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Alpha 1 adrenergic receptor-mediated inflammatory responses in human testicular peritubular cells

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ABSTRACT

Stress activates the sympathetic nervous system and is linked to impaired fertility in man. We hypothesized that catecholamines by acting on testicular cells have a role in these events, possibly by fostering an inflammatory environment. The cells of the wall of seminiferous tubules, human testicular peritubular cells (HTPCs), express adrenergic receptors (ADRs) *α1B, α1D, β1* and *β2*. A selective α1-ADR agonist, phenylephrine, increased intracellular Ca^{2+}-levels in cultured HTPCs and induced *COX-2*, *IL-6* and *MCP-1* mRNA expression without affecting *IL-1β* mRNA. These changes were paralleled by a significant increase in the secretion of IL-6 and MCP-1. Epinephrine was also effective, but salbutamol, a selective β2-ADR agonist was not. Our results suggest that stress-associated elevation of catecholamines may be able to promote inflammatory events by targeting peritubular cells in the human testis. Blockage of α1-ADRs may therefore be a novel way to interfere with stress-related impairment of male reproductive functions.

1. Introduction

Stress involves activation of the hypothalamus-pituitary-adrenal axis, leading to elevated levels of glucocorticoids and to the activation of the sympathetic nervous system (Chrousos, 2009). Human adrenal medullary cells produce and release epinephrine (80%) and norepinephrine (20%) into the bloodstream (Gerra et al., 2001; Scanzano and Cosentino, 2015; Schoder et al., 2000). Normally, catecholamine blood levels are reported in the low nanomolar range (Goldstein et al., 2003) but during stress they reach 0.53–1 nM (epinephrine) and 3.60–10 nM (norepinephrine) (Clutter and Cryer, 1980; Paran et al., 1992; Sofuoglu et al., 2001). Corresponding concentrations in different organs are not well established. While Campos et al. (1990) described catecholamine levels in different rodent testicular compartments, and Mayerhofer et al. (1996) reported on norepinephrine in rhesus monkey testes, to our knowledge, human testicular concentrations are not known.

Several testicular cells possess adrenergic receptors and thus are potential targets of catecholamines (Patrao et al., 2008). For example, alpha adrenergic receptors (α-ADRs) and beta adrenergic receptors (β-ADRs) were described in Leydig cells, Sertoli cells, early spermatocytes and myoid cells of the seminiferous tubules of different species (Huo et al., 2012; Jacobus et al., 2005; Mayerhofer et al., 1991, 1993; Miyake et al., 1986; Skinner and Heindel, 1990; Stojkov et al., 2014). *In vitro* studies mainly performed in animal testicular tissues or isolated cells with selective adrenergic agonists or antagonists, indicated that α-ADRs and β-ADRs regulate for example androgen production in Leydig cells (Frungieri et al., 2000, 2002a; Huo et al., 2012; Mayerhofer et al., 1989, 1993, 1996, 1999; Mhaouty-Kodja et al., 2007; Stojkov et al., 2012).

Little is known about human testicular ADRs and their roles, but

Abbreviations: ADRs, adrenergic receptors; ATP, adenosine triphosphate; COX-2, cyclooxygenase 2; ERK1/2, extracellular signal-regulated kinase; HTPCs, human testicular peritubular cells; IDO, indoleamine 2,3- dioxygenase; IL, interleukin; LMD, laser microdissection; TLRs, toll like receptors; MAPKs, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein 1; PTX 3, pentraxin 3; TNF-α, tumor necrosis factor α.

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there is evidence that the cells forming the wall of the human seminiferous tubule, express α- and β-ADRs. These peritubular ("myoid") cells, have characteristics of smooth muscle cells (Maekawa et al., 1996; Mayerhofer, 2013). Miyake et al. (1986) monitored the intra-tubular pressure changes *in vitro* and concluded that isolated human seminiferous tubules are capable of undergoing contractions specifically after norepinephrine stimulation. In the testis, peritubular cells are in close proximity to sympathetic nerve fibers and microvessels, i.e. the sources of norepinephrine and epinephrine. These catecholamines therefore are likely physiological regulators of contractions and sperm transport.

Human testicular peritubular cells (HTPCs) are also the only human testicular cell type that can be isolated, cultured and studied *in vitro* (Albrecht et al., 2006; Mayerhofer, 2013). Results from recent studies indicate that their roles go beyond sperm transport and include immunological functions. They express, for example, Toll-like receptors (TLRs), which can be activated by extracellular matrix factors, specifically biglycan. They produce pro-inflammatory factors, such as interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1) and others, and they are targets e.g. for tumor necrosis factor α (TNF-α) (Albrecht et al., 2006; Mayer et al., 2016; Mayerhofer, 2013; Schell et al., 2008). Consequently, via inflammatory factors, peritubular cells may be involved in inducing and/or maintaining a state of inflammation, which is observed frequently in the testes of infertile patients and which may be a key factor in male infertility.

We explored, which ADR subtypes are present in human testicular peritubular cells, and investigated the potential impact of catecholamines on inflammatory processes in these testicular cells.

2. Materials and methods

2.1. Human testicular peritubular cells isolation/cell culture experiments

Human Testicular Peritubular Cells (HTPCs) were isolated from testicular biopsies of men with normal spermatogenesis, as previously described (Albrecht et al., 2006; Schell et al., 2008). The local Ethics Committee has approved the study (Ethikkommission, Technische Universität München, Fakultät für Medizin, München, project number 5158/11) and the patients gave written informed consent. All methods for this study were carried out in accordance with the approved guidelines. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose + 10% foetal calf serum (FCS; both from GE Healthcare; Freiburg, Germany) + 1% penicillin/streptomycin (PANBiotech; Aidenbach, Germany) under standard conditions (37 °C, 5% CO_2, 95% humidity) to 90–100% confluence, unless indicated otherwise, and were used from passages 5–12. Stimulations with epinephrine (1 μM), phenylephrine (10 μM), salbutamol (10 μM) or prazosin (100 nM, 1 μM and 10 μM) (all from Sigma-Aldrich, Deisenhofen, Germany) were performed in FCS-free medium for 24 h for cell viability assays, live cell imaging studies and/or human XL cytokine array. Stimulations with epinephrine (1 μM), phenylephrine (10 μM), salbutamol (10 μM) or prazosin (100 nM) were performed in FCS-free medium for 3 h for qPCR experiments and for 6 h for IL-6 and MCP-1 immunoassays. Ascorbic acid (Sigma-Aldrich) was used as a solvent for epinephrine, phenylephrine, salbutamol and prazosin and was included in all controls (basal) as well.

2.2. Human testicular biopsies and immunohistochemistry

Human testicular biopsies of men with normal spermatogenesis were fixed in Bouin's solution, embedded in paraffin and processed for immunohistochemical studies, as previously described (Welter et al., 2013). A rabbit polyclonal anti-alpha 1 adrenergic receptor (α1-ADR) serum (1:100; Abcam, Cambridge, United Kingdom) was used to detect the immunological expression of α1-ADR. Negative controls included omission of the primary antibody and incubation with non-immune serum. The samples were slightly counterstained with hematoxylin to reveal cellular details and spermatogenesis.

2.3. RT- PCR and qPCR

Total RNA from cultured HTPCs and three testicular biopsies (serving as a positive control) was prepared as described (Windschuttl et al., 2015) using the Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany). A total amount of 500 ng of RNA was subjected to reverse transcription, using random primers (15-mer) and SuperScript II Reverse Transcriptase, 200 U/μl (Invitrogen GmbH, Darmstadt, Germany). PCR steps consisted of pre-incubation at 95 °C for 150 s, followed by 30–40 cycles of 94 °C for 60 s, 55–60 °C (annealing temperature) for 60 s, 72 °C for 60 s, and a final incubation at 72 °C for 300 s. For information on primer sequences, see Table 1. For control purposes either RT was omitted or water was used instead of cDNA. PCR amplification using nested primers was used when required. PCR products were visualized by Midori Green Advance DNA (Nippon Genetics Europe GmbH, Dueren, Germany) staining in 2% agarose gels and the amplified products were sequenced to verify their correct identity (GATC, Konstanz, Germany).

The qPCR studies were performed as published in Windschuttl et al. (2015). The reactions were conducted using the QuantiFast SYBR Green PCR Master Mix 2x (Qiagen). The mathematical model of Pfaffl (2001) was used to determine the relative levels of mRNA expression for each sample as previously described (Rossi et al., 2016). The sequences of the primers used for qPCR are described in Table 1.

2.4. Laser microdissection (LMD)

Paraffin-embedded human testicular biopsy sections (5 μm) were mounted onto a thin polyethylene naphthalene membrane, as previously described (Frungieri et al., 2002b). The sections were deparaffinized, briefly stained with hematoxylin and subjected to laser microdissection (LMD; P.A.L.M. GmbH Mikrolaser Technologie, Bernried, Germany) to excise the tubular wall and then the material was catapulted into a microcentrifuge tube. The isolated material was kept frozen (−80 °C) until use. The extraction of RNA was performed using the RNeasy Micro Kit (Qiagen) and subjected to RT-PCR using the Sensiscript Reverse transcription Kit (Qiagen). For information about primer sequences see Table 1. A second PCR amplification using nested primers was performed when required. PCR products were visualized by Midori Green Advance DNA (Nippon Genetics Europe GmbH) staining in 2% agarose gels and the amplified products were sequenced to verify their identity (GATC, Konstanz, Germany).

2.5. Viability assay

Metabolic activity was determined by measuring the cellular amounts of ATP using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany). The ATP levels are directly proportional to the number of metabolically active cells, meaning viable cells in culture. Approximately 10×10^3 HTPCs per well were plated on 96 well plates in sextuples and incubated overnight in DMEM + 10% FCS. The stimulation was performed in DMEM without phenol red and without FCS, in the presence or absence of

Table 1
Information about sense (forward) and antisense (reverse) oligonucleotide primers and amplicon size used for RT-PCR and RT-qPCR experiments.

Gene	Primer-sequence (5′–3′)	Amplicon size	Protocol
18S[a]			
Forward	5′- GGA GGT TCG AAG ACG ATC AG -3′	149 bp	RT-PCR
Reverse	5′- TCA GCT TTG CAA CCA TAC TCC -3′		
α1A-ADR		339 bp	RT-PCR
Forward	5′- GGC TCC TTC TAC CTG CCT CT -3′		
Reverse	5′- AGG GCT TGA AAT CAG GGA AG -3′		
α1B-ADR (first set)		148 bp	RT-PCR
Forward	5′- TCA TCT TGT GCT GGC TAC CC -3′		
Reverse	5′- GCT GGA GCA TGG GTA GAT G -3′		
α1B-ADR (second set)		79 bp	RT-PCR
Forward	5′- TTC TTC ATC GCT CTA CCG CT -3′		
Reverse	5′- AGC CAG AAC ACC ACC TTG AA -3′		
α1D-ADR		115 bp	RT-PCR
Forward	5′- TCT GCT GGT TCC CTT TCT TC -3′		
Reverse	5′- CAC GCA GCT GTT GAA GTA GC -3′		
α2A-ADR		689 bp	RT-PCR
Forward	5′- AAA CCT CTT CCT GGT GTC TC -3′		
Reverse	5′- GAC GAG CTC TCC TCC AGG T -3′		
α2B-ADR		627 bp	RT-PCR
Forward	5′- TGG CCT CCA GCA TCG GAT -3′		
Reverse	5′- CAG AGC ACA AAA ACG CCA -3′		
α2C-ADR		572 bp	RT-PCR
Forward	5′- GTG GTG ATC GCC GTG CTG A -3′		
Reverse	5′- GTT TTC GGT AGT CGG GGA C-3′		
β1-ADR (first set)		201 bp	RT-PCR
Forward	5′- CCC ACA ATC CTC GTC TGA AT -3′		
Reverse	5′- TCA CAC GCA CAC ACA AAA GA -3′		
β1-ADR (second set)		79 bp	RT-PCR
Forward	5′- GCA AAG AGA AAA GCC ACG GA -3′		
Reverse	5′- CAA GGA ACA TCA GCA AGC CAC -3′		
β2-ADR		287 bp	RT-PCR
Forward	5′- CAC AGC CAT TGC CAA GTT CG -3′		
Reverse	5′- CGG GCC TTA TTC TTG GTC AG -3′		
β3-ADR		122 bp	RT-PCR
Forward	5′- CCT GGC TGT GAC CAA C -3′		
Reverse	5′- ACT GGC TCA TGA TGG GC -3′		
RPL19[b]		198 bp	qPCR
Forward	5′- AGG CAC ATG GGC ATA GGT AA -3′		
Reverse	5′- CCA TGA GAA TCC GCT TGT TT-3′		
COX2		131 bp	qPCR
Forward	5′- CTT ACC CAC TTC AAG GGA -3′		
Reverse	5′- GCC ATA GTC AGC ATT GTA AG -3		
IL-6		158 bp	qPCR
Forward	5′- AAC CTG AAC CTT CCA AAG ATG G -3′		
Reverse	5′- TCT GGC TTG TTC CTC ACT ACT -3′		
IL-1β		75 bp	qPCR
Forward	5′- TAC CTG TCC TGC GTG TTG AA -3′		
Reverse	5′- TCT TTG GGT AAT TTT TGG GAT CT -3′		
MCP-1		108 bp	qPCR
Forward	5′- GCC TCC AGC ATG AAA GTC TC -3′		
Reverse	5′- AGG TGA CTG GGG CAT TGA T -3′		

[a] 18S: 18S ribosomal RNA.
[b] RPL19: ribosomal protein L19.

epinephrine (10 nM, 100 nM, 1 μM and 100 μM), phenylephrine (10 μM), salbutamol (10 μM) or prazosin (100 nM, 1 μM and 10 μM) for 24 h. ATP assays were repeated at least three times in different patients.

2.6. Cell counting

With an automated cell counting device (Casy System, Schärfe Systems, Reutlingen, Germany) the total number of cells was calculated. After 24 h of incubation the cells were trypsinized and measured following the manufacturer's instructions.

2.7. Live cell imaging studies

HTPCs were grown overnight in a culture dish (μ-Dish, Ø 35 mm, high; Ibidi, Martinsried, Germany) under standard conditions. Then, cells were incubated in the presence or absence of epinephrine (1 μM) in DMEM without phenol red and without FCS in a humid atmosphere with 5% CO_2 at 37 °C for 24 h. Optimal incubation conditions (5% CO_2, 37 °C, 95% relative humidity) were created by using an Ibidi heating system and an Ibidi gas incubation system. Time-lapse series were generated by taking a picture every 20 min for 24 h with a ProgRes MF camera (Jenoptik, Jena, Germany), the Micro-Manager 1.3 Microscopy Software (Ron Vale's Laboratory at UCSF, San Francisco, CA, USA) and a transmitted light microscope (Axiovert 135; Zeiss, Oberkochen, Germany).

2.8. Measurements of intracellular Ca^{2+} concentrations

Approximately 6–10x10^4 HTPCs were incubated overnight in

Ibidi μ-Dishes for 24 h. Briefly, the medium was replaced by fresh DMEM + 10% FCS containing 5 μM Fluoforte (Enzo Life Sciences (ELS) AG, Lausen, Switzerland) and cells were loaded for 45 min at 37 °C and 5% CO_2. Then, the cells were washed in PBS and measured in serum-free DMEM. Acute stimulation was performed every 4 min for 30 s. Changes in fluorescence intensity were monitored for 20 min every 2 s at excitation and emission wavelengths of 488 nm and 520 nm, respectively with a confocal microscope (Axiovert 200M; Zeiss, Göttingen, Germany) and the program AIM 4.2 (Carl Zeiss Laser Scanning microscope 510). Ca^{2+} measurements were performed with cells of three different patients.

2.9. IL-6 and MCP-1 immunoassays

ELISA measurement of IL-6 (Affymetrix eBioscience, CA USA) and MCP-1 (R&D Systems, Wiesbaden-Nordenstadt, Germany) were performed following the manufacturer's instructions. The levels of IL-6 and MCP-1 in the culture media were determined after 6 h of incubation with epinephrine (1 μM), salbutamol (10 μM), phenylephrine (10 μM), with or without prazosin (100 nM). For IL-6 the minimum detectable immunoassay concentration was 0.92 pg/ml. Intra-assay and inter-assay coefficient of variation were less than 5.2% and 3.4%, respectively. For MCP-1 the minimum detectable immunoassay concentration was 1.7 pg/ml. Intra-assay and inter-assay coefficient of variation were less than 5.0 % and 5.1 %, respectively. For immunoassays, samples from four different patients were used and the levels of IL-6 and MCP-1 were expressed as femtogram (fg)/μg protein.

2.10. Proteome profiler human XL cytokine array

The human XL cytokine array (R&D Systems) is a membrane based sandwich immunoassay. Capture and control antibodies are spotted in duplicate on nitrocellulose membranes. The capture antibodies on nitrocellulose membranes bind to specific target proteins present in the HTPCs culture media after 24 h of incubation, in the presence or absence of phenylephrine (10 μM). Captured proteins were detected with biotinylated detection antibodies and then visualized using chemiluminescent detection reagents. The signal detected at each spot corresponded to the amount of protein bound. The average signal (pixel density) was quantified by densitometry, then the averaged background was subtracted and density was normalized to the control conditions (basal) per microgram (μg) of protein. The average signal of each spot was quantified by densitometry using ImageJ (U.S. National Institutes of Health, Bethesda, MA, USA, http://imagej.nih.gov/ij/). Data are expressed as mean of each duplicate spot.

2.11. Data analysis and statistics

Data analyses were performed using GraphPad Prism® 5.03 (GraphPad Software, San Diego, CA, USA). Statistical analyses were performed using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons, or Student's *t*-test for comparison of two groups. Data are expressed as mean ± S.E.M. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Adrenergic receptor expression in human testes, human peritubular wall and HTPCs

RT-PCR experiments revealed the expression of *α1A-, α1B-, α1D-, β1- β2-*and *β3-ADRs* in human testes biopsies (Fig. 1A). In HTPCs we detected *α1B-, α1D-, β1-*and *β2-ADRs*. These were also detectable in tubular wall samples, isolated by LMD from men with normal spermatogenesis (Fig. 1A and B). Neither in cultured HTPCs, nor in whole human testis, did we find evidence for *α2A-, α2B-, α2C-ADRs* mRNA (data not shown). Immunohistochemistry revealed the expression of α1-ADR in several testicular cells, including Leydig cells and peritubular cells (Fig. 1C). These results imply that catecholamines, namely epinephrine and norepinephrine, can target among others testicular peritubular cells.

3.2. Functional α1-adrenergic receptors in HTPCs

To analyze functionality of α1-ADR in HTPCs we monitored intracellular Ca^{2+} levels. The selective α1-ADR agonist phenylephrine (10 μM) rapidly and transiently increased intracellular Ca^{2+} levels (Fig. 2A). Epinephrine (1 μM) also increased intracellular Ca^{2+} levels (Supplementary Fig. 1). The specificity of the phenylephrine action was confirmed by the action of α1-ADR inverse agonist prazosin (10 μM), which prevented the phenylephrine-induced elevation in Ca^{2+} (Fig. 2B).

3.3. Effects of epinephrine, phenylephrine and salbutamol on cell viability in HTPCs

HTPCs were incubated for 24 h in the presence of different concentrations of epinephrine (10 nM, 100 nM, 1 μM and 10 μM) and the results revealed that epinephrine exerted a trophic effect and increased ATP levels (Supplementary Fig. 2A). Live cell imaging performed in the presence or absence of epinephrine (1 μM) for 24 h, showed no obvious changes in the cellular morphology of HTPCs (data not shown). Incubation studies performed for 24 h showed that not only epinephrine (1 μM), but also the selective agonist of α1-ADR phenylephrine (10 μM) and the selective agonist of β2-ADRs salbutamol (10 μM) increased cell viability (Supplementary Fig. 2B), as well as cell number (Supplementary Fig. 2C; control 100%, epinephrine 1 μM: 132 ± 3.5* %, salbutamol 10 μM: 190.5 ± 1.2* %, phenylephrine 10 μM: 279.5 ± 0.27* %; n = 3, *$p<0.05$). These results indicate that epinephrine, phenylephrine and salbutamol, at the concentrations used in this study, are not toxic for HTPCs, but rather have trophic actions. Prazosin at all concentrations tested (100 nM, 1 μM and 10 μM) for 24 h did not influence ATP levels (Supplementary Fig. 2D).

3.4. Role of epinephrine and phenylephrine on inflammatory state in HTPCs

HTPCs incubated in the presence of epinephrine (1 μM) or in the presence of the selective α1-ADR agonist phenylephrine (10 μM) for 3 h significantly increased mRNA expression levels of *COX-2, IL-6* and *MCP-1*, but did not change *IL-1β* mRNA expression levels (Fig. 3). HTPCs incubated for 3 h in the presence of the selective β2-ADR agonist salbutamol (10 μM), did not show changes in the mRNA expression levels of *COX-2, IL-6, MCP-1* and *IL-1β* (Fig. 3). These results suggest that the stimulatory effect of epinephrine on the mRNA expression levels of the pro-inflammatory factors *COX-2, IL-6*, and *MCP-1* is exerted via α1-ADR.

In order to further test this hypothesis, HTPCs were incubated for 3 h in the presence or absence of phenylephrine (10 μM) with or without the inverse agonist of α1-ADR prazosin (100 nM). The results showed that prazosin completely abolished the stimulatory effect of phenylephrine on *COX-2, IL-6*, and *MCP-1* mRNA expression levels (Fig. 4).

Increased *IL-6* and *MCP-1* mRNA expression levels correlated with increments in the protein levels of IL-6 and MCP-1 in the culture media after 6 h of incubation in the presence of epinephrine (1 μM) or the selective α1-ADR agonist phenylephrine (10 μM) as

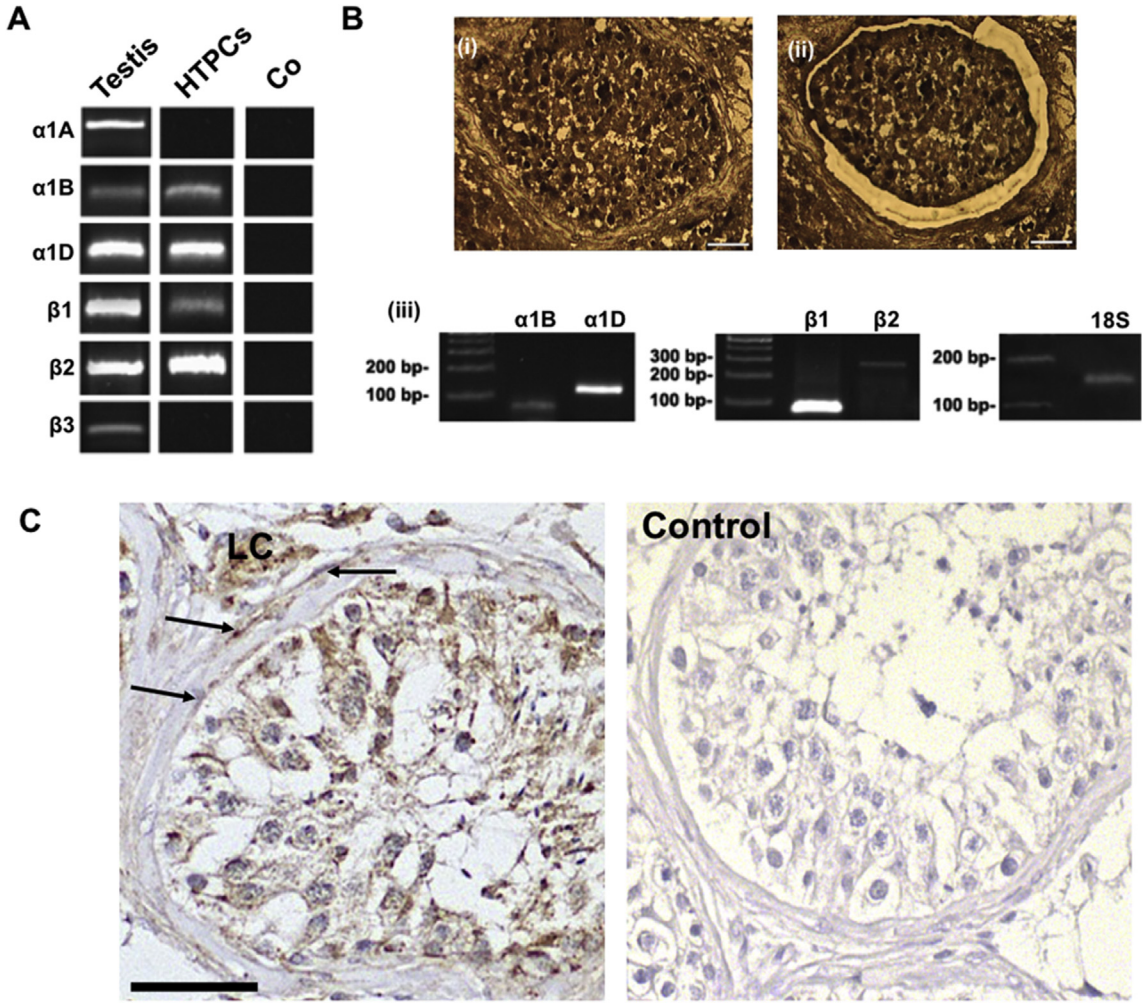

Fig. 1. Human testis, HTPCs and cells of the tubular wall of seminiferous tubules express adrenergic receptors.
(A) Adrenergic receptors (ADRs) subtype α1A-ADR (339 bp), α1B-ADR (148 bp), α1D-ADR (115 bp), β1-ADR (201 bp), β2-ADR (287 bp) and β3-ADR (122 bp) were detected in a human testicular biopsy sample. In addition, human testicular peritubular cells (HTPCs) express α1B-ADR (148 bp), α1D-ADR (115 bp), β1-ADR (201 bp) and β2-ADR (287 bp) detected by RT-PCR. (B) Cells in the tubular wall (i) areas excised by laser microdissection (LMD; ii) and subjected to RT-PCR studies (iii) also express α1B-ADR (79 bp), α1D-ADR (115 bp), β1-ADR (79 bp) and β2-ADR (287 bp) mRNA. Scale bar: 40 μm. After sequencing the amplicons they were shown to correspond to human α1B-ADR, α1D-ADR, β1-ADR and β2-ADR. Control (Co) was run without mRNA input and 18S ribosomal RNA (18S; 149 bp) was used as the reference gene in RT-PCR reaction. The gel shows results obtained from one of five different patients examined that yielded comparable results.
(C) Immunohistochemistry of α1-ADR in biopsies of patients with normal spermatogenesis showed positive staining in Leydig cells (LC), cells of the germinal epithelium and of peritubular cells (black arrows). Control omitted the first antiserum. Scale bar: 50 μm.

measured by immunoassays (Fig. 5). Finally, experiments performed in HTPCs isolated from two different patients showed that the phenylephrine (10 μM) induced IL-6 secretion into the cell culture medium was blocked by the inverse agonist of α1-ADR prazosin (at 100 nM; 6 h of incubation; Supplementary Fig. 3).

To explore whether other pro-inflammatory factors may be secreted by HTPCs, a proteome profiler study was performed using the culture media of HTPCs incubated with or without phenylephrine (10 μM) for 24 h. The obtained results confirmed the data on IL-6 and MCP-1 and, in addition, showed increased levels of Pentraxin 3 (PTX 3) after phenylephrine treatment. Again, IL-1β was not altered and other cytokines were not measurably affected (Supplementary Fig. 4).

4. Discussion

In this study, we provide evidence that epinephrine can promote the secretion of pro-inflammatory factors via acting on α1-ADR of human peritubular cells. In the human testis, the peritubular cell compartment is innervated and in close proximity to microvessels. Stress evokes an increase in the release of norepinephrine from nerve endings (Rivier and Rivest, 1991) and in increased levels of circulating catecholamines. Hence, we propose a link between stress-related elevations of adrenergic neurotransmitters and testicular inflammatory events, which may lead to impairment of male fertility.

A previous report (Miyake et al., 1986) revealed functional ADRs in the cells of human seminiferous tubules. The study concluded that they are involved in the regulation of contractility. The ADR subtypes were not studied previously, but three major types of ADRs exist, alpha1 (α1-ADR), alpha 2 (α2-ADR) and beta (β-ADR). Each type is further divided into subtypes, namely α1A-ADR, α1B-ADR, α1D-ADR, α2A-ADR, α2B-ADR, α2C-ADR, β1-ADR, β2-ADR and β3-ADR (Dixon et al., 1986; Langer, 1998; McCune et al., 1993; Nagatomo and Koike, 2000; Scanzano and Cosentino, 2015). In human testicular samples, we now found *α1A-, α1B-, α1D-, β1-, β2* and *β3*-ADRs. Moreover, cultured HTPCs, as well as, the cells of the tubular wall isolated by LMD, express the ADR subtypes *α1B, α1D, β1* and *β2*.

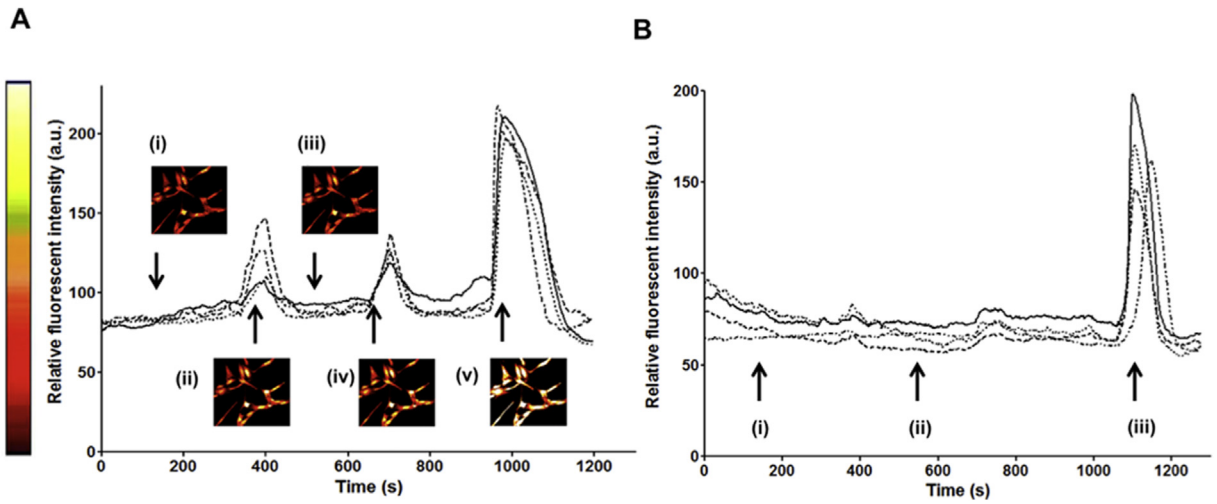

Fig. 2. *HTPCs respond to the selective α1-ADR agonist phenylephrine (10 μM) with transient intracellular Ca^{2+} elevation.*
(A) A representative graph on the response of HTPCs to Ca^{2+} concentration shows that phenylephrine (10 μM) led to an increase in intracellular Ca^{2+} levels. Color changes from red to yellow represent increased intracellular Ca^{2+} concentrations. Line plot graph shows the time-course of intracellular Ca^{2+} changes for four representative cells sequentially treated with medium (basal conditions; i and iii), 10 μM phenylephrine (ii and iv), and 0.005% trypsin (v) used as a positive control.
(B) A representative graph on the response of HTPCs to Ca^{2+} concentration shows that prazosin, the inverse agonist of α1-ADR (10 μM) blocked the increased intracellular Ca^{2+} levels. Line plot graph shows the time-course of intracellular Ca^{2+} changes for four representative cells sequentially treated with 10 μM phenylephrine +10 μM prazosin (i), 10 μM phenylephrine (ii) and 0.005% trypsin (iii). Data are expressed as relative fluorescence intensities corresponding to a pseudo color scale from red to yellow in arbitrary units (a.u.). All experiments were performed with cells from three different patients.

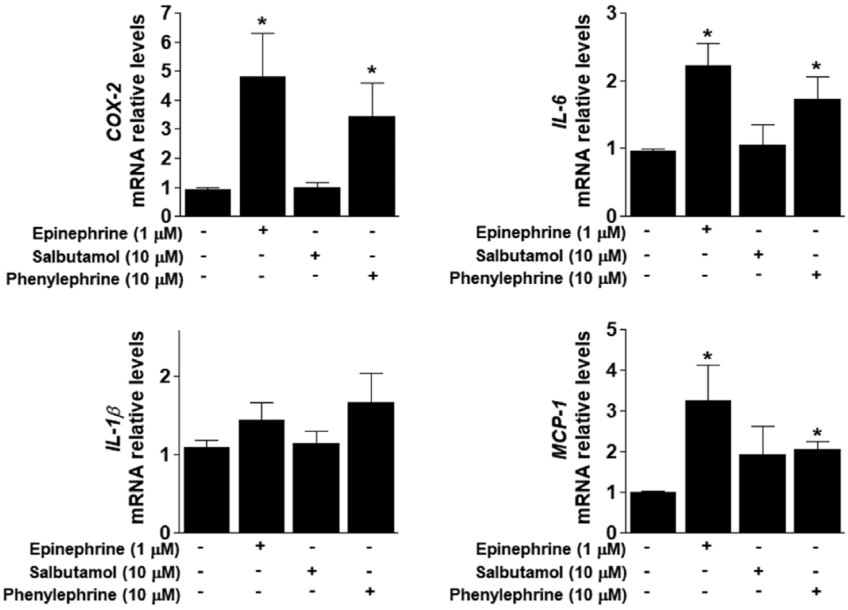

Fig. 3. *Stimulatory effect of epinephrine and phenylephrine on inflammatory factors mRNA expression levels in HTPCs.*
COX-2, *IL-6*, *IL-1β* and *MCP-1* mRNAs were analyzed in HTPCs incubated in the presence or absence of epinephrine (1 μM), the selective β2-ADR agonist salbutamol (10 μM) or the selective α1-ADR agonist phenylephrine (10 μM) for 3 h. A significant increase in the levels of *COX-2*, *IL-6* and *MCP-1* was observed in the presence of epinephrine and phenylephrine. The experiments were performed using HTPCs from five different patients with normal spermatogenesis and the relative levels of mRNA expression were determined with the ribosomal protein *L19* (*RPL19*) as the reference gene. Bar graphs represent the mean ± SEM. $^{*}p < 0.05$ (treated versus control; Student's t-test). Control (untreated basal conditions).

Our immunohistochemical analysis, which does not discriminate between subtypes, nevertheless revealed the expression of α1-ADR by different human testicular cells, including Leydig cells, cells of the tubular compartment and peritubular cells, and thus implies a spectrum of testicular catecholamine targets. While previous studies are focused on rodent Leydig cells (see introduction) these are not readily available from the human. However, human peritubular cells can be studied and they are in the center of the present study.

ADRs are activated by their physiological ligands, epinephrine and norepinephrine, with different affinity (Scanzano and Cosentino, 2015). Activation of β-ADRs increases the second messenger cAMP (Krizanova et al., 2016) and regulates MAPKs, (Crespo et al., 1995; Magocsi et al., 2007; Turner et al., 2003; Zheng et al., 2000). We found evidence for functional β-ADRs, as salbutamol (a selective agonist of β2-ADRs) elevated proliferation of HTPCs (Lin et al., 2012; Sun et al., 2015). This implies functional β2-

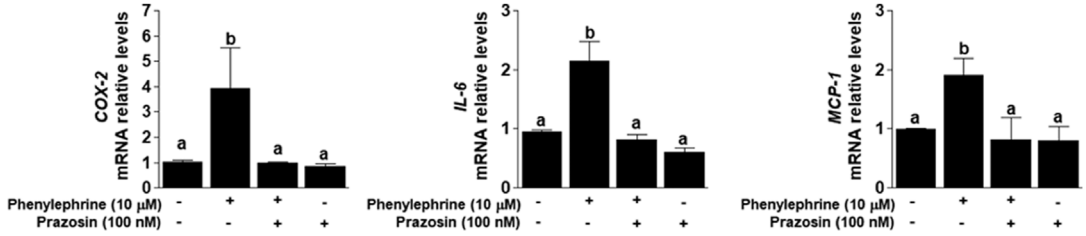

Fig. 4. *The inverse agonist of α1-ADR (prazosin) blocks phenylephrine actions on inflammatory factors in HTPCs.*
The mRNA expression levels of *COX-2, IL-6* and *MCP-1* were analyzed in HTPCs and incubated in the presence or absence of the selective α1-ADR agonist phenylephrine (10 μM) with or without prazosin (100 nM) for 3 h. A significant induction of mRNA expression relative levels of *COX-2, IL-6* and *MCP-1* induced by phenylephrine was blocked in the presence of prazosin. The experiments were performed using HTPCs from three different patients with normal spermatogenesis and the relative levels of mRNA expression were determined with the *ribosomal protein L19* (*RPL19*) as the reference gene. Results were analyzed using the mathematical model of Pfaffl (2001). Bar graphs represent the mean ± SEM. Different letters denote a statistically significant difference between groups. $p < 0.05$; Student-Newman-Keuls test.

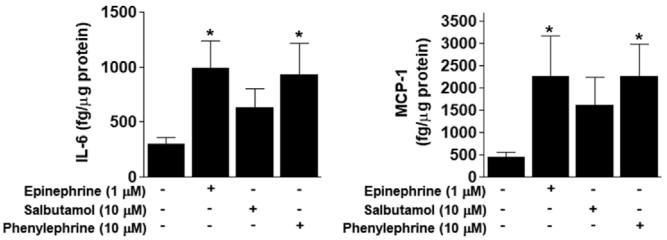

Fig. 5. *Stimulatory effect of epinephrine and phenylephrine on IL-6 and MCP-1 production in HTPCs.*
Immunoassays were used to quantify secreted amounts of IL-6 and MCP-1 protein in HTPCs culture media from four patients. The HTPCs were incubated in the presence or absence of epinephrine (1 μM), the selective β2-ADR agonist salbutamol (10 μM) or the selective α1-ADR agonist phenylephrine (10 μM) for 6 h. A significant induction of IL-6 and MCP-1 was observed in the presence of epinephrine and phenylephrine but not in the presence of salbutamol. The results were normalized to total cellular protein and are given as femtogram (fg)/μg of protein. Bar graphs represent the mean ± SEM. $^{*}p < 0.05$ (treated versus control). Control (untreated, basal conditions). Student's *t*-test.

ADRs on HTPCs. The mechanisms, by which β2-ADRs lead to cell proliferation, remain to be elucidated and were not within the scope of this study.

The stimulation of α1-ADRs by epinephrine or phenylephrine (a selective agonist of α1-ADRs) leads to the activation of Gq protein, coupled to phospholipase C, which catalyzes the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol, thereby causing a release of internal Ca^{2+} as second messenger (Kong et al., 2015). We demonstrated that HTPCs possess functional α1-ADRs, as phenylephrine evoked elevations of intracellular Ca^{2+}, an effect which was blocked by prazosin (an inverse agonist of α1-ADR). In addition, phenylephrine stimulated cell proliferation, as did epinephrine. However, the mechanisms by which α1-ADRs cause cell proliferation, remain to be examined in future studies.

A role of catecholamines in the regulation of human immune cells is emerging. For example, the incubation of human macrophages with epinephrine led to increased levels of COX-2 (a key enzyme in the synthesis of prostaglandins), IDO (indoleamine 2,3-dioxygenase, first and rate-limiting enzyme of tryptophan catabolism) and IL-10 (Muthuswamy et al., 2017). Norepinephrine also induces IL-6 generation in macrophages via β-ADR signaling pathway (Li et al., 2015). In addition, ADRs are involved in the production of MCP-1 in circulation, liver, spleen and subcutaneous adipose tissue (Cox et al., 2014).

While HTPCs are not immune cells, they produce IL-6, MCP-1 and other immunologically relevant factors (Mayer et al., 2016). Our results demonstrated that epinephrine and phenylephrine increased the mRNA expression levels of *COX-2, IL-6* and *MCP-1* in HTPCs without affecting the mRNA expression level of *IL-1β*. No such effects were observed in the presence of salbutamol (the selective agonist of β2-ADR). Subsequent studies, using epinephrine or phenylephrine and a blocker of α1-ADRs (prazosin), confirmed that the production of inflammatory factors in HTPCs is regulated via α1-ADRs. These results were further confirmed by measurements of IL-6 and MCP-1 using a commercial available immunoassays and a cytokine profiler assay. The later revealed that other inflammatory factors were not markedly increased, with the exception of PTX 3, which was previously identified in HTPCs (Flenkenthaler et al., 2014; Mayer et al., 2016).

The reported factors mainly act as pro-inflammatory agents. Inflammation of the human testis is of potential relevance to male infertility. In the testes of men suffering from idiopathic infertility due to impaired spermatogenesis, structural changes in the tubular wall of the seminiferous tubules can be often witnessed. A fibrotic thickening of the wall of the seminiferous tubules (Adam et al., 2012; Mayerhofer, 2013; Welter et al., 2013) and the high density of testicular immune cells (macrophages and mast cells), present in such patients with idiopathic infertility, are clear signs of inflammation (Frungieri et al., 2002b; Meineke et al., 2000). The results obtained in our study suggest that via α1-ADRs, epinephrine can contribute to the inflammatory state in the testis.

Sustained elevations of catecholamines occur during chronic stress. It is becoming clear that specifically chronic stress situations are linked to a number of human conditions, such as obesity and the metabolic syndrome, to alterations of the immune system, and interestingly, also to impaired testicular function (Furtado and Katzman, 2015; Hardy et al., 2005; Menacho-Marquez et al., 2013; Nargund, 2015; Qi and Ding, 2016). A recent study showed that chronic stress is negatively associated with semen quality in otherwise healthy men (Nordkap et al., 2016).

Clearly, consequences of stress on reproductive functions may be due to a number of changes, including deranged hypothalamic-pituitary functions. Yet, our results in combination with previous studies in human testes and HTPCs (Mayer et al., 2016; Schell et al., 2008) may provide a partial explanation for this observation, since they link inflammation to male infertility. If this can be confirmed, the results may also lead to new treatment options. Several α1-ADRs inverse agonists and/or antagonists are already being used to treat nightmares associated with post-traumatic disorder and hypertension, or in combination to treat benign prostatic hyperplasia, as well as, disrupted sexual health, including erectile and ejaculatory dysfunctions (Cao et al., 2016; Hendrickson and Raskind, 2016; Seftel et al., 2007; Stojkov et al., 2013). The impact of α1-ADR antagonists on male (in)fertility and/or their future as potential therapeutic targets should therefore be further considered.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mce.2018.01.027.

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