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Upregulation of TLR2 and TLR4 in the human adrenocortical cells differentially modulates adrenal steroidogenesis.

[1]Waldemar Kanczkowski, [1]Piotr Tymoszuk, [1]Triantafyllos Chavakis, [2]Volker Janitzky, [2]Torsten Weirich, [3]Kai Zacharowski, [1]Monika Ehrhart-Bornstein and [1]Stefan R. Bornstein.

[1] Technical University Dresden, Department of Medicine III, 01307 Dresden, Germany;
[2] Pirna General Hospital, Department of Urology, 01796 Pirna, Germany;
[3] Clinic of Anaesthesiology, Intensive Care Medicine and Pain Therapy, 60595 Frankfurt am Main, Germany;

Corresponding author:

Kanczkowski Waldemar*
Department of Medicine III
Technical University Dresden
University of Technology, Dresden
Fetscherstrasse 74, 01307 Dresden, Germany
E-Mail: Waldemar.kanczkowski@uniklinikum-dresden.de
Tel: +49-351-458-6606
Fax: +49-351-458-6336

* To whom reprint requests should be addressed

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Abstract

Rapid activation of adrenal steroid release plays a pivotal role in an organism's first line of defense during sepsis. Adrenal gland function is often suppressed in critically ill patients and negatively impacts the overall survival rate. Increasingly, experimental and clinical evidence suggests that Toll-like receptors (TLRs), components of the innate immune system, play a key role in the mediation of systemic responses to invading pathogens during sepsis. In the present study, we aimed to elucidate the effect of TLR2, TLR4 and CD14 upregulation on adrenocortical cell steroidogenesis. We found that TLR4 and CD14 but not TLR2 overexpression in NCI-H295R cells inhibited basal and acute cortisol and aldosterone production. This effect could be partially explained by reduced expression of enzymes involved in the synthesis of latter steroids - CYP11B1 and CYP11B2. Together, these data suggest that TLR upregulation in the steroid producing cells may be involved in the adrenal gland dysfunction during sepsis.

Key words:
Adrenal insufficiency, adrenal dysfunction, steroidogenesis, immune-adrenal crosstalk, pattern recognition receptors.

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1. Introduction

Sepsis and septic shock still remain a major cause of death in intensive care units stations worldwide despite recent improvements in their management and treatment. According to recent estimates, the incidence of severe sepsis in the United States of America accounts for 710.000 patients per year with a mortality rate of 28% (Angus, 2001). Sepsis is a clinical syndrome resulting from a complex interaction between host and infectious agents; bacterial or viral infections are its primary cause. It is characterized by systemic activation of multiple inflammatory pathways, including cytokine networks and coagulation (van der Poll, 2001) and by activation and dysfunction of innate and adaptive branches of the immune system (Russell, 2006). Recently, an increased frequency of sepsis and septic shock due to infections with Gram positive bacteria has been reported (Angus, 2001). Increasing evidence suggests that Toll-like receptors (TLRs) play a key role in the mediation of systemic responses to invading pathogens during sepsis. TLRs are components of the innate immune system and are responsible for recognizing a variety of microbial pathogens and the initial induction of immune and inflammatory responses. TLRs sense various conserved pathogen-associated molecules expressed by microorganisms such as lipopolysaccharide (LPS) of Gram negative bacteria (sensed by TLR4), or lipoteichonic acid frequently found on cellular walls of Gram-positive bacteria (detected by TLR2). To date, 13 TLRs have been identified in mammals (Leulier, 2008). Due to their ability to initiate an overt inflammation, TLRs are often associated with destruction of various tissues, e.g. during septic shock; which has initiated the pharmaceutical development of many TLR antagonists (Hennessy et al., 2010). Increasing experimental and clinical evidence has demonstrated importance of TLR expression on both immune and non-immune cells during sepsis. Significant upregulation of TLRs in various organs e.g. lung, liver, kidney or periphery blood has been reported in different models of sepsis and in critically ill patients (El-Achkar et al., 2006; Tsujimoto et al., 2005; Williams et al., 2003).

The hypothalamic-pituitary adrenal axis is a key component of the host response to sepsis and adrenal glands are the main effector part of this system (Beishuizen, 2003; Gallo-Payet et al., 2008). Indeed, rapid activation of adrenal steroid release plays a pivotal role in an organism's first line of defense during this form of severe illness. The critical role of adrenal derived hormones during stress has been demonstrated using adrenolectomized animals, which demonstrated a higher mortality rate then sham operated controls (McCallum, 1982). Multiorgan dysfunction syndrome (MODS) is the main cause of death due to sepsis; however the adrenal gland seldom suffers irreversible anatomical damages due to bilateral necrosis and hemorrhage as reported originally by Waterhouse and Friderichsen (Waterhouse, 1911). It is rather, a transient functional impairment in hormone synthesis which is a more common complication of severe sepsis (Prigent et al., 2004). Indeed, during sepsis relative adrenal gland insufficiency occurs frequently and is responsible for increased mortality (Koo et al., 2001; Sprung et al., 2008). Several factors may be responsible for the incidence of adrenal insufficiency in septic

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patients such as a suppression of the HPA axis by exogenous glucocorticoids, viral infections or certain medication (Bornstein, 2009).

During stress conditions such as sepsis, adrenal hormone production is shifted toward glucocorticoid production as a result of increased ACTH release from pituitary. The generalized suppressive influence of glucocorticoids protects the host from a hyperactive inflammatory response, which may be deleterious to cell and organ functions and ultimately in the outcome of critically ill patients (Prigent et al., 2004). Furthermore, a substantial drop in aldosterone production during sepsis can be identified, regardless of the rise in renin levels, suggesting that additional factors such as elevated cytokines or nerve regulation substantially modulate adrenal steroidogenesis (Prigent et al., 2004). Although levels of dehydroepiandrosterone (DHEA) and its sulfated form (DHEAS) decrease in the plasma of septic patients, resulting in the increased cortisol to DHEA ratio, the function of androgens during sepsis is not clear. Indeed, androgen supplementation, as opposed to glucocorticoid replacement does not influence the mortality rate of intensive care unit (ICU) patients (Marx et al., 2003). We have previously shown expression of several TLRs in mouse and human adrenal glands and in primary cultures of adrenocortical cells (Bornstein et al., 2004a; Kanczkowski et al., 2009). Moreover, we found that TLR2, TLR4 or TLR9 ligands when administered to mice induce a strong systemic and local inflammation of adrenal gland with upregulation of the major proinflammatory cytokines such as IL-1 β, IL-6 and TNF- α (Bornstein et al. 2004b). Interestingly, in the latter study we have found un upregulation of both TLR2 and TLR4 expression in the adrenal glands from mice that received a single dose of LPS or lipoteichonic acid (Bornstein et al., 2004b). However, we also found that TLR2 and TLR4 deficiency results in a dysregulation of adrenal glucocorticoid production and increased mortality of TLR2 and TLR4 deficient mice. Since the latter observation was paralleled by dysregulation of plasma cytokines and an impairment of adrenocortical cells ultrastructure an important link between intact TLR expression and a proper adrenal function has been postulated (Zacharowski et al., 2006).

In the current study, we sought to evaluate the importance of TLR2 and TLR4 upregulation previously seen in mice models using human adrenocortical cells with induced TLRs overexpression. Understanding of the role which TLR2 and TLR4 play in the adrenocortical cells during sepsis is important because an overt activation of TLRs and related cytokine and oxidative stress may directly interfere with the adrenal cortex hormone synthesis leading to adrenal insufficiency often diagnosed in critically ill patients.

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2. Materials and Methods

2.1. Cell culture and stimulation

NCI-H295R cells were grown in DMEM-F12 medium (GibcoBRL/Life Technologies GmbH) supplemented with 1% ITS plus (Gibco-Invitrogen, Carlsbad, CA, USA), 5% Nu-Serum I (Becton Dickinson, Heidelberg, Germany) and either 100 U/mL penicillin G/100 µg/mL streptomycin sulfate (normal cells) or 5 µg/ml of blasticidine S (cell clones). An equal number of cells were seeded into 24 well plates and grown until 80% confluence was reached. 24h prior to stimulation cells were grown in medium without any supplements and antibiotics. For stimulation cells were incubated in supplement-free medium together with angiotensin II (AII) (10^{-7} M) or forskolin (FSK), (10^{-6} M) for 24h.

2.2. Western blot

Cell proteins were extracted using Cell Lysis Buffer (Cell Signalling Technology, Denvers, MA, USA) according to the manufacturer's instructions. Protein concentration was measured with Bio-Rad Protein Assay Reagent (Bio-Rad, München, Germany) and separated in polyacrylamide gels and electro-transferred onto PVDF membranes. After blotting polyclonal anti-sera against human TLR2 (concentration 1µg/ml, ProSci. Inc.); monoclonal antibody against CD14 (clone 5A3B11B5) and polyclonal antibody recognizing TLR4 (H80) (both form Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at 1:250. Signal was developed using Supersignal West Femto Substrate (Thermo-Pierce) and visualized using LAS 3000 Bioimager (Fuji, Tokyo, Japan).

2.3. Stable transfection of the NCI-H295R cells with TLR2 and TLR4-CD14 encoding plasmids

NCI-H295R cells were transfected with expression plasmids encoding for TLR2, TLR4-CD14 or with an empty plasmid as a negative control (MOCK) (InvivoGen, San Diego, CA, USA) using Lipofectamine™ LTX PLUS transfection reagent (Invitrogen Life Technologies, Karlsruhe, Germany). NCI-H295R cell clones stably expressing TLR2, TLR4-CD14 or containing an empty plasmid were selected using blasticidin S (5 µg/ml; InvivoGen).

2.4. RNA isolation and cDNA synthesis

RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For reverse transcription reaction (RT), 1 µg of mRNA was incubated at 37 °C for 1 h in buffer containing 200 U of M-MLV reverse polymerase, oligo$(dT)_{15}$ primers mix and dNTPs. All reagents were from Promega (Madison, USA).

2.5. Quantitative PCR

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mRNA levels of human cholesterol side-chain cleavage enzyme (CYP11A1) and steroid acute regulatory protein (StAR) genes were determined using the Quantitec SYBR® PCR Master Mix (Qiagen, Hilden, Germany) and Light Cycler® 2.0 PCR thermocycler (Roche Applied Science, Basel, Switzerland). The amplification protocol, primer sequences and annealing temperatures were used as reported previously (Kanczkowski et al., 2009). Total cDNA copy numbers of CYP11A1 and StAR genes were calculated as a ratio of copy numbers of amplified gene of interest to copies of internal standard and adjusted to the copies of β-actin. Quantitative standard curves were generated applying dilutions over seven log scales (10^1–10^8) per capillary of the pCRII-TOPO vector (Invitrogen Life Science) containing PCR-fragment of the gene of interest and determined by the Light Cycler® quantification software version 3.5.3 (Roche Applied Science). Due to the high homology rate between 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) genes, a relative changes in number of both transcripts were evaluated using Taqman PCR Master Mix (Roche) and transcript specific FAM-TAMRA labeled probe. Samples were measured in duplicate and expression was calculated relative to the housekeeping gene hypoxanthine ribosyl transferase 1 (HPRT1) using ΔCT method. Primers and probe sequences used were already reported (Chai et al., 2010).

2.6. Steroid hormone measurement

H295R cells were treated with 10^{-7} M AII or 10^{-6} M FSK in DMEM-F12 medium. Concentration of secreted adrenal steroids in cell supernatants were measured using specific radioimmunoassays: DSL for aldosterone and DHEA (DSL, Sinsheim, Germany) and DPC for cortisol (DPC Biermann/ Siemens Healthcare Diag., Eschborn, Germany).

3. Results

3.1. Generation of NCI-H295R cell clones with stable enhanced expression of TLR2 or TLR4 proteins.

To elucidate the effect of TLR2 and TLR4 upregulation on adrenocortical cell steroidogenesis and inflammation, we generated several cell clones stably expressing the corresponding proteins as well as containing an empty plasmid (MOCK). Western blot analysis confirmed upregulation of TLR2 and TLR4 expression. TLR2 was found to be successfully upregulated in 100% (14/14) of randomly analyzed clones, TLR4-CD14 in 42% (3/7). For further experiments 3 clones with the highest expression of TLRs representing each group were used. A Western blot analysis of TLR2 expression obtained from a representative cell clone is depicted in Figure 1A. Densitometrical quantification of TLR2 expression performed on 2 representative NCI-H295R cell clones showed a 3.75-fold (clone

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#89) and a 3.25-fold (clone #102) upregulation of protein expression as compared to the MOCK transfected clone (Figure 1B). Successful generation of stably transfected NCI-H295R cell clones with an upregulation of TLR4 (1.52-fold) and CD14 by 13.56-fold was recently reported (Kanczkowski et al., 2010).

3.2. Differential effect of TLR2 and TLR4 overexpression on human adrenocortical cell steroidogenesis

To determine whether an increased expression of TLR observed in septic adrenal glands modulates adrenal steroidogenesis, levels of aldosterone, cortisol and DHEA were measured in supernatants of the NCI-H295R cell clones with stable upregulation of TLR2 and TLR4-CD14 proteins before and after incubation with 10^{-7} M of AII or 10^{-6} M of FSK. Our results showed that enhanced TLR2 expression mediated a significant 2.5-fold increase in basal aldosterone secretion as compared to MOCK control (89.89 ± 8.02 vs. 35.29 ± 3.27 pg/mL; $p < 0.01$) (Fig.2A). Although the potential of AII to enhance aldosterone production did not differ substantially between MOCK and NCI-H295R cells with TLR2 upregulation (18.7-fold vs. 17.7-fold), the resulting concentrations of this mineralocorticoid after incubation with AII varied significantly and was almost 2.7-fold higher in TLR2 than in MOCK group (1680 ± 286 pg/mL vs. 624.4 ± 196.6 pg/mL, $p < 0.05$) (Fig. 2B). Interestingly, contrary to TLR2 an enhanced expression of TLR4-CD14 protein reduced basal (5 ± 2.23 vs. 35.29 ± 3.27 pg/mL, 7-fold, $p < 0.05$) and AII-mediated aldosterone production (24.34 ± 8.4 vs. 624.4 ± 196.6 pg/mL, 4.8-fold, $p < 0.05$) Fig.2A. In contrast to aldosterone, basal levels of cortisol varied slightly but not significantly (1.18-fold increase) between NCI-H295R cells with an enhanced TLR2 expression and MOCK control cells (89.86 ± 8.48 vs. 75.97 ± 9.78 nmol/L) (Fig. 2C). No significant difference was observed after FSK treatment between the latter mentioned 2 cell groups. Basal (6.7 ± 1.57 vs. 75.97 ± 9.78 nmol/L, $p < 0.01$) and FSK-mediated cortisol production (15.0 ± 3.09 vs. 406 ± 108 nmol/L, 27-fold, $p < 0.01$) were strongly reduced in NCI-H295R cells with enhanced TLR4-CD14 expression as compared to the MOCK control cells (Fig. 2D). Basal DHEA levels increased in NCI-H295R cells after enhanced expression of both TLR2 (8.94 ± 2.2 vs. 5.46 ± 1.64 ng/mL, $p < 0.01$) and TLR4-CD14 proteins (11.61 ± 1.65 vs. 5.46 ± 1.64 ng/mL) (Fig. 2E). However, despite this initial increase in FSK-mediated DHEA production was lower in both TLR2 (24.49 ± 6.83 vs. 42.9 ± 14.26 ng/mL) and in TLR4-CD14 (33.83 ± 3.54 vs. 42.9 ± 14.26 ng/mL) as compared to MOCK transfected cells (Fig. 2F).

3.3. Expression of steroidogenic enzymes and proteins involved in cholesterol utilization

To evaluate the effect of the enhanced expression of TLR2 and TLR4 on adrenal steroidogenesis, expression of key enzymes involved in cholesterol transport (StAR) as well as steroid biosynthesis (CYP11B1, CYP11B2 and CYP11A1) were assessed (Fig. 3). Our results show that in comparison to

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control cells, stimulation with AII induced CYP11B2 gene expression in NCI-H295R cells with enhanced TLR2 expression (62.04 ± 8.265 vs. 50.99 ± 6.29; 1.2-fold), whereas in cells with enhanced TLR4-CD14 expression it caused an opposite effect (21.55 ± 2.316 vs. 50.99 ± 6.29; 2.36-fold, $p < 0.05$) (Fig.3A). Enhanced upregulation of both TLR2 (8.359 ± 2.55 vs. 14.57 ± 3.72, 1.7-fold) and TLR4 (2.55 ± 0.48 vs. 14.57 ± 3.728; 5.78-fold, $p < 0.05$) reduced FSK-induced CYP11B1 expression as compared to the MOCK control Fig. 3B. Analysis of CYP11A1 expression showed that NCI-H295R cells with upregulation of TLR2 tend to have higher copy numbers of this gene (0.41 ± 0.1 vs. 0.22 ± 0.026) as compared to MOCK and to cells with upregulation of TLR4-CD14 (0.41 ± 0.1 vs. 0.08 ± 0.008, $p< 0.05$). In fact, basal expression of CYP11A1 gene was significantly reduced in NCI-H295R cells after TLR4-CD14 upregulation compared with TLR2 upregulation (0.41 ± 0.1 vs. 0.08 ± 0.008, $p < 0.05$). Finally, an upregulation of TLR2 in NCI-H295R cells resulted in higher StAR copy number as compared to the MOCK control, however the differences were not significant (0.263 ± 0.0137 vs. 0.19 ± 0.002, 1.38-fold) Fig. 3D. On the contrary, enhanced expression of TLR-CD14 resulted in reduced StAR mRNA levels (0.14± 0.016 vs. 0.19 ± 0.002, 1.36-fold) Fig. 3D. Basal levels of StAR mRNA were significantly higher in TLR2 (1.8-fold, $p < 0.05$) than in TLR4-CD14 clones of NCI-H295R cells.

4. Discussion

Severe sepsis seldom causes irreversible anatomical damages to the adrenal gland leading to primary adrenal failure. Instead, a transient impairment in the hormone synthesis is a commonly found complication (Prigent et al., 2004). Compelling evidence strongly implicates expression of various TLRs and TLR-signaling in many cell types with the progression of sepsis and septic shock (Salomao et al., 2009). Being the most important group of the innate immunity receptors, TLRs are primarily responsible for pathogen recognition and initiation of an overt inflammatory responses which often culminate in systemic inflammatory response syndrome and septic shock. Although sepsis may be caused by various microorganisms, both Gram positive and Gram negative bacteria, recognized by either TLR2 or TLR4, are the most frequently isolated pathogens from the blood of septic patients. TLR overexpression in certain tissues during course of sepsis is not unexpected. Indeed, during sepsis and septic shock an enhanced TLR expression was already documented e.g. TLR2 and TLR4 in liver (Tsujimoto et al., 2005) or TLR2, TLR4 and TLR9 in peripheral blood (Brandl et al., 2005). This finding is in line with our previous results showing an upregulation of TLR2 and TLR4 in the adrenal glands of mice after injection of lipotechonic acid and LPS (Bornstein et al., 2004b; Zacharowski et al., 2006). In order to see whether an increased expression of TLRs in adrenocortical cells may impact adrenal steroidogenesis, we have stably transfected NCI-H295R cell with expression plasmid encoding either for TLR2 or TLR4-CD14 and tested basal and AII or FSK-stimulated hormone synthesis in these transfected cells. Upregulation of TLR2 results in enhanced basal and AII-mediated aldosterone

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synthesis, having no effect on cortisol or DHEA levels. Interestingly, upregulation of TLR4 in NCI-H295R cells strongly reduced aldosterone and cortisol synthesis at basal conditions and after stimulation with either FSK or AII. However, at the same time it showed no effect on DHEA production. These results suggest that overexpression of TLRs may differentially modulate adrenocortical steroidogenesis in a direct manner which is very interesting especially considering our *in vivo* data on TLR-upregulation in mouse adrenal glands during SIRS. Moreover, it shows that not only TLR4-deficiency but also TLR4 enhanced expression affects adrenal glucocorticoid production. This seemingly contradictory observation could be explained by differences between *in vivo* and *in vitro* conditions. TLR4-deficient mice have an increased basal cortisol production due to most probably elevated levels of proinflammatory cytokines and marked hyperplasia of the adrenal cortex (Zacharowski et al., 2006). We have tested NCI-H295R cells, since these cells are the only human adrenocortical cell line which expresses all enzymes necessary to synthesize three major adrenal steroids aldosterone, cortisol and DHEA (Rainey et al., 2004). However, using a malignant transformed cell line has some disadvantages such as insensitivity to adrenocorticotropic hormone action or reduced expression of certain TLRs (Kanczkowski et al., 2009). In fact we found reduced expression of TLR2 and abrogation of TLR4-CD14 in NCI-H295R cells compared to primary cell cultures (Kanczkowski et al., 2010). Since antibiotic selection or transfection conditions had minimal effect on the steroidogenesis of NCI-H295R cells transfected with TLR2 or an empty control plasmid; it is therefore unlikely that these conditions could be responsible for the observed TLR4-CD14-mediated aldosterone and cortisol reduction. Thus, one of the possible mechanisms involved in this effect may be reduced expression of enzymes involved in biosynthesis of cortisol (CYP11B1) and aldosterone (CYP11B2). Indeed, our results show that compared to MOCK or TLR2 transfected cells, NCI-H295R cells with enhanced expression of TLR4-CD14 had reduced capacity to induce the expression of both enzymes. Finally, reduced steroidogenesis may also result from impairment in transport and metabolism of the main steroid substrate cholesterol. To test this possibility, we analyzed the expression of 2 main enzymes involved in cholesterol utilization: StAR and CYP11A1. Although the mRNA levels of the latter enzymes did not differ between MOCK and TLR4CD14 transfected NCI-H295R cells, it was significantly reduced when compared to cells transfected with TLR2. This observation suggests that at least in part, the differences in aldosterone and cortisol synthesis between TLR2 and TLR4 upregulation may arise from the different expression level of the mentioned cholesterol metabolizing enzymes. We did not, however, find any correlation between the upregulation of TLR2 and StAR expression on protein level (data not shown). It has already been reported that LPS-mediated activation of TLR4 in primary rodent adrenocortical cells reduces basal and ACTH-mediated corticosterone synthesis; this effect was attributed to an increased level of reactive oxygen species (Garcia et al., 1999). Since the primary rodent adrenocortical cells may contain some amount of the immune cells, the involvement of the latter cells in reactive oxigen species-mediated steroidogenesis dysfunction could not be excluded. Therefore, our data using NCI-H295R cell line which is a pure

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population of adrenocortical cells could partially support the notion that LPS-mediated TLR4 upregulation in steroid producing cells may negatively impact adrenal steroidogenesis. Whether reactive oxygen species are involved in an observed by us TLR4-mediated downregulation of adrenal steroidogenesis is the subject of undergoing studies in our laboratory.

In conclusion, our results demonstrate that increased expression of TLR4 but not TLR2 in NCI-H295R cells can negatively impact adrenocortical cell hormone synthesis and expression of enzymes involved in steroidogenesis and cholesterol metabolism. In addition these data suggest that an overt activation of the TLR system in the steroid producing cells may be potentially involved in the development of the critical illness related corticosteroid insufficiency in the long-term course of sepsis.

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Figure 1. Generation of NCI-H295R clones with stable upregulation of TLR2 and TLR4-CD14.
(A) Western blot examination of TLR2 expression in TLR2- and MOCK-transfected NCI-H295R cells. TLR2 expression is strongly upregulated in both representative cell clones as compared with MOCK – control cells. **(B)** Densitometrerical analysis of Western blot results showing 3.75-fold (#89) and 3.25-fold (#102) upregulation of TLR2 protein expression in 2 representative NCI-H295R cell clones. Expression of TLR2 was adjusted to expression of tubulin.

Figure 2. The effect of TLR2 and TLR4-CD14 upregulation on adrenal gland steroidogenesis.
Stable upregulation of TLR2 in NCI-H295R cells resulted in an increased **(A)** basal and **(B)** angiotensin (AII)-induced aldosterone production. Conversely, TLR4-CD14 upregulation resulted in a decreased **(A)** basal and **(B)** FSK-induced aldosterone production and strongly reduced **(C)** basal and **(D)** FSK-mediated cortisol production. Neither TLR2 nor TLR4 upregulation did modulate adrenal **(E)** basal or **(F)** FSK-mediated production of DHEA as compared to MOCK control cells. Absolute values of hormone levels were evaluated in 3 different clones of NCI-H295R cells with the highest upregulation of either TLR2 or TLR4-CD14. Pooled values from three independent experiments are depicted as mean ± S.E.M. (*) $p<0.05$, (**) $p<0.01$.

Figure 3. Effect of TLR2 and TLR4-CD14 upregulation on the expression of steroidogenic enzymes and proteins involved in cholesterol utilization.
Stable upregulation of TLR4-CD14 but not TLR2 in NCI-H295R cells resulted in decreased **(A)** angiotensin II (AII) **(B)** and FSK-induced CYP11B2 and CYP11B1 gene expression. Relative values of CYP11B2 and CYP11B2 expression have been adjusted to the expression of house-keeping gene HPRT-1. Effect of TLR2 and TLR4 upregulation on the absolute mRNA levels of **(C)** CYP11A1 and **(D)** StAR genes normalized to expression of beta actin gene. Values from three independent experiments depicted as mean ± S.E.M. (*) $p<0.05$, (**) $p<0.01$.

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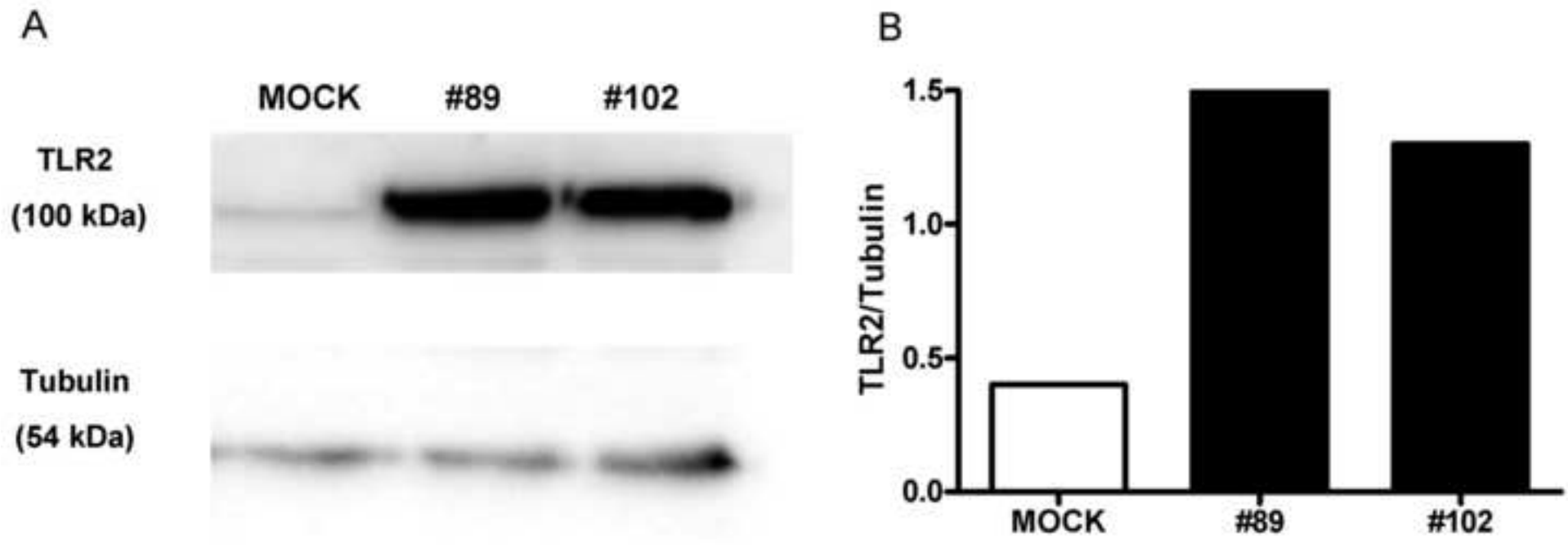

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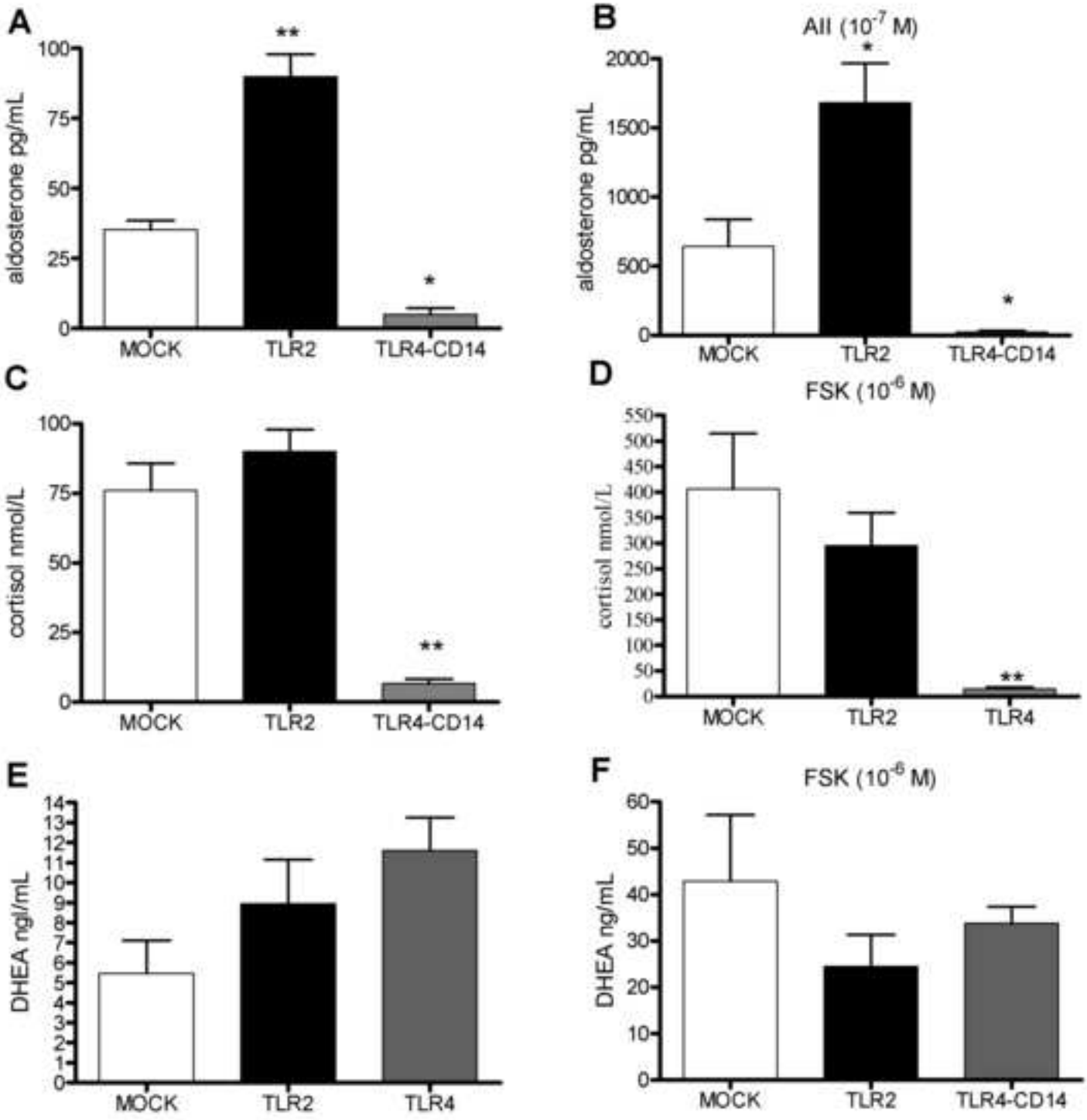
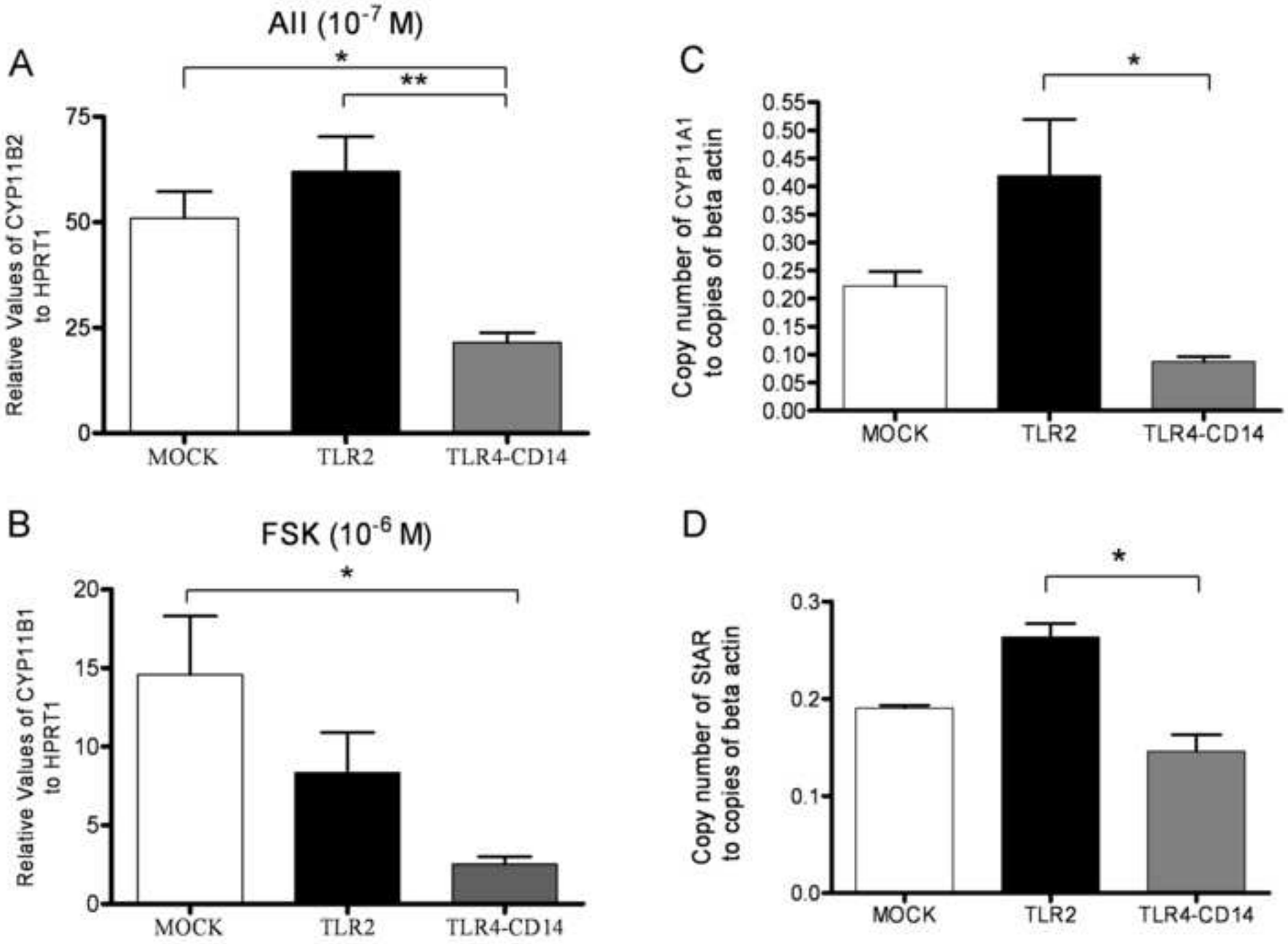
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