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Chen CD, Sawyers CL. (2002) NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. Mol Cell Biol 22:2862-2870. DOI: 10.1128/mcb.22.8.2862-2870.2002

Bourman et al., 2005 (not 2004)

Immunotoxicology

Effect of estrogen-active compounds on the expression of RACK1 and immunological implications

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Abstract

We previously demonstrated the existence of a balance among steroid hormones, i.e. glucocorticoids and androgens, in RACK1 (receptor for activated C kinase 1) expression and innate immunity activation, which may offer the opportunity to use RACK1 expression as marker to evaluate immunotoxicity of hormone-active substances. Because of the existence of close interconnections between the different steroid hormone receptors with overlapping ligand specificities and signaling pathways, in this study, we wanted to investigate a possible effect of estrogenic active compounds, namely 17β-estradiol, diethylstilbestrol, and zearalenone, on RACK-1 expression and innate immune responses using THP-1 cells as experimental model. All compounds increased RACK1 transcriptional activity as evaluated by reporter luciferase activity, mRNA expression as assessed by real time-PCR and protein expression by western blot analysis, which paralleled an increase in LPS-induced IL-8, TNF-α production, and CD86 expression, which we previously demonstrated to be dependent on RACK1/PKCβ activation. As the induction of RACK1 expression can be blocked by the antagonist G15, induced by the agonist G1 and by the non-cell permeable 17β-estradiol conjugated with BSA, a role of GPER (previously named GPR30) activation in estrogen-induced RACK1 expression could be demonstrated. In addition, a role of androgen receptor (AR) in RACK1 transcription was also demonstrated by the ability of flutamide, a nonsteroidal antiandrogen, to completely prevent diethylstilbestrol-induced RACK1 transcriptional activity and protein expression. Altogether, our data suggest that RACK1 may represent an interesting target of steroid-active compounds, and its evaluation may offer the opportunity to screen the immunotoxic potential of hormone-active substances.

AQ1

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Article Highlights

1. RACK1 expression is induced by estrogenic active compounds.
2. Increased RACK1 levels correlate with increased response to LPS.
3. RACK1 evaluation offers the opportunity to screen the immunotoxic potential of steroid hormone-active substances.

Keywords

Endocrine-disrupting chemicals
Estrogens
Immune system
Hormones
Cytokines
Signal transduction

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00204-020-02756-9) contains supplementary material, which is available to authorized users.

Introduction

After the second world war, industrialized countries have faced a significant increase of diseases, such as cancer, allergy, autoimmunity, and neurological disorders, that can be all linked to neuro–immune–endocrine network alterations. Environmental factors, including hormonally active substances, are believed to be a major factor responsible for such increased prevalence (Nahta et al. 2015; Kravchenko et al. 2015). Growing evidence clearly indicates that endocrine-disrupting compounds (EDCs) can interfere with the immune system in human and wildlife (Kuo et al. 2012). This is a relatively new area of research, because most of the studies on hormonally active substances primarily focused on reproductive and developmental toxicity.

EDCs are perceived as a serious public health issue due to their potency, constant, and universal human exposure. Known endocrine disruptors derive mainly from industrial and agricultural sources and include

synthetic chemicals and their by-products (e.g. polychlorinated biphenyls, polybrominated biphenyls, and dioxins), plasticizers (e.g. phthalates and bisphenol A), pesticides (e.g. methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane or DDT), fungicides (e.g. vinclozolin), and pharmaceutical agents (e.g. diethylstilbestrol). Next to the anthropogenic known endocrine disruptors, we must remember that we are also exposed through diet to hormonally active substances of natural origin, including phytoestrogens, such as genistein, daidzein and coumestrol, and mycotoxins such as zearalenone produced by numerous species of *Fusarium* (UNEP and WHO 2013; Nesic et al. 2014).

Endogenous steroid hormones are important for keeping a competent and healthy immune system, where they can modulate the functions of immune cells, influencing the initiation of the immune response and the maintenance of peripheral tolerance to self-antigens (Rubinow 2018). Steroid hormones, including estrogens, androgens, progestogens, and glucocorticoids may profoundly affect all leukocytes, e.g. influencing dendritic cells differentiation, maturation, and function leading to either a pro-inflammatory or an anti-inflammatory (or tolerogenic) phenotype (Bouman et al. 2005; Nadkarni and McArthur 2013; Laffont et al. 2017; Cain and Cidlowski 2017; Cari et al. 2019; Strehl et al. 2019). If not properly regulated, these processes can lead to immunotoxicity (Corsini et al. 2018).

The action of estrogens is mediated by interaction with the classical estrogen receptors (ER), ERα and ERβ, and G-protein-coupled receptor 30/G protein estrogen receptor (GPR30/GPER-1) (Takada et al. 1997; Revankar et al. 2005; Barton et al. 2018). Estrogens act via these different estrogen receptors affecting both gene transcription and rapid signaling pathways in a complex interplay. To a different extent, all immune cells, including neutrophils, monocytes, dendritic cells, NK cells, as well as B and T cells express ERs and GPER (Bouman et al. 2005; Pierodominici et al. 2010; Nadkarni and McArthur 2013).

The identification of the regulatory elements in the promoter region of the human *rack1* gene [also known as guanine nucleotide binding protein, beta polypeptide 2-like 1 (GNB2L1) gene], encoding RACK1 (receptor for activated C kinase 1), sheds some light on the transcriptional modulation of RACK1 (Del Vecchio et al. 2009). We demonstrated the existence of a

complex hormonal balance among cortisol, 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, representing a link between the endocrine and the immune system (reviewed by Racchi et al 2017). We showed in immune cells that RACK1 expression is negatively regulated by glucocorticoids (Del Vecchio et al. 2009; Buoso et al. 2011; Corsini et al. 2014a, b), whereas dehydroepiandrosterone (DHEA) via androgen receptor (AR) (Corsini et al. 2016; Buoso et al. 2017a, b, c), endogenous androgens (testosterone, dihydrotestosterone) or anabolic compounds increase RACK1 expression, resulting in increased immune cells activation (Corsini et al. 2005; Buoso et al. 2011; Buoso et al. 2017a, b, c).

The protein RACK1 has been identified in the early nineties and so named because of its interaction with protein kinase C (PKC) (Ron et al. 1994). Due to its seven-bladed β-propeller structure, RACK1 is now recognized as a multiple target scaffolding protein involved in several key biological events, including development, immune response, neuronal activity, and cancer (reviewed by Adams et al. 2011; Ron et al. 2013; Li et al. 2015). Due to its plethora of interaction proteins, over 80 binding proteins have been reported, RACK1 controls essential cellular processes, such as transcription and translation, cell proliferation and growth, as well as cell spreading and cell–cell interactions (Adams et al. 2011). Our working hypothesis is that RACK1 may be a relevant target to study the immunotoxicity of steroid-active compounds.

The purpose of this work was to explore if also estrogens and estrogen-active compounds could modulate RACK1 expression. As a proof of concept, 17β-estradiol (the endogenous hormone), diethylstilbestrol (a synthetic estrogen), and zearalenone (an estrogen-active compound of natural origin) were used as reference compounds. As experimental model, the human promyelocytic cell line THP-1 was used. THP-1 is a widely used model for primary human monocytes/macrophages, and represents a valuable, simple and reproducible in vitro tool to investigate immunotoxic compounds. Results obtained support our working hypothesis: also estrogen-active compounds, via GPER and AR activation, can target RACK1, and as a consequence of the increased levels, an increase in the response to LPS was observed. Altogether, our data suggest that RACK1

may represent a relevant target of steroid-active compounds (including glucocorticoids, androgens, and estrogens), and the evaluation of its expression may offer the opportunity to screen the immunotoxic potential of hormone-active substances.

Materials and methods

Chemicals

17β-estradiol (Cas N° 50-28-2, purity 98%), diethylstilbestrol (Cas N° 56-53-1, purity 99%), zearalenone (Cas N° 17924-92-4, purity 99%), and flutamide (Cas N° 13311-84-7, purity 99%) were obtained from Sigma Aldrich Italia (Merck Life Science SRL, Milano, Italy). Compounds were dissolved in DMSO (Sigma Aldrich Italia, Cas N° 67-68-5, purity 99.9%) at concentration of 50 mM and frozen at − 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 < 0.2%). Control cells were treated with the same amount of DMSO. G1 (Cas N° 881639-98-1, purity 98%), G15 (Cas N° 1161002-05-6, purity 99%), and Bay 11–7085 (Cas N° 196309-76-9, purity ≥ 99%) were obtained from Tocris Bioscience (Bio-Techne SRL, Milan, Italy). 17β-estradiol-BSA was obtained from Cloud-Clone Corp (Houston, TX, USA). Cell culture media and all supplements were from Sigma-Aldrich. Lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (LPS) was from Sigma-Aldrich. Mouse anti-human RACK1 monoclonal antibody (Cat. N° 610177) and mouse monoclonal anti-β-tubulin (Cat. N° 612656) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Host specific peroxidase conjugated IgG secondary antibody (Cat. N° 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.

Cells

For experiments, THP-1 cells, obtained from Istituto Zooprofilattico di Brescia (Italy), were diluted to 10^6 cells/mL 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 cultured at 37 °C in 5% CO_2 incubator. Preliminary experiments were

conducted to identify non-cytotoxic concentrations [cell viability > 80% (CV80)]. Cytotoxicity was assessed by propidium iodine staining, and the CV80 determined for all compounds: 17β-estradiol > 100 μM; diethylstilbestrol 20 μM; zearalenone 13 μM. For the different experiments, cells were then treated with increasing concentrations of 17β-estradiol (0.001–10 μM), diethylstilbestrol (0.002–20 μM), zearalenone (0.001–10 μM), or DMSO as vehicle control (final concentration of DMSO in culture medium < 0.2%), as reported in the legends.

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 Quantus™ Fluorometer (Promega, Madison, WI). 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 incubated at 37 °C in 5% CO_2 and then treated with the selected compounds 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).

Real-time PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, 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-Man™ PCR technology was used. Primers were purchased from Applied Biosystems. PCRs were performed 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 reference and the quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method (Livak et al. 2002).

Western blot analysis

The expression of RACK1 in cell homogenates was assessed by western blot analysis. Briefly, after treatment, cells harvested, washed once with PBS, 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 10% 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) and β-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 (Clarity western ECL blotting substrates Bio-Rad). The image of the blot was acquired with the Molecular Imager Gel Doc XR (BioRad). The optical density of the bands was calculated and analyzed by means of the Image Lab program for digital image processing (Version 4.0, Bio-Rad Laboratories).

Cytokine production

Cytokine production was assessed in cell-free supernatants by specific sandwich ELISAs, commercially available (ImmunoTools, Friesoythe, Germany). Cell-free supernatants obtained by centrifugation at 1200 rpm

for 5 min were stored at − 20 °C until measurement. Limits of detection were IL-8 2.6 pg/mL for IL 8, and 22 pg/mL for TNF-α. Results are expressed in pg/mL.

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 200 µL of PBS. Cells were stained in the dark for 30 min with specific FITC-conjugates antibodies against CD86 (ImmunoTools) or with isotype control antibody (ImmunoTools) at 4 °C, 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. The intensity of fluorescence and the percentage of positive cells were analyzed using a Novocyte 3000 flow cytometer and data were quantified using Novocyte software (Acea Bioscience Inc.). 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:

$$\mathrm{SI} = \mathrm{MFIt}/\mathrm{MFIc}.$$

MFIt stand for chemical-treated cells, whereas MFIc for the untreated ones.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using InStat software version 7.0 (GraphPad Software, La Jolla, 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 $p \leq 0.05$.

Results

Effects of 17β-estradiol on RACK1 expression

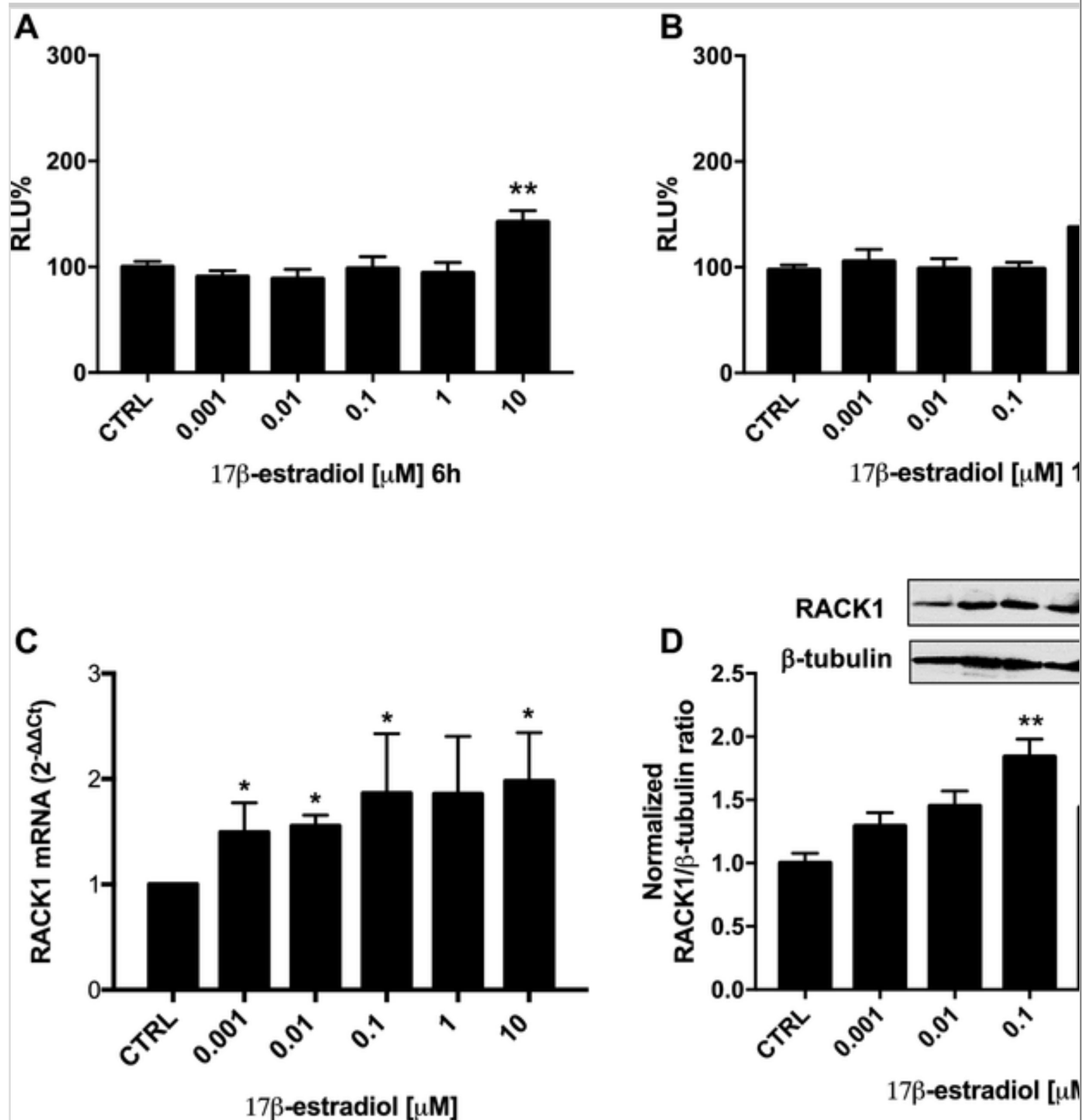
The effects on RACK1 expression were evaluated by means of reporter

luciferase activity using the human *rack1* gene promoter, mRNA expression using real-time PCR, and protein level by western blot analysis. THP-1 cells were treated for 6 h, 16 h, 18 h or 24 h with increasing concentrations of 17β-estradiol (0.001–10 μM) or DMSO as vehicle control. These times were chosen from previous experiments as optimal for DHEA and cortisol-induced transcriptional activity, mRNA, and protein expression (Buoso et al. 2011). As shown in Fig. 1a and b, 17β-estradiol induced a concentration-related increase of RACK1 transcriptional activity at 6 h and 16 h, which reached statistical significance at concentrations ≥ 1 μM. Increase of RACK1 mRNA expression at 18 h was observed at all concentrations tested (Fig. 1c), with statistical significance at 0.001, 0.1 and 10 μM, while the concentration-related increase in RACK1 protein evaluated at 24 h already appreciable from the concentration of 0.001 μM, reached a statistical significance at concentrations ≥ 0.1 μM (Fig. 1d). Overall, these results indicate the capacity, also at physiologically relevant concentrations (in adult women the levels of 17β-estradiol can reach concentrations > 2 nM), of 17β-estradiol to induce the expression of RACK1, with the awareness that the most clear effects are observed at supraphysiological concentrations (≥ 1 μM).

Fig. 1

Effects of 17β-estradiol on RACK1 expression. A/B. Effect of 17β-estradiol on the 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**) or 16 h (**b**) with increasing concentration of 17β-estradiol (0.001–10 μM) 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 ± SEM, $n = 3$ independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett’s multiple comparison test with $^{*}p < 0.05$ or $^{**}p < 0.01$ vs control (CTRL). **c** Effect on mRNA expression. THP-1 cells were treated for 18 h 17β-estradiol (0.001–10 μM) or DMSO as vehicle control. 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 $^{*}p < 0.05$ versus control. **d** Effect on RACK1 protein expression. THP-1 cells were treated for 24 h with increasing concentrations of 17β-estradiol

(0.001–10 μM) or DMSO as vehicle control (CTRL). β-tubulin expression was used to normalize RACK1 expression. The image is a representative Western blot. Each value represents the mean ± SEM, $n = 3$ independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$ or $**p < 0.01$ versus control (CTRL)



Effects of diethylstilbestrol on RACK1 expression

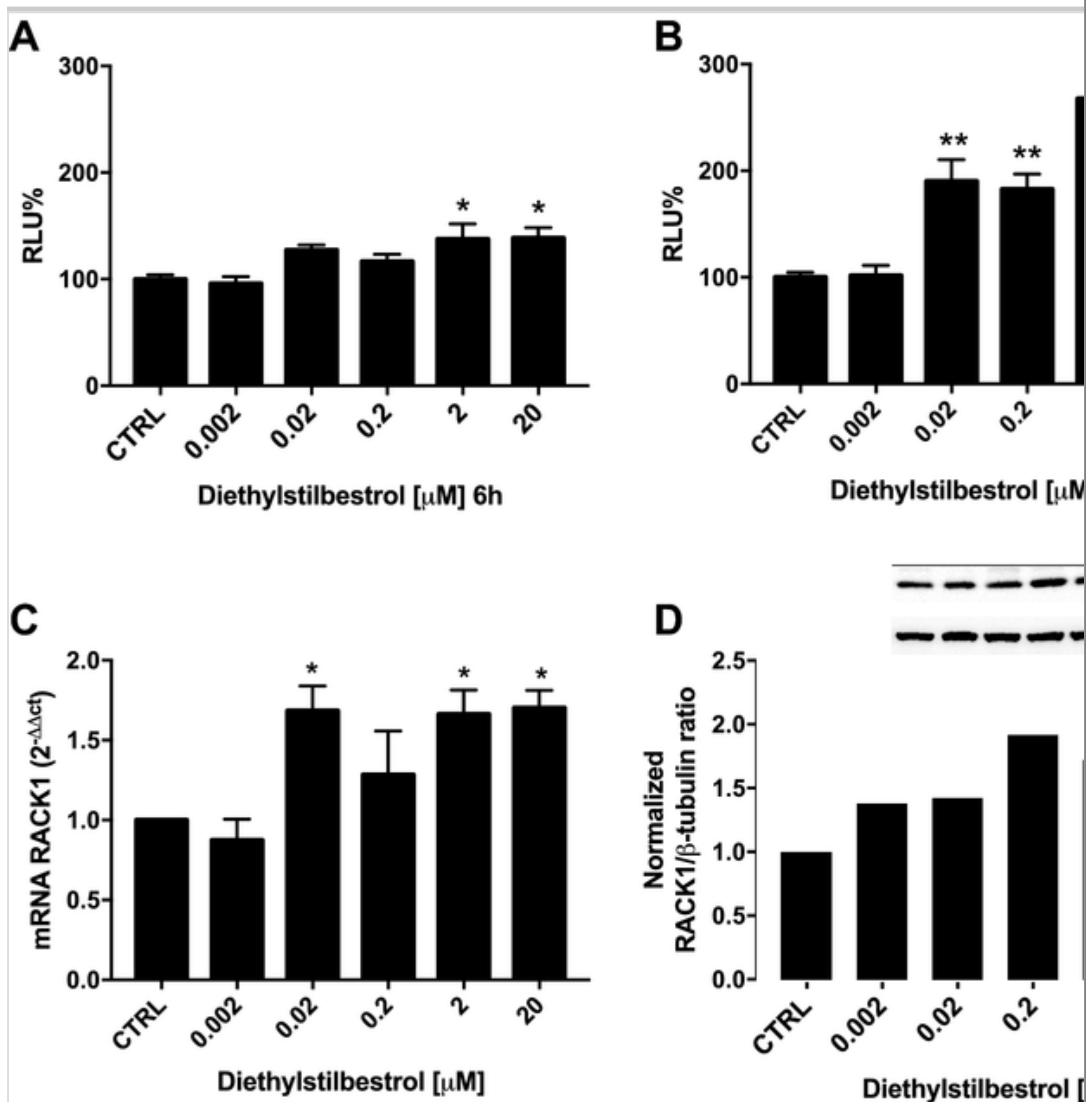
Diethylstilbestrol is a synthetic nonsteroidal estrogen developed to supplement a woman's natural estrogen production during pregnancy to prevent miscarriages and premature births, which was stopped in 1971

because it was linked to a rare vaginal cancer in female offspring (Veurink et al. 2005). To assess its effects on RACK1 expression, THP-1 cells were treated for 6 h, 16 h, 18 h or 24 h with increasing concentrations of diethylstilbestrol (0.002–20 μM) or DMSO as vehicle control. As shown in Fig. 2a and b, diethylstilbestrol induced a concentration-related increase of RACK1 transcriptional activity at 6 h and 16 h, which reached statistical significance at concentrations ≥ 0.02 μM. Statistical significant increase of RACK1 mRNA expression was observed at concentrations > 0.02 μM (Fig. 2c), while the concentration-related increase in RACK1 protein evaluated at 24 h already appreciable from the concentration of 0.002 μM, reached a statistical significance at concentrations ≥ 0.2 μM (Fig. 2d). Overall, these results indicate the capacity of diethylstilbestrol to induce the expression of RACK1. Plasma levels of diethylstilbestrol have been reported to be in range between 6 and 22 nM (Kemp et al. 1981), the lowest effect on RACK1 expression was observed at 0.02 μM (20 nM); therefore, effects were observed at in vivo relevant concentrations.

Fig. 2

Effects of diethylstilbestrol on RACK1 expression. **a**/**b** Effect of diethylstilbestrol on the 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**) or 16 h (**b**) with increasing concentration of diethylstilbestrol (0.002–20 μM) 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 ± SEM, $n = 3$ independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with $^{*}p < 0.05$ or $^{**}p < 0.01$ vs control (CTRL). **c** Effect on mRNA expression. THP-1 cells were treated for 18 h diethylstilbestrol (0.002–20 μM) or DMSO as vehicle control. The effect on mRNA levels was evaluated by real-time PCR using 18S as an endogenous reference. Each value represents the mean ± SEM, $n = 3$ independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $^{*}p < 0.05$ versus control. **d** Effect on RACK1 protein expression. THP-1 cells were treated for 24 h with increasing concentrations of diethylstilbestrol (0.002–20 μM) or DMSO as vehicle control (CTRL). β-tubulin expression was used to normalize RACK1 expression. The image is a representative western blot. Each value represents

the mean ± SEM, $n = 3$ independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $**p < 0.01$ versus control (CTRL)



Effects of zearalenone on RACK1 expression

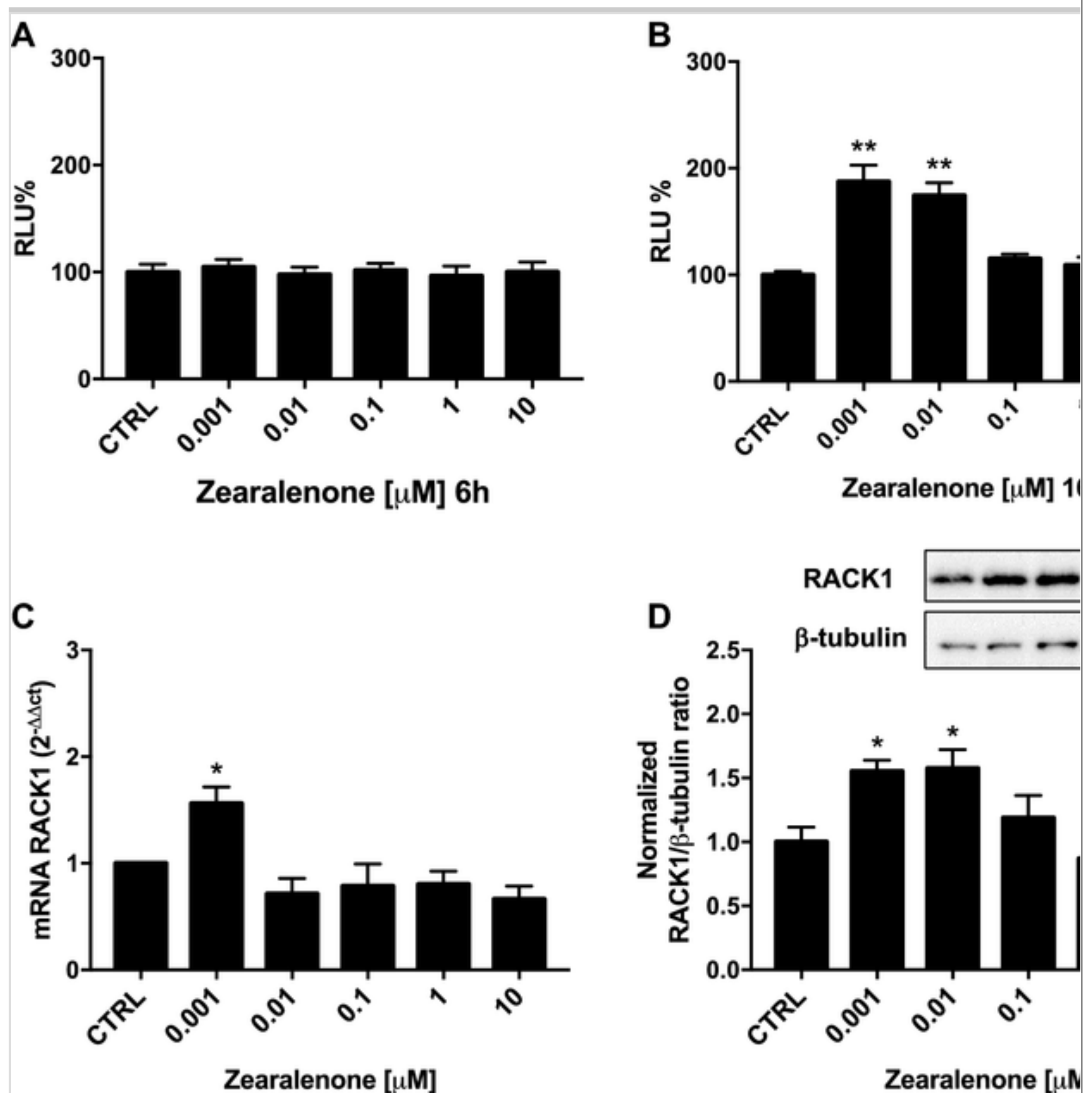
Finally, we investigated the effect of the potent estrogen-active mycotoxin zearalenone on RACK1 expression. To assess its effects on RACK1 expression, THP-1 cells were treated for 6 h, 16 h, 18 h or 24 h with increasing concentrations of zearalenone (0.001–10 μM) or DMSO as vehicle control. As shown in Fig. 3a and b, zearalenone induced a statistically significant increase of RACK1 transcriptional activity only at

16 h at the lowest concentrations tested (0.001 and 0.01 μM). Similarly, statistically significant increase of RACK1 mRNA expression was also observed at the lowest concentration of 0.002 μM (Fig. 3c), and the increase in RACK1 protein was also observed at the two lowest concentrations tested 0.001 and 0.01 μM (Fig. 3d). Overall, with a non-monotonic dose–response, also zearalenone induced the expression of RACK1. The lack of effects at the highest concentrations tested may be due to a possible glucocorticoid action resulting from the ability of zearalenone to inhibition of 11β-hydroxysteroid dehydrogenase 2, with IC50 values in the range 32–49 μM (Li et al. 2015), which may counter act with AR in the induction of RACK1, as we demonstrated for cortisol and androgens. Regarding the relevance of the concentration used, in animals fed with contaminated oats, plasma levels of zearalenone in the range of 10–19.6 nM have been reported (Songsermsakul et al. 2013). Thus, also in this case, in vitro effects were observed at in vivo relevant concentrations.

Fig. 3

Effects of zearalenone on RACK1 expression. A/B. Effect of zearalenone on the 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**) or 16 h (**b**) with increasing concentration of zearalenone (0.001–10 μM) 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 ± SEM, $n = 3$ independent experiments performed in quadruplicate. Statistical analysis was performed with Dunnett's multiple comparison test with $^{**}p < 0.01$ vs control (CTRL). **c** Effect on mRNA expression. THP-1 cells were treated for 18 h zearalenone (0.001–10 μM) or DMSO as vehicle control. The effect on mRNA levels was evaluated by real-time PCR using 18S as an endogenous reference. Each value represents the mean ± SEM, $n = 3$ independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $^{*}p < 0.05$ versus control. **d** Effect on RACK1 protein expression. THP-1 cells were treated for 24 h with increasing concentrations of zearlanenone (0.001–10 μM) or DMSO as vehicle control (CTRL). β-tubulin expression was used to normalize RACK1 expression. The image is a representative western blot. Each value represents the mean ± SEM, $n = 3$ independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with $^{*}p < 0.05$ versus

control (CTRL)



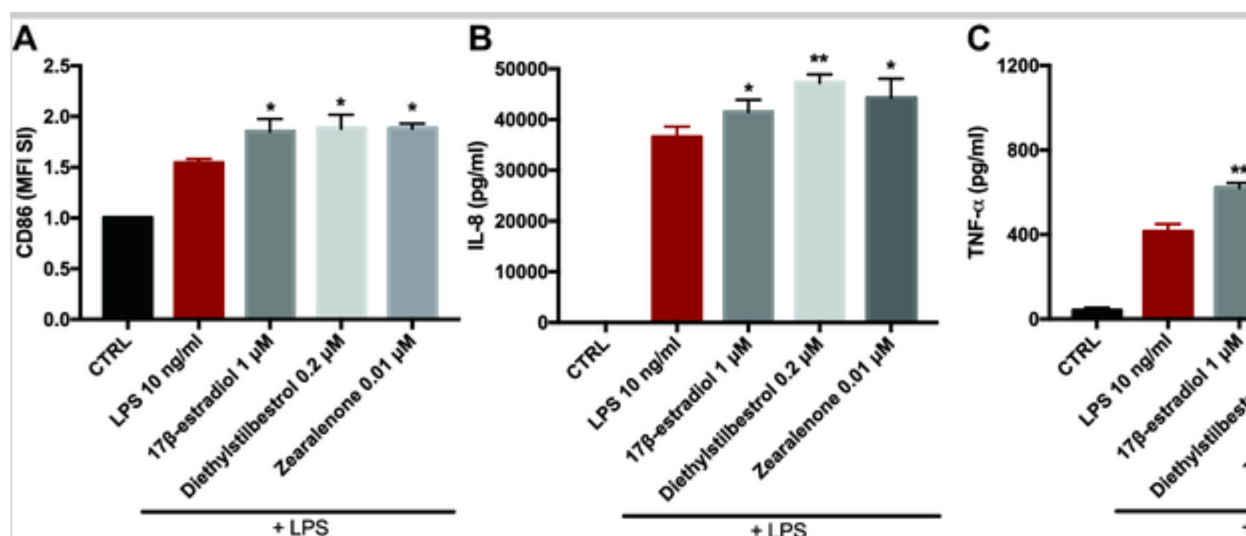
The increased RACK1 expression following exposure to estrogen-active compounds paralleled increased response to LPS

We have previously demonstrated a role of RACK1/PKCβ in LPS-induced cytokine production and CD86 expression both in THP-1 cells and primary human dendritic cells (Corsini et al. 2014a, b). In particular, we demonstrated using a selective inhibitor of PKCβ, a complete block of LPS-induced CD86 expression and a 50% reduction in IL-8 and TNF-α release, and using RACK1 pseudosubstrate to directly activate PKCβ a

concentration-dependent increase in CD86 expression and IL-8 release. Therefore, we focused on these parameters to verify that the increased levels of RACK1 following exposure to estrogen-active compounds could result in increased response to LPS. THP-1 cells were treated for 24 h with 17β-estradiol (1 μM), diethylstilbestrol (0.2 μM), and zearalenone (0.01 μM) at concentrations able to increase RACK1 protein levels (Figs. 1d, 2d, 3d), and then exposed to LPS 10 ng/mL. 24 h later, supernatants were collected for cytokine release as assessed by ELISA, while cells stained for CD86 expression and FACS analysis. As shown in Fig. 4, a statistically significant increase in CD86 expression (Fig. 4a), IL-8 (Fig. 4b), and TNF-α release (Fig. 4c) compared to control cells treated with LPS alone was observed, indicating that the increased levels of RACK1 induced by estrogen-active compounds predispose cells to an increased response to pro-inflammatory stimuli. It is important to note that compounds alone did not induce CD86 expression or cytokine production (data not shown).

Fig. 4

Effects of the selected estrogen-active compounds on immune parameters. THP-1 cells were treated for 24 with 17β-estradiol (1 μM), diethylestradiol, (0.2 μM) and zearalenone (0.01 μM) or DMSO as vehicle control, then LPS (10 ng/ml final concentration) was added for additional 24 h. A. Effect on LPS-induced CD86 expression. CD86 expression was evaluated by FACS analysis. Results are expressed as MFI SI. Each value represents the mean ± SD, $n = 3$ independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with $*p < 0.05$ versus LPS alone. **b**/**c** Effect on LPS-induced IL-8 (**b**) and TNF-α (**c**) release. Cytokine release was assessed by ELISA. Results are expressed as pg/ml. Each value represents the mean ± SD, $n = 3$ independent experiments. Statistical analysis was performed with unpaired *t* test with $*p < 0.05$, $**p < 0.01$ versus LPS alone



Role of GPER in estrogen-active compound-induced RACK1 expression

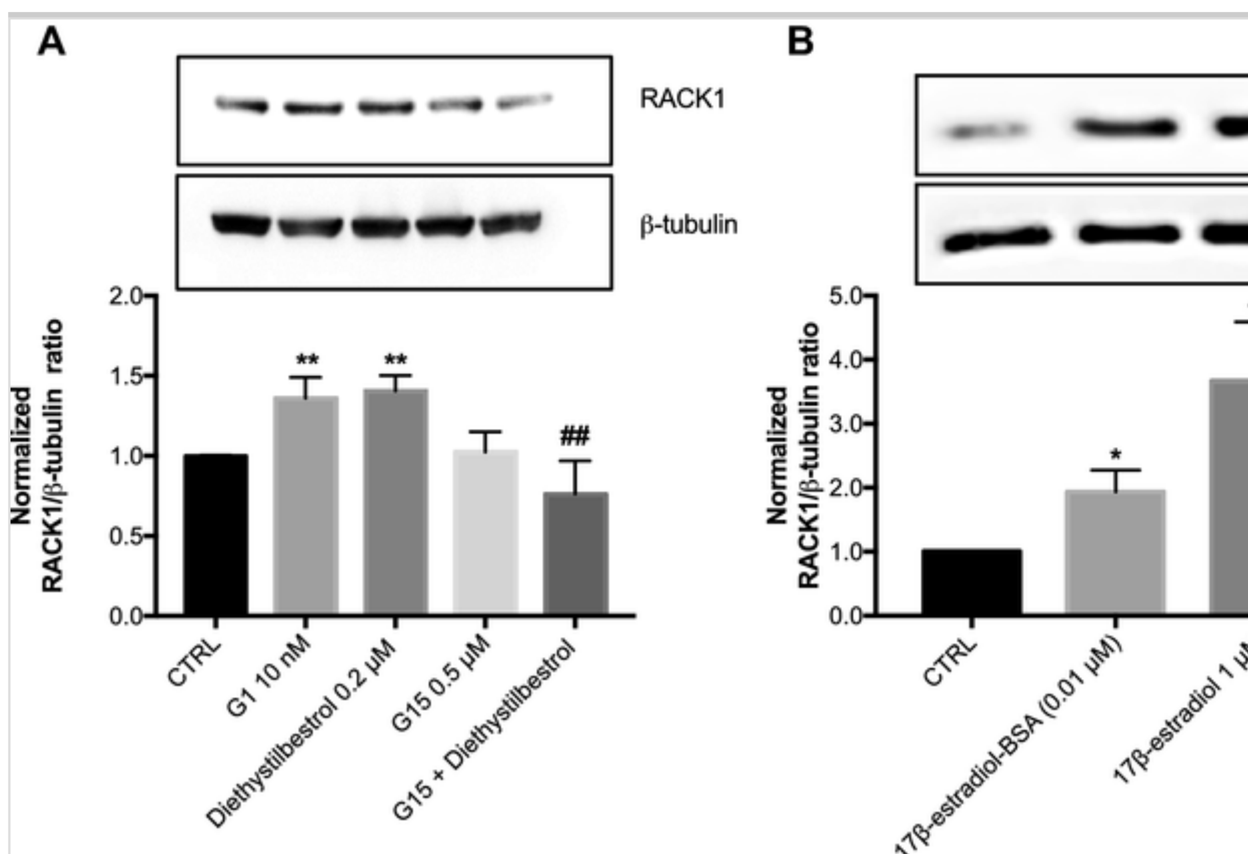
Five reasons led us to investigate the role of GPER rather than the nuclear estrogen receptors in the observed effect: (1) naive unstimulated THP-1 cells, as primary human monocytes, do not express ERα66 or ERβ at the protein level, while they express ERα46, ERα36, and GPER (Cutolo et al. 2001; Pelekanout et al. 2016; Galván-Ramírez et al. 2019; in house unpublished data); (2) the RACK1 promoter does not contain a nuclear estrogen receptor element (Del Vecchio et al. 2009); (3) the effects of 17β-estradiol on RACK1 are more evident at higher concentrations than the physiological values, in agreement with a possible involvement of GPER that is typically associated with higher 17β-estradiol concentrations (Prossnitz and Barton 20141); (4) DHEA, the first compound we have identified able to modulate the levels of RACK1 (Corsini et al. 1999), has been reported to interact at physiological relevant concentration with GPER with effects mediated in part by AP-1 and AR (Teng et al. 20145); (5) the binding and activation of GPER by environmental estrogens are also emerging (Thomas and Dong 2006).

The study of the role of GPER was possible due to the availability of selective agonist and antagonist for this receptor. We used diethylstilbestrol as a reference estrogenic active compound. THP-1 cells were treated with the selective GPER agonist G1 (10 nM), or with diethylstilbestrol (0.2 μM) in the presence or absence of the G15-GPER antagonist (0.2 μM). As clearly shown in Fig. 5a, G1 was able to induce RACK1 expression as

assessed by western blot analysis, while G15 completely blocked the effect of diethylstilbestrol, demonstrating the role of GPER in the observed effects. To further support the involvement of GPER, we used commercially available 17β-estradiol conjugated with bovine serum albumin (17β-estradiol-BSA), incapable of penetrating the cell membrane. As shown in Fig. 5b, 17β-estradiol-BSA (0.01 μM) was able to induce RACK1 expression as assessed by western blot analysis. 17β-estradiol (1 μM) as used in parallel.

Fig. 5

Role of GPER in RACK1 induction. **a** THP-1 cells were treated for 24 with the GPER agonist G1 (10 nM), and diethystilbestrol (0.2 μM) or DMSO as vehicle control in the presence or absence of the GPER inhibitor G15 (0.5 μM). RACK1 expression was evaluated by western blot analysis. β-tubulin 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 Tukey's multiple comparison test with $^{**}p < 0.01$ versus control and $^{\#\#}p < 0.01$ versus diethylstilbestrol alone. **b** Effect of 17β-estradiol-BSA conjugated on RACK1 expression. THP-1 cells were treated for 24 h with 17β-estradiol-BSA conjugated (0.01 μM), 17β-estradiol (1 μM) or DMSO as vehicle control (Control). RACK1 expression was evaluated by western blot analysis. β-tubulin 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 $^{*}p < 0.05$ or $^{**}p < 0.01$ versus control



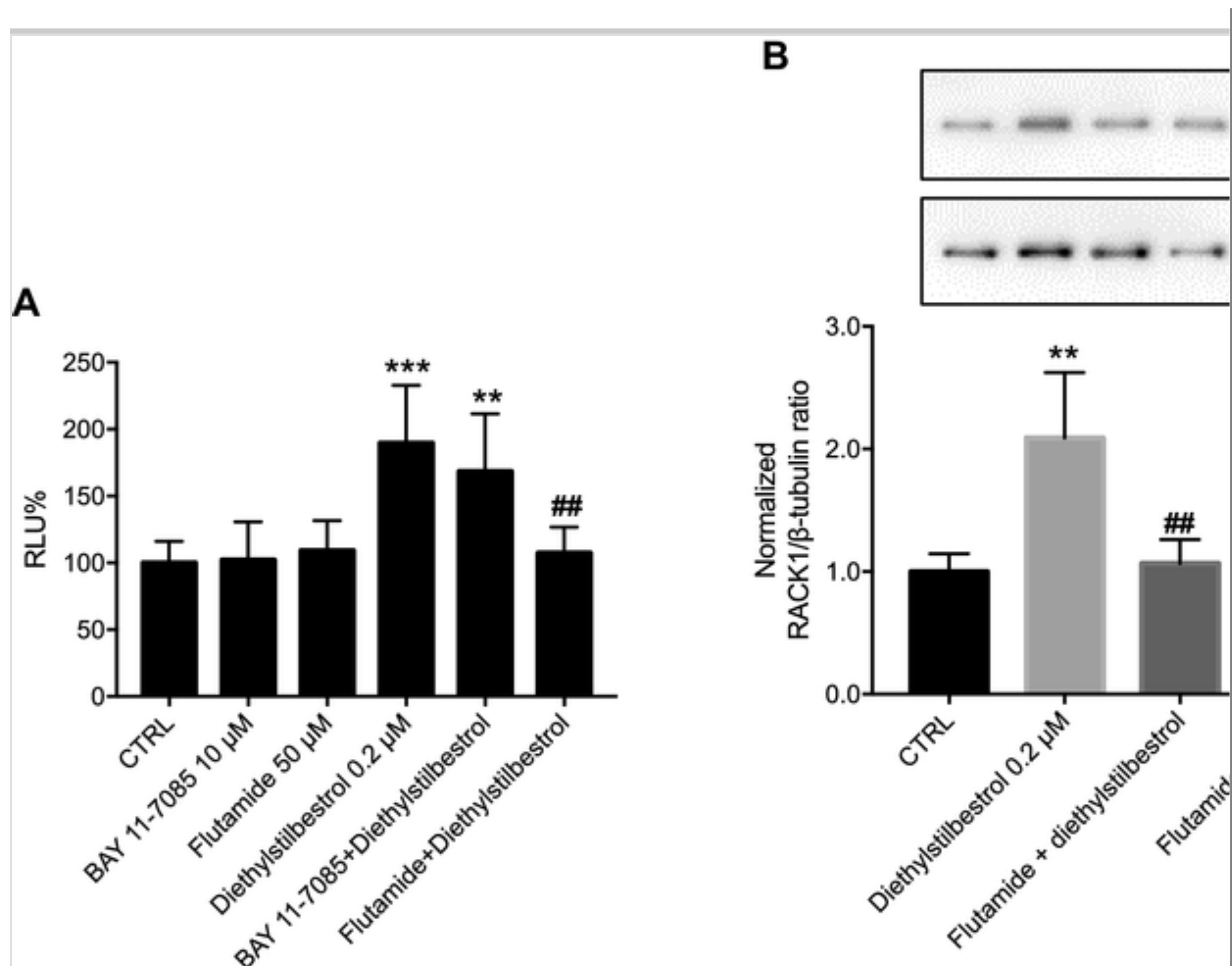
Role of AR in diethylstilbestrol-induced RACK-1 expression

To link the results with the regulatory elements identified in the promoter region of the *rack1* gene, which include c-Rel (NF-κB), Pax-4, Oct-1, Elk-1, and a glucocorticoid response element (Del Vecchio et al. 2009), we investigated possible role of AR and NF-κB in the observed effect. The AR antagonist flutamide and the IκB degradation inhibitor Bay 11-7085 were used. Cells were treated for 30 min with flutamide (50 μM) or Bay 11-7085 (10 μM) and then diethylstilbestrol (0.2 μM) or DMSO as vehicle control were added for 16 h (luciferase activity) or 24 h (western blot analysis). As shown in Fig. 6a, flutamide completely blocked the effect of diethylstilbestrol on RACK1 transcriptional activity, while the inhibition of NF-κB pathway did not significantly affected diethylstilbestrol-induced RACK1 transcriptional activation. We then investigated the effect of flutamide on diethylstilbestrol-induced RACK1 protein expression, and as shown in Fig. 6b, flutamide completely prevented RACK1 induction, demonstrating the role of AR in the observed effects.

Fig. 6

Role of AR in diethylstilbestrol- induced RACK1. A. Effect on the 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 16 h with diethylstilbestrol (0.02 μM) or DMSO as vehicle control (CTRL) in the presence or absence of the NF-κB inhibitor Bay 11-7085 (10 μM) or the AR antagonist flutamide (50 μM). 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 ± SEM, $n = 3$ independent experiments performed in quadruplicate. Statistical analysis was performed with Tukey's multiple comparison test with $^{**}p < 0.01$, $^{***}p < 0.001$ vs control (CTRL)., and $^{\#\#}p < 0.01$ versus diethylstilbestrol alone. B. Effect of flutamide on diethylstilbestrol-induced RACK1 expression. THP-1 cells were treated for 24 h with diethylstilbestrol (0.02 μM) or DMSO as vehicle control (CTRL) in the presence or absence of flutamide (50 μM). β-tubulin expression was used to normalize RACK1 expression. The image is a representative western blot. Each value represents the mean ± SEM, $n = 3$ independent experiments. Statistical analysis was performed with Tukey's multiple comparison test with $^{**}p < 0.01$ versus control (CTRL) and $^{\#\#}p < 0.01$ versus diethylstilbestrol alone



Discussion

Our working hypothesis is that EDCs can target RACK1 expression in immune cells, and that its modulation, demonstrated in the current and previously published manuscripts for glucocorticoid, androgen, and estrogen-active compounds, may offer the opportunity to screen the immunotoxic potential of steroid hormone-active substances. The purpose of this study was to investigate the effects of estrogen-active compounds on RACK1 expression and LPS-induced cell activation. As a proof of concept, the endogenous estrogen 17β-estradiol, the synthetic drug diethylstilbestrol, and the mycotoxin zearalenone were used, all with clear estrogenic activity. All compounds increased RACK1 transcriptional activity, mRNA expression, and protein expression, which were followed by an increase in LPS-induced IL-8, TNF-α production and CD86 expression. These immune parameters were chosen as we previously demonstrated to be dependent upon RACK1/PKCβ activation (Corsini et al. 1999, 2005, 2014a, b, 2016; Racchi et al. 2006, 2017; Buoso et al. 2017). Regarding the mechanism of action, we could demonstrate a role of plasma

membrane GPER, using selective agonist and antagonist and by the use of 17β-estradiol-BSA, incapable of penetrating the cell membrane, and AR, as demonstrated using flutamide, in the observed effects. Overall, results support our working hypothesis, indicating that RACK1 also represents a relevant target of estrogenic active compounds.

It is well established that estrogens, besides their role on sexual differentiation and reproduction, can act on the immune system through estrogen receptor-dependent and -independent mechanisms (Bouman et al. 2005). Furthermore, it is emerging that the immune system can be a relevant target for EDCs (Corsini et al. 2018; Novak et al. 2019). In this context, we focused on RACK1 because of its role in immune cell activation (Corsini et al. 1999, 2005, 2014a, b, 2016; Racchi et al. 2006, 2017; Buoso et al. 2017), and the role of hormones in the control of its expression (Corsini et al. 2016; Buoso et al. 2011, 2017b, c).

All started from the observation of the presence on RACK1 promoter of a glucocorticoid responsive element (GRE) (Del Vecchio et al. 2009), which is responsible of cortisol and corticosteroids action at the transcriptional level resulting in RACK1 expression modulation (Buoso et al. 2011; Corsini et al. 2014a, b). The anti-glucocorticoid effect of dehydroepiandrosterone (DHEA) on RACK1 expression was also demonstrated (Buoso et al. 2011; Pinto et al. 201~~6~~5). DHEA, through the conversion to active androgens (Corsini et al. 2016), and endogenous androgens, namely androstenedione, testosterone, and dihydrotestosterone, have an opposite effect on RACK1 expression and on the regulation of PKC activity involved in immune cell activation (C~~a~~orsini et al. 2002, 2005; Buoso et al. 2011). A more specific indication came from data demonstrating that the effect of DHEA on RACK1 expression could be completely prevented 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 and LPS-induced cytokine production. Therefore, modulation of AR is a key step in the mechanism supporting RACK1 expression (Corsini et al. 2016). Indeed, it is known that the AR and GR can interact at the transcriptional level and that this interaction is related to their ability to form heterodimers at a common DNA site since identical P boxes of GR, AR, mineralocorticoid receptor (MR), and progesterone receptor (PR) allow them to bind similar

hormone response element (HRE). These receptors are all capable of activating reporter genes by recognizing a similar palindromic sequence usually termed as a canonical androgen/glucocorticoid response element (ARE/GRE) (Pihlajamaa et al. 2015).

Although, an ER-specific site was not identified on RACK1 promoter, our data show a significant increase of RACK1 promoter activity after treatment with 17β-estradiol, diethylstilbestrol, and zearalenone, thus, indicating they act at transcriptional level. We demonstrated that the observed effects are mediated by the plasma membrane GPER and AR activation, as flutamide can completely prevent diethylstilbestrol-induced RACK1 expression. In addition to the AR activation, the involvement of NF-κB activation was investigated using the selective inhibitor (Pelekanou et al. 2015~~5~~6). The rational being that similar to those found in the mouse and porcine genes, four c-Rel binding sites were identified in the human RACK1 promoter (Chou et al. 1999; Del Vecchio et al. 2009; Buoso et al. 2013). c-Rel is a member of the NF-κB transcription factor family, whose involvement in RACK1 regulation was demonstrated in cells of immune origin using LPS (Del Vecchio et al. 2009). In addition, potential interplay between pro-inflammatory and androgen signaling for gene regulation correlated with the crosstalk between AR and NF-κB (Chen ~~et al.~~ and Saywers, 2002; De Bosscher et al. 2006; Malinen et al. 2017, Khurana and Sikka, 2018). While our results suggest a role for AR in the diethylstilbestrol-induced RACK1 expression, also consistent with similar result observed in diethylstilbestrol-induced programming of prostate differentiation where flutamide treatment blocked both branching and prostate growth induced by diethylstilbestrol (Gupta 2000), no modulation of RACK1 promoter activity was observed in the presence of the inhibitor Bay 11-7085, ruling out a role of NF-κB activation in the transcriptional effect observed.

AQ3

AQ4

GPER-1 is a seven transmembrane G-protein-coupled receptor with no known alternative spliced isoforms detected at the plasma membrane and intracellular membranes, mainly the endoplasmic reticulum. The degree to which GPER-1 influences ERs signaling depends on cell type, developmental stage, and health status (Romano and Gorelick 2018).

GPER-1 is considered a low-capacity receptor for estrogen, requiring higher concentrations to be activated compared to ERs: ERs binding affinities for 17β-estradiol are typically in the subnanomolar range [dissociation constant (Kd) = 0.1–1.0 nM]; while for GPER a Kd = 3.3 nM has been reported (reviewed in Filardo and Thomas 2012). Unlike ER knockout mice, GPER-1 knockout mice are fertile and show no overt reproductive anomalies. However, they do exhibit thymus atrophy, impaired glucose tolerance, and altered bone growth (reviewed in Filardo and Thomas 2012). Unstimulated THP-1 cells, as primary human monocytes, do not express ERα66 or ERβ at the protein level, while they express GPER, and shown the presence of a low-affinity estrogen-receptor activity (Cutolo et al. 2001; Pelekanout et al. 2016; Galván-Ramírez et al. 2019). Membrane-localized ERs rapidly signal through kinase cascades, calcium, and other second messengers (Zimmerman et al. 2016; Barton et al. 2018). It physically engages and activates discrete Gα subunits, which triggers rapid signaling through calcium and cAMP (Gαs), protein kinase C, and Src kinases (Gq), leading to extracellular-regulated kinases (ERK) or PI3 activation, impacting the phosphorylation and regulation of enzymes, phosphorylation and recruitment of co-activator proteins, contributing to cell proliferation and survival, and many other aspects of cellular function (Levin and Pietras 2008).

AR can undergo extensive post-translational modifications including phosphorylation, acetylation, sumoylation, ubiquitination, and methylation that modulate its function (Coffey and Robson 2012). As the kinase cascade is the main mechanism through which GPER signals, AR phosphorylation is a plausible mechanism. We are currently investigating the mechanism through which GPER induces AR activation, speculating its phosphorylation via one of kinase pathways activated by GPER as the main mechanism. At present, we can exclude a role of NF-kB, on the other hand, we have preliminary evidence of a possible involvement of PI3K (phosphoinositide 3-kinase) in light of the inhibitory effect of wormannin on diethylstilbestrol effect on RACK1 expression.

As a consequence of the increase in RACK1 expression, an increase in the response to LPS was observed, as measured by the release of the proinflammatory cytokines TNF-α and IL-8, and the up-regulation of the costimulatory molecule CD86, important for full activation of T cells. We

have previously demonstrated the role of RACK1 and PKC in immune cell activation, as previously described. Thus, the observed immunostimulation is consistent with previous results (Corsini et al. 1999, 2002, 2005, 2014a, b, 2016; Racchi et al. 2006, 2017; Buoso et al. 2017). We are currently investigating if similar results can be obtained in human primary cells. Preliminary results (Supplementary Fig. 1) seem to suggest that indeed 17β-estradiol, diethylstilbestrol, and zearalenone can induce RACK1 expression and increase the response to LPS. Similarly, in macrophage-differentiated THP-1 cells, Couleau et al. (2015) found that bisphenol A, dibutyl phthalate and 4-tert-octylphenol, while reducing phagocytosis, they increased LPS-induced TNF-α, and bisphenol A also IL-8, in agreement with our findings. Highlighting how the immunomodulatory role of estrogens in inflammation is complex, as both pro- and anti-inflammatory effects have been described, and a uniform concept to the action of estrogen cannot be found for all inflammatory diseases. This dualistic action can be explained by the concentration of circulating estrogen, and differential expression of ERs in different cell types (Straub 2007). In term of hazard identification, our results indicate that exposure to estrogen-active compounds is associated in increased immunostimulation, which should be considered indicative of immunotoxicity.

Exposure to estrogen-active compounds predispose cells to an enhanced response, which, depending on the context, could be detrimental, i.e. it can favor the onset of autoimmune diseases, allergic reactions to unrelated antigens, misregulated inflammation to mention some possible consequences. The ability of estrogens to activate the immune response is also the basis of the evidence that, in general, males are more susceptible to infectious diseases (e.g. bacterial, parasitic, and viral infections), than females (Guerra-Silveira and Abad-Franch 2013). Nonagenarian females produce more vigorous humoral immune responses and a more prominent pro-inflammatory response, making them more resistant to infections, but they suffer a higher incidence of autoimmune disorders and allergy as compared to males (Bouman et al. 20045; Marttila et al. 2013; Laffont et al. 2017).

AQ5

To conclude, in the assessment of the immunotoxic potential of endocrine-active compounds, we propose an in vitro 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/or 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, can be investigated by evaluating immune functions, e.g. cytokine production, surface markers upregulation, to mention some of the possible targets. The other advantage in the use of the expression of this protein with respect to the evaluation of the effect on a single hormonal receptor, is that its expression can capture the complex interplay of transcriptional and non-transcriptional events associated with exposure to hormonally active compounds targeting more than one hormonal systems, and the resulting biological consequence, as we previously demonstrated for cortisol and DHEA (Buoso et al. 2011).

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

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary file1 (DOCX 879 kb)

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