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Experimental Investigations

NLRs CHALLENGE IMPACTS TIGHT JUNCTION CLAUDINS IN SERTOLI CELLS

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АКТИВАЦИЯ NLRs ВОЗДЕЙСТВУЕТ НА КЛАУДИНЫ В ПЛОТНЫХ МЕЖКЛЕТОЧНЫХ КОНТАКТАХ В КЛЕТКАХ СЕРТОЛИ

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ABSTRACT

Aim: The present study aims to investigate the NALP3 system and its effect on claudins in Sertoli cells using a mouse adult Sertoli cell line as a model. We focus on the Sertoli cell biology looking for the possible implications for male reproductive functions. **Materials and methods:** Adult Sertoli cells were transfected with NAPL3 siRNA and treated with NOD1 (ie-DAP) and NOD2 (MDP) receptor ligands. Two dimensional gel electrophoresis was performed on lysates of non-challenged and MDP-treated Sertoli cells. **Results:** There were positive claudin-5 and claudin-11 expression levels on transcript (RT-qPCR) levels. Specific protein spots in 2D gels were detected after bioinformatics analysis. This study demonstrates direct induction of tight-junction proteins probably favouring junction stability. **Conclusions:** The innate immunity and tight-junction pathway integration probably have a protective role for both blood-testis immune barrier and spermatogenesis compartmentalisation maintained by the very same barrier. This integration also points the way for mechanistic research of the disturbances inflicted during an inflammatory response in the testis niche.

Key words: *Sertoli cells, tigbht-junctions, claudins, NALP3*

РЕЗЮМЕ

Цель: Целью данного исследования является прослеживание воздействия системы NALP3 на клаудины в клетках Сертоли с применением мышиной линии клеток Сертоли взрослой особи в качестве модели. Основными целями исследования являются биология клеток Сертоли с предполагаемым значением для репродуктивных функций мужчин. **Материалы и методы**: Сертоли клетки взрослой особи были трансфицированы малой интерферирующей РНК-а от NAPL3 и обработаны с применением рецепторных лигандов NOD1 (ie-DAP) и NOD2 (MDP). Разделение протеинов в лизатах необработанных и обработанных посредством MDP Сертоли клеток проводили с помощью двумерного гель-электрофореза. **Результаты:** Установлена позитивная экспрессия клаудина 5 и клаудина 11 на уровне транскриптов (RT-qPCR). Специфические протеиновые пятна в двумерных гелях были детектированы после биоинформатического анализа. Исследование установило прямую индукцию протеинов плотных контактов по всей вероятности усиливающих стабильность межклеточных контактов. **Выводы**: Интеграция сигнальных путей врождённого иммунного ответа и плотных межклеточных контактов вероятно осуществляет протективную роль как в отношении гематотестикулярного барьера, так и в отношении компартментализации сперматогенеза, поддерживаемой тем же самым барьером. Данная компартментализация указывает также направление механистических исследований нарушений, вызванных воспалительными ответами в области ниши стволовой клетки яичка.

Ключевые слова: *Сертоли клетки, плотные межклеточные контакты, клаудины, NALP3*

INTRODUCTION

The normal function of Sertoli cells is tightly connected to the reproductive status of males. Sertoli cells form tight-junctions (TJ) as well as other structures responsible for the selective permeability of the blood-testis barrier.[1] This barrier mechanically segregates germ cell autoantigens and creates a microenvironment that protects germ cells from both interstitial and ascending invading pathogens.[2] Innate immunity resembles an ancient signalling system that deeply impacts the fundamental functions of the cells, and it is currently becoming evident as being involved in cell fate, cell death, apoptosis and autophagy, as well as antigen presentation. Over the past few years the field of innate immunity has undergone a revolution with the discovery of pattern recognition molecules (PRMs) and pattern recognition receptors (PRRS) and their role in microbe detection. Signalling PRRs include the large families of membrane-bound Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs).[3] Several TLRs are expressed in Sertoli cells and can trigger testicular innate responses after activation by ligands. NLRs have emerged as key microbial sensors that participate in the global immune responses to pathogens and contribute to the resolution of infections. The system of NOD1, NOD2 receptors and their downstream targets – NFκB and NALP3 pathways are part of the inflammasome and are currently under study for being implicated in many autoimmune disease in men.[4]

AIM

The present study aims to investigate the role of the NOD1/2/NALP3 system in the Sertoli cells using a mouse Sertoli cell line as a model. The main goal was to validate whether this system is operational in Sertoli cells, and to look for a possible impact of its actions on their biology, with possible implications for male reproductive functions.

MATERIALS AND METHODS

Reagents: The minimal bioactive peptidoglycan motif common to all bacteria and NALP3 activator conjugated with FITC dye muramyl dipeptide (MDP, Ac-(6-O-stearoyl)-muramyl-Ala-D-Glu-NH$_2$) and γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) - a dipeptide present in the peptidoglycan (PGN) of all Gram-negative and certain Gram-positive bacteria, were purchased from Invivogen. Both ligands were found to be effective in 4 µg/ml in 24h for ie-DAP, and 20 µg/ml in 24h for MDP. These concentrations were used throughout the study.

Cell cultures induction with innate inflammatory ligands: The established Sertoli cell line 15P-1 (ATCC), derived from 6-month-old adult male transgenic mice was maintained at 32°C in DMEM media supplemented with 10% FBS, sodium pyruvate.

Mouse NALP3 siRNA transfection and knockdown: The gene silencing experiments were carried out using HiPerfect transfection reagent (Qiagen) and 5 nmol of DsiRNA, with the following sense sequence: 5'-AAC CUG CUU CUC ACA UGU CGU CUG UAC-3'. The knockdown efficiency was confirmed using RT-qPCR with specific mouse primer pair for NALP3 amplification, and the specificity was proved using a control - scrambled siRNA or death siRNA (Qiagen).

Real-time PCR analyses: Total RNA was isolated from 15P-1 Sertoli cells using RNeasy mini kit (Qiagen). First strand cDNA was synthesized using Sensiscript Reverse Transcriptase (Qiagen), by adding RT mix, random hexamers and oligo-dT (1:1) to 1 µg of total RNA per experimental condition, transcription for 1h at 42°C in a 20-µl reaction following the manufacturer's protocol. Quantitative amplification was performed on a MX3500P cycler real-time RT-PCR system (Stratagene). After setup of 20 µL DNAse-reaction mix containing primer set, 1 µL cDNA template and EvaGreen Mastermix (Geneaxxone), the PCR amplification was done using initial denaturation step at 94°C for 10 min, 45 cycles of denaturation (94°C for 15s), annealing (50°C for 30s), and synthesis (72°C for 30s) were performed. The melting curve of each PCR product was checked for specific amplification. Data were normalised for the expression of β-Actin (ACTB) in each experiment. Data are presented as relative expression (RE) = $2^{(\Delta CtC0 - \Delta CtSample)}$ with $\Delta Ct = Ct_{gene\ of\ interest} - Ct_{ACTB}$ (where Ct = threshold cycle), C_0 – referred to as "calibrator" (control subject), and Sample – referred to as "tested subject/specimen", following the ΔΔCt method. The method assumes equal amplification efficiency, and the last was optimised in a set of preliminary experiments. The primers were:

nlrp3: 5'-ACTGACCATCCCGCATAAGGAG-3'
5'- TGAGAGGCTGCCACAAACCTTC-3';
cldn5: 5'-AACTGCCGCGAACAGTTCCTAC-3'
5'-AGCAGGTGCCACTGGACATTAAG-3';
cldn11: 5'-TGCTGACTTGGGTGTGGATTGG-3'
5'-AGGCTTCCACTGTCGTTGGTAAC-3';
actb: 5'-CTGGGAGTGGGGGAGGC-3'
5'-TCAACTGGTCTCAGTG-3'

2D PAGE: The isoelectrofocusing was performed using strips with pH gradient 3-10. The pharmalytes (pH range 3-10, Pharmacia Fine Chemicals) were added to the rehydration buffer. The protein samples (non-treated and treated with MDP Sertoli cells) were loaded on the strips and the isoelectrofocusing was carried out on a Multiphore apparatus (Pharmacia Biotech) in two steps: 30 min at 500V, 14mA, 15W and 90 min at 2000V. The strips were equilibrated in equilibration buffer in two steps and the proteins were distributed by their molecular weights using SDS-PAGE method (Laemley) on 12% PAGE. The gels were silver stained, using silver staining kit (Amersham). The results are presented after the processing of the gel images using the software for 2D PAGE and DIGE analysis - ImageMaster 2D Platinum 7.0 DIGE (developed by the Swiss Institute of Bioinformatics in collaboration with GeneBio and GE Healthcare).

Statistical Analysis: The data were generated from three independent experiments, each performed in triplicates. One-way ANOVA tests with Dunnett multiple comparison post-hoc was used to compare the control group to differentially treated groups in the experiments. GraphPad Prism 6 (CA, USA) software used for the statistics in this study applied Geisser-Greenhouse correction for the *p* values for the main effect computing. P < 0.01 was considered significant.

RESULTS

Silver stained 2D PAGE gels from mouse Sertoli cells, non-treated (gel 1), or treated with MDP ligand (gel 2) were subjected to image software analysis (Fig. 1a, 1b). Corresponding spots from each gel were auto-numbered and spots intensity scatterplot showed several significant over-expressed putative protein targets. From the significantly different spots (Fig. 1c), only those deterministic for classifying the gels as either *non-treated* or *MDP-treated* were selected using Factor Analysis (Fig. 1d). Selected spots properties pI and MW and protein source species were used to predict the corresponding proteins as registered in the Swiss Prot 2D Gel database, using the web based tool TagIdent. The resulting predicted proteins were up to hundreds per a single
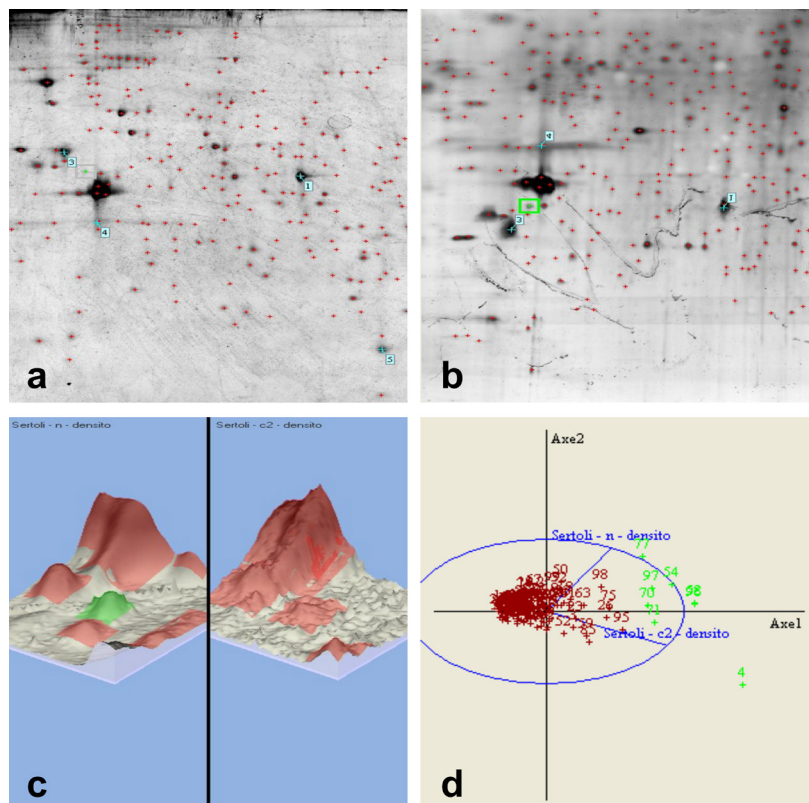

Figure 1. Silver stained gels from non-treated (gel 1, a), and MDP treated (gel 2, b) Sertoli cells, were subjected to 2D PAGE differential expression software image analysis (ImageMaster 2D Platinum). Corresponding spots from each gel were auto numbered and spots intensity (3D Gaussian fitting) showed several significant over expressed putative protein targets. (c) Factor Analysis was used to determine which of significantly different spots (evaluated by intensity, size, shape) are deterministic for the gel classification as either non-treated (n) or MDP treated (c2) (d).

Table 1. Predicted by TagIdent Proteins using differential spot properties (pI, MW), enriched signal pathways and predicted molecules mapped in ID - Spot IDs, P# - number of predicted by (pI, MW) proteins, C# - corresponding proteins (via DAVID

ID	P#	C#	Significant Enriched Pathways	Specific Molecules of Interest
4	5	3	positive regulation of transport; G-protein coupled receptor protein signaling pathway	tachykinin 4 (hemokinin)
54	637	333	SNARE interactions in vesicular transport; Allograft rejection; Antigen processing and presentation; Viral myocarditis; Type I diabetes mellitus; Autoimmune thyroid disease; Cell adhesion molecules (CAMs);	CD14; CD40; major histocompatibility complex; mannan-binding lectin serine peptidase 2; sialophorin;
58	530	332	G protein-coupled receptor; gluconeogenesis; peroxisome; lipid synthesis; glycolysis; Acyltransferase; Tight junction; immunoglobulin;	adipocyte-specific adhesion molecule; claudin-16; immunoglobulin superfamily, member 5;
70	439	220	Intermediate filament; phosphoprotein; gtp-binding; microtubule; coiled coil; Redox-active center; autophagy; acetylation; heparan sulfate; Apoptosis; voltage-gated channel; nucleotide-binding; transcription factor; potassium transport; potassium channel;	ATG4 autophagy related 4 homolog A; KIAA0652; beclin 1, autophagy related; potassium voltage-gated channel, shaker-related subfamily, members 2,6, 10
97	727	120	SNARE interactions in vesicular transport; Long-term depression; Allograft rejection; Type I diabetes mellitus; Tight junction; Endocytosis	guanine nucleotide binding protein (G protein); par-6 partitioning defective 6 homolog beta; protein phosphatase 2, catalytic subunit;
99	365	100	calcium ion binding; ATP-dependent protein binding; carbohydrate binding; ion channel activity; metal ion transmembrane transporter activity; passive transmembrane transporter activity; voltage-gated ion channel activity;	ATP binding domain 4; potassium channel tetramerisation domain containing 2;

spot. We used Functional and Pathway Enrichment software DAVID to infer the putative signal pathways affected by the ligand challenge (Table 1).

Following pathways enrichment we decided to investigate two claudins important for tight junction. Sertoli cells subjected to challenge with specific ligands – ie-DAP and MDP, were used for RT-qPCR assay (Fig. 2). Data were presented as the relative mRNA expression fold change, assessed using the ΔΔCt method, and normalised by the expression of the constitutive housekeeping gene Actin-β. We observed an increased gene expression of claudin-5 and -11 in Sertoli cells after ie-DAP treatment and after combinational treatment using both ligands. Low expression levels were detected after MDP treatment for both investigated claudins (Fig. 2).

The silencing of NALP3 in adult Sertoli cell line 15P-1 using transfection with validated efficiency DsiRNA against its exon 5 CDS resulted in decreased claudin-5 and -11 transcripts, compared to transfection with negative siRNA. The results showed up-regulation of claudin-5 and claudin-11 after combination of both ligands (ie-DAP and MDP) treatment and NALP3 siRNA silencing. Decreased levels of expression for claudin-5 and increased expression for claudin-11 after MDP treatment and NALP3 siRNA silencing were detected. The induction with ie-DAP after transfection with NALP3 siRNA resulted in non-changed mRNA levels for both investigated proteins (Fig. 3).

DISCUSSION

Sertoli cells nourish the developing sperm cells and act as phagocytes, consuming the residual cytoplasm during spermatogenesis. Infections of the testis are relatively rare in comparison with more distal tissues of the male reproductive tract.[2] This may be due to an increased reliance on innate immunity, and there has been a steady increase of interest in the role of innate immunity in testicular function
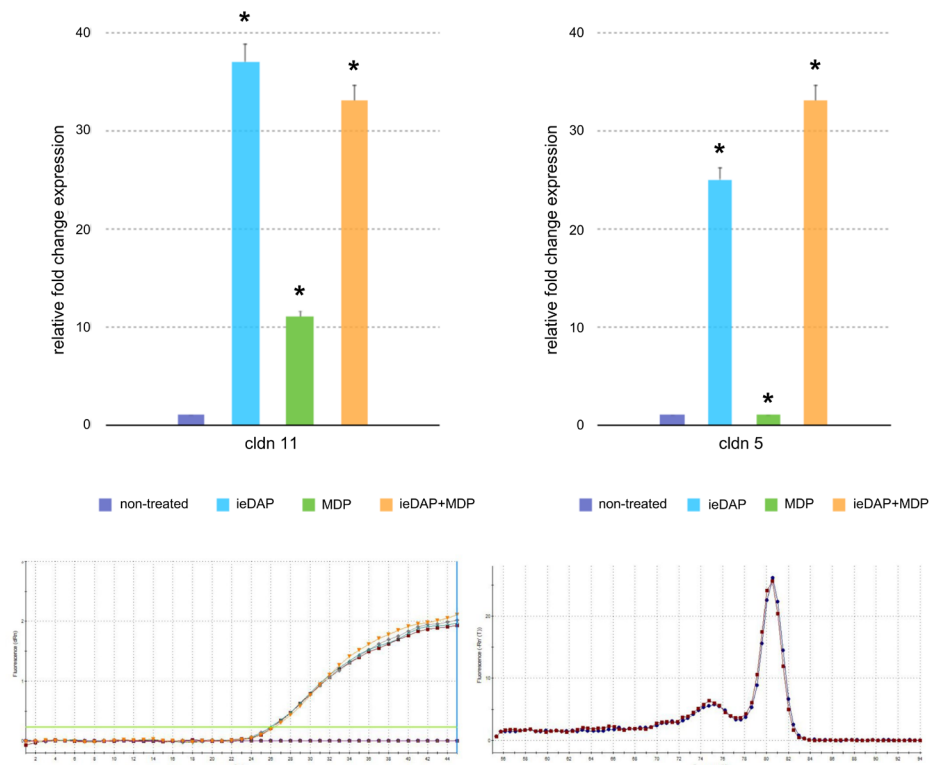
Figure 2. Differential impact on some of the major tight-junction protein mRNAs after challenge with ie-DAP and MDP, respectively. qPCR using transcript specific primers was performed. Y-axis represents the relative mRNA expression fold change, assessed using the ΔΔCt method, and normalised by the expression of the constitutive housekeeping gene Actin-β. In Sertoli cells, NOD1 ligand ie-DAP induction increased claudin-11, claudin-5 mRNAs compared to control cells. Typical Amplification curve (qPCR amplification cycle [n] vs. Signal Fluorescence [arbitrary units]) and Dissociation curve (Temperature [C] vs. Fluorescence (-Rn'(T))) of the gene of interest are presented.
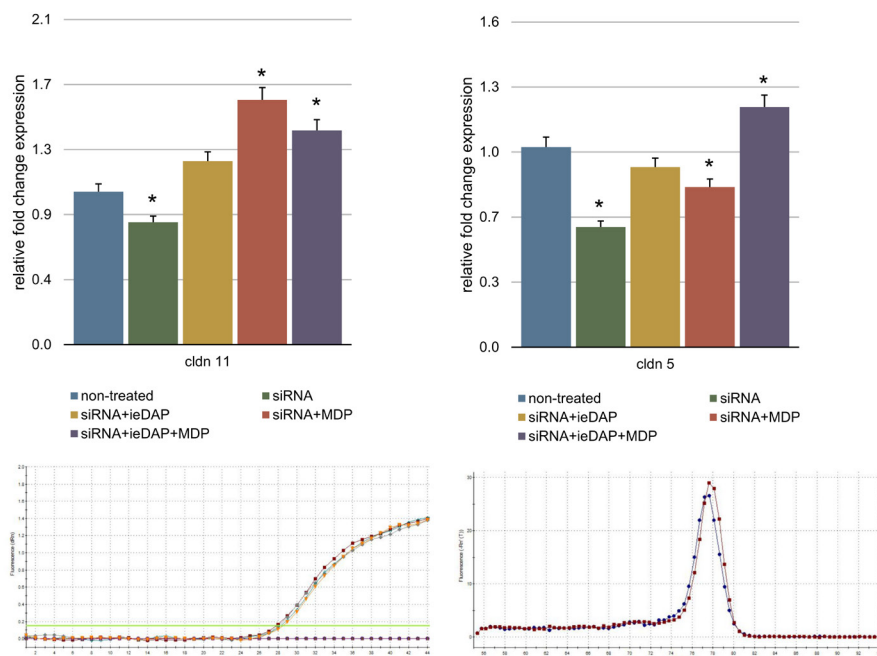

Figure 3. RT-qPCR analysis. The silencing of NALP3 in adult Sertoli cell line 15P-1 using transfection with validated DsiRNA against its exon 5 CDS, resulted in a decrease of the tight-junction proteins claudin-11 and claudin-5 mRNAs, while induction with either ie-DAP or MDP had differential effect on claudin-11 vs. claudin-5 mRNA levels; the induction using concomitant incubation with both ligands upregulated claudin-11 molecules. Typical Amplification curve (qPCR amplification cycle [n] vs. Signal Fluorescence [arbitrary units]) and Dissociation curve (Temperature [C] vs. Fluorescence (-Rn'(T))) of the gene of interest are presented.

recently.[5-7] The system of NOD1, NOD2 receptors and their downstream targets – NALP3 are part of the inflammasome and have been implicated so far in many autoimmune diseases in men.[8] Claudins and occludin were the first tight-junction integral membrane proteins that were identified.[9] In our study, the blood-testis barrier crucially important tight-junction proteins – claudin-5 and claudin-11 were differentially influenced upon pro-inflammatory pathways induction by innate immunity NOD1/2 stimuli. NOD1 receptor ligand ie-DAP was more effective on modulating both investigated molecules. Interesting phenomenon was observed after NALP3 silencing with combination with additional stimuli (MDP and ie-DAP). NOD2 receptor ligand MDP caused different functional behaviour of claudin-5 and claudin-11. This study demonstrates direct induction of TJ proteins probably favouring junction stability. Interestingly, this is only possible under steady state NALP3 expression, suggesting that pro-inflammatory signalling is closely interconnected with cell motion and cell-cell interaction behaviour pathways. Switching off the inflammasome signalling actually deregulates the production of TJ proteins.

CONCLUSIONS

The modulation of tight-junction pathways by innate immunity although serving a protective role to some extent points the way for mechanistic explanation of how the junction deregulation could impact both testis immune barrier and spermatogenesis compartmentalisation maintained by the very same barrier.

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