{,}5 \times 10^5$ cells were seeded in DMEM medium without phenol red. Transfections were carried out using Lipofectamine 2000 following manufacturer's instructions. Each luciferase-reporter construct plasmid DNA was co-transfected with pRL- TK Renilla luciferase expressing vector to measure transfection efficiency (Promega, Madison, WI). During transfection MDA-MD-231 cells were incubated at 37 °C in 5% CO_2 and then treated with 0.1 μM cortisol for 6 h. Cells were lysed with Passive Lysis Buffer provide by Dual-Luciferase Reporter Assay System following manufacturer's specifica- tions (Promega, Madison, WI). The luminescence was measured with a 20/20n Luminometer with 10s of integration (Turner BioSystems, Sunnyvale, CA).

2.5. RT-PCR and real-time PCR

To analyze mRNA expression of our interest genes and GR minigene, 2×10^6 cells were plated in a Petri dish 60 mm. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. RNA quantification was obtained by QuantusTM Fluorometer (Promega, Madison, WI). Following manufacturer's specifications, QuantiTect reversion transcription kit (Qiagen, Valencia, CA) was used for cDNA synthesis. To analyze alternative splicing of GR minigene we used specific primers; GRα and GRβ exogenous forward was F:(T7): 5′-AATACGACTCACTATAGGGAGACC-3′. GRα exogenous reverse was R:(9aR) 5′-GATGACGACTCAACTGCTT CTG-3′ whereas GRβ exogenous reverse was R:(9bR) 5′-TTGTCGATGA GCATCAGTTGAC-3′ also reported in ref. [38]. Amplification was obtained by polymerase chain reaction (PCR) using GoTaq®G2 DNA polymerase (Promega, Madison, WI). Real-time PCR was performed by QuantiTect Syber Green PCR kit and for gene expression analysis of SRSF3 (SRp20), RACK1, NR3C1 (GR) and RpL6, primers were provided by Qiagen as indicate in Ref. [32]. GRα and GRβ primers were custom designed and synthesized by Primm (Milan, Italy) and the nucleotide primer sequences are indicated in ref [31]. The RpL6 (ribosomal protein L6) RNA transcription was used as endogenous reference [32,39] and the quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method [40].

2.6. Subcellular fractionation

Cellular fractionation protocol was obtained as described in ref [39]. Briefly, 3×10^6 MDA-MB-231 were seeded in 100 mm2 dishes and treated for 6 h with 0,1 μM cortisol, 30 μM mifepristone or pre- treated for 1 h with 30 μM mifepristone and subsequently 0,1 μM cortisol was added; afterwards the medium was removed, and cells were washed with PBS. These cells were subsequently homogenized 15 times using a Teflon glass homogenizer in 0.32M sucrose buffered with 20 mM Tris–HCl (pH 7.4) containing 2 mM EDTA, 10 mM EGTA, 50 mM β-mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin. The homogenate was centrifuged at 3600×g for 5 min to obtain the nuclear fraction. The supernatant was centrifuged at 100,000×g for 30 min; the supernatant obtained represented the cytosolic fraction. The pellet was sonicated in the same homogenization buffer supplemented with 0.2% (vol/vol) Triton X-100. The sample was incubated at 4◦C for 45 min and centrifuged at 100,000×g for 30 min. The supernatant was separated and represents the membrane fraction. The pellet represents cytoskeleton, which was resuspended in frac- tioning buffer. Aliquots of the fractions were used for protein assay by the Bradford method and the remaining was boiled for 5 min after di- lution with sample buffer and subjected to polyacrylamide gel elec- trophoresis and immunoblotting as described.

2.7. Western blot analysis

The expression of GRα, SRSF3, RACK1 and α-tubulin in cell homogenates was assessed by Western blot analysis. Briefly, cells were treated and then collected, washed twice with PBS, centrifuged and lysed in 100 μL of homogenization buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitor mix). The protein content was measured using the Bradford method (Bradford, 1976). Western blotting samples were prepared mixing the cell lysate with sample buffer (125 mM Tris–HCl pH 6, 8.4% SDS, 20% glycerol, 6% β-mercaptoethanol, 0.1% bromophenol) and denaturing at 95 °C for 5 min. Equivalent amounts of extracted protein (10 μg) were electrophoresed into an appropriate % SDS-PAGE under reducing conditions. The proteins were then transferred to a PVDF membrane (Amersham, Little Chalfont, UK) which was blocked in 5% w/v BSA, 1X TBS, 0.1% Tween-20 for 1 h with gentle shaking. The proteins were visualized using primary antibodies diluted in 5% w/v BSA, 1X TBS, 0.1% Tween-20 for RACK1 (1:1000), GRα (1:1000), SRSF3 (1:500) and α-tubulin (1:1000) as indicated in Refs. [31,32]. In all experiments, immuno-reactivity was measured using host specific secondary IgG peroxidase conjugated antibodies (1:7000 diluted) and developed using enhanced chemiluminescence reagent (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

2.8. Small interference RNA (siRNA)

To evaluate the role of SRSF3 in GRα and RACK1 expression, silencing experiments were conducted. The effect of inducing RNA interference on SRSF3 and RACK1 was assessed using commercially available reagents from Life Technologies [29,32]. siRNA transfection was performed with Lipofectamine RNAiMAX Transfection Reagent following manufacturer's instructions. Forty-eight hours after transfection cells were treated as previously described.

2.9. Scratch wound healing assay

Scratch wound healing assay was performed as described in Ref. [41]. Briefly, cells were seeded in a six-well plate and grown to confluence. Confluent cells were grown in serum-free DMEM medium for 24 h before experimentation. The monolayer cells were scratched and plates were washed twice to remove floating cells and then cells were incubated with medium containing 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) with cortisol (0.1 μM) or vehicle. Cells migrating from the leading edge were photographed at 0, 3, 6 and 24 h. Percentage open wound area (percentage of an image that is not con- sidered as occupied by cells) was calculated using ImageJ (NIH).

2.10. Statistical analysis

Following acquisition of the Western blot or RT-PCR images, the optical density of the bands was calculated and analyzed with Scion Image program for digital image processing (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD). The relative densities of the bands were expressed as arbitrary units and normalized to data obtained from control sample run under the same conditions. All experiments were performed at least three times, with representative results shown. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). The data were analyzed by analysis of variance (ANOVA) followed by an appropriate post-hoc comparison test as indicated in figure legend. Effects were indicated as significant if $p < 0.05$.

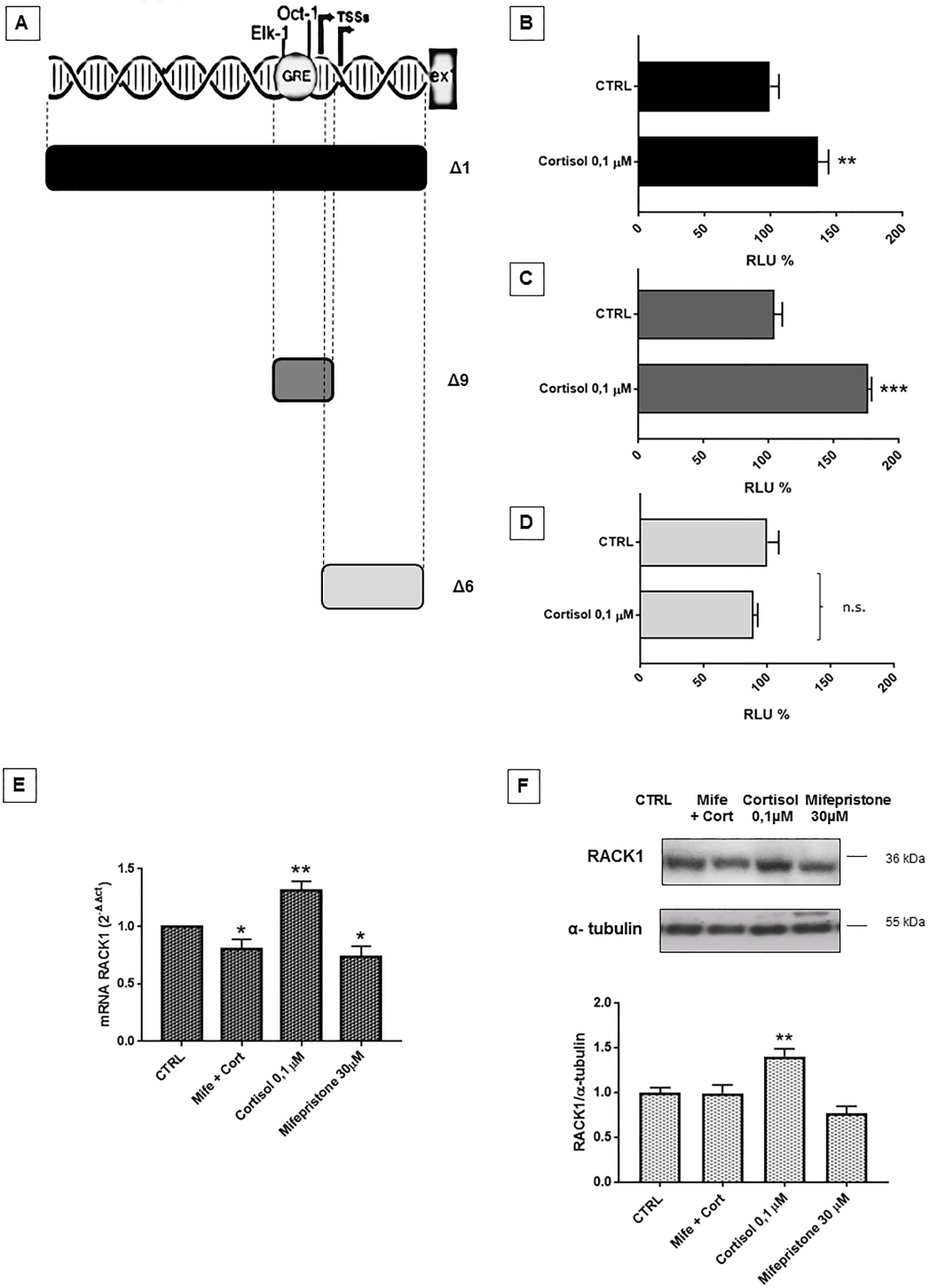
3. Results

3.1. Cortisol induces RACK1 expression in MDA-MB-231 cells

The presence of a GCs responsive GRE site in RACK1 promoter prompted us to investigate the transcriptional regulation of cortisol on RACK1 gene promoter. MDA-MB-231 cells were transiently transfected with three luciferase reporter constructs, Δ1, Δ6 and Δ9 (Fig. 1A) and treated for 6h with the physiological concentration of cortisol, according to our data showing that increasing concentration of cortisol (0.1, 1 and 10 μM) significantly up-regulated RACK1 (Fig. S1A). Luciferase reporter constructs containing GRE site, Δ1 and Δ9, showed a significant increase in luciferase activity (Fig. 1B and C) whereas in Δ6, cortisol had no effect and luciferase activity was comparable to transfected control cells (Fig. 1D). Data concerning transcriptional effect on Δ1 and Δ9 reporter constructs are paralleled by mRNA and protein expression results (Fig. 1E and F) suggesting that in MDA-MB-231 cells, RACK1 expression is significantly up-regulated by glucocorticoids. Cortisol role in RACK1 expression was confirmed by GR inhibitor mifepristone (RU486), which abolished cortisol-induced RACK1 mRNA and protein up-regulation (Fig. 1E and F). The same results were also obtained with low serum level (1% DCC-FBS) (data not shown) thus highlighting that RACK1-increased expression involves GR-induced gene transcription. Moreover, the presence of only mifepristone induced a modest reduction of RACK1 mRNA expression thus suggesting that RACK1 basal regulation could be dependent to the high level of GR expression, as previously discussed. Indeed, after a 24 h treatment with different concentrations of mifepristone, RACK1 protein expression was significantly down-regulated in a dose-dependent manner (Fig. S1B).

MDA-MB-231 cells treated with cortisol also showed a significant up-regulation of GRα at both mRNA and protein level, which was prevented by mifepristone treatment (Fig. 1G and H). Mifepristone also reduced GRα expression suggesting that GRα could be involved in RACK1 regulation. Suppressed GR expression could be part of mife- pristone mechanism of action as reported by literature [42].

Data presented here demonstrate that RACK1 is a GR target gene and this regulation is a cell specific event. In our previous work we demonstrated that, in THP-1 cells used as models of monocyte immune functions, cortisol and other corticosteroids induce RACK1 down-regulation. This is paralleled by significant reduction of cytokine release following cell stimulation and affecting basal immune responses [29]. Therefore, GR-depended RACK1 transcriptional regulation seems to be related to cellular environment and to specific cofactors that determine the final net effect of GR on gene transcription. This matter will require further specific investigation to understand the complex involvement of RACK1 in different forms of cancer and cancer cell types.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Mifepristone 30 μM

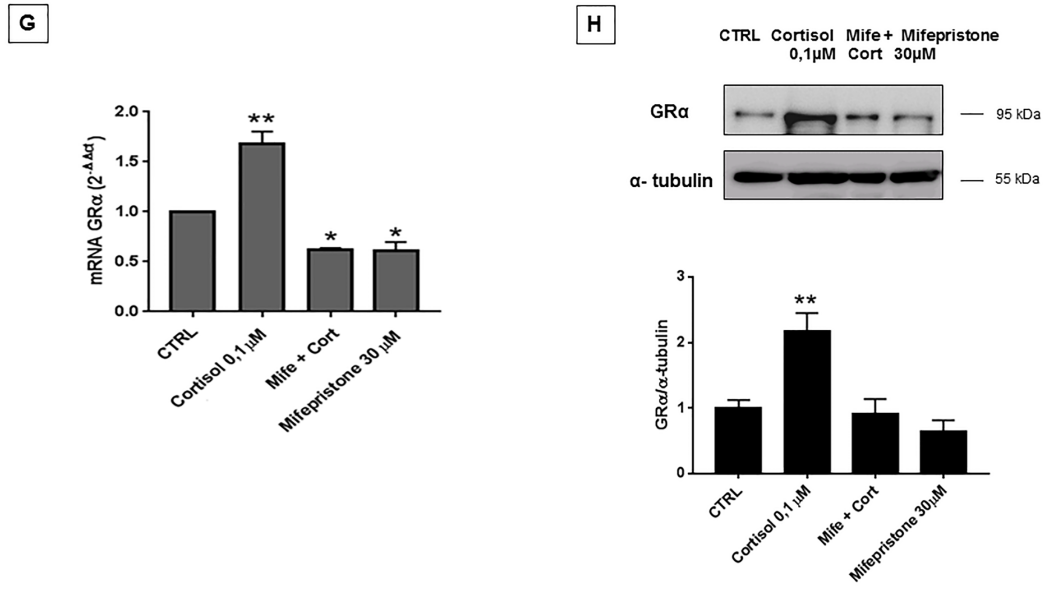


Fig. 1. Effect of cortisol on RACK1 expression through GRα modulation. **A.** Scheme of the genomic promoter region of the human RACK1 gene modified from Ref. [26]. The figure also presents the luciferase reporter constructs of RACK1 promoter region used for this study and described in Ref. [28]; Δ1 represented the entire 2-kb region 5′ of the human rack1 gene, Δ6 was a promoter fragment that did not include the GRE sequence whereas Δ9 construct included only the GRE sequence. **B–D.** MDA-MB-231 cells were transfected with Δ1, Δ9 or Δ6 reporter constructs, and subsequently treated with 0.1 μM cortisol for 6 h. For each reporter con- struct, luciferase activity was expressed as RLU% and compared to CTRL values assumed at 100%. Each bar represents the mean ± SEM of three independent ex- periments, in triplicate. Significance was set at $p < 0.05$ by the Student's t-test (**$p < 0.01$, ***$p < 0.001$). In **E-H**, MDA-MB-231 cells were treated for 6 h with 0,1 μM cortisol, 30 μM mifepris- tone or pretreated for 1 h with 30 μM mifepristone and subsequently 0,1 μM cortisol was added. **E–F.** RACK1 mRNA and protein expression. Value bars represent the mean ± SEM of six independent experiments. **G–H**. GRα mRNA and protein expression. Value bars represent the mean ± SEM of three independent ex- periments. In **E** and **G**, real-time PCR for RACK1 and GRα was performed as described in materials and methods. In **F** and **H**, immunoblotting was performed with the indicated antibodies described in Refs. [31,32]. In **E-H**, statistical analysis was performed with Dunnett's multiple comparison test, with *$p < 0.05$, **$p < 0.01$

3.2. Cortisol modulates GRα expression through SRSF3 involvement

Recently, we have demonstrated that in THP-1 cells cortisol induced GR up-regulation with specific increase of GRα isoform via induction of splicing and SRSF3-induced expression [32]. Cortisol also promoted a significant increase of SRSF3 at mRNA and protein level in MDA-MB- 231, which was abolished by mifepristone treatment (Fig. 2A and B) thus suggesting cortisol involvement in GR alternative splicing and in GRα up-regulation. To investigate the role of SRSF3 in cortisol-induced GRα splicing, its expression was silenced for 48 h. SRSF3 knockdown blocked cortisol effect on SRSF3 expression (Fig. 3A and B) and consequently on GRα mRNA and protein expression (Fig. 3C and D). Therefore,

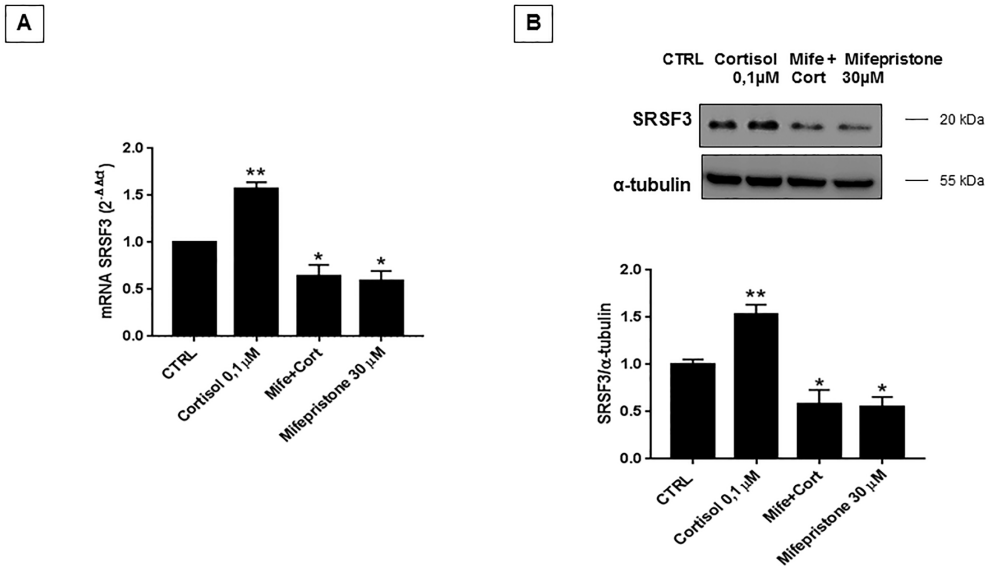


Fig. 2. Effect of cortisol on SRSF3 expression. MDA-MB-231 cells were treated for 6h with 0,1 μM cortisol, 30 μM mifepristone or pre- treated for 1 h with 30 μM mifepristone and subsequently 0,1 μM cortisol was added. RNA total extracts and cellular extracts were ana- lyzed by real-time PCR (**A**) and Western blot (**B**) respectively. In A–B value bars represent the mean ± SEM of three independent ex- periments. Statistical analysis was performed with Student's t-test, with **$p < 0.01$.

cortisol effect is driven by SRSF3 modulation, which in turn, influences GR splicing in favor of α isoform. SRSF3 down-regulation also affected cortisol-induced RACK1 expression (Fig. 3A and E) thus highlighting a possible interpretation of a chain of events that link the effect of cortisol on RACK1 expression, depending on GRα expression levels, which are induced by the effect of the splicing factor SRSF3. GRα and RACK1 expression was also affected in untreated SRSF3 silenced cells (see Fig. 3A) suggesting a basal level of control of this splicing factor over their expression, similar to the data obtained with mifepristone treatment (Fig. S1B).

Considering that dexamethasone in MDA-MB-231 cells was able to increase total GR expression [1], we investigated whether this cortisol- induced GRα over-expression could be the results of a GRα/GRβ ratio increase [43]. Our data showed that cortisol significantly promoted GR total expression (Fig. 4A) without affecting GRβ mRNA levels, which remained similar to control cells (Fig. 4B). Therefore, we observed a GRα/GRβ ratio increase, which is mainly due to the up-regulation of GRα mRNA (Fig. 4C). A similar mechanism of action was also observed in THP-1 cells treated with dehydroepiandrosterone (DHEA) where the hormone increased total GR expression. In this case the effect was through the induction of SRSF9, another splicing factor which increased GRβ isoform without affecting GRα expression [31,32].

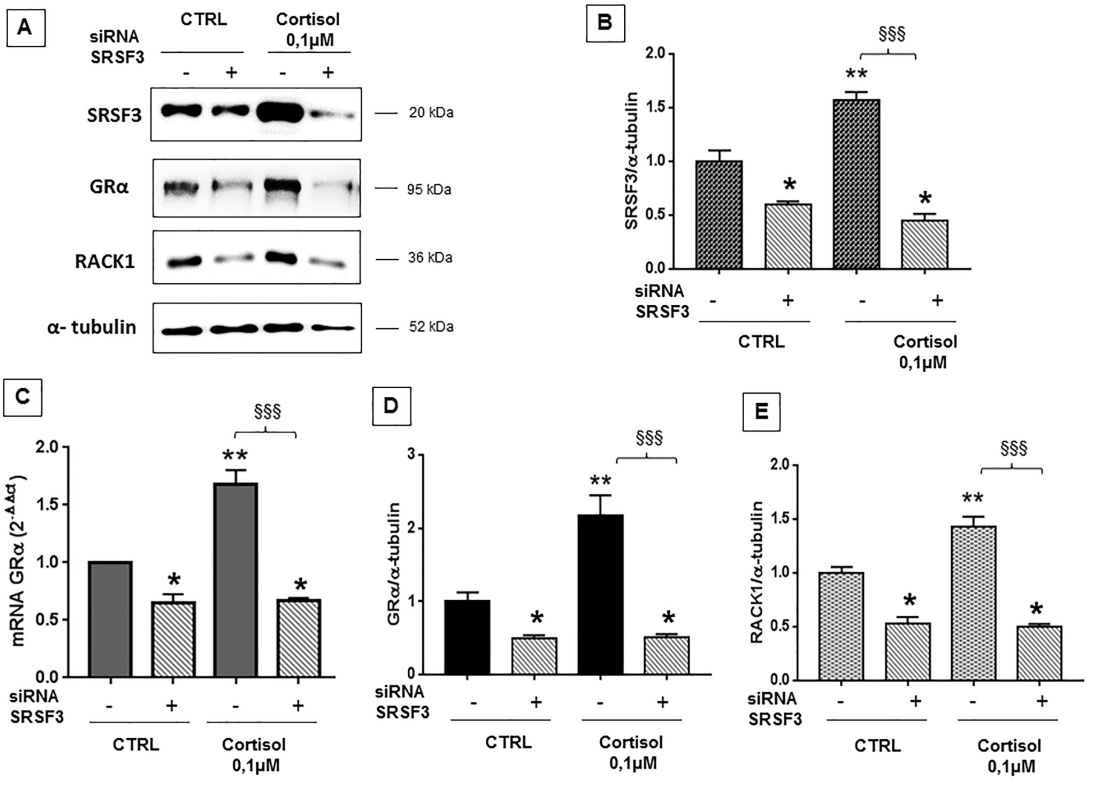


Fig. 3. Prevention of cortisol effect on SRSF3, GRα and RACK1 expression in SRSF3 silenced cells **A**. MDA-MB-231 cells silenced for 48 h with SRSF3 siRNA were treated for 6 h with 0,1 μM cortisol. The image is a representative Western Blot result. **B**. Results are shown as ratio SRSF3/α-tubulin ± SEM of four independent experiments. Tukey's multiple comparison test with *p < 0.05, **p < 0.01 vs CTRL and with §§§ p < 0.001 vs SRSF3 siRNA. **C**. GRα mRNA expression analysis was performed by real-time PCR as described in materials and methods. Statistical analysis was performed with Tukey's multiple comparison test with *p < 0.05, **p < 0.01 vs CTRL and with §§§ p < 0.001 vs SRSF3 siRNA. Each value in the graph represents the mean ± SEM of three independent experiments. In **D-E**, quantitative analysis of GRα and RACK1 protein. Each value in the graph represents the mean ± SEM of at least three independent experiments (GRα n = 3; RACK1 n = 4). Statistical analysis was performed with Tukey's multiple comparison test with *p < 0.05, **p < 0.01 vs CTRL and with §§§ p < 0.001 vs SRSF3 siRNA.

3.3. Characterization of cortisol effect on GRmini-MDA231 cells

To confirm the involvement of cortisol in the alternative splicing of exon 9 in GR pre-mRNA, we constructed a GR minigene vector in order to generate a stable MDA-MB-231 cell line (GRmini-MDA231) (Fig. 5A). Cortisol caused a significant increase of GRα exogenous mRNA, which was abolished in GRmini-MDA231 SRSF3-silenced cells thus confirming that SRSF3 is a critical splicing factor to generate GRα (Fig. 5B). Hence, cortisol treatment induces an over-expression of SRSF3, suggesting that modulation of the spliceosome proteins involved in GR mRNA splicing can be a significant mechanism for regulation of glucocorticoid activities. However, GRβ exogenous splicing trend (data not

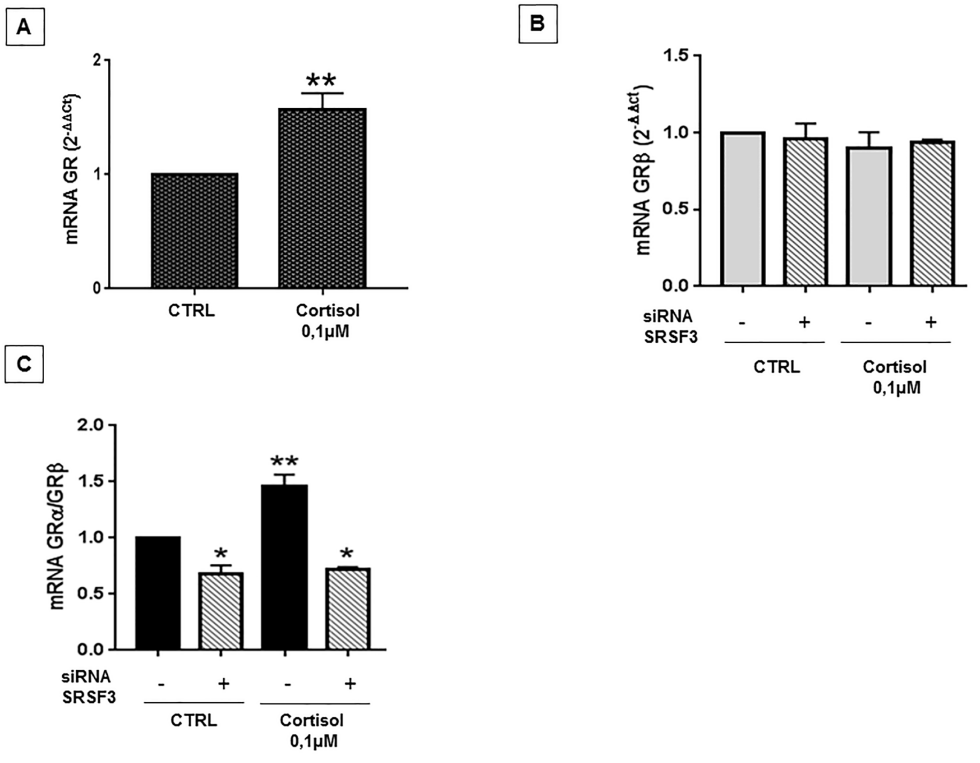


Fig. 4. Cortisol-induced increase GRα/GRβ ratio through the regulation GR total mRNA expression **A**. MDA-MB-231 cells were treated for 6 h with 0,1 μM cortisol and subsequently RNA total extracts were analyzed by real-time PCR to evaluate GR total mRNA expression. Value bars represent the mean ± SEM of three independent experiments. Statistical analysis was performed with Student's t-test, with ** $p < 0.01$. **B**. MDA-MB-231 cells silenced with SRSF3 siRNA were treated with for 6 h with 0,1 μM cortisol and subsequently RNA total ex- tracts were analyzed by real-time PCR by spe- cific primer as detailed in materials and methods. Each value in the graph represents the mean ± SEM of three independent ex- periments. Statistical analysis was performed with Tukey's multiple comparison test, not significant. In **C**, statistical analysis was per- formed with Dunnett's multiple comparison test with *$p < 0.05$, **$p < 0.01$.

shown) is comparable to not transfected cells (Fig. 4B). We can speculate that splicing factors involved in GRβ generation are significantly down- regulated in order to prevent GRβ action as, in different cell lines, GRβ was demonstrated to acts as a dominant negative on GRα [44,45]. Consequently, it is also possible that more time is necessary to observe a significant modulation of GRβ. However, we observed that GRα/GRβ ratio was increased in untransfected-minigene cells (Fig. 4C) where cortisol did not influence GRβ mRNA levels (Fig. 4B). However, considering the possible GRβ antagonist role towards GRα, we recognize that future studies are need to better understand GRβ modulation and expression in this cell line.

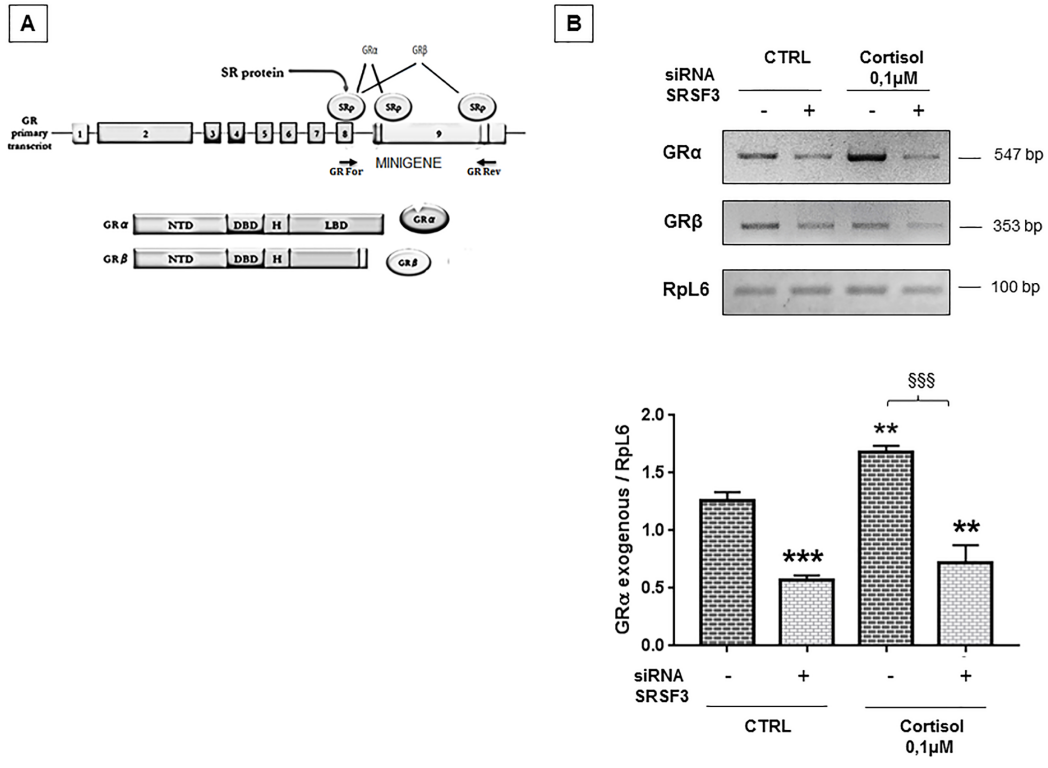
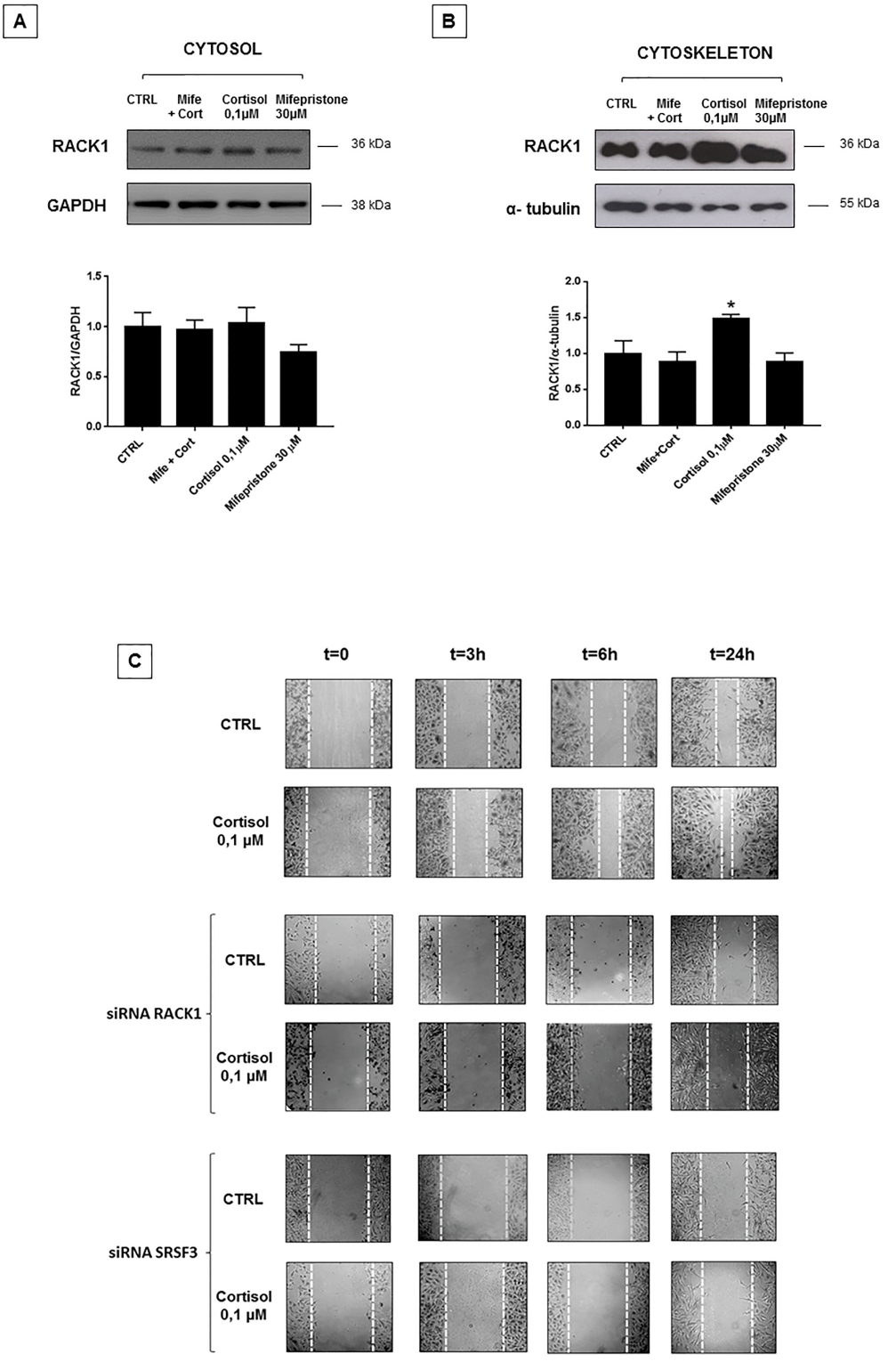


Fig. 5. Depletion of SRSF3 to prevent cortisol-induced GRα splicing in GR minigene **A**. Schematic representation of GR alternative splicing to generate α and β isoforms. Black arrows indicate GR forward and reverse primers used to construct GR minigene in order to amplify GR genomic region including exon 8, intron H exon 9α, intron J and exon 9β as described in materials and methods. Alternative splicing in exon 9 generates two highly homologous isoforms, GRα and GRβ. The two kinds of receptors share the first 727 amino acids at their N terminus coded by first eight exons (NTD, N-terminal domain, DBD, DNA binding domain and H, hinge region), and differ only at their carboxyl-terminus (LBD, ligand binding domain): GRα has an additional 50 amino acids coded by exon 9α but GRβ has only another 15 amino acids coded by exon9β. As a result of these differences, GRβ is unable to bind GC and can not trans-activate GC-sensitive genes. **B**. GRmini-MDA231 cells silenced with SRSF3 siRNA were treated for 6 h with 0,1 μM cortisol. To analyze alternative splicing of GR minigene we used GRα and GRβ exogenous specific primers, reported in Ref. [35]. The image is a representative RT-PCR result. Each value in the graph represents the mean ± SEM of four independent experiments for GRα minigene splicing. Statistical analysis was performed with Tukey's multiple comparison test with $^{**}p < 0.01$, $^{***}p < 0.001$ vs CTRL and with §§§ $p < 0.001$ vs SRSF3 siRNA.

3.4. Cortisol-induced RACK1 expression promotes MDA-MD-231 cell migration

Considering the established role of RACK1 in cancer cell migration and invasion [25] we decided to evaluate whether cortisol-induced RACK1 could influence MDA-MB-231 cell motility. Indeed, MDA-MB- 231 is a highly invasive cell line, used in the identification of genes and pathways that are potential mediators of metastasis to specific sites, lung, brain and bones [46–50].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CYTOSOL
CTRL
Mife + Cort
Cortisol 0,1µM
Mifepristone 30µM
RACK1
36 kDa
GAPDH
38 kDa
RACK1/GAPDH
1.5
1.0
0.5
0.0
CTRL
Mife + Cort
Cortisol 0,1 µM
Mifepristone 30 µM
B
CYTOSKELETON
CTRL
Mife + Cort
Cortisol 0,1µM
Mifepristone 30µM
RACK1
36 kDa
α- tubulin
55 kDa
RACK1/α-tubulin
2.0
1.5
1.0
0.5
0.0
*
CTRL
Mife+Cort
Cortisol 0,1µM
Mifepristone 30µM
C
t=0
t=3h
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siRNA SRSF3
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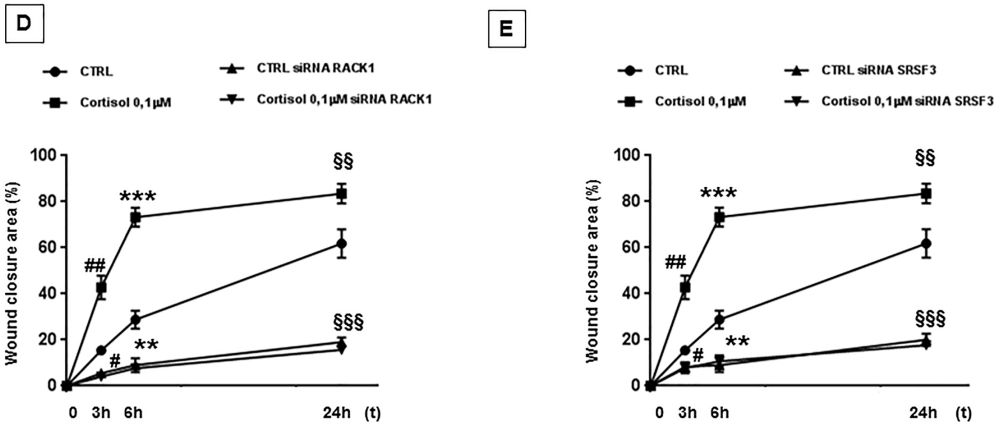

Fig. 6. Inhibition of cortisol-induced cell migration in SRSF3 and RACK1 silenced MDA-MB-231 cells. **A–B**. Analysis of RACK1 expression in cytosolic and cytoskeleton fractions of MDA-MB-231 cells treated for 6 h with 0.1 μM cortisol or 30 μM mifepristone or pretreated for 1 h with 30 μM mifepristone before 0,1 μM cortisol addition. The image is a representative Western blot result. Each value in the graph represents the mean ± SEM of five independent experiments. Statistical analysis was performed with Dunnett's multiple compar- ison test, with $^{**}p < 0.01$. **C-E**. Effect of cortisol on cellular migration in RACK1 or SRSF3 silenced and unsilenced cells by scratch wound healing assay, performed as described in materials and methods. Silenced and unsilenced cells were cultured without (CTRL) or with 0.1 μM cortisol and subsequently migration was evaluated at 3, 6 and 24h. In C, the image is a representative result. In **D-E**, value bar in the graph represents the mean ± SEM of three independent experiments, in duplicate of wound healing area in RACK1 and SRSF3 silenced and unsilenced MDA-MB-231 cells. The analysis was performed by two-way ANOVA with Bonferroni's multiple comparisons test with # $p<0.05$, ## $p<0.01$ vs CTRL at t=3h, $^{***}p < 0.01$ vs CTRL at t = 6 h, §§ $p < 0.01$, §§§ $p < 0.001$ vs CTRL at t=24h.

First, we evaluated the subcellular localization of cortisol-induced RACK1 protein. Cells were treated with cortisol (in the absence or presence of mifepristone) and then we performed a fractionation protocol separating cytosol and cytoskeletal compartments. In cortisol- treated cells, we found a significant increase of RACK1 in cytoskeleton compartment, which is consistent with a possible effect on cell migration (Fig. 6A and B). We next performed a scratch wound healing assay. As shown in Fig. 6C, cells incubated with cortisol exhibited a significant increase in cell migration of about 45% at 6 h while migration slope is constant. After 24 h, in cells treated with cortisol we could observe that 80% of wound area was healed whereas only 60% was covered in untreated control cells (Fig. 6C). In line with literature data, we also demonstrated that mifepristone blocked MDA-MB-231 cells migration both in presence and in absence of cortisol (data not shown). Finally, cell migration was almost completely blocked in cells where SRSF3 or RACK1 expression was down-regulated by specific siRNA. (Fig. 6C–D and E for quantitative analysis).

Overall, we confirm the important role of SRSF3 in MDA-MB-231 cell migration [45] and demonstrate that GRα isoform is one of the products of the activity of this splicing factor. We

show a mechanism in which in MDA-MB-231, SRSF3 modulates GRα splicing, which is essential for RACK1 transcriptional regulation, a scaffolding protein that is important in cell migration according to literature data [12]. Finally, we also established that this mechanism can be positively controlled by cortisol administration with consequent cell migration increase.

4. Discussion

GR seems to have a pivotal role in TNBC progression and drug resistance, as previously discussed, these data give a further support in considering SRSF3 and RACK1 as potential target for TNBC therapy [45]. Our data demonstrated that in MDA-MB-231 cells, SRSF3 is essential for GRα splicing and consequently for cells migration; therefore, in line with literature, these results confirm that SRSF3 is involved in splicing events correlated with cell migration [45]. Moreover, we give the first evidence that GRα isoform is one of the products of SRSF3 activity; indeed, SRSF3 promotes GRα isoform, which is essential for RACK1 transcriptional regulation thus highlighting that SRSF3 involvement in cell migration implies its role in controlling different pathways. According to our data, it has been shown that the potent GR inhibitor mifepristone inhibits migration of MDA-MB-231 cells suggesting GR-regulated genes involvement on cell migration [37]. Therefore, considering that mifepristone down-regulated RACK1 in a concentration-dependent manner (Fig. S1B) and that, this protein was involved in cell migration, our data further support the relationship among SRSF3, GRα and RACK1 in MDA-MB-231 cells. RACK1 has been identified as one of the major transcript in TNBC [10] and subsequent investigations in breast cancer patients suggested that RACK1 can be proposed as a highly powerful predictor of poor outcome [13]. It was also observed that multiple genes involved in cell migration and invasion can be modulated by glucocorticoids [51]. More specifically GR antagonism can reverse expression of these genes and it was proposed that GR antagonism could be exploited as additive chemotherapy to improve the likelihood of response in patients at high risk [6].

The data presented here also contribute to the evidence about the negative effect of stress hormones reported by literature data [1]. We demonstrated that SRSF3 up-regulation induced by cortisol was responsible of cell migration increase through its effect on RACK1 expression and localization. We found that in cortisol treated cells, RACK1 protein was significantly increased in cytoskeleton where it could improve focal adhesion assembly [24,37]. It is interesting to note that GCs were reported to be able to promote fibronectin deposition, focal adhesion-dependent activation of Src and the remodeling of the actin cytoskeleton [52] thus giving a further support to our finding. In MDA- MB-231 cells, stress hormones also reduced paclitaxel efficacy through induction of DNA damage, which blocked the cell cycle in G1 phase [1]. Since we observed that cortisol induced cell migration, we suggest that MDA-MB-231 cells increased migration could be another mechanism activated by GCs to induce drug resistance. Accordingly, it has been observed that breast cancer cell migration is faster in the G1 phase of the cell cycle [51,53].

Our data provide the first evidence that RACK1 can be transcriptionally regulated by GR in MDA-MB-231 cells, the most widely used model for GR-positive TNBC as previously discussed. This also demonstrate that cortisol-induced RACK1 regulation is correlated with the migration potential of MDA-MB-231 cells and may lead to possible drug targeting considering that GR antagonism is being advocated as addition to chemotherapy and in a

more distant perspective considering that many of the RACK1 signalling partners have been identified. Finally, we provide the demonstration that RACK1 expression modulation is connected to the expression of SRSF3 splicing factor thus highlighting that, in GR-positive TNBC, new players have to be considered in cell migration and drug resistance.

5. Conclusion

Breast cancers (BCs) lacking expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 are termed triple-negative breast cancers (TNBCs) and are classified in six subtypes: basal-like-1, basal- like-2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor (LAR) [54]. Patients with TNBC are the subgroup with the worst outcome: no specific targeted therapy is currently available and consequently cytotoxic chemotherapy offers the only systemic treatment option [55,56]. However, development of multidrug resistance has led to the search of chemosensitizer drugs in order to enhance the efficacy of standard chemotherapy [57]. Glucocorticoid receptor (GR) antagonism was demonstrated to sensitize cells to chemotherapy-induced cytotoxicity in TNBC [4,9]. A phase I clinical trial conducted in advanced breast cancer patients suggests that GR can be a useful biomarker and a promising target in TNBC [5,58]. Indeed GR is a corticosteroid receptor, with both transcription factor and chromatin remodeling functions, involved in the regulation of genes involved in cell survival and migration functions [59]. Indeed, it has been demonstrated that in ER negative BC patients, ligand-dependent genomic binding of GR to GRE is the predominant mechanism in the regulation of genes associated with drug resistance and unfavorable clinical characteristics and outcomes [6,7,13]. Hence, to improve clinical outcome, the network of GR target genes, including RACK1, may be a better indicator of GR activity in TNBC rather than GR expression alone. Therefore, GR activity signature may be useful for patient stratification in order to identify individual ER-negative early-stage patients with a relatively increased risk of relapse that could benefit from adding GR antagonism to adjuvant chemotherapy. The observation that RACK1 expression is modulated as a consequence of GRα activation by SRSF3 opens the opportunity to further study about the connections between glucocorticoids, GR positive-TNBC and SFSF3 splicing factor. Therefore, our work allows not only to confirm and integrate literature data but also highlight that new players have to be considered in cell migration and drug resistance.

Conflict of interest

The authors declare that they have no affiliations with or involve- ment in any organization or entity with any financial interest or non- financial interest in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2019.03.008.

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Estrogen Deficiency Promotes Hepatic Steatosis via a Glucocorticoid Receptor-Dependent Mechanism in Mice

Matthew A. Quinn, Xiaojiang Xu, Melania Ronfani, John A. Cidlowski

Cell Reports

(March, 2018)

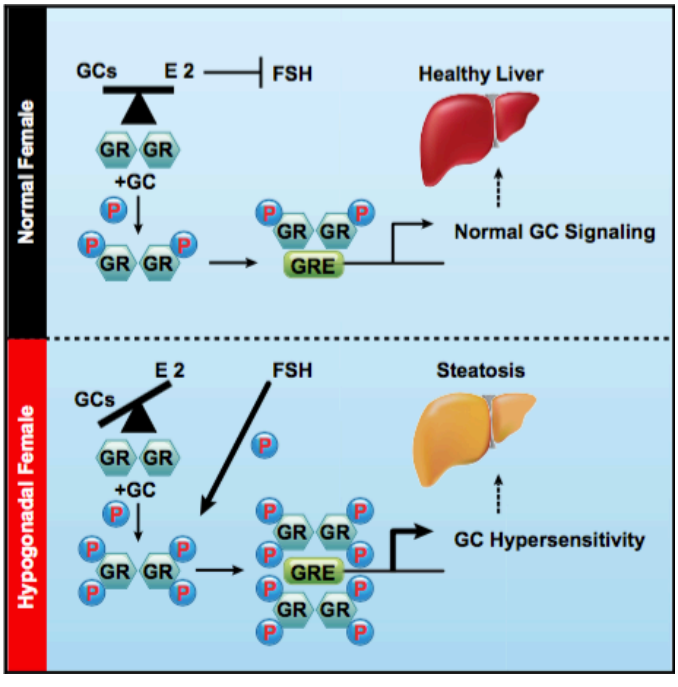
C H A P T E R 6

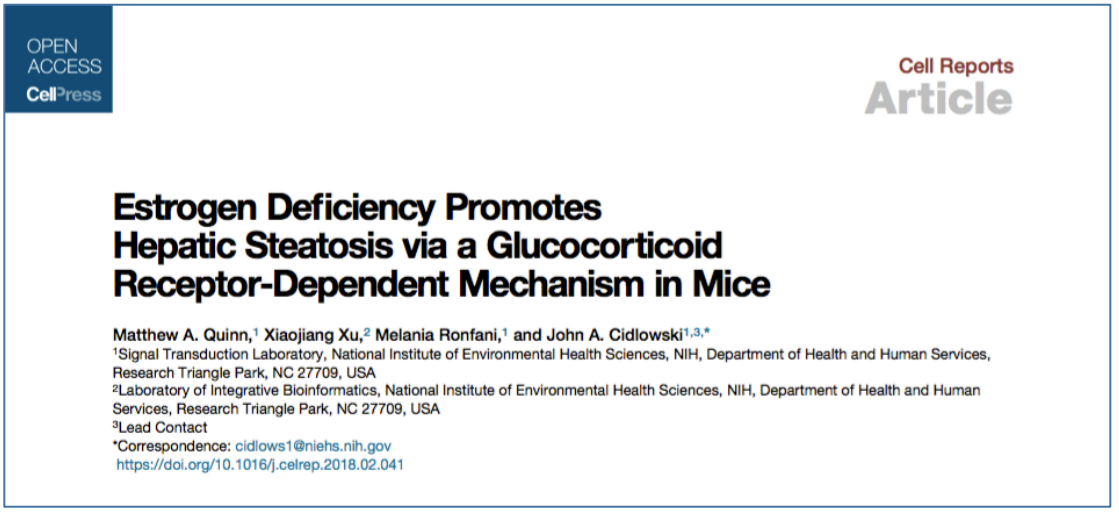
My PhD time abroad

During the second semester of my first year of PhD I had the great opportunity of spending three months at the laboratory of Signal Trasduction (Molecular Endocrinology group) of the National Institute of Environmentale Health and Siences (NIEHS) in Durham, North Carolina.

The laboratory is run by Professor John A. Cidlowski and is focused on the study of steroid hormones and on their ability in regulating the expression of tissue-specific genes via receptor-dependent intracellular signal transduction pathways. Indeed the major areas of research of the laboratory are: glucocorticoid receptors and their actions on the inflammatory response, regulation of apoptosis in normal and neoplastic cells and genetically modified animal models for studying glucocorticoid actions. Given my studying on cortisol-induced regolation of RACK1 expression and GR pre-mRNA splicing, all the experiments carried out in the laboratory were for me of great interest.

During my staying in the laboratory I had the chance to learn new techniques, performed my first *in vivo* experiments, interact with many scientists and looked at GR function and regulation within different tissutal contexts. I spent the majority of the time assisting Matthew A. Quinn, a post-doc involved in studying of glucocorticoid-induced hepatic steatosis in a murine model of menopause consisting of ovariectomized mice. The following paper is the results of our experiments.





ABSTRACT

Glucocorticoids (GCs) are master regulators of systemic metabolism. Intriguingly, Cushing's syndrome, a disorder of excessive GCs, phenocopies several menopause-induced metabolic pathologies. Here, we show that the glucocorticoid receptor (GR) drives steatosis in hypogonadal female mice because hepatocyte-specific GR knockout mice are refractory to developing ovariectomy-induced steatosis. Intriguingly, transcriptional profiling revealed that ovariectomy elicits hepatic GC hypersensitivity globally. Hypogonadism-induced GC hypersensitivity re- sults from a loss of systemic but not hepatic estrogen (E2) signaling, given that hepatocyte-specific E2 receptor deletion does not confer GC hypersensitivity. Mechanistically, enhanced chromatin recruitment and ligand-dependent hyperphosphorylation of GR underlie ovariectomy-induced glucocorticoid hypersensitivity. The dysregulated glucocorticoid-mediated signaling present in hypogonadal females is a product of increased follicle-stimulating hormone (FSH) production because FSH treatment in ovary-intact mice recapitulates glucocorticoid hypersensitivity similar to hypogonadal female mice. Our findings uncover a regulatory axis between estradiol, FSH, and hepatic glucocorticoid receptor signaling that, when disrupted, as in menopause, promotes hepatic steatosis.

Introduction

Menopause occurs during the aging process in women and is characterized by a steady decline in ovarian function. Several pathologies accompany the onset of menopause, such as metabolic syndrome and fatty liver disease (Lobo et al., 2014). Although the molecular events giving rise to menopause-associated pathologies are largely unknown, loss of ovarian function is thought to be the underlying mechanism. This notion has led to hormone replacement therapy (HRT) being the mainstay clinical treatment for menopausal symptoms (Kaunitz and Manson, 2015). Although HRT is efficacious at alleviating the symptoms of menopause, it increases the risk for breast cancer (Chlebowski et al., 2003; Li et al., 2003). Furthermore, recent studies suggest that lack of female sex hormones, particularly estradiol, cannot fully explain the metabolic manifestations of menopause (Bingol et al., 2010; Fenkci et al., 2003; Mittelstrass et al., 2011; Song et al., 2011; Stampfer and Colditz, 1991; Turner et al., 2011; Wang et al., 2011).

Glucocorticoids (GCs) are primary stress hormones secreted in response to either physiological or psychological stressors. GCs mediate their physiological effects through the GC receptor (GR), a ligand-activated transcription factor. Because GCs are potent regulators of a variety of biological processes, their synthesis and secretion are tightly regulated to avoid insufficient or excessive production, leading to Addison's disease or Cushing's syndrome respectively. GCs are well known modulators of metabolism and have been linked pathogenically to obesity and steatosis (Arnaldi et al., 2010; Lemke et al., 2008; Macfarlane et al., 2014; Patel et al., 2011; Sun et al., 2013). Intriguingly, menopause phenocopies a spectrum of pathologies observed in Cushing's syndrome patients such as osteoporosis, insulin resistance, and steatosis. However, the clinical link between GCs and menopause has not been studied empirically, despite human studies indicating trends toward higher cortisol levels during the menopausal transition (Woods et al., 2009). We show here that circulating GCs are elevated in a mouse surgical model of menopause via ovariectomy (OVX). Moreover, the rise in systemic GCs directly promotes metabolic syndrome and steatosis in ovariectomized mice because adrenalectomy (ADX) or genetic deletion of GR from hepatocytes blocks these metabolic abnormalities. Intriguingly, we discovered that hypogonadism in female mice promotes hepatic GC hypersensitivity. Furthermore, we have uncovered a mode of estradiol antagonism of hepatic GR signaling independent of liver-expressed estrogen receptor alpha (ERa), involving estradiol inhibition of follicle-stimulating hormone (FSH) production, leading to decreased GC-dependent phosphorylation of GR and diminished recruitment of GR to chromatin. Collectively, our data illuminate a role for GR signaling in the pathogenesis of metabolic syndrome/steatosis in estradiol-depleted mice and indicate that this pathway may be an alternative therapeutic target for the treatment of menopause-induced metabolic dysfunction.

Results

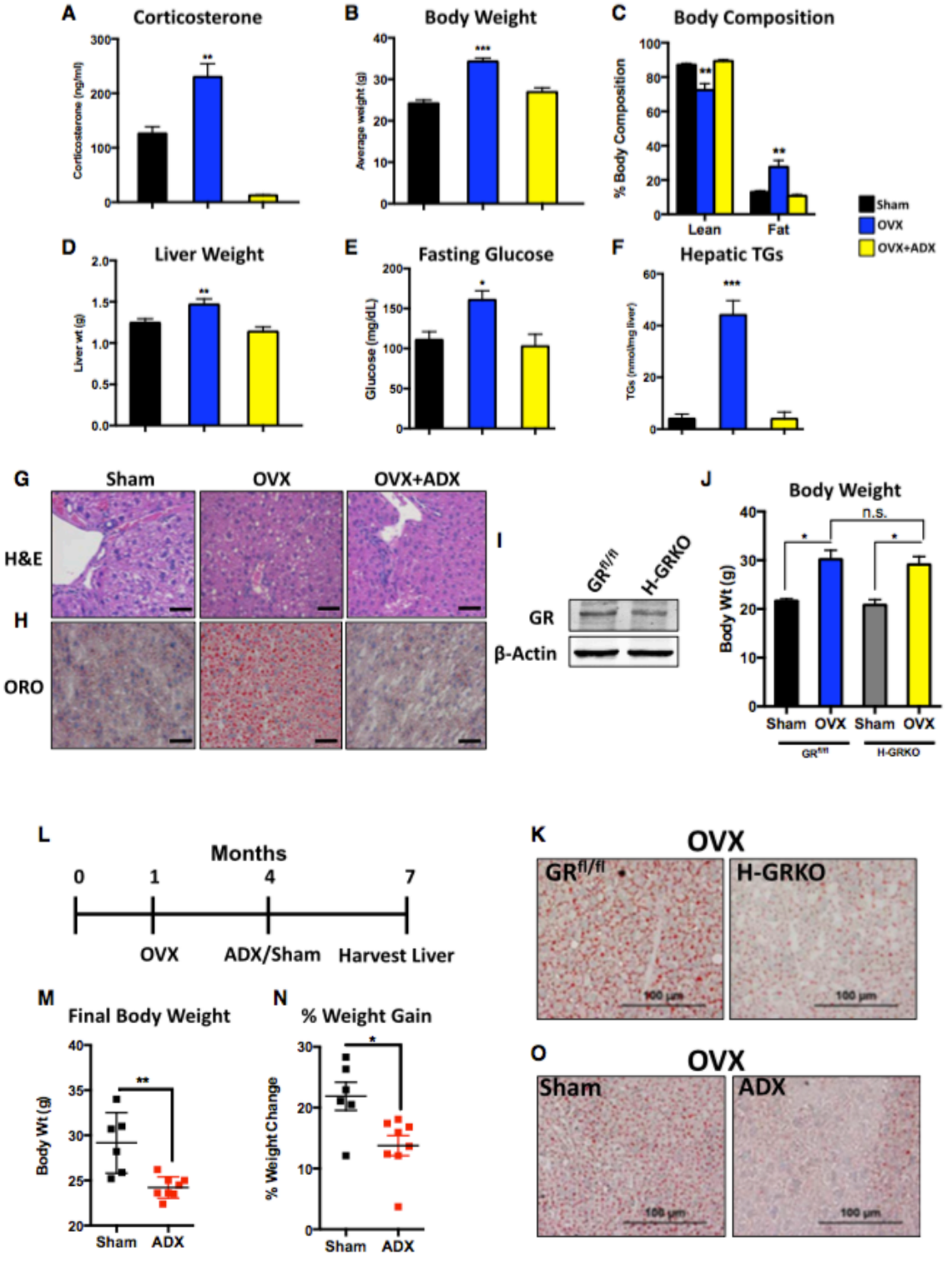
Loss of Ovarian Function Results in Cushing-like Syndrome in Mice

Given the clinical similarities between menopause and Cushing's syndrome, we wanted to determine whether GCs drive metabolic syndrome/steatosis in a murine model of menopause. Consistent with Cushing's syndrome, we observed significantly elevated corticosterone levels in ovariectomized mice compared with sham controls (Figure 1A). Furthermore, OVX promotes increased body weight coupled with a higher fat mass and lower lean mass, a phenomenon driven by adrenal hormones (Figures 1B and 1C).

The metabolic abnormalities associated with menopause can manifest in the liver as steatosis and insulin resistance (Brady, 2015; Stefanska et al., 2015). We found that ovariectomized mice have increased liver weights compared with both sham and ovariectomized/adrenalectomized mice (Figure 1D). Furthermore, hyperglycemia was observed in fasted ovariectomized mice but not ovariectomized/adrenalectomized mice (Figure 1E). Triglyceride (TG) levels were measured, revealing the presence of steatosis selectively in ovariectomized mice but not ovariectomized/adrenalectomized mice (Figure 1F). Livers were examined histologically with H&E and oil red O staining to mark neutral lipid deposition, which confirmed the presence of steatosis only in ovariectomized mice (Figures 1G and 1H). Intriguingly, we observed increased protein expression of all five mitochondrial oxidative phosphorylation complexes specifically in ovariectomized mice but not sham and OVX+ADX mice (Figure S2A), suggesting that over mitochondrial dysfunction is likely not the primary pathogenic mechanism promoting hypogonadism-induced steatosis. No overt metabolic phenotype was present in mice receiving ADX alone (Figure S1).

Because ADX removes an array of hormones, we wanted to gauge whether hepatic GR signaling was responsible for OVX-induced steatosis. To determine this, we utilized hepatocyte-specific GR knockout mice (H-GRKO) (He et al., 2015; Quinn and Cidlowski, 2016; Figure 1I). Ovariectomized $GR^{fl/fl}$ and H-GRKO mice had comparable increases in body weight between the two strains (Figure 1J), indicating that hepatocyte-specific GR signaling is not the underlying mechanism for systemic weight gain in hypogonadal female mice. However, OVX-induced steatosis was attenuated by deleting GR from hepatocytes (Figure 1K). These data indicate that OVX-induced steatosis is due to inherent GR signaling within the liver and not a secondary phenotype to obesity and altered adipose tissue lipolysis.

We found that we could also reverse OVX-induced metabolic syndrome by targeting the GR pathway 3 months after OVX (Fig- ure 1L). Adrenalectomizing ovariectomized mice led to a significant decrease in body weight and percent weight gained in response to OVX (Figures 1M and 1N). This resulted in a reduced hepatic TG burden (Figure 1O).


A
Corticosterone
Corticosterone (ng/ml)
B
Body Weight
Average weight (g)
C
Body Composition
% Body Composition
Lean
Fat
Sham
OVX
OVX+ADX
D
Liver Weight
Liver wt (g)
E
Fasting Glucose
Glucose (mg/dL)
F
Hepatic TGs
TGs (nmol/mg liver)
G
Sham
OVX
OVX+ADX
H&E
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ORO
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GRfl/fl
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Body Weight
Body Wt (g)
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Sham
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Sham
OVX
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0
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% Weight Gain
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Figure 1. GCs Drive Metabolic Dysfunction and Steatosis in Ovariectomized Mice (**A**) Serum corticosterone levels in sham-operated, ovariectomized, and OVX+ADX mice for 3 months. n = 4 per group. (**B**) Body weight in sham, ovariectomized, and OVX+ADX mice. n = 5 per group. (**C**) Body composition measured by dual-energy X-ray absorptiometry (DEXA)-scan of sham, ovariectomized, and OVX+ADX mice. n = 3 per group. (**D**) Liver weight of sham, ovariectomized, and OVX+ADX mice. n = 4 per group. (**E**) Glucose levels in sham, ovariectomized, and OVX+ADX mice fasted overnight. n = 3 per group. (**F**) TG levels measured from sham, ovariectomized, and OVX+ADX mice. n = 3 per group.(**G**) Representative H&E staining of livers from sham, ovariectomized, and OVX+ADX mice. Scale bars represent 50 mm.(**H**) Representative images of oil red O-stained livers from sham, ovariectomized, and OVX+ADX mice. Scale bars represent 50 mm. (**I**) Immunoblot for GR in $GR^{fl/fl}$ and H-GRKO mice. (**J**) Body weight of sham and ovariectomized $GR^{flox/flox}$ and H-GRKO mice. n = 3–8 per group. (**K**) Representative oil red O staining of livers from ovariectomized $GR^{flox/flox}$ and H-GRKO mice 3 months after OVX. Scale bars represent 100 mm.(**L**) Schematic of the experimental design for ADX rescue of ovariectomized mice. (**M**) Final body weight at the end of the rescue experiment. n = 6–8 mice per group. (**N**) Percent weight gain at the end of the rescue experiment. n = 6–8 mice per group. (**O**) Representative oil red O staining of livers from ovariectomized mice receiving either sham or ADX surgery 3 months after OVX. Scale bars represent 100 mm. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

These findings implicate the hepatic GR pathway as a pathogenic driver of steatosis in hypogonadal female mice, which may be of therapeutic relevance in reversing hypogonadism-induced steatosis.

GR-Governed Hepatic Lipogenic Pathways Are Reprogrammed in Hypogonadal Female Mice

We next determined the hepatic GR transcriptome in normal and hypogonadal female mice via RNA sequencing (RNA-seq). A correlation heatmap of our RNA-seq datasets revealed a low correlation score for ovariectomized mice treated with the synthetic GC dexamethasone compared with dexamethasone-treated ovary-intact mice (Figure 2A). Furthermore, principal-component analysis shows clear separation between dexamethasone-treated normal and hypogonadal mice, further confirming an altered transcriptional response to GCs in the absence of ovarian hormones (Figure S3A). To determine the transcriptional activity of GR, we plotted the fold change elicited by GC treatment in normal and hypogonadal mice. This revealed a large cohort of genes that respond differentially to GR activation in the absence of ovarian hormones (Figure 2B). Notably, approximately 600 GC-upregulated genes were hyper-induced (Figure 2B). Network enrichment analysis of the biological pathways significantly regulated by hormone treatment in ovary-intact and hypogonadal female mice revealed that GCs regulate similar physiological processes, such as cell cycle regulation, translation/transcription regulation, and axon guidance, regardless of the ovarian hormonal milieu (Figures S3B and S3C).

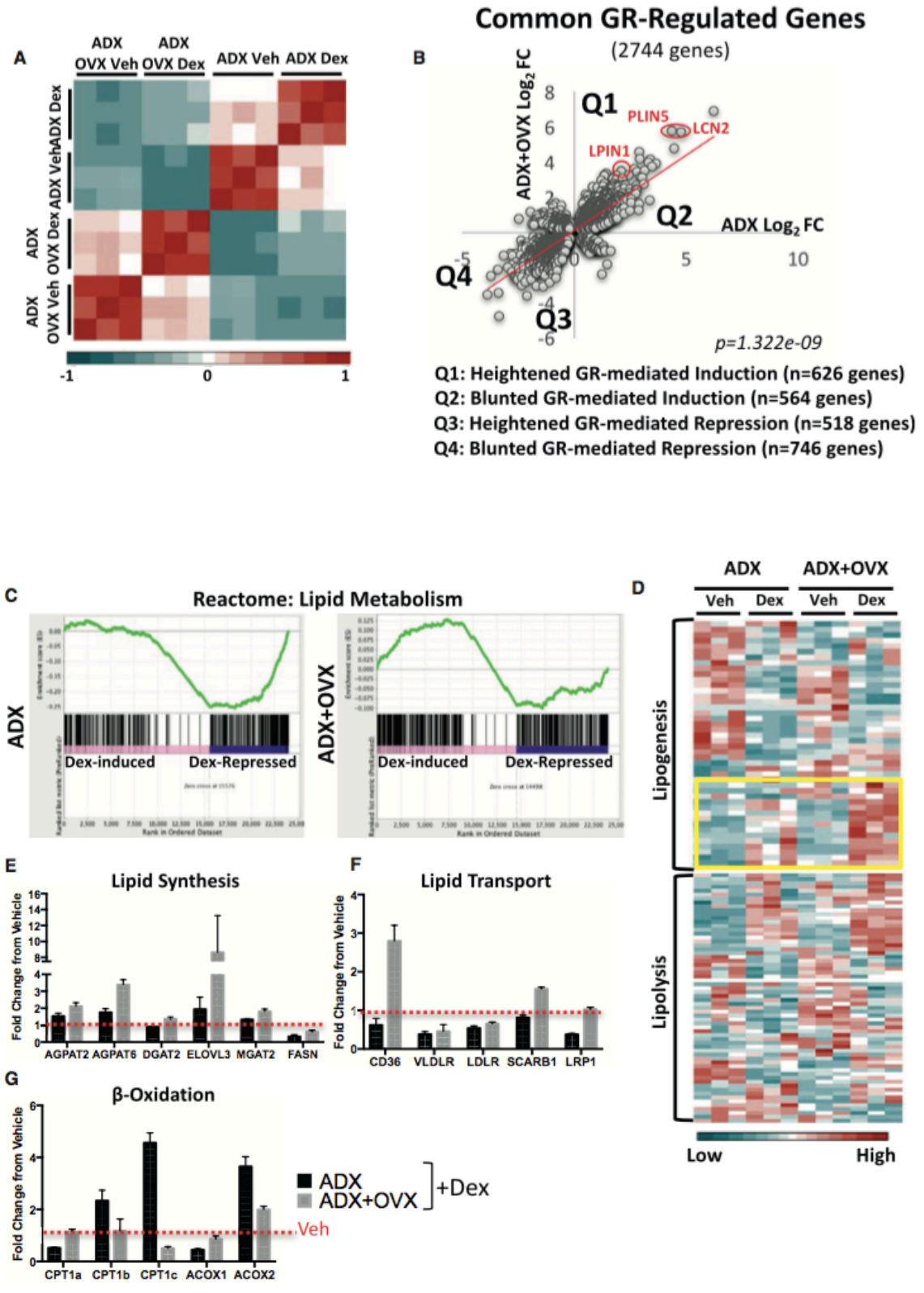
A
ADX OVX Veh
ADX OVX Dex
ADX Veh
ADX Dex
-1
0
1
B
Common GR-Regulated Genes
(2744 genes)
ADX+OVX Log2 FC
ADX Log2 FC
Q1
Q2
Q3
Q4
PLIN5
LCN2
LPIN1
p=1.322e-09
Q1: Heightened GR-mediated Induction (n=626 genes)
Q2: Blunted GR-mediated Induction (n=564 genes)
Q3: Heightened GR-mediated Repression (n=518 genes)
Q4: Blunted GR-mediated Repression (n=746 genes)
C
Reactome: Lipid Metabolism
ADX
ADX+OVX
Dex-induced
Dex-Repressed
D
ADX
ADX+OVX
Veh
Dex
Lipogenesis
Lipolysis
Low
High
E
Lipid Synthesis
Fold Change from Vehicle
AGPAT2 AGPAT6 DGAT2 ELOVL3 MGAT2 FASN
F
Lipid Transport
CD36 VLDLR LDLR SCARB1 LRP1
G
β-Oxidation
CPT1a CPT1b CPT1c ACOX1 ACOX2
ADX
ADX+OVX
+Dex
Veh

Figure 2. The GR-Governed Hepatic Lipid Metabolism Network Is Reprogrammed in Hypogonadal Female Mice (**A**) Correlation heatmap of adrenalectomized ovary-intact and ADX+OVX mice treated with vehicle or dexamethasone for 6 hr. (**B**) Scatterplot of dexamethasone-regulated genes commonly regulated between ovary-intact and ovariectomized mice. Each dot represents a gene. The x axis represents the dexamethasone (dex)-induced fold change in ADX alone, and the y axis represents the dexamthasone-induced fold change in ADX+OVX mice. (**C**) GSEA of the GC-controlled lipid metabolism reactome in adrenalectomized and ADX+OVX mice treated with dexamethasone. (**D**) Heatmap of fragments per kilobase per million (FPKM) values from adrenalectomized and ADX+OVX mice treated with vehicle or dexamethasone, showing the lipogenic (top) (GO_0008610) and lipolytic pathways (bottom) (GO_0016042). Hyper-induced GC targets are outlined in a yellow box.(**E–G**) FPKM fold change elicited by GCs in ADX and ADX+OVX mice of genes involved in lipid synthesis (**E**), lipid transport (**F**), and b-oxidation (**G**).

Despite no appreciable differences in the pathways regulated by GCs between normal and hypogonadal mice, GR-governed lipid metabolism networks are highly sensitive to dysregulation in hypogonadal female mice (Figures S3B and S3C, red circles). Gene set enrichment analysis (GSEA) indicated a negative enrichment score for ovary-intact dexamethasone-treated mice (Figure 2C). In stark contrast, dexamethasone-treated ovariectomized mice had a positive enrichment score for the lipid metabolism reactome (Figure 2C). This demonstrates that OVX alters the function of GR in regulating hepatic lipid metabolism. In-depth analysis of the lipolytic pathway (GO_0016042) indicated negligible differences in GC regulation of this pathway between normal and hypogonadal mice (Figure 2D). However, the lipogenic pathway (GO_0008610) displayed altered GC regulation, characterized by transcriptional hypersensitivity (Figure 2D, yellow box). *De novo* motif analysis of hypersensitive lipogenic genes revealed a high number of putative GC response elements (GREs) within their loci, indicating that these genes may be subject to direct regulation by GR (Table S1). To gain a better understanding of the net physiological effect GCs exert on regulating hepatic lipid metabolism, we surveyed genes involved in lipid synthesis, transport, and fatty acid β-oxidation in ovaryintact and ovariectomized mice. We found a gene expression profile consistent with the accumulation of lipids in the liver. The steatotic gene program was characterized by higher induction of genes involved in lipid synthesis and transport following GC treatment in hypogonadal mice (Figures 2E and 2F). Inversely, GC upregulation of β-oxidation genes in ovary-intact mice is severely dampened in ovariectomized mice, whereas mitochondrially encoded genes were not subject to GC regulation (Figure 2G). PPARα is the master regulator of hepatic fatty acid β-oxidation and has previously been shown to crosstalk with GR, which alters their transcriptional activity (Bougarne et al., 2009; Lee et al., 2015). Global analysis of the PPARα pathway indicates altered GR/PPARα crosstalk in ovariectomized mice (Figure S4, green and red boxes). Moreover, 3 GR target genes identified by RNA-seq as being transcriptionally hypersensitive to GC treatment in ovariectomized mice (PLIN5, LCN2, and LPIN1) are all transcriptionally upregulated in the long-term OVX model of steatosis in a GC-dependent manner (Figure S5).

OVX Enhances GR Transcriptional Activity and Recruitment to Chromatin

We used *PLIN5*, *LCN2*, and *LPIN1* as model genes to further examine the molecular mechanism underlying GC hypersensitivity in hypogonadal mice, with particular emphasis on *PLIN5* given its role in hepatic lipid storage. Real-time PCR confirmed hyper-induction of *PLIN5*, *LCN2*, and *LPIN1* following GC treatment in ovariectomized mice (Figure 3A). We tested whether GC hypersensitivity was due to enhanced GR recruitment to GRE-containing loci. A chromatin immunoprecipitation (ChIP) assay revealed enhanced occupancy of GR to the GREs within the *PLIN5*, *LCN2*, and *LPIN1* loci following GC treatment in ovariectomized compared with normal mice (Fig- ure 3B). These data suggest that increased GR recruitment to target loci underlies GC hypersensitivity in hypogonadal female mice.

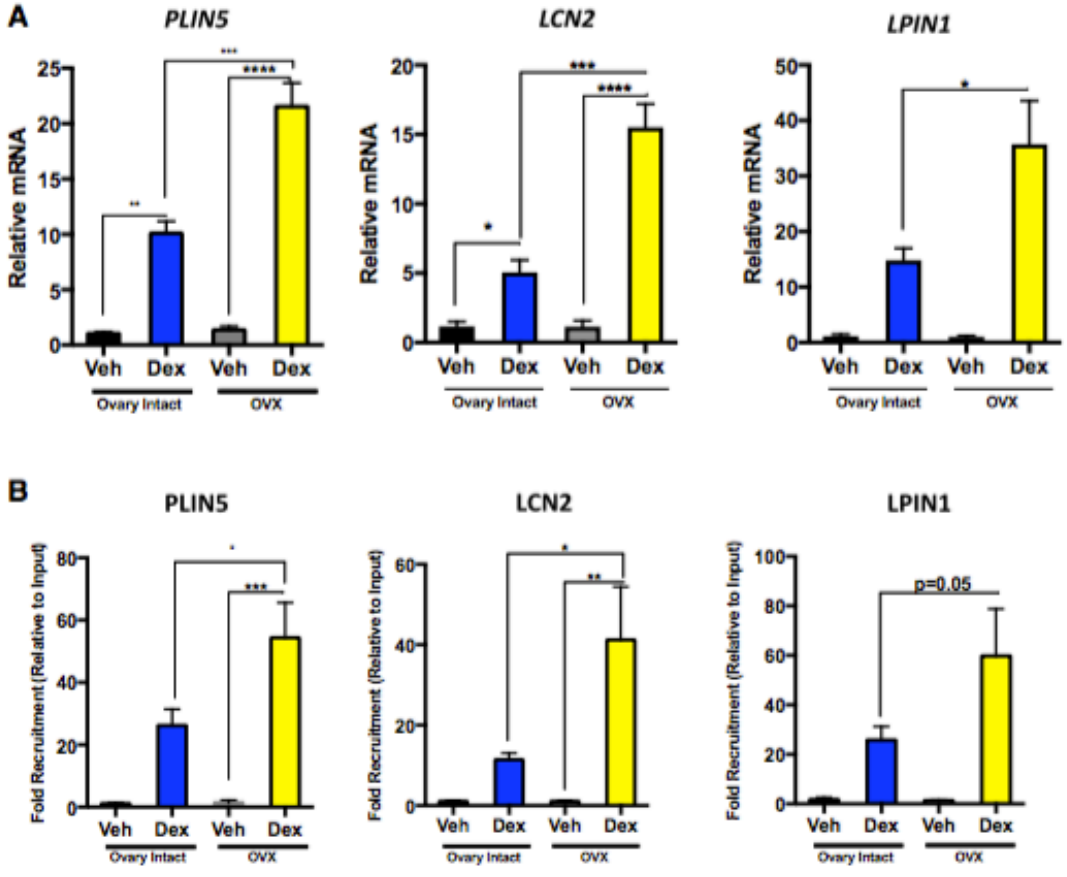

Figure 3. Ovariectomy Promotes Enhanced Chromatin Recruitment of GR (**A**) qPCR of *PLIN5*, *LCN2*, and *LPIN1* mRNA in adrenalectomized ovary-intact and ADX+OVX mice treated with either vehicle or dexamethasone for 6 hr. *PLIN5*, *LCN2*, and *LPIN1* were normalized to *PPIB* mRNA; n = 3 per group. Data are expressed as relative mRNA to *PPIB* ± SEM. (**B**) Chromatin immunoprecipitation of GR to putative GREs in the PLIN5, LCN2, and LPIN1 loci in response to hormone treatment for 1 hr in adrenalectomized ovary-intact and ADX+OVX mouse livers. n = 4 per group. Data are expressed as fold recruitment of GR over adrenalectomized ovary-intact vehicle-treated mice ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Estradiol Antagonizes Hepatic GR Signaling Independent of Hepatic ERα

Our findings indicate that removal of ovarian hormones promotes hepatic GC hypersensitivity. We hypothesized that OVX alters the chromatin architecture of target loci, allowing increased GR recruitment following dexamethasone treatment. We performed formaldehyde-assisted isolation of regulatory elements (FAIRE) to survey the chromatin architecture in ovary-intact and ovariectomized mice treated with dexamethasone to test this hypothesis. FAIRE analysis of the *PLIN5* loci revealed increased accessibility of this GRE in both ovary-intact and hypogonadal female mice following GC treatment; however, no differences were seen between normal and ovariectomized mice (Figure S5A). This result indicates that enhanced GR recruitment to chromatin in hypogonadal female mice is not due to increased accessibility to the DNA. We also did not observe any differences in the epigenetic marks H3K27me3 or H3K27ac between groups (Figure S5B).

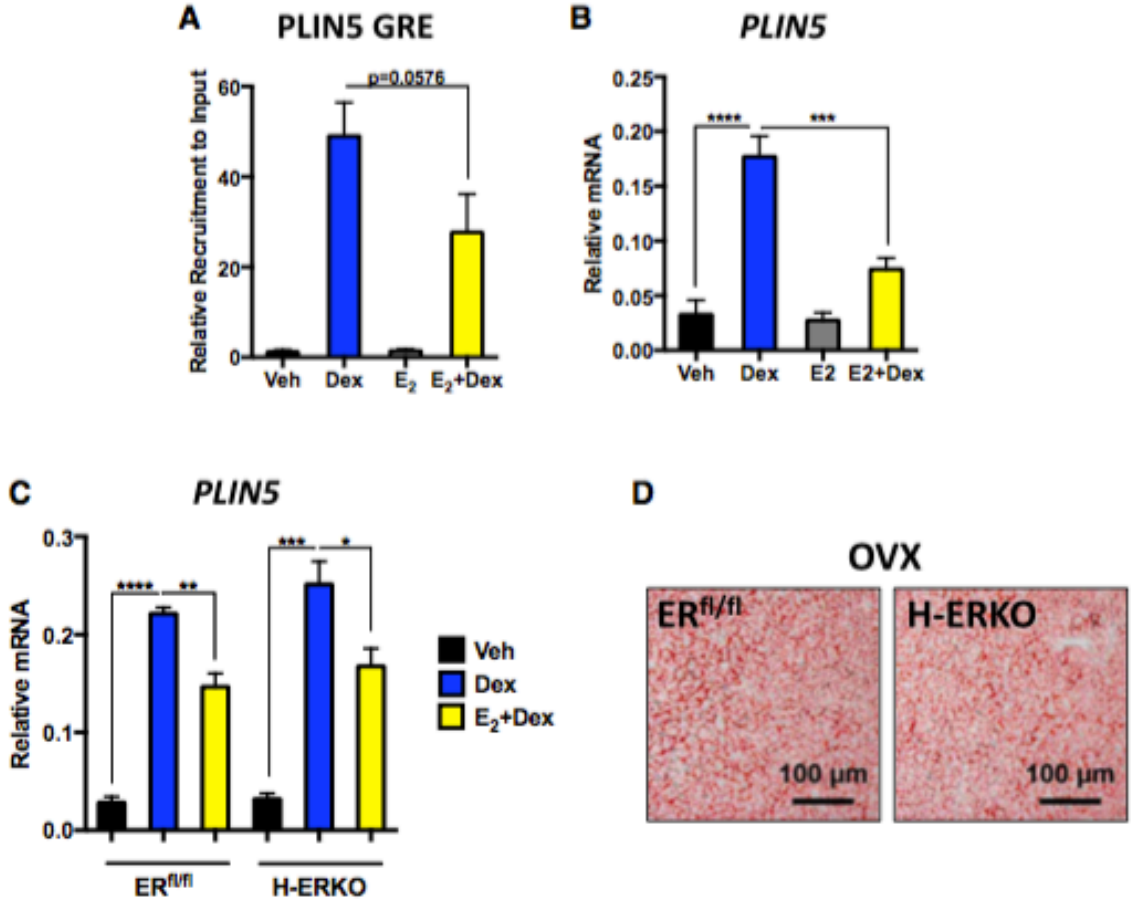

Figure 4. Systemic but Not Hepatic Estro- gen Signaling Deficiency Promotes GC Hypersensitivity (**A**) Chromatin immunoprecipitation of GR to the GRE in the PLIN5 loci in hypogonadal female mice treated with dexamethasone for 1 hr, primed with and without estradiol for 72 hr. n = 3–4 animals per group. Data are expressed as fold recruitment of GR over vehicle-treated ovariectomized mice ± SEM. (**B**) *PLIN5* mRNA in ovariectomized mice with and without estradiol priming for 72 hr following 6 hr dexamethasone treatment. Data are expressed as relative *PLIN5* mRNA normalized to PPIB mRNA ± SEM. n = 4 animals per group. (**C**) *PLIN5* mRNA in vehicle- and dexamethasone- treated ER-floxed and H-ERKO mice with and without estradiol priming. Data are expressed as relative *PLIN5* mRNA normalized to *PPIB* mRNA ± SEM. n = 3 independent animals per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (**D**) Representative oil red O staining of livers from ovariectomized $ER^{flox/flox}$ and H-ERKO mice 2 months after OVX. Scale bars represent 100 mm. n = 3–4 mice per group.

Because OVX does not increase chromatin accessibility at the *PLIN5* loci or alter the H3K landscape, we postulated that ER competes with GR for DNA binding, limiting the recruitment of GR to chromatin in ovary-intact mice. To test this, we primed hypogonadal female mice with either vehicle or estradiol for 3 days to activate ER before treatment with dexamethasone. Estradiol re-administration blunted GR recruitment to the PLIN5 loci compared with vehicle-primed hypogonadal mice (Figure 4A). We next assayed *PLIN5* mRNA to see whether the reduced chromatin recruitment resulted in diminished tran- scriptional output to dexamethasone. Indeed, estradiol priming significantly decreased GC induction of *PLIN5* (Figure 4B). These data indicate that activation of ER could potentially be a mechanism to limit GR recruitment to chromatin and transcriptional activity. We focused our attention on ERα given the lack of a metabolic phenotype in the global ERβ KO (Bryz- galova et al., 2006). Moreover, ERβ expression was undetectable in livers of mice in our RNA-seq dataset. To test whether ERα competes with GR for chromatin binding, we generated hepatocyte-specific ERα KO mice (H-ERKO) by crossing $ER^{fl/fl}$ mice (Hewitt et al., 2010) with Alb-cre mice. Repeating our estradiol replacement experiment in $ER^{fl/fl}$ and H-ERKO mice revealed that estradiol still retained its ability to antagonize hepatic GR signaling even in the absence of hepatic ERα (Figure 4C). Furthermore, we hypothesized that if ERα is playing a protective role against GC hypersensitivity, then a greater steatotic pathology would be observed in ovariectomized H-ERKO mice. Oil red O staining of liver sections from ovariec- tomized $ER^{fl/fl}$ and H-ERKO mice revealed a comparable hepatic TG burden between the genotypes (Figure 4D). These data are consistent with lack of a basal phenotype previously reported in H-ERKO mice (Hart-Unger et al., 2017). Our data demonstrate that hepatic ERα is dispensable for mediating the favorable metabolic effects of estradiol and indicate that the antagonistic effects of estradiol on hepatic GR signaling are mediated via an extrahepatic ERα mechanism.

Estradiol Deficiency Promotes Hyperphosphorylation of Serine 211 in GR

Collectively, our data indicate that estrogen deficiency results in GR transcriptional hypersensitivity associated with increased GR recruitment to chromatin. Because the chromatin architecture at the *PLIN5* loci did not appear to be altered following OVX, and

ERα does not limit GR binding by competing with GR for chromatin binding, we speculated that estradiol deficiency promotes these effects via an alternative mechanism, perhaps in a multi-organ fashion.

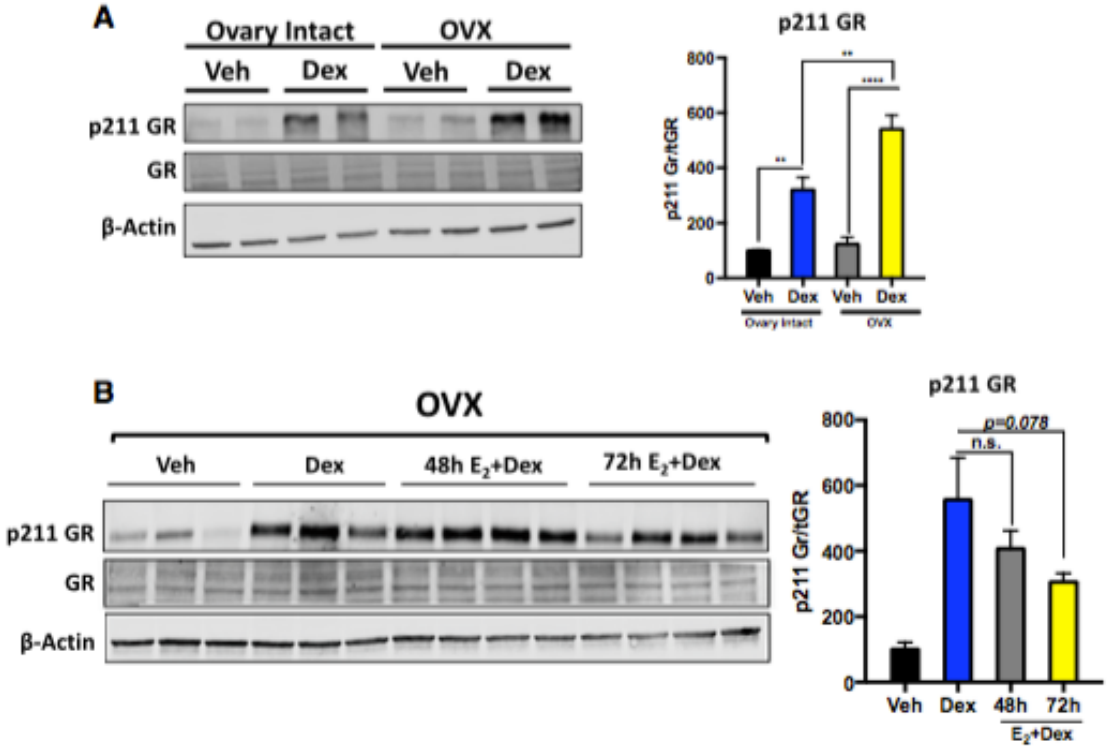

Figure 5. Systemic Estrogen Deficiency Promotes Ligand-Dependent Phosphoryla- tion of GR at Serine211 in the Liver (**A**) Western blot analysis of phospho-serine 211 of GR in vehicle- and dexamethasone-treated (1 hr) adrenalectomized ovary-intact and ADX+OVX mice. Data are expressed as percent of vehicle- treated adrenalectomized ovary-intact mice of p211 normalized to total GR ± SEM. n = 4 indi- vidual animals per group. (**B**) Immunoblot for phospho-serine 211 of GR in vehicle- and dexamethasone-treated (1 hr) ovari- ectomized mice with and without estradiol priming for 48/72 hr. Data are expressed as percent of vehicle-treated adrenalectomized ovary-intact mice of p211 normalized to total GR ± SEM. n = 5–6 individual animals per group. $^{**}p < 0.01$, $^{****}p < 0.0001$.

One primary mechanism regulating the transcriptional activity of GR is through post-translational modifications such as phosphorylation (Galliher-Beckley and Cidlowski, 2009). Most notably, phosphorylation of GR at serine 211 in response to ligand has been shown to increase transcriptional output following hormone treatment (Chen et al., 2008). Furthermore, phosphorylation of GR on serine 211 has also been shown to promote recruitment to GRE-containing loci in response to GC treatment (Blind and Garabedian, 2008). We therefore

hypothesized that GC hypersensitivity in hypogona- dal female mice is a product of enhanced ligand-dependent phosphorylation of GR at serine 211. Indeed, serine 211 of GR was hyperphosphorylated following dexamethasone treatment in the livers of hypogonadal mice compared with ovary-intact mice (Figure 5A). We next wanted to determine whether phosphorylation of serine 211 was also subject to estradiol antagonism. Estradiol readministration to hypogonadal female mice led to a trend in decreased ligand-dependent phosphorylation of serine 211 of GR ($p = 0.07$) (Figure 5B). These data indicate that hyperphosphorylation of serine 211 of GR could be the potential mechanism underlying the increased recruitment of GR to chromatin and subsequent transcriptional hypersensitivity elicited by GCs in estrogen-deficient mice.

FSH Enhances GC-Mediated Signaling

Our results suggest that the antagonistic effects of estradiol on hepatic GR signaling occur via an extra-hepatic mechanism. One of the primary endocrine manifestations of losing ovarian function is aberrant production of FSH and luteinizing hormone (LH) in both humans and rodents (Parlow, 1964; Wise and Rat- ner, 1980; Yen and Tsai, 1971). We therefore speculated that enhanced production of these pituitary peptides in hypogonadal mice may be the underlying mechanism driving ligand-induced hyperphosphorylation of GR. We observed detectable levels of FSH receptor protein, but not LH receptor, in the livers of female mice (Figure 6A); therefore, we focused our efforts on determining whether FSH could promote GC hypersensitivity. Consistent with previous reports, we observed a significant increase in circulating FSH levels following OVX (Figure 6B). Moreover, circulating FSH levels decreased only at the time point when we observed the antagonistic effects of estradiol on GR phosphorylation (72 hr) (Figure 6B). To determine whether FSH has the capacity to enhance GR-mediated signaling *in vivo*, we pre-treated ovary-intact mice with a bolus of FSH 5 min prior to administering dexamethasone and measured the phosphorylation levels of serine 211 of GR. This revealed that FSH does indeed have the ability to boost the ligand-dependent phosphorylation of GR both *in vivo* (Figure 6C) as well as *in vitro* (Figure S6). We did not observe FSH-elicited phosphorylation of serine 211 of GR in the absence of dexamethasone (Figure S6). This likely points to a conformational change in GR elicited by ligand binding, exposing serine 211 to FSH-mediated enhanced phosphorylation. Importantly, enhanced phosphorylation of serine 211 of GR elicited by FSH pre-treatment *in vivo* resulted in a subsequent increase in GR-mediated induction of PLIN5, LCN2, and LPIN1 mRNA (Figure 6D). Our data reveal a regulatory loop between estradiol regulation of FSH production to maintain proper hepatic GR signaling.

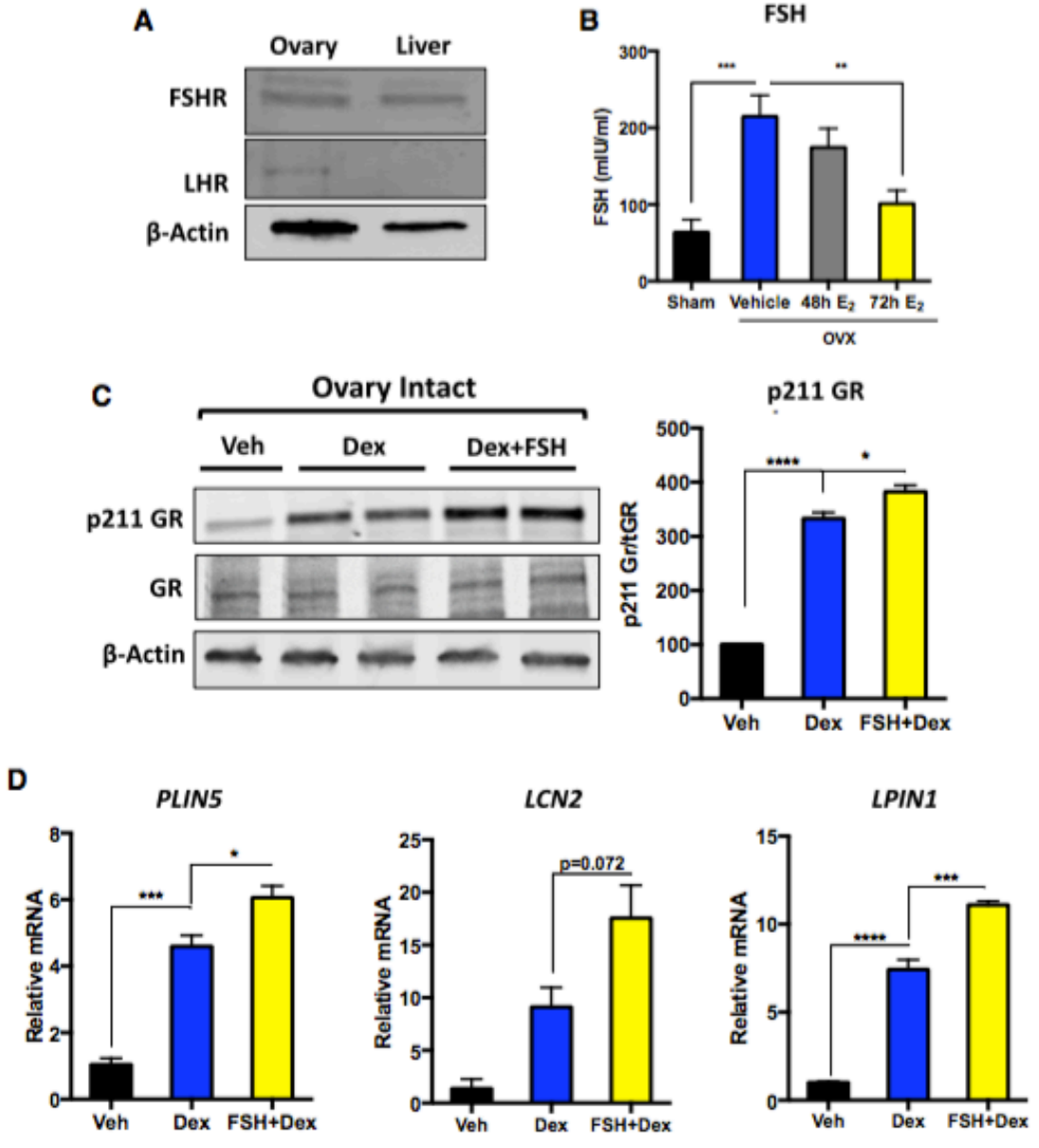

Figure 6. FSH Enhances GR Phosphoryla- tion and Transcriptional Induction of Target Genes (**A**) Immunoblot of FSHR and LHR in female mouse livers. b-Actin was used as a loading control, and mouse ovary was used as a positive control. (**B**) Circulating FSH levels in sham-operated mice and ovariectomized mice treated with either vehicle or estradiol for 48 and 72 hr. Data are ex- pressed as milli-international units per milliliter of FSH ± SEM. n = 5–9 individual animals per group. (**C**) Immunoblot for p211 GR and total GR in livers of vehicle-, dexamethasone-, and FSH+dex- amethasone-treated adrenalectomized ovary- intact mice. FSH was administered 5 min prior to dexamethasone injection, and livers were har- vested 1 hr after dexamethasone injection. Data are expressed as percent of vehicle-treated adrenalectomized ovary-intact mice of p211 normalized to total GR ± SEM. n = 3–4 animals per group. (**D**) *PLIN5*, *LCN2*, and *LPIN1* mRNA in vehicle-, dexamethasone-, and FSH+dexamethasone- treated adrenalectomized ovary-intact mice. FSH was administered 5 min prior to dexamethasone injection, and livers were harvested 1 hr after dexamethasone injection. Data are expressed as relative mRNA to *PPIB* mRNA ± SEM. n = 3–8 in- dividual mice per group. *p < 0.05, **p < 0.01, ***p < 0.001.

Discussion

It is well established that aging promotes metabolic disturbances that manifest most evidently in females during the menopausal period. The molecular drivers of menopause-induced metabolic dysfunction are largely unknown; however, estrogen depletion is thought to be the underlying pathological mechanism. Here we report that GCs are the primary pathogenic driver of metabolic dysfunction and steatosis in estrogen-depleted mice. Targeting the GR pathway via ADX or genetic deletion of GR from the liver protects against OVX-induced steatosis. Furthermore, altered global transcriptional responses following GR activation was observed in estrogen-depleted mice. Transcriptional hypersensitivity of GC-governed lipogenic genes is a pathogenic mechanism underlying OVX-induced steatosis in mice. GC hypersensitivity in hypogonadal females is associated with enhanced GR:DNA binding and heightened ligand-dependent phosphorylation of GR on serine 211. The heightened ligand-dependent phosphorylation of GR in hypogonadal females is at least in part due to aberrant production of FSH and subsequent signaling in hepatocytes. Lowering FSH levels *in vivo* by estradiol re-administration reversed the heightened GR responsiveness in ovariectomized mice and diminished the transcriptional response elicited by GCs.

Here we show that hypogonadism dramatically reprograms the hepatic GR transcriptome in female mice. Analyzing the physiological pathways regulated by GCs in normal and hypogonadal mice revealed that the hepatic lipid metabolism network was subject to the most profound changes in transcriptional regulation. Interestingly, genes within the lipogenic pathway exhibited heightened dexamethasone-mediated induction, suggesting that hypersensitivity to GCs on this pathway is a pathophysiological mechanism driving steatosis in hypogonadal female mice.

Increasing evidence indicates that ER and GR crosstalk to alter the transcriptome of one another (Whirledge and Cidlowski, 2013; Whirledge et al., 2013; Gong et al., 2008; West et al., 2016). In the uterus, estradiol can antagonize the transcriptional effects of GR activation, a mechanism requiring ERα (Whirledge and Ci- dlowski, 2013; Whirledge et al., 2013). These studies provide support for the notion that GC hypersensitivity in hypogonadal females is due to loss of estradiol/ERα antagonism of the GR pathway. We tested this hypothesis through a multi-tiered approach, including estradiol co-administration with dexamethasone and H-ERKO mice. Our findings revealed that loss o hepatic estrogen signaling was not the underlying cause promoting enhanced GC sensitivity in ovariectomized mice, indicating that estradiol depletion alters the hepatic GR transcriptome through extrahepatic actions. Our results support previous findings in which the protective metabolic effects of es- trogens are mediated via ERa in non-hepatic tissues (Hart-Unger et al., 2017; Matic et al., 2013). Estrogen is a potent repressor of FSH synthesis in the pituitary to limit its own synthesis in a negative feedback loop. Following estrogen depletion, FSH is increased systemically. We discovered that long-term estradiol readministration could lower FSH levels and, consequently, antagonize the transcriptional activity of GR. Furthermore, administration of

FSH to ovary-intact mice could promote GC hypersensitivity, indicating that this pituitary peptide has the capacity to potentiate hepatic GR signaling.

FSH is now being appreciated for its extragonadal actions, most notably its effects in metabolic tissues. In bone, for example, FSH, acting through the FSH-R, mediates hypogonad- ism-induced bone loss (Sun et al., 2006). Recently, the metabolic effects of FSH have been extended beyond bone to adipose tis- sue, where administration of this peptide to young chickens leads to increased abdominal fat mass by increasing lipid biosynthesis (Cui et al., 2012). In the liver, FSH has been shown to have multiple functions. For example, cholangiocytes, through a paracrine loop, utilize FSH-R signaling to regulate proliferation (Mancinelli et al., 2009). In their immunohistochemical staining, Mancinelli et al. (2009) showed FSH-R-positive staining not only in cholangiocytes but also in hepatocytes, suggesting that FSH may also elicit biological effects in the liver outside of the biliary tract. A recent report confirmed the expression of FSH-R in hepatocytes and showed that this signaling cascade results in the downregulation of LDL-R in the liver, resulting in increased circulating total cholesterol (Song et al., 2016). OVX-induced hypercholesterinemia could be abated by the use of a GNRH receptor agonist to systemically lower FSH (Song et al., 2016). Furthermore, FSH-R-haploinsufficient female mice, also known as FORKO mice, have diminished estradiol production and mimic human menopause by developing a dysregulated menstrual cycle and reproductive senescence (Danilo- vich et al., 2000). These mice develop metabolic dysfunction and obesity, although, not as severely as the global ERa KO mouse (Heine et al., 2000), which, in conjunction with ablated estrogen signaling, has increased FSH production (Emmen and Korach, 2003). The FORKO mouse suggests that a mere lack of estrogen is not sufficient to recapitulate the full metabolic phenotype observed in the global ERa KO mouse, and perhaps elevated FSH levels are required for the pathogenesis of metabolic syndrome in hypogonadal females. This can somewhat explain the lack of a basal phenotype observed in our H-ERKO mice and why the transcriptional response to GC treatment is similar to that of $ER^{fl/fl}$, where FSH levels are presumed to be normal. Our data support a model in which hepatic ERa signaling is dispensable for proper GC regulation of gene expression, but, rather, hepatic FSH signaling can alter the course of the GC response. This has revealed a potential mechanism by which elevated FSH levels may dysregulate hepatic lipid metabolism through amplification of the transcriptional effects of GCs. These findings also support a role for FSH signaling beyond the reproductive tracts as a regulator of metabolism. Our discovery is highlighted by the recent finding that targeting FSH through an FSH-neutralizing antibody could correct metabolic dysfunction in ovariectomized mice and promote beiging of adipose tissue (Liu et al., 2017). Our current findings indicate that dysregulation of the GR pathway may be the underlying molecular mechanism promoting metabolic dysfunction in response to aberrant FSH production. In a physiological context, we hypothesize that this crosstalk between FSH and GCs in regulating hepatic lipid meta- bolism is to initiate a unique maternal metabolic program to sup- port the implanting embryo and subsequent pregnancy and is an active area of ongoing research.

Serine 211 is a promiscuous residue on GR and can be phosphorylated by an array of different protein kinases. Furthermore, FSH has been shown to activate a number of similar protein kinases known to phosphorylate GR at serine 211. For example, FSH-R has been shown to activate both p38 mitogen-activated protein kinase (MAPK) and SGK1, which also has the ability phosphorylate serine 211 of GR. Other potential mechanisms, including FSH-mediated inhibition of protein phosphatases, could also contribute to the hyperphosphorylation of GR mediated by FSH. The mechanism linking the hepatic effects of FSH to GR signaling is of serious clinical significance and is a subject of our future studies. In the clinical context, our data illuminate the GR pathway as a potential alternative therapeutic target for the treatment of post- menopausal steatosis as an alternative to traditional therapies such as HRT and selective estrogen receptor modulators. Furthermore, selective blockage of GCs could prevent weight gain and alterations in body composition, suggesting a patho- genic role for GCs in adipose tissue in hypogonadal females. We did not evaluate the role of GR in adipose tissue in the present study; however, there are many reports implicating stress hor- mones in the development of obesity (reviewed in Baudrand and Vaidya, 2015; Lee et al., 2014). These findings suggest that anti- GC therapy may have beneficial effects during menopause-induced metabolic syndrome beyond the liver. The second clinical implication our study highlights is that GR activation differs in females depending on the ovarian hormone milieu/HPG axis function. Given the GC hypersensitivity to genes involved in lipo- genesis, we speculate that post-menopausal women on cortico-steroid therapy may potentially be more sensitive to developing the metabolic side effects associated with chronic steroid use more rapidly than a woman in her reproductive years per se. In fact, development of adverse side effects during long-term corticosteroid therapy occurs in approximately 80% of autoimmune hepatitis patients after 2 years of therapy (Czaja, 2008; Summerskill et al., 1975; Uribe et al., 1984), which is the most common reason for premature drug withdrawal (Czaja, 2008; Czaja et al., 1984; Manns et al., 2010). Understanding if and how certain populations, such as postmenopausal women, may be more sensitive to developing adverse side effects while on corticosteroid therapy could prove beneficial in developing strategies to combat these side effects, such as initiating combination therapy.

In conclusion, we report that GCs drive metabolic abnormalities, specifically steatosis, in hypogonadal female mice. More- over, we have uncovered a regulatory loop of extrahepatic estradiol inhibition of hepatic GR signaling involving estradiol antagonism of FSH production, which, in turn, limits the tran- scriptional activity of GCs by reducing GR:DNA binding via decreased ligand-dependent phosphorylation of GR. These findings challenge the current dogma that the metabolic syndrome accompanied by hypogonadism in females is merely due to a loss of ovarian hormones. We suggest that the GR pathway is responsible for the metabolic phenotype in hypogonadal females, and it may prove to be a more clinically efficacious target than classical HRT for the treatment of metabolic complications during menopause in humans.

Experimental procedures

Materials

FSH was purchased from Sigma (St. Louis, MO). Dexamethasone and 17b-estradiol were purchased from Steraloids (Newport, RI).

Animal Experiments

Female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H-GRKO mice were generated by crossing $GR^{flox/flox}$ mice (Oakley et al., 2013) with mice expressing CRE-recombinase under the liver-specific promoter albumin (Quinn and Cidlowski, 2016). H-ERKO mice were generated in a similar fashion as the H-GRKO mice by crossing $ER^{flox/flox}$ mice (a kind gift from Dr. Kenneth Korach, National Institute of Environmental Health Sciences (NIEHS)/NIH) to albumin-CRE mice. Female homozygous floxed CRE mice were used as controls, and homozygous floxed CRE+ animals were used as experimental groups in all experiments. All animals were subject to a 12:12 hr light/dark cycle and had *ad libitum* access to standard mouse chow and drinking water. Bilateral OVX was performed as a surgical model of menopause in both wild-type and H-GRKO mice. To reduce circulating GCs, bilateral ADX was performed in wild-type mice. Adrenalectomized mice were maintained on 0.9% saline to maintain salt levels. Surgically altered mice were used between the ages of 5 and 7 months. To determine GC-regu- lated genes, dexamethasone (1 mg/kg) was injected, and livers were harvested 6 hr after injection. Dexamethasone-treated mice were used at 3 months of age. For ChIP and FAIRE assays, mice were treated with dexamethasone, and livers were harvested 1 hr after injection. Estradiol was injected at 100 mg/kg daily up to 3 days. FSH was injected at 60 mg/kg 5 min prior to dexamethasone injection. All experiments utilizing non-adrenalectomized mice were performed at zeitgeber time 3 (ZT3) during the nadir of circadian GC release. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee at the NIEHS, NIH.

Corticosterone and FSH Measurement

Circulating corticosterone and FSH were measured via colorimetric ELISAs (corticosterone at ZT3, Arbor Assays, Ann Arbor, MI; FSH, Enzo Life Sciences, Farmingdale, NY).

Body Composition

Body composition was determined in sham, ovariectomized, and OVX+ADX mice via dual X-ray absorptiometry (DEXA) (Lunar Piximus 2, Fitchburg, WI) according to the manufacturer's protocol.

Glucose Measurements

Glucose levels were measured in mice fasted overnight using the TrueTrack glucometer (CVS Pharmacy, Durham, NC).

Hepatic TG Measurement

TGs were determined with a TG colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). Oil red O staining (Sigma) was used to stain lipids in frozen liver sections as described previously (Mehlem et al., 2013).

qPCR

One hundred nanograms of total RNA was reverse-transcribed and amplified using the iScript One-Step RT-PCR kit for probes (Bio-Rad, Hercules, CA). Real-time qPCR was performed with the Bio-Rad CFX96 sequence detection system using predesigned primer/probe sets against PLIN5, LCN2, and LPIN1. ERa and PPIB were from Applied Biosystems (Foster City, CA). The relative fluorescence signal was normalized to PPIB using the DCT method.

Chromatin Immunoprecipitation Assay

Approximately 100 mg of liver from vehicle- and dexamethasone-treated mice was cross-linked, and isolated nuclei were subjected to sonication (15 cycles on high, 30 s on, 30 s off; Diagenode Bioruptor, Denville, NJ). Sonicated DNA was immunoprecipitated with rabbit anti-GR monoclonal antibody (Cell Signaling Technology, Danvers, MA), followed by isolation using the Magna- ChIP kit (Millipore, Billerica, MA). Isolated DNA was purified via the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and eluted in 50 mL of elution buffer. The primers used are listed in Table 1.

Table 1. ChIP Primers

Loci	Forward	Reverse
PLIN5	5′- CCCACTGCAAGCT CTGT-3′	5′-CAGCTGCGAGAG GACATT-3′
LCN2	5′-TAGACAGGGAAGA AGAGGACA-3′	5′-GGCTCAAGGTATT GGACACTT-3′
LPIN1	5′-GTTTGTGACGAAAG CTGAGAAA-3′	5′-ACATGCTGCTCCA ACACT-3′

Western Blotting and Immunohistochemistry

Protein lysates were prepared from livers of mice by homogenization in SDS sample buffer (Bio-Rad, Hercules, CA) containing b-mercaptoethanol (Sigma). Approximately 30 mg of total protein was resolved on a 4%–20% Tris-glycine gel (Bio-Rad) and transferred onto a 0.2 mM nitrocellulose membrane (Bio- Rad). Membranes were blocked with blocking buffer (LI-COR Biosciences, Lincoln, NE) and incubated overnight with either anti-GR 59 (Oakley et al., 2017), anti-phospho-serine 211 GR (Cell Signaling Technology), anti-FSHR (Abcam), anti-hCGR (LHR, Abcam), or anti-b-actin (Millipore). Total GR, phos- phorylated GR, FSHR, and LHR were used at 1:1,000, and b-actin was used at 1:10,000. Protein was detected via fluorescent secondary antibody detection (1:10,000) (LI-COR Biosciences) and imaged on the LI-COR Odyssey (LI-COR Biosciences). Densitometry was performed with LI-COR Odyssey software, and b-actin was used to normalize loading.

RNA Sequencing

RNA was extracted from livers of mice with Qiazol, and purification of total RNA was performed with the QIAGEN RNeasy RNA isolation kit (Redwood City, CA) according to the manufacturer's protocol. RNA-seq libraries were generated with 1 mg of RNA as input using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) and poly(A)-enriched according to the TruSeq proto- col. Indexed samples were sequenced using the 100-bp paired-end protocol via the NextSeq500 (Illumina) according to the manufacturer's protocol. Reads (38–55 million reads per sample) were aligned to the University of California Santa Cruz (UCSC) mm9 reference genome using TopHat2. The quantification results from htseq-count were then analyzed with the Bioconductor package DESeq2, which fits a negative binomial distribution to estimate technical and biological variability. Comparisons were made between vehicle- and dexamethasone-treated adrenalectomized ovary-intact and ADX+OVX mice. A gene was considered differentially expressed when the p value for differential expression was less than 0.01. The correlation heatmap was generated using the R software package (version 3.3.3) with the ''ggplot2'' package. GSEA was performed using GSEA v2.2.2 software. Genes were pre-ranked based on the fold change of gene expression. This application scores a sorted list of genes with respect to their enrichment of selected functional categories (Kyoto Encyclopedia of Genes and Genomes [KEGG], Biocarta, Reactome, and gene ontology [GO]). The significance of the enrichment score was assessed using 300 permutations. Benjamini and Hochberg's false discovery rate (FDR) was calculated for multiple testing adjustments. $q < 0.05$ was considered significant. The resulting enriched pathways were visualized using the Cytoscape (v3.3.0) Enrichment map plugin.

Statistical Analysis

Statistical significance was detected between groups by GraphPad Prism 7 (La Jolla, CA) using a Student's t test when comparing two groups or two-way ANOVA when comparing three or more groups, followed by a Sidak *post hoc* test to correct for multiple comparisons. A normality test was performed on the t test to determine whether data were normally distributed, and when failing the normality test, a Mann-Whitney *post hoc* test was performed. Statistical signif- icance was defined as $p < 0.05$.

DATA AND SOFTWARE AVAILABILITY

The accession number for the data reported in this paper is GEO: GSE99309.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.041.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.A.Q. and J.A.C.; Methodology, M.A.Q. and M.R.; Soft- ware, Formal Analysis, M.A.Q. and X.X.; Investigation, M.A.Q. and X.X.; Data Curation, X.X.; Writing – Original Draft, M.A.Q., X.X., and J.A.C.; Visualization, M.A.Q. and X.X.; Supervision, J.A.C.; Funding Acquisition, J.A.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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