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Prostaglandin E_2 (PGE_2) is a testicular peritubular cell-derived factor involved in human testicular homeostasis

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ABSTRACT

In man, blockage of prostaglandin (PG)-production e.g. by non-steroidal anti-inflammatory drug (NSAIDs) may have negative testicular side effects, implying beneficial actions of PGs in the testis. We examined human testicular samples and isolated human testicular peritubular cells (HTPCs) to explore sites of PG-synthesis and targets. HTPCs express cyclooxygenase 1 (COX1) and secrete PGE_2. Receptors (EP1, 2, 4) were specifically identified in peritubular cells. In HTPCs PGE_2 significantly increased mRNA levels of the contractility protein calponin, but did not induce contractions. PGE_2, as well as EP1 and EP4 receptor agonists, significantly increased glia cell line derived neurotrophic factor (GDNF) mRNA and/or protein levels. Importantly, the NSAID ibuprofen reduced PGE_2 and this action also lowered SMA and calponin mRNA levels and levels of secreted GDNF protein. The results reveal an unknown PGE_2 system in the human testis, in involving peritubular cells, which may be prone to interference by NSAIDs.

1. Introduction

Several studies suggested that prostaglandins (PGs) have important and multiple roles in the testis (see Rossitto et al., 2015, Frungieri et al., 2015, Moniot et al., 2014). Consequences of the actions of analgesics, i.e. drugs that inhibit PG-synthesis, including aspirin, paracetamol (acetaminophen), indomethacin and ibuprofen, have recently ignited the interest in testicular prostaglandin systems. They belong to the heavily used group of non-steroidal anti-inflammatory drugs (NSAIDs), which block the rate limiting enzymes of PG-synthesis, namely the cyclooxygenases 1 and 2 (COX1/2). Studied in ex vivo cultures and xenograft systems provided evidence that they may interfere with testicular function and development in the human (Mazaud-Guittot et al., 2013) (Ben Maamar, et al., 2017). For example, acetaminophen reduced testosterone production by human fetal testes in a xenograft model (van den Driesche et al., 2015). Differences in the potential of different NSAIDs and windows of sensitivity became apparent and ibuprofen showed the most robust negative actions on Leydig cell and Sertoli cell function (Ben Maamar, et al., 2017). A summary of the evidence for a potential role of in-utero exposure to these drugs in reproductive disorders of male offspring was recently published (Hurtado-Gonzalez and Mitchell, 2017).

Drugs, which block PG-synthesis, may also affect adult human testes (Albert et al., 2013). Paracetamol, aspirin or indomethacin caused multiple endocrine disturbances in explants of the adult human testes.

In this context another study showed that urinary paracetamol in men was correlated with longer time to pregnancy intervals in couples (Smarr et al., 2016). This implies that the use of this COX-

Abbreviations: COX, cyclooxygenase; ECM, extra cellular matrix; EP, prostaglandin E2 receptor; GDNF, glial cell line derived neurotrophic factor; HTPCs, human testicular peritubular cells; MRPs, multiple drug resistance proteins; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PTGES2/3, PGE_2 synthase variants 2 and 3; SMA, smooth muscle actin; SSC, spermatogonial stem cell.

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blocker interferes with male reproductive functions in yet unknown ways. Furthermore, there is evidence that certain environmental factors may interfere with PG synthesis (Kristensen et al., 2011). Hence, although PGs and their actions in the human testis are of emerging importance, the sources and the possible roles of PGs in the human testis during development and adulthood, as well as in health and disease, are not well known.

Two COX isozymes exist and catalyze the first and common steps in the synthesis of all PGs. PGH_2 is generated first and then utilized by specific enzymes, which synthesize the main types of PGs. By RT-PCR low levels of expression of both the constitutively expressed COX1 and of the inducible isozyme COX2 were found in adult human testicular samples (O'Neill and Ford-Hutchinson, 1993). Hase et al. (2003) did not report COX1/2 expression in the normal human testis, but described both COX1 and COX2 in testicular cancer. COX2 at mRNA and protein level was also readily observed in testicular samples stemming from men with impairments of spermatogenesis and associated infertility. In these cases, several testicular cell types were identified to express COX2, including Leydig cells, Sertoli cells, peritubular (myoid; smooth-muscle like) cells (Frungieri et al., 2002, Welter et al., 2011, Schell et al., 2008, Matzkin et al., 2010) and testicular immune cells (mast cells and macrophages) (Welter et al., 2011, Matzkin et al., 2010, Rossi et al., 2014) (see review by Frungieri et al., 2015). The inducible isozyme COX2 in male infertility is likely linked to inflammatory events in the testes of these men, and consequently PGs generated may play roles in testicular pathologies. This topic was in the focus of above-mentioned studies and consistent with such a role, blockers of PG synthesis may be useful in the treatment of infertility patients. Indeed, the PG synthesis-blocker diclofenac was suggested for the treatment of oligospermia in men (Moskovitz et al., 1987, Moskovitz et al., 1988). Thus, inducible COX2 appears to play a role in testicular pathologies, but a possible expression of the constitutive enzyme COX1 by cells of the normal human testis has not yet been fully explored. Likewise, which specific PG-synthases are expressed and active, and which PG-types are consequently produced in the adult human testis in health and disease, are only rudimentary known (see for example Carpenter et al, 1978). PGD_2 and its metabolite, 15d-PGJ_2, were found in testicular samples suffering from idiopathic infertility (Kampfer et al., 2012, Rossi et al., 2016). PGE_2 was reported in explants of human testes (Albert et al., 2013).

The mentioned explant and xenograft culture methods are experimental approaches to study the human testis. In addition, isolated human testicular peritubular cells (HTPCs), together with human samples, provide a cell-focused window into the human testis. HTPCs can be derived from very small testicular samples of adult men with normal spermatogenesis (Albrecht et al., 2006, Mayerhofer, 2013). Peritubular "myoid" cells have smooth-like characteristics and *in vivo* are very slender, inconspicuous cells. It is thought that their major role is to transport sperm (see review by Mayerhofer, 2013, Maekawa et al., 1996). In addition they produce extracellular matrix (Adam et al., 2011) and factors important in testicular homeostasis, including glial cell line derived neurotrophic factor, GDNF, crucial for spermatogonial stem cell (SSC) renewal (Spinnler et al., 2010) and others (Windschuttl et al., 2015). A previous proteomic study report identified the large repertoire of cellular and secreted proteins in HTPCs (HTPCs; Flenkenthaler et al., 2014).

The list of cellular proteins of HTPCs includes also the rate-limiting enzyme for PG synthesis COX1. To our knowledge there is only one report, which previously has described COX1 in testicular peritubular cells of adult alpaca testes (Zerani et al., 2011). In HTPCs, PGE_2 synthase variants 2 and 3 (PTGES2/3) were further identified (Flenkenthaler et al., 2014). If present *in vivo*, it appears possible that these cells are therefore a source of PGE_2 and that NSAIDs drugs may target these cells and impair physiological PGE_2 actions within the human testis.

We aimed to examine the expression of COX1 and PGE_2 synthesis by HTPCs and then to elucidate the expression of PGE_2 receptors in human testis. We next performed cellular studies to explore the actions of PGE_2. We also tested the ability of a NSAID to interfere with PGE_2 actions. Our results imply that PGE_2 synthesis and actions occur *in vivo* and are likely involved in testicular homeostasis.

2. Material and methods

2.1. Cells/human samples

Procedures of isolation and the explant culture, as well as the characterization of HTPCs were performed as described (Albrecht et al., 2006, Windschuttl et al., 2015). Cells from up to 12 different patients with normal spermatogenesis (undergoing reconstructive surgery of the vas deferens or ICSI patients) were used. Cells stem from passages 5–12. The patients had granted written Informed Consent. The local Ethical Committee (Ethikkommission, Technische Universität München, Fakultät für Medizin, München, project number 5158/11) has approved the study. Experiments were performed in accordance with the relevant guidelines and regulations (including laboratory and biosafety regulations). Based on pilot studies, in which we examined possible toxic actions (ATP-assays), or previous reports, the treatment duration and drug concentrations were chosen. Stimulations with PGE_2 (Sigma-Aldrich, Deisenhofen, Germany), ibuprofen (Sigma-Aldrich) were performed for 24 h in serum free media. The concentration for this NSAID is based on the reported blood levels of this group of drugs, which are high as 10^{-4} M (Albert et al., 2013). Testicular concentrations are unknown and we used 10^{-6} M, which also did not alter cell morphology or cell number or viability, as determined in pilot studies using video monitoring and cell counting. For gel contraction studies, HTPCs were plated in a collagen matrix as described (Schell et al., 2010), or were observed by video imaging as described (Welter et al., 2014). The following EP1-4 agonists were used: 17-phenyl trinor PGE_2 and L-902,688 (Biomol GmbH; Hamburg, Germany), Sulprostone and (*R*)-Butaprost (Sigma-Aldrich (Deisenhofen, Germany). DMSO for Sulprostone (Sigma-Aldrich, Deisenhofen, Germany) and ethanol for PGE_2, 17-phenyl trinor PGE_2, L-902,688 and (*R*)-Butaprost (Carl Roth GmbH, Karlsruhe, Germany) were used as a solvents and were included as controls.

2.2. Immunohistochemistry/immunocytochemistry

Testicular samples (n = 8), embedded in paraffin, stem from a set of samples previous studied and were used and processed for immunohistochemical studies as mentioned (Welter et al., 2014). Primary antibodies against COX1 (1:100; polyclonal affinity isolated rabbit anti-human; Thermofisher Scientific, Waltham, MA, USA; Cat #RB-10687-P0), EP1 (1:200; polyclonal affinity isolated rabbit anti-human; Alpha Diagnostic, San Antonio, TX, USA; Cat #EP11-A), EP2 (1:100; polyclonal affinity isolated rabbit anti-human; Sigma-Aldrich; Cat # SAB2900118), EP3 (1:500; polyclonal affinity isolated rabbit anti-human; Biomol, Hamburg, Germany; Cat # LSA975), EP4 (1:100; polyclonal affinity isolated rabbit anti-human; Sigma-Aldrich; Cat # HPA012756) were applied. For negative controls we incubated the sections with the corresponding IgG isotype instead of the antiserum, we omitted the antiserum and used normal serum instead of the antiserum. In case of EP1, pre-adsorption with the peptide, which was commercially available, was used as a

further control. In some cases, sections were counterstained with hematoxylin. For immunocytochemistry, the same COX-1 rabbit polyclonal antibody (1:300), as used for immunohistochemistry and Western blotting, was applied overnight at 4 °C. For control purposes, the antibody was omitted. An alexa-fluor 488 labeled goat-anti rabbit secondary antibody (1:900 in PBS, Thermo Fisher Scientific, Darmstadt, Germany) was applied for 1.5 h at room temperature. The cells were counterstained for 3 min with the nuclear stain DAPI (2 μg/ml in PBS, Thermo Fisher Scientific, Darmstadt, Germany). The cells were rinsed with water, mounted on fluoromount (Leica, Nussloch, Germany) and viewed with a Zeiss Observer Z1; Zeiss, Göttingen, Germany). Pictures were taken with Axiocam 506 (Zeiss, Munich, Göttingen, Germany).

2.3. Western blot and ELISAs

Western blots of HTPC lysates were performed as described (Frungieri et al., 2002a,b) using the COX1 (1:250) antibody mentioned above. ELISA-measurement of GDNF (Promega, Fitchburg, WI, USA) and PGE_2 (Enzo Life Sciences (ELS) AG, Colleton Cres, Exeter, United Kingdom) were performed following the instructions of the manufacturers. The levels of GDNF in the culture supernatants were determined in media after 72 h (Spinnler et al., 2010). The levels of PGE_2 were measured in culture supernatants after 24 h. The time points were determined in pilot studies and samples were assayed in duplicates. Results were normalized to the protein content of cell homogenates.

2.4. First strand cDNA synthesis RNA, conventional polymerase chain reaction (PCR) and qPCR

Total RNA from sub-confluent HTPCs was prepared as described (Windschuttl et al., 2014) using Qiagen RNeasy Micro-Kit (Qiagen, Hilden, Germany). First strand cDNA synthesis followed by conventional RT-PCR for EP1-4, COX1, GDNF and L19 was described previously. The identities of the amplicons were confirmed by sequence analysis (GATC, Konstanz, Germany). Primer design (http://primer3.wi.mit.edu) and synthesis (Metabion, Munich, Germany), as well as sequence analysis (GATC), were done as described (Welter et al., 2014).

Real time PCR (LightCycler® 96 System, Roche Diagnostics, Penzberg, Germany) using the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) was performed as described earlier (Windschuttl et al., 2014). Changes in gene expression of Calponin, SMA and GDNF were normalized to the ribosomal gene L19 as housekeeping gene and calculated according to the $2^{-\Delta\Delta ct}$ method. Human Calponin, SMA, GDNF expression levels are depicted relative to untreated controls. Information about primer sequences and qRT-PCR conditions are described in Table 1.

2.5. Collagen gel contraction assays/video monitoring

The methods were performed as described (Schell et al., 2010, Welter et al., 2014). In brief, 100.000 cells were seeded onto gel lattices. PGE_2 (1 μM, solved in ethanol and diluted in serum free medium), ethanol (0.01% in serum free medium) and FCS were added. After 1 h, stressed matrices were released from the surrounding brim of the wells. Pictures of free-floating collagen gel lattices were taken immediately after release, as well as 30 min, 1, 3, 24 and 48 h later. Areas of collagen gel size were analyzed with ImageJ (U.S. National Institutes of Health, Bethesda, MA, USA, http://imagej.nih.gov/ij/) and normalized to the area of the well. The degree of contraction was evaluated by determining the area of the gel matrix before and after treatment. Results are presented as absolute values, as percentage of change between groups, or normalized to the respective untreated control group, as indicated. Cellular morphology was screened by light microscopy. All assays were performed with cells from three different patients. In independent studies, video monitoring of HTPCs exposed to PGE_2 and untreated controls was performed for up to 24 h, as previously described (Welter et al., 2014).

Table 1
List of oligonucleotide primers used for RT-PCR experiments.

Gene of interest	Primer-sequence	Amplicon size
COX1		96 bp
Forward	5′-TCC ATG TTG GTG GAC TAT GG-3′	
Reverse	5′-GTG GTG GTC CAT GTT CCT G-3′	
EP1		152 bp
Forward	5′-TAT CAT GGT GGT GTC GTG CAT-3′	
Reverse	5′-TGT ACA CCC AAG GGT CCA GG-3′	
EP2		101 bp
Forward	5′-ACC ACC TCA TTC TCC TGG CTA-3′	
Reverse	5′-TTC CTT TCG GGA AGA GGT TTC-3′	
EP3		104 bp
Forward	5′-TAT GGG GAT CAT GTG CGT GC-3′	
Reverse	5′-TGT GTG TCT TGC AGT GCT CA-3′	
EP4		105 bp
Forward	5′-CAT CTG CTC CAT CCC GCT C-3′	
Reverse	5′-ATT CGG ATG GCC TGC AAA TC-3′	
GDNF		93 bp
Forward	5′-GCA GAC CCA TCG CCT TTG AT-3′	
Reverse	5′-ATC CAC ACC TTT TAG CGG AAT G-3′	
SMA		110 bp
Forward	5′-ACC CAG TGT GGA GCA GCC C-3′	
Reverse	5′-TTG TCA CAC ACC AAG GCA GT-3′	
Calponin		183 bp
Forward	5′-CGA AGA CGA AAG GAA ACA AGG T-3′	
Reverse	5′-GCT TGG GGT CGT AGA GGT G-3	
L19		199 bp
Forward	5′-AGG CAC ATG GGC ATA GGT AA-3′	
Reverse	5′-CCA TGA GAA TCC GCT TGT TT-3′	

2.6. Cell viability assay

Viability of HTPCs was evaluated by measuring cellular ATP content, as described previously (Saller et al., 2010).

2.7. Statistics

Results are expressed as individual measurements or as mean + SEM. The effects of ibuprofen on the medium-levels of PGE_2, of PGE_2 or the PGE_2-agonists' actions, and consequences of ibuprofen treatment on GDNF, SMA and calponin mRNA levels, were calculated using *t*-test (Prism, GraphPad Software (version 4.0a), Inc., San Diego, CA, USA). A probability value of at least $p < 0.05$ was regarded as significant.

3. Results

3.1. HTPCs express COX1 and produce PGE_2

Western blots confirmed that HTPCs express COX1 (Fig. 1A). Immunocytochemistry for COX1 using the same antibody as for Western blotting showed that most, but not all HTPCs express COX1 (Fig. 1B; see asterisk for a COX1-negative cell; insert: control without antibody). Immunohistochemistry of human testicular samples implicated *in vivo*-relevance (Fig. 1C) and identified COX1 in peritubular cells of the human testis. ELISA measurements showed that HTPCs (from 5 individual patient derived samples) constitutively secrete PGE_2 into the medium (Fig. 1D). The amounts varied somewhat between the cells from individual donors.

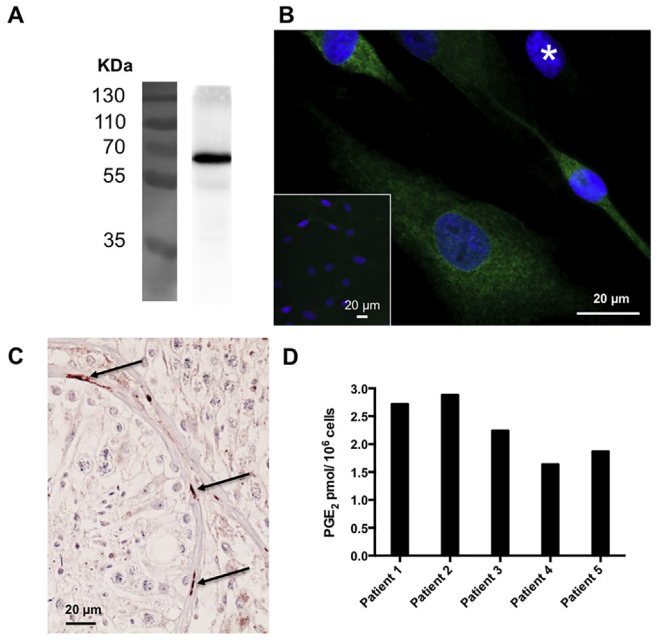

Fig. 1. HTPCs express COX1 and produce PGE_2.
A: Example of a Western blot experiment, which confirmed that HTPCs express COX1. Note that one single band was observed of about 69 kDa.
B: Immunocytochemistry using the same antibody as for Western blotting showed that most, but not all HTPCs express COX1 (green color; asterisk denotes the blue, DAP-stained nucleus of a COX1 negative cell). Insert: Control without primary antibody.
C: Immunohistochemistry (IHC) for COX1 in human testicular samples revealed *in vivo*-relevance. Arrows point to peritubular cells.
D: ELISA measurements revealed that HTPCs constitutively secrete PGE_2 into the media, as shown in 5 individual samples after 24 h. Note that the levels vary in individual donor-derived cells.

3.2. Identification of testicular PGE_2 receptors EP1- 4

The expression of the receptors for PGE_2, EP1-4, in human testis and HTPCs was examined next. EP1, EP2 and EP4 were revealed in HTPCs *in vivo* and *in vitro* by RT-PCR and/or immunohistochemistry (Fig. 2A and B**)**. Staining intensity was marked in peritubular cells for EP1, -2 and 4. Further testicular cells were immuno-positive, including interstitial cells and cells in the tubular compartment. EP3 was specifically detected in cells residing at the perimeter of the tubules, i.e. typical spermatogonia (Fig. 2B**)**. The results imply that interactions of PGE_2 with different human testicular cells can occur *in vivo*. Except peritubular cells, most human testicular cells can, however not be isolated and further studied *in vitro*. Hence we did attempt to further identify immunoreactive testicular cell types. Rather, we focused on isolated peritubular cells, HTPCs.

3.3. Influence of PGE_2 on the contraction and regulation of smooth muscle markers in HTPCs

To explore its actions, PGE_2 (1 μM) was added to HTPCs. Results of collagen gel contractility assays indicate that within up to 48 h it does not induce contraction, which was however induced by 10% FCS, as shown previously (Schell et al., 2010) **(**Fig. 3A). Likewise, video monitoring did not provide evidence for an acute contractile effect of PGE_2 (not shown). When PGE_2 was added to HTPCs for 3 h it statistically significantly ($p < 0.05$) increased mRNA levels for the contractility protein calponin in several independent experiments (Fig. 3 B). This treatment did not change smooth muscle actin (SMA) levels.

3.4. PGE_2 and EP agonists increase GDNF secretion by HTPCs

PGE_2 elevated mRNA levels for GDNF around two fold ($n = 5$; $p < 0.05$) after 3 h (Fig. 4A**)**. When HTPCs were exposed for 72 h to PGE_2, increased GDNF protein levels in the media were found (Fig. 4 B; $n = 7$; $p < 0.05$).

When EP agonists were (Fig. 4C) tested for 3 h in HTPCs from $n = 4$ donors, we found that the EP3 agonist Sulprostone (1 μM; not shown) and EP2 agonist (*R*)-Butaprost (1 μM) were not able to increase levels of GDNF. However, EP1 agonist 17-phenyl trinor PGE_2 at the same concentration (1 μM) and the EP4 agonist (L-902,688,1 μM) statistically significantly ($p < 0.05$) stimulated GDNF levels.

3.5. Ibuprofen effects on GDNF, SMA and calponin

The NSAID ibuprofen (1 μM; 24 h) reduced PGE_2 levels in HTPC supernatants ($n = 3$; $p < 0.05$) by about half (Fig. 5 A; $p < 0.05$). ELISA measurements of GDNF in cellular supernatant after 72 h of exposure to ibuprofen (1 μM) showed significantly lower levels (Fig. 5 B; $n = 4$; $p < 0.05$). This treatment also robustly and significantly reduced the mRNA levels of two smooth muscle markers RNA ($n = 3$; $p < 0.05$; Fig. 5 C.

4. Discussion

PGE_2 was detected previously in the human testis, yet the cellular sources were not well defined (Albert et al., 2013). PGE_2 synthesis requires first COX1 or COX2, which produce PGH_2, and then PGE_2-synthases. The analysis of the proteome of HTPCs had revealed COX1 and two PGE_2-synthesising enzymes (Flenkenthaler et al., 2014) and this now led to the identification of this testicular cell type as a physiological source of PGE_2.

Previous studies did not report immunoreactive COX1-positive cells in normal adult human testes (see Frungieri et al., 2002a,b), possibly due to low sensitivity of antibodies available at that time and the inconspicuous nature of extremely flat peritubular cells. Yet low levels of COX1 and COX2 were found when RT-PCR was employed in adult human testicular samples (O'Neill and Ford-Hutchinson, 1993). In our present study we found immunoreactive peritubular cells *in vivo* and *in vitro*, yet not all peritubular cells appeared to be stained. The specificity of the antibody used was further supported by results of Western blot experiments, in which it yielded a single band of expected size (around 69 KD). The results identify HTPCs as a source of PGE_2.

PGE_2 is considered to be a short-lived molecule, which acts via membrane receptors EP1-4 (Abramovitz et al., 2000). We used commercially available antibodies to investigate their presence in human testicular samples. Only for one (recognizing EP1) pre-adsorption was possible, but all additional controls performed for the others were negative. In general, immunoreactive cells were noted in the tubular wall, inside the tubular compartment and in interstitial areas. Except for peritubular cells, human testicular cells can not be isolated and further studied in culture, hence we made no attempt to further identify other potential receptor bearing cells. EP3 was detected in typical spermatogonia, residing at a basal position in the basal compartment of the seminiferous tubules. EP3 was also the only receptor not found in peritubular cells *in situ* by immunohistochemistry. RT-PCR studies confirmed the results and identified EP1, 2 and 4 in HTPCs. Thus, these cells are targeted by PGE_2 and can be studied *in vitro*. We focused on two peritubular cellular functions of importance, smooth-muscle properties and production of GDNF.

Cycles of contractions and relaxations of peritubular cells transport immotile sperm (Mayerhofer, 2013, Maekawa et al., 1996)

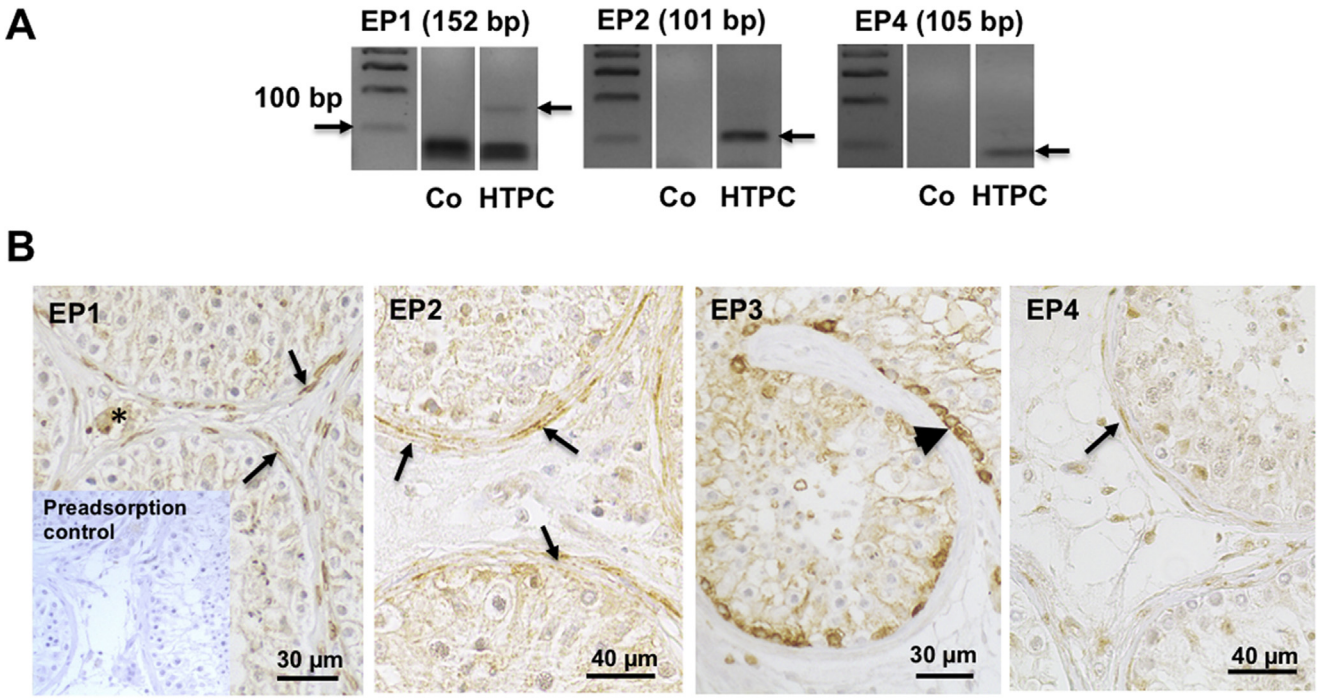

Fig. 2. Testicular receptors for PGE_2.
A: EP1, EP2 and EP4 were revealed in HTPCs by RT-PCR and identities were confirmed upon sequencing (markers are indicated, as well as a control without input cDNA; pictures were cropped from a larger gel).
B: EP1, EP2 and EP4 were also found by immunohistochemistry in peritubular cells *in situ*. Note that besides peritubular cells (arrows) other testicular cells were also immune-positive, including interstitial cells (asterisk). Pre-adsorption for EP1 yielded negative results. Germ cells located close to the basement membrane of the seminiferous tubules, presumably spermatogonia, were immunopositive for EP3 (arrowheads).

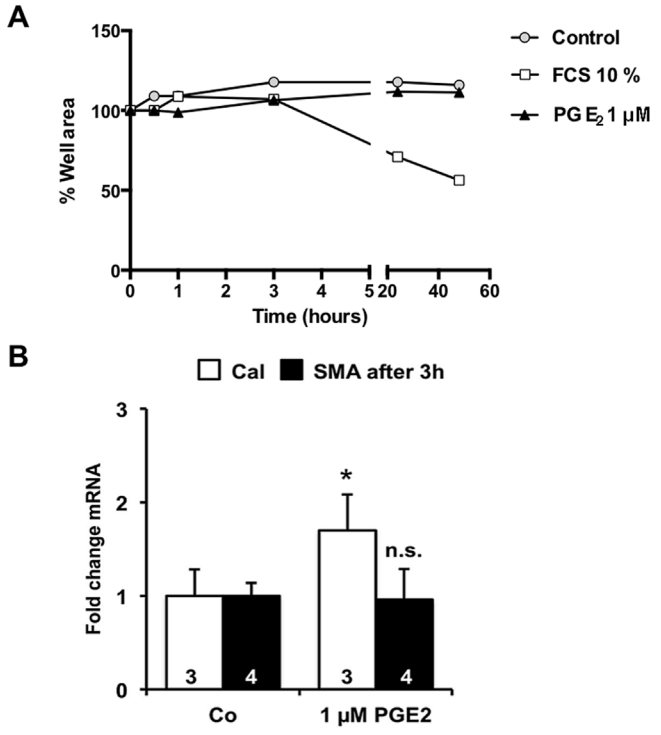

Fig. 3. Influence of PGE_2 on contractility and smooth muscle markers.
A: Result of a gel contraction assay. Only FCS, used as a positive control, was effective, while PGE_2 did not cause a change in the size. The experiment was repeated twice with cells from three patients with a similar result.
B: When PGE_2 was added to HTPCs from 3 to 4 different donors for 3 h it increased the mRNA levels for the contractility proteins smooth muscle actin (SMA) and calponin. (* indicates statistically significant differences; $p < 0.05$).

and in infertility patients smooth muscle markers, including SMA and calponin, are reduced or absent (Kampfer et al., 2012, Welter et al., 2013). We found that PGE_2 is able to increase the mRNA levels of the important smooth muscle protein, calponin, while at least under the experimental conditions SMA levels were only slightly but not significantly changed. The NSAID tested in our study, ibuprofen, reduced PGE_2 as well as SMA and calponin. Hence, PGE_2 may be a physiological factor involved in the maintenance of their smooth muscle phenotype. Based on the uterotonic action of PGE_2 and PGF2alpha observed in myometrial cells (Sugimoto et al., 2015) we tested actions of PGE_2 on cellular contraction, in an approach previously employed (Schell et al., 2010). The gel contractility assay did however not reveal contraction-promoting actions and likewise video monitoring (Welter et al., 2014) did not hint to acute contraction.

Work from our group previously reported that HTPCs, in addition to Sertoli cells, secrete GDNF in a constitutive manner (Spinnler et al., 2010). The importance of peritubular cell-derived GDNF was demonstrated by recent studies in rodents. They elegantly confirmed not only the expression of GDNF by mouse peritubular cells, but also showed that the peritubular cell-derived GDNF is crucial for normal spermatogenesis (Chen et al., 2014, Chen et al., 2016). In our study HTPCs, derived from individual men, responded to PGE_2 and this resulted in elevated GDNF mRNA and GDNF secretion. Thus, the view that GDNF is constitutively released by HTPCs, described previously, must be extended. Taken to the testis, this implies paracrine action of peritubular cell-derived PGE_2 acting within the tubular wall, and furthermore points to an indirect role in the maintenance of the spermatogonial stem cell niche.

The actions of PGs are complex and determined among others by the levels of receptors and the repertoire of subtypes of receptors of PGs (Steinke, 2012). We obtained some insights by using

commercial EP1-4 agonists in our studies with HTPCs (Markovic et al., 2017). While the EP2 and EP3 agonists lacked action, EP1 and EP 4 agonists significantly increased GDNF mRNA levels. This implies that mainly these receptors are involved in the stimulation of GDNF by PGE_2. Clearly, only one concentration of agonists was used in a set of individual patient-derived cells. Thus additional studies are required, also to elucidate the signaling pathways involved (Markovic et al., 2017). Metabolism and the cellular transport mechanism of PGs, which facilitate release/import from the cells (prostaglandin transporter SLCO2AI; multiple drug resistance proteins (MRPs) for a summary see Duffy, 2015), are important points, as well. They are also beyond the scope of this paper, as are possible further testicular PG-systems, involving other PGs and their receptors, which all may be prone to interference by NSAIDs or endocrine disruptors. It is important to note that actions of endocrine disruptors seen in rodent cells may not be readily transferred to the human situation (Habert et al., 2014). Therefore it appears mandatory that PG-systems and actions of NSAIDs or endocrine disruptors be studied in human, rather than in rodent cells.

In our study we tested ibuprofen for its ability to interfere with PGE_2-induced actions in HTPCs. Although we also tested indomethacine (data not shown), we turned to this NSAID because a recently published study, which compared different NSAIDs, reported that among the NSAIs examined ibuprofen produced the most robust effects in fetal human testes (see Ben Maamar et al., 2017). We used a concentration that is likely of relevance, as in human plasma typical, effective NSAIDs levels range between 10^{-4} to 10^{-5} M (see summary in Albert et al., 2013). The testicular levels are unknown but the concentration of 10^{-6} M, employed can be assumed to represent a pharmacologically relevant concentration. We found that 10^{-6} M ibuprofen reduced PGE_2 by about 50%, did not negatively affect cell viability or number but reduced GDNF levels in cell culture supernatant and reduced smooth muscle gene expression in HTPCs.

In summary, our study provides novel evidence for a cellular site of the synthesis of PGE_2 in the human adult testis. It identified receptor-mediated roles of PGE_2, which are of relevance for the maintenance of cellular homeostasis. Ibuprofen, a prototype NSAIDs, by reducing its production, as seen *in vitro*, may thus also target this physiological PGE_2-system in the testis.

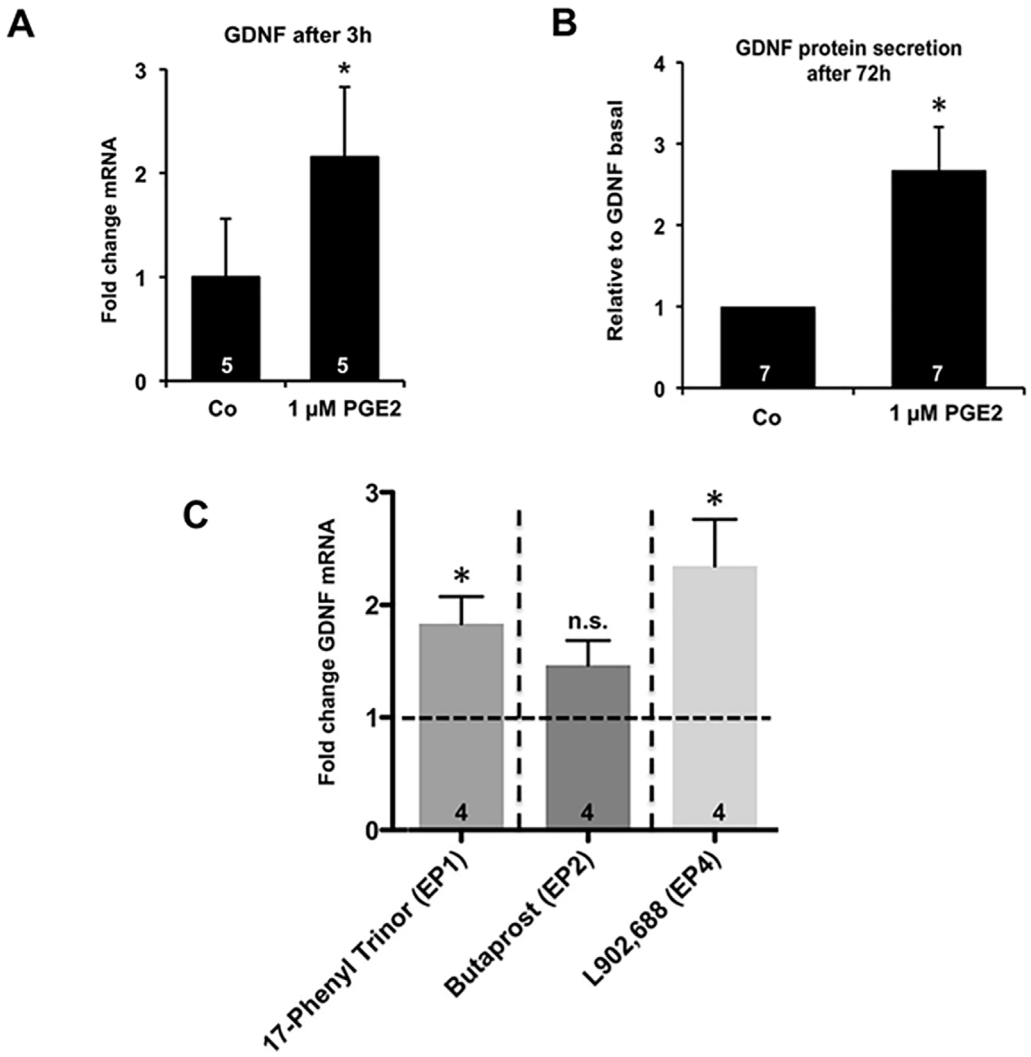

Fig. 4. PGE_2 and agonists of PGE_2 receptors EP1 and EP4 increase GDNF in HTPCs.
A: HTPCs produce GDNF and PGE_2 increased the levels of GDNF mRNA after 3 h in HTPCs (qPCR; n = 5 individual, patient-derived cell-preparations (*p < 0.05).
B: PGE_2 significantly (*p < 0.05) increased GDNF protein accumulation in medium of HTPCs after 72 h (n = 7 individual, patient-derived cell-preparations).
C: Significantly higher levels of GDNF mRNA after 3 h in HTPCs (n = 4) were found upon treatment with the EP1 agonist (17-phenyl trinor PGE_2, 1 µM) and EP4 agonists (L-902,688,1 µM; qPCR; *p < 0.05). The EP2 agonist (butaprost) treatment was not effective. Results are expressed as increases over respective controls (dotted line).

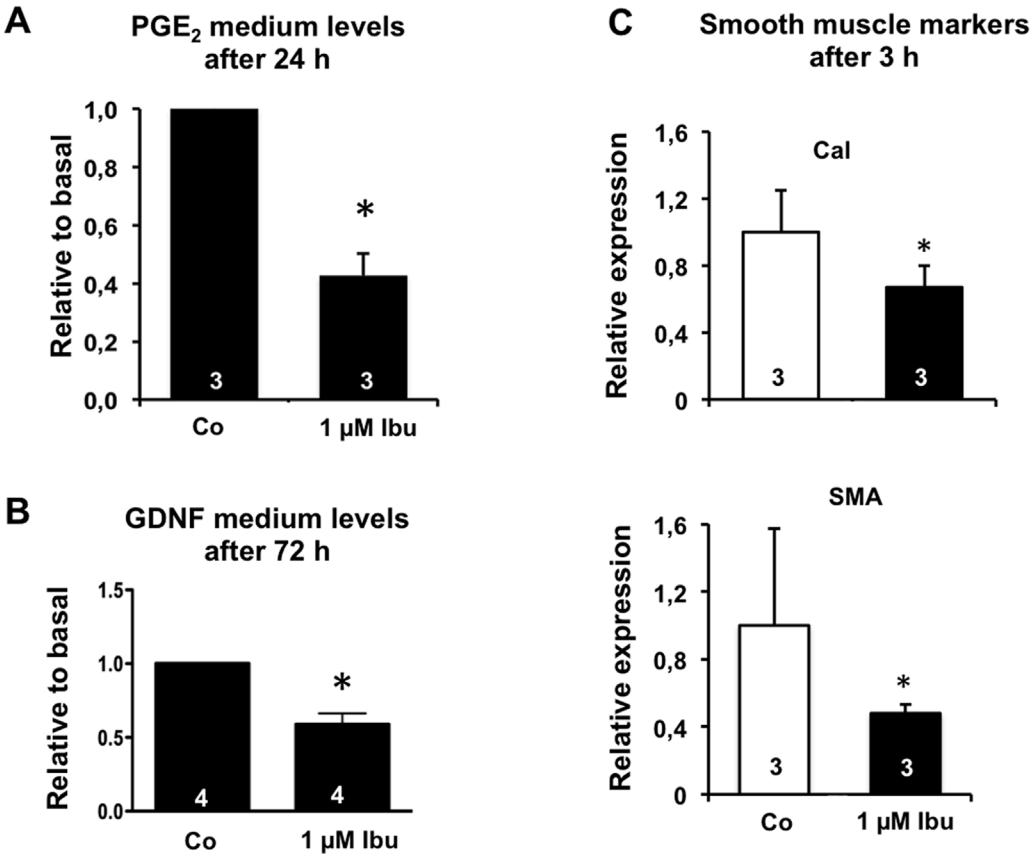

Fig. 5. Ibuprofen reduced PGE_2 and mRNA levels of SMA, Calponin and GDNF.
A: Result of a PGE_2 -ELISA showing that 1 µM ibuprofen (Ibu) statistically significantly lowered PGE_2 in medium of HTPCs from 3 donors compared to the untreated control group (Co; 24 h; $^*p < 0.05$).
B. Result of GDNF-ELISA, showing that when ibuprofen was added to HTPCs, GDNF levels in the supernatant decrease significantly (72 h; n = 4 different donor-derived cells; $^*p < 0.05$).
C. When ibuprofen was added to HTPCs from 3 different donors for 3 h it significantly reduced SMA, calponin mRNA levels (qPCR; $^*p < 0.05$).

Author's roles

R-AV participated in the study design, was involved in planning of the experiments, and supervision of the study and data analysis. RS performed contractility measurements and was involved in analysis of the data. DK performed ELISA, Western and qPCR-studies, KFM and SJU provided human samples and critical discussions. WH was involved in the execution of immunohistochemical and immunocytochemical studies and helped in the analysis of the results. FMB participated in the study design and together with MA conceived of the study. MA supervised all experiments and data analysis and drafted the paper. All authors contributed to the final version of the manuscript and approved it.

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Conflicts of interest

The authors declare no conflict of interest.

Note added in proof

A recently published study showed that ibuprofen affects human testicular cells and their functions and this includes peritubular cells. Kristensen DM, Desdoits-Lethimonier C, Mackey AL, Dalgaard MD, De Masi F, Munkbøl CH, Styrishave B, Antignac JP, Le Bizec B, Platel C, Hay-Schmidt A, Jensen TK, Lesné L, Mazaud-Guittot S, Kristiansen K, Brunak S, Kjaer M, Juul A, Jégou B. Ibuprofen alters human testicular physiology to produce a state of compensated hypogonadism. Proc Natl Acad Sci U S A. 2018 Jan 23; 115 (4):E715-E724.

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