# PATHOMECHANISM OF TESTICULAR INFLAMMATION IN RAT INVOLVES ACTIVATION OF PROTEINASE ACTIVATED RECEPTOR 2

IOAN RADU IOSUB

**INAUGURALDISSERTATION**
zur Erlangung des Grades eines
Doktors der Humanbiologie
des Fachbereichs Medizin der
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# LIST OF ABBREVIATIONS

| | |
|---|---|
| bp | Base pair |
| b.w. | Body weight |
| BSA | Bovive serum albumin |
| cDNA | Complementary DNA |
| COX-2 | Cyclooxygenase-2 |
| DAPI | 4', 6'-diamidino-2-phenylindole, dihydrochloride |
| DMEM | Dulbecco's Minimal Essential Medium |
| DMSO | Dimethyl-sulfoxid |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTPs | 2'-deoxynucleoside-5'-triphosphates |
| EAO | Experimental autoimmune orchitis |
| EDTA | Ethylene diaminetetraacetic acid |
| FCS | Fetal calf serum |
| GAPDH | **G**lycerin-**A**ldehyd-**P**hosphat-**D**ehydrogenase |
| HEPES | (2-**H**ydroxy**e**thyl)-1-**p**iperazine**e**thane**s**ulphonic acid |
| HRP | Horse radish peroxidase |
| iNOS | Inducible nitric oxide synthase |
| kD | Kilodalton |
| LPS | Lipo-polysacharide |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MPO | Myeloperoxidase |
| NaCl | Sodium Chloride |
| $PAR_2$ | Proteinase activated receptor-2 |
| $PAR_2$-AP | $PAR_2$ activating peptide (SLIGRL-$NH_2$) |
| $PAR_2$-f-AP | $PAR_2$ modified activating peptide ([2-furoyl]-LIGRLO-$NH_2$) |
| $PAR_2$-RP | $PAR_2$ reverse peptide (LSIGRL-$NH_2$) |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |

| | |
|---|---|
| RT-PCR | Reverse transcription |
| SDS | Sodium-dodecyl- sulphate |
| sma | Smooth muscle actin |
| TAE | Tris-acetate-EDTA buffer |
| TE | Tris-EDTA |
| TGFß-2 | Transforming growth factor-betta-2 |
| Tris | Tris(hydroxymethyl)-amino-methane |
| U | Unit |
| UV | Ultraviolet |

# 1 INTRODUCTION

## *1.1 Morphology of the testis*

The male reproductive system has to fulfill three major functions: steroidogenesis (production of male sex steroid hormones), spermatogenesis (production of the male gametes) and delivery of the male gametes to the female reproductive tract. The testes are anatomically and functionally compartmentalized to accomplish the first two of these tasks. Histologically the organ is separated into an endocrine (interstitial) and a gametogenic (tubular) compartment. The male gonad is contained in a tough fibrous capsule called tunica albuginea. In the human, the testis is partitioned by connective tissue septa into discrete lobules containing the loops of the seminiferous tubules, which are connected at both ends to a reservoir termed rete testis located along one pole of the testis in the mediastinum. In contrast, rodent species such as rat and mouse display only free intertubular connective tissue with no distinct septa separating the seminiferous tubules (Huckins and Clermont, 1968, Fawcett et al., 1973)

### 1.1.1 Functional organization and hormonal regulation of the testis

The interstitial compartment completely surrounds the seminiferous tubules and contains the androgen producing Leydig cells. Moreover, in the interstitial tissue the vasculature, lymphatic vessels and nerves of the testis are found.
The seminiferous tubules are the gamete forming compartment of the testis. The seminiferous tubules are covered by a circumferential layer mainly consisting of peritubular myoid cells and the acellular components of the basal lamina (basement membrane), which together form the limiting tissue to the interstitial compartment on which the Sertoli cells and the most basally located spermatogenic cells rest.

Male reproduction is regulated and maintained by pulsatile secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus under the control of the central nervous system. GnRH stimulates concordant pulses of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (Fig. 1.1.1) (Leung and Steele, 1992, Huhtaniemi, 1995). LH

stimulates Leydig cell development, morphology and secretion of androgens, mainly testosterone (Coquelin and Desjardins, 1982, Ellis and Desjardins, 1982, Sisk and Desjardins, 1986). Testosterone and FSH bind to specific receptors on Sertoli cells to regulate spermatogenesis and Sertoli cell functions directly such as secretion of inhibin. Spermatogenic cells do not respond to testosterone and FSH as they do not express the respective receptors. In turn, androgens and inhibin exert a negative feedback loop at the pituitary and hypothalamic level to regulate LH and FSH production (Coquelin and Desjardins, 1982, Ellis and Desjardins, 1982, Leung and Steele, 1992).

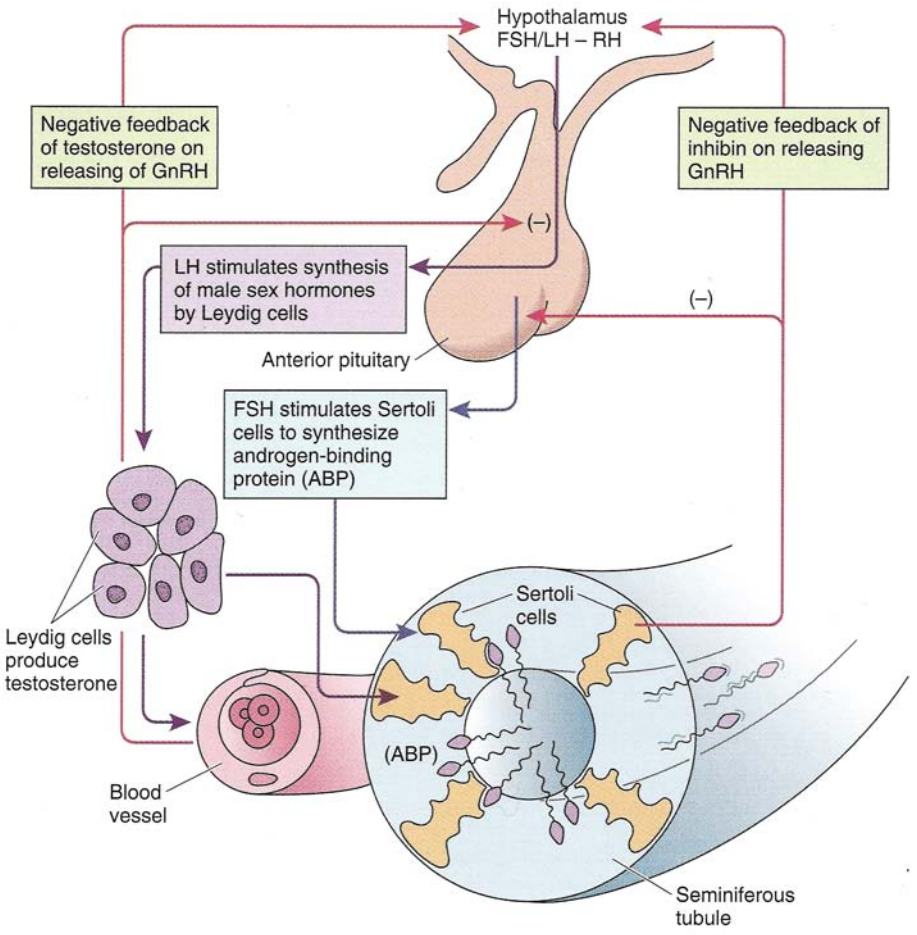



**Fig. 1.1.1 Hormonal regulation of the testis (Gartner L. P., 2001)**

## 1.1.2 The tubular compartment and spermatogenesis

The Sertoli cells provide the structural framework for the organization of the seminiferous epithelium, but also play a crucial role in supporting and directing the development of the spermatogenic cells. These cells remain in

intimate contact with their adjacent Sertoli cells at all times during the spermatogenetic process, with junctional and membrane specializations providing physical contact and communication and forming the so called "blood-testis barrier" (Cheng and Mruk, 2002).

The spermatogenic cells start out as mitotically dividing precursors called spermatogonia, sitting on the basal lamina (Fig. 1.1.2). At puberty these mitotically dividing cells enter into meiosis at regular intervals, moving from the periphery of the tubule to the luminal part and becoming primary and later secondary spermatocytes. Meiosis leads to chromosomal rearrangements leading to production of haploid round spermatids (early spermatids), that subsequently undergo structural differentiation to become mature or elongated spermatids (late spermatids). Once these cells are released by the Sertoli cell into the lumen of the tubule they are called spermatozoa and the fluid secreted by the Sertoli cells sweeps the released immotile spermatozoa to the rete testes.

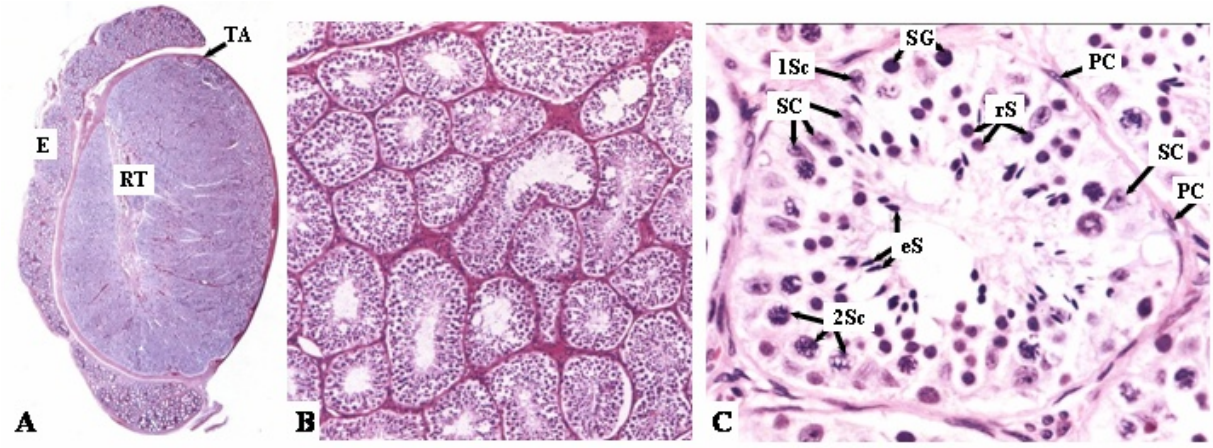



**Fig. 1.1.2 Histology of the testis (J. W. Heath, 2000) A) longitudinal section through the testis stained with hematoxilin-eosin (HE) E-epididimis, RT-rete testis, TA-tunica albuginea; B) section through testicular parenchima HE-stained; C) HE staining of a cross-sectioned seminiferous tubule PC-peritubular cell, SC- Sertoli cell nucleus, SG-spermatogonia, 1Sc-primary spermatocyte, 2Sc-secondary spermatocytes, rS-round spermatids, eS-elongated spermatids.**

Tight junctions between adjacent Sertoli cells and their associated membrane specializations form an intercellular barrier, which is completely impermeable for even small molecules (Whitehead, 1999, Cheng and Mruk, 2002). This blood-testis barrier separates the spermatogonia and early meiotic cells in the basal region of the seminiferous epithelium from the adluminal spermatocytes and spermatids. In this way a large majority of the developing germ cells are

sequestered behind a physical barrier and effectively isolated from the immune system.

### 1.1.3 The interstitial compartment

The interstitium of the testis consists of connective tissue cells, steroidogenic Leydig cells, the vasculature and immune cells.

The vascular supply to the testis arises from the abdominal aorta and in species with scrotal testes this results in a comparatively long and highly coiled spermatic artery. The arterioles, capillaries and venules of the testis completely permeate the interstitial tissue. The venous drainage of the testis via the spermatic veins is very closely associated with the arterial supply and involves a very effective counter-current heat and solute exchange structure called the pampiniform plexus. The lymphatics of the testis are variable between species, from irregular channels incompletely bounded by endothelial cells in rodents, to large discrete lymphatic vessels in humans (Ghinea and Milgrom, 1995).

#### 1.1.3.1 Leydig cells

Leydig cells are representing the majority of interstitial cells in the testis and are responsible for testosterone production. Various growth factors (interleukin 1alpha, transforming growth factor beta, inhibin, insulin-like growth factors I and II, vascular endothelial growth factor, and relaxin-like growth factor) modulate Leydig cell differentiation, regeneration, and steroidogenic capacity (Haider, 2004). Resident macrophages in the interstitial tissue of the testis are important for differentiation and endocrine function of Leydig cells (Hales, 2002). Acute testicular and systemic inflammation is accompanied by a decrease in the spermatogenic function of Leydig cells (Gow et al., 2001, Hedger et al., 2005) and the numbers of Leydig cells are considerably lower in EAO testis (Suescun et al., 1994, Suescun et al., 2001) probably due to apoptosis of Leydig cells which can be induced by cytotoxins.

#### 1.1.3.2 Immune cells in the testis

The testis in spite of its immune privileged status is not isolated from the immune system (Hedger and Culler, 1997). Macrophages are observed in the interstitium of most species and many testes also contain variable numbers of

eosinophils. In addition to the resident macrophages, mast cells are found adjacent to subcapsular blood vessels (Nistal et al., 1984, Gaytan et al., 1989). Less numerous, but ubiquitous are the intratesticular lymphocytes (Dym and Romrell, 1975). As in most other tissues, circulating immune cells, including T lymphocytes, also have relatively free access to the testis (Hedger and Meinhardt, 2000), and the testis has an efficient and effective lymphatic drainage to regional lymph nodes. Therefore, locally produced cytokines as well as those in the general circulation have the potential to exert effects at the testicular level.

The testicular macrophages

In rat and mouse the ratio of macrophages to Leydig cells is 1:4 (Hume et al., 1984). Macrophages display a close physical and functional relationship with the Leydig cells described as highly-specialized cytoplasmic interdigitations (Bambino and Hsueh, 1981).

The majority of testicular macrophages express a marker for tissue-resident macrophages (ED2, CD163). However, about 15-20% of the total testicular macrophages do not synthesize ED2. These cells can be identified by the expression of the lysosomal antigen CD68, recognized by antibody ED1 (Mahi-Brown et al., 1987, Wang et al., 1994, Gerdprasert et al., 2002a, Hedger, 2002). Moreover, 50% of the ED2+ cells are ED1- indicating the existence of several populations of macrophages in the rat testis, putatively representing different stages of development and/or functional stages (Hedger, 2002). ED1+ monocytes are recruited by chemokines into the testis where they become resident macrophages (ED2+) loosing the CD68 (ED1) marker. During this process, at a certain intermediate developmental step, these cells express both markers.

An increased number of ED1+ and ED2+ macrophages has been reported even at early stages of orchitis and more pronounced at later time points (Lustig et al., 1993).

Testicular dendritic cells

In spite of the common lineage, dendritic cells are morphologically distinct from macrophages and lack the phagocytic and killing capabilities of macrophages, whereas they are much more effective as antigen presenting

cells (APCs) (Banchereau and Steinman, 1998). Dendritic cells have been observed in the testis of rat (Head and Billingham, 1985) mouse (Itoh et al., 1995, Hoek et al., 1997) and human (Becker et al., 1987, Derrick et al., 1993). The functional role of these cells in the testis remains unclear although it appears that they direct immune responses within the testis and adjacent lymph nodes.

Testicular lymphocytes

In rat and human testis, lymphocytes represent 10-20% of the total leukocyte population. T cells ($CD4^+$ and $CD8^+$) and natural killer (NK) cells comprise the specific subsets of lymphocytes that have been described in the normal rat, mouse and human testis (Ritchie et al., 1984, Pollanen and Niemi, 1987, Pollanen and Maddocks, 1988, Tompkins et al., 1998). The number of testicular lymphocytes are expanded in the testes of man with infertility and sperm autoimmunity. The T cells tend to recirculate throughout tissues where they initially encountered an antigen (Picker and Butcher, 1992), suggesting that expanded T cell populations in human infertile testes may be specific to testicular autoantigens.

Testicular mast cells

The distribution of granulocytes within the testis is species-specific. Mast cells are almost absent from the testicular interstitium of rat, mouse, cat, bull, dog and deer. They are associated with blood vessels at the periphery of the testes directly under the capsule. Contrary to the mentioned species, mast cells are found throughout the interstitial tissue in human testis (Nistal et al., 1984, Yamanaka et al., 2000a). It is most likely that mast cells play a role in local innate immunity. In humans, testicular mast cell numbers increase in various types of male infertility (Yamanaka et al., 2000a), but decline with increasing age (Nistal et al., 1984).

In the adult rat, mast cells proliferate dramatically throughout the testicular parenchyma following ablation of Leydig cells by EDS (Wang et al., 1994). The degree of proliferation appears to be under the control of Leydig cells, suggesting that these cells produce an inhibitor of the mast cell activity.

Tryptase is found in mast cells of the human adult testis and is present in interstitial and peritubular mast cells, both in testes with normal spermatogenesis and in infertile testes. Mast cells are significantly increased in testes with spermatogenesis defects, suggesting that tryptase released from mast cells is a factor involved in male infertility (Meineke et al., 2000). Therefore tryptase appears as an interesting target in the treatment of male infertility.

Testicular neutrophils

In contrast to mast cells and eosinophils, neutrophils are absent from the interstitium of normal testis. They are found in the testis only in conditions of testicular inflammation or damage (Kohno et al., 1983, Gerdprasert et al., 2002a). They are chemoattracted by MCP-1, but it seems that higher levels of MCP-1 are required to chemoattract neutrophils, as compared to the levels required for the recruitment of macrophages.

### 1.1.4 The testicular immune response

Inflammation occurs in response to numerous stimuli and involves activation of monocytes, macrophages and mast cells, leading to production of pro-inflammatory cytokines, prostaglandins and cytotoxic reactive oxygen species, up-regulation of adhesion molecules, recruitment of immune cells, changes in blood flow and increased capillary permeability. If foreign antigen is present, an immunological response may also be triggered, involving antigen-specific T and B-lymphocytes, which also respond to specific cytokines.

Numerous clinical and experimental studies have shown that both local and systemic infection cause a transient down-regulation of androgen production, and disruption of germ cell production (O'Bryan et al., 2000). Several inflammatory mediators including cytokines are produced within the normal testis, where they are believed to be involved in regulating Leydig cell function and spermatogenic development (Hales et al., 1999, Hedger and Meinhardt, 2003). Production of these mediators is increased by inflammatory stimuli. The apparent overlap between testicular and immune regulatory mechanisms could provide the key to understanding both the processes leading to inflammation-mediated damage of testicular function and the phenomenon of

immune privilege in the testis. Interestingly, the non-immune cells of the testis such as Sertoli, Leydig and peritubular myoid cells are found to contribute significantly, to the overall production of cytokines.

## MCP-1 in the testis

MCP-1 belongs to a family of proteins called chemotactic cytokines which are important regulators of the leukocyte recruitment process and play an important role in inflammation (Kunkel and Butcher, 2002). Structurally, chemokines are proteins with low molecular mass characterized by four conserved cysteines forming two disulfide bridges. The position of the first two cysteines has been used to divide the chemokine family into two main subfamilies, the CXC chemokines and CC chemokines (Proost et al., 1996). MCP-1 is a member of the CC subfamily and is the chemokine that most potently acts on mononuclear cells, both monocytes and lymphocytes and on neutrophils (Proost et al., 1996).

It has been reported that in autoimmune diseases such as multiple sclerosis, arthritis and psoriasis, chemokines might be responsible for leukocyte migration to the inflamed tissue (Baggiolini and Dahinden, 1994) and for the local stimulation of leukocytes to release proteases. Concerning the testis, it was demonstrated in vitro production of MCP-1 by peritubular and Leydig cells where MCP-1 expression was markedly stimulated by some cytokines and LPS (Aubry et al., 2000). Recent studies (Gerdprasert et al., 2002a, Gerdprasert et al., 2002b) suggested that in a LPS inflammatory model the increase in the number of intratesticular macrophages was stimulated by MCP-1. Large amounts of MCP-1 are released during experimental autoimmune orchitis (Guazzone et al., 2003), but the underlying mechanisms are not elucidated.

## TGFßs in the testis

The transforming growth factor-ß (TGFß) family members are dimeric cytokines with predominantly immunosuppressive and anti-inflammatory activities. TGFß is produced by macrophages and lymphocytes. In testis TGFß isoforms (1–3) are highly expressed by Sertoli cells, peritubular cells and Leydig cells in the foetal and immature testis, although production declines dramatically post-puberty (Mullaney and Skinner, 1993, Avallet et al.,

1994). In addition, in the post-pubertal testis they also have been localised to the developing germ cells in a developmentally specific pattern of expression (Caussanel et al., 1997). The receptors for TGFß are found in both somatic and germ cells (Le Magueresse-Battistoni et al., 1995). Consequently, these cytokines have been involved in controlling both Leydig cell and seminiferous tubule development (Kohno et al., 1983). A precise role in the adult testis has yet to be established, although TGFß has been proposed to enhance immune privilege in the cryptorchid rat testis (Pollanen et al., 1993), and has been implicated in the immuno-protective activity of Sertoli cells in co-transplantation studies (Suarez-Pinzon et al., 2000).

## Inducible nitric oxide synthase

NO is synthesized from L-arginine by the action of NO synthase (NOS), an enzyme existing in three isoforms. Brain NOS (bNOS) or neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), also referred to as constitutive NOS (cNOS), are responsible for the continuous basal release of NO and both require calcium/calmodulin for activation (Griffith and Stuehr, 1995, Snyder, 1995). A third isoform is an inducible calcium-independent form (iNOS or NOS2) that is expressed only in response to inflammatory cytokines and lipopolysaccharides (LPS); reviewed in (Nussler and Billiar, 1993, Morris and Billiar, 1994). Several studies are reporting constitutive expression of iNOS in rat testes and increased iNOS levels associated with LPS induced acute systemic inflammation (O'Bryan et al., 2000, Gerdprasert et al., 2002a). More recent reports are showing a significantly statistical correlation between the iNOS immunoreactivity and mast cell numbers in the testis of man with impaired spermatogenesis (Sezer et al., 2005).

## Cyclooxygenase 2

Cyclooxygenase 2 (COX-2) is constitutively expressed in the rat testis (Neeraja et al., 2003b) and overexpressed in testes of men with impaired spermatogenesis and in testicular cancer biopsies. Coculture studies of a human mast cell line and human orbital fibroblasts have indicated up-regulation of cyclooxygenase 2 in fibroblasts (Smith and Parikh, 1999) and production of $PGE_2$. Other reports indicated that IL-1ß (Inoue et al., 1997) or secretory phospholipase A (Tada et al., 1998) are involved in COX-2 induction

and prostaglandins synthesis. A recent study showed that mast cell tryptase, activating $PAR_2$ is responsible for the induction of COX-2 expression in human testis (Frungieri et al., 2002).

## *2.2 Male infertility*

The autoimmunity in the form of antisperm antibodies and epididymo-orchitis is a significant cause of sub- and infertility in men. Inflammation due to reproductive tract infections, even systemic infection and illness, can lead to a failure of testicular androgen and sperm production (Bohring and Krause, 2003). The obvious capacity of the testis for inflammatory responses is contrasting with the fact that it is also one of a very few organs of the body capable of sustaining foreign grafts for extended periods without evidence of rejection (Head and Billingham, 1985). The so-called “immunological privilege” of the testis is believed to arise from the need to prevent immune responses against the auto-antigens of the meiotic and haploid germ cells, which first appear in the testis at the time of puberty, long after the establishment of self-tolerance in the perinatal period. The testis, in spite of its immune privileged status, is not isolated from the immune system (Hedger, 1997) having a population of resident macrophages as well as numerous mast cells adjacent to subcapsular blood vessels (Nistal et al., 1984, Anton et al., 1998). A variety of acute or chronic animal models were used to mimic the immunological male factor infertility, and many of them brought important contribution in understanding the underlying pathophysiological mechanisms.

## *1.3 Models of immunological infertility*

Experimental autoimmune orchitis

Acute or chronic inflammation of the male genital tract may result in alterations of spermatogenesis, steroidogenesis and fertility (Bohring and Krause, 2003). Chronic orchitis in men usually occurs as a consequence of different injuries induced by trauma or infectious agents. The interaction of immune cells with spermatic antigens is one of the pathogenic mechanisms involved in testis autoimmunity. Different models of experimental autoimmune orchitis (EAO) have been useful in understanding testicular cell interactions under pathological conditions. EAO has been induced in different species by active immunization with spermatic antigens, by adoptive T cell transfer or by

neonatal thymectomy (Tung and Teuscher, 1995). EAO was induced in mouse (Kaneko et al., 2003, Watanabe et al., 2005) and rat (Doncel et al., 1989, Lustig et al., 1993, Guazzone et al., 2003), with most studies being performed in the rat EAO model.

Testicular damage resulting from EAO is characterized initially by a mild interstitial lymphomononuclear cell infiltrate and focal damage to the seminiferous epithelium. As the disease progresses, substantial interstitial immune cell infiltrates can be observed, spermatogenesis cessation, sloughing of the tubules and granuloma formation take place. The main target of the immunological attack of EAO are the germ cells that undergo apoptosis (Theas et al., 2003).

Acute orchitis

Acute testicular inflammation is studied in different experimental models, either as part of a systemic inflammatory process (Aubry et al., 2000, O'Bryan et al., 2000, Gerdprasert et al., 2002a, Gerdprasert et al., 2002b) or as isolated testicular inflammation induced by local traumatism or by testicular torsion (Ozturk et al., 2003). High levels of MCP-1 and iNOS, as well as increased numbers of ED1+ macrophages are reported in acute orchitis. In humans, the most frequent causes of acute testicular inflammation are mechanical injuries (trauma, testicular torsion) and mumps orchitis.

## *1.4 Proteinase-activated receptors*

The protease-activated receptors (PARs) belong to a large superfamily of G-protein-coupled receptors and mediate the cellular actions of certain serine proteases. Four members, PARs 1 to 4, have been cloned within the past decade. PAR-1, -3 and -4 are alternative thrombin receptors with different tissue distributions; roles for these three distinct thrombin receptors in the activation of platelets have been well described. $PAR_2$ is activated by trypsin, mast cell tryptase and coagulation factors VIIa and Xa, but not by thrombin.

These proteolytic enzymes cleave an N-terminal peptide at a specific site, and the newly exposed N-terminal end binds to the second extracellular loop of the receptor (Nystedt et al., 1994, Lerner et al., 1996, Al-Ani et al., 1999) (Fig. 1.4.2). Thus, the new receptor N-terminus functions as a ‘tethered ligand’ and

activates the receptor, resulting in intracellular signaling mainly by $G_q$ proteins (Vergnolle et al., 2001, Steinhoff et al., 2005).

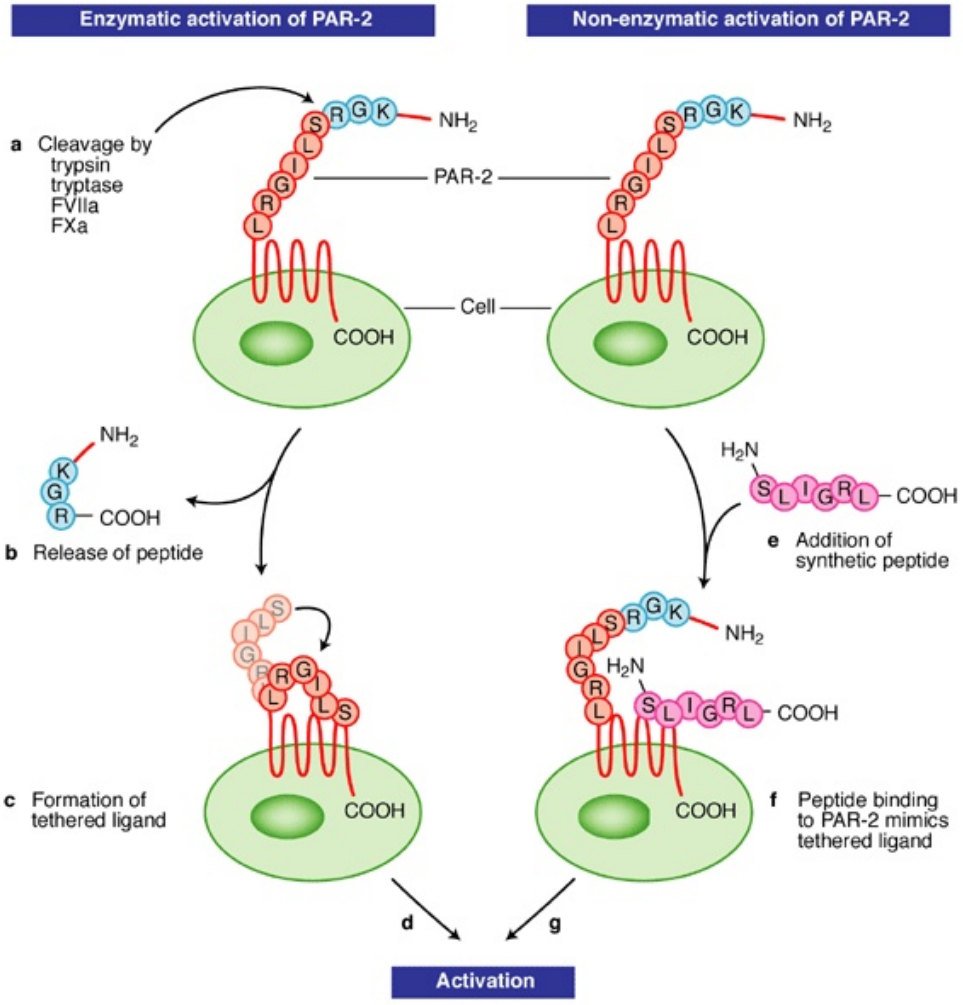



**Fig. 1.4.2 Enzymatic and non-enzymatic $PAR_2$ activation, taken from (Kawabata, 2002).**

Enzymatic $PAR_2$ activation

The N-terminal cleavage/activation site of $PAR_2$ can be cleaved by trypsin, mast cell tryptase and coagulation factors VIIa and Xa, yielding the new N-termini $NH_2$-SLIGRL for murine $PAR_2$ and $NH_2$-SLIGKV for human $PAR_2$. Tryptase is secreted by activated mast cells during inflammation and is a strong endogenous activator of human $PAR_2$ (Molino et al., 1997, Compton et al., 2001). Factors VIIa and Xa might become available as agonists for $PAR_2$ expressed by the vascular endothelium in conditions such as disseminated intravascular coagulation (Camerer et al., 2000) and might also activate $PAR_2$ in other tissues following tissue injury or during inflammation.

Non-enzymatic $PAR_2$ activation

Non-enzymatic activation of $PAR_2$ is achieved by synthetic peptides as short as five or six amino acids, such as SLIGRL-$NH_2$ and SLIGKV-$NH_2$, based on the receptor-activating sequence of the tethered ligands (Fig.2.4.2). These are capable of binding directly to the body of $PAR_2$, mimicking the actions of the endogenous activators. Recently a newly derived $PAR_2$ activating peptide 2-Furoyl-LIGLRO-$NH_2$ ($PAR_2$-f-AP) was reported to have a markedly increased activity, an enhanced selectivity for $PAR_2$ and to show more stability in an *in vivo* environment as compared to classical $PAR_2$-AP. This compound should prove to be very useful for studies done in intact animals that are aimed at elucidating the potential patho-physiological responses due to $PAR_2$ when activated *in vivo* by locally generated proteinases (McGuire et al., 2004a).
Development of $PAR_2$ antagonists would not only dramatically facilitate understanding of the physiological and pathophysiological roles of $PAR_2$, but might also be valuable to the clinical treatment of certain human diseases.

Activation of $PAR_2$ induces assembly of a mitogen-activated protein kinases (MAPK) signaling module activating the extracellular-signal-regulated kinases (ERK1/2). By the other hand $PAR_2$ couples to $G_{ßq/11}$, resulting in activation of phospholipase C, production of inositol 1,4,5-trisphosphate and diacylglycerol, mobilization of $Ca^{2+}$ and activation of protein kinase C. These signaling events are rapidly attenuated and desensitized after repeated stimulation, indicative of receptor desensitization and down-regulation. $PAR_2$ activation induces translocation of ß-arrestins to the plasma membrane, where they interact with $PAR_2$ to mediate both desensitization and endocytosis (Dery et al., 1999, DeFea et al., 2000). At one extreme, PARs, which are activated by irreversible proteolysis and are thus single-use receptors, traffic to lysosomes, a process termed down-regulation (Bohm et al., 1996). The down-regulation of receptors is of fundamental importance in terminating signaling. However, little is known about the molecular mechanisms of post-endocytic sorting that target receptors for degradation. Sustained signaling requires the mobilization of $PAR_2$ from prominent stores in the Golgi apparatus or synthesis of intact receptors (Bohm et al., 1996).

### 1.4.3 $PAR_2$ in inflammation

Numerous studies suggest that $PAR_2$ contributes to the development of inflammation. $PAR_2$ activation leads to increased vascular permeability (Kawabata et al., 1998, Vergnolle et al., 1999), blood vessel relaxation (Al-Ani et al., 1999, Sobey et al., 1999), systemic hypotension, bronchoconstriction (Ricciardolo et al., 2000), granulocyte infiltration (Vergnolle et al., 1998b) and leukocyte adhesion and margination (Vergnolle, 1999). These inflammatory responses, which can be induced by surgical exposure of tissues, are delayed in $PAR_2$ deficient mice (Lindner et al., 2000), suggesting that $PAR_2$ is an important receptor in inflammation and is necessary for early inflammatory responses *in vivo*.

Neurogenic inflammation is a form of inflammation that depends on primary spinal afferent neurons with cell bodies in the dorsal root ganglia close to spinal cord and projections to the spinal cord and peripheral tissues (McDonald et al., 1996). Inflammatory agents trigger the release of the neuropeptides substance P (SP) and calcitonin gene related peptide (CGRP) from the peripheral endings of these neurons. SP interacts with the neurokinin 1 receptor (NK1R) on endothelial cells of post-capillary venules to cause plasma extravasation and granulocyte infiltration, and CGRP interact with its type 1 receptor on arteriolar smooth muscle to cause vasodilatation.

$PAR_2$ agonists can also trigger processes that protect against inflammation. $PAR_2$ agonists improve cardiac function and reduce tissue damage after myocardial ischemia (Napoli et al., 2000). $PAR_2$ agonists can also serve to protect the airway by acting as bronchodilators in certain species (Cocks et al., 1999). Systemic injection of $PAR_2$ activating peptide to mice protects from colitis and decreases pro-inflammatory cytokine synthesis and lethality (Fiorucci et al., 2001). $PAR_2$ activation protects also the gastric mucosa against the injurious effect of non-steroidal anti-inflammatory drugs (Kawabata et al., 2001). According the physiological or pathophysiological environments, $PAR_2$ may be involved in different pro- or anti-inflammatory processes.

### 1.4.4 $PAR_2$ in the testes

Initially $PAR_2$ expression has been reported in cells of the testicular germinal epithelium (D'Andrea et al., 1998). Later studies described $PAR_2$ immunoreactivity on the acrosome of ejaculated spermtozoa (Weidinger et al., 2003) and in a fraction of the interstitial cells in the human testis. However, the precise nature of these $PAR_2$-positive interstitial cells was not specified (Frungieri et al., 2002). $PAR_2$ activation by mast cell tryptase was found to be responsible for the proliferation of peritubular cells in infertile testis and thus leading to the fibrotic thickening of the seminiferous tubules wall.

## *1.5 Aim of the project*

Inflammation due to reproductive tract infections, even systemic infection and illness, can lead to a failure of testicular androgen and sperm production (Bohring and Krause, 2003). Immunological factor male infertility is one of the major causes of infertility in human and certain forms of male infertility have been found to be associated with increased numbers of tryptase containing testicular mast cells (Meineke et al., 2000). Moreover, recent advances are beginning to unravel the intriguing role mast cells play both in various autoimmune diseases (Benoist and Mathis, 2002, Robbie-Ryan and Brown, 2002) and in acute inflammation (Cocks and Moffatt, 2001, Roche et al., 2003b).

Proteinase-activated receptor-2 ($PAR_2$), a G-protein coupled receptor important to injury responses, was shown to be activated by mast cell tryptase. This study aimed to investigate the involvement of mast cells and $PAR_2$ in the development and/or aggravation of acute and chronic testicular inflammation.

# 3 MATERIALS AND METHODS

## *3.1. Materials*

### 3.1.1 Buffers and solutions

**10X DNase I buffer:**

- 0.1M TRIS (pH 8.3)
- 0.5M KCl
- 15mM $MgCl_2$

**6X DNA loading buffer:**

- 0.25% (w/v) bromophenol blue
- 30% glycerol in $H_2O$

**HEPES buffer salt solution (1l, pH 7.4, sterile filtered)**

- 418mg KCl
- 7.97 g NaCl
- 1ml $MgCl_2x6H_2O$
- 2.2ml $CaCl_2x2H_2O$
- 2.18g Glucose
- 2.38g HEPES

**Michaelis buffer (0.1M, 1l, pH 7.4):**

- 20.6g sodiumbarbital
- 58.1ml HCl (1N)

**50X TAE electrophoresis buffer (1l, pH 8.0):**

- 242g of Tris base
- 57.1ml of glacial acetic acid
- 100ml of 0.5M EDTA

**TE8:**

- 10 mM Tris (pH 8.0)
- 1 mM EDTA (pH 8.0)

**Toluidin blue (stock solution):**

1% Toluidin blue O (Waldeck, Münster, Germany) (w/v) in 70% ethanol (v/v)

**Toluidin blue (working solution):** Dilute the stock solution 1:10 in freshly prepared 1% NaCl (v/v)

**10X PBS (1l):**

- 87.6g NaCl
- 22.8g $K_2HPO_4$
- 6.8g $KH_2PO_4$

### 3.1.2 Oligonucleotides

All oligonucleotides were designed using Primer3 software available online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Primer sequences, annealing temperatures and other technical details for PCR setup are presented in Table 3.1.2 All nucleotides were supplied by MWG-Biotech, Ebersberg, Germany. All primers were diluted at 100 pM/µl in 0.2x TE8 buffer and stored as stock solution at -20°C.

**Table 3.1.2:**

| Gene | Primer sequences | [primer] (µM) | Annealing Temperature (°C) | Annealing Time (s) | $[Mg^{2+}]$ (mM) | Amplicon Length (bp) | Gene Accession Number |
|---|---|---|---|---|---|---|---|
| GAPDH | Fw: 5'-CATTGTTGCCATCAACGACC-3'<br>Rw: 5'-TCACACCCATCACAAACATG-3' | 2 | 55 | 20 | 2.5 | 320 | NM_017008 |
| $PAR_2$ | Fw: 5'-GGGAGGTATCACCCTTCTGG-3'<br>Rw: 5'-TGTCCAATCTGCCAATCAGA-3' | 2 | 60 | 40 | 2.5 | 101 | NM_007974 |
| MCP-1 | Fw: 5´-CAGGTCTCTGTCACGCTTCT-3'<br>Rw: 5´-AGTATTCATGGAAGGGAATAG-3' | 1 | 55 | 40 | 2.5 | 525 | M57441 |
| TGFß-2 | Fw: 5´-CCGGAGGTGATTTCCATCTA-3'<br>Rw: 5´-GCGGACGATTCTGAAGTAGG-3' | 2.4 | 61 | 30 | 2.5 | 201 | NM_031131 |
| iNOS | Fw: 5'-ACCTGAAAGAGGAAAAGGAC-3'<br>Rw: 5'-ACTCTTGGAGTTCATGATGG-3' | 2 | 60 | 30 | 2.5 | 314 | X76881 |
| COX-2 | Fw: 5'-TACCCGGACTGGATTCTACG-3'<br>Rw: 5'-AGTCATCAGCCACAGGAGGA-3' | 2 | 48 | 25 | 2.5 | 302 | NM_017232 |

### 3.1.3 Animals

Male inbred Wistar rats (Charles River, Sulzfeld, Germany) were used for all aspects of this study. Peritubular cells (PTC) were prepared from 19-day-old rats while for the *in-vivo* experiments and for the isolation of macrophages adult 200g rats were used. Experimental procedures were approved by the local authority (Regierungspraesidium Giessen) and conform to the Code of Practice for the Care and Use of Animals for Experimental Purposes.

## *3.2 Methods*

### 3.2.1 Induction of experimental autoimmune orchitis (EAO)

Decapsulated testes from 200g rats were homogenized under sterile conditions at 4°C in an equal volume of isotonic saline buffer (500mg/ml wet weight) and stored at –20°C prior to use. Thirty rats 180-220g body weight (b.w.) were anaesthetized by intraperitoneal administration of 100mg/kg b.w. Ketamine (Ketavet, Pharmacia GmbH, Erlangen, Germany) and 10mg/kg b.w. Xylazine (Rompun, Bayer Vital GmbH, Leverkusen, Germany) and then immunized with 0.4ml syngenic testicular homogenate mixed with 0.4ml complete Freund's adjuvant (Sigma-Aldrich, USA). Testes homogenates were mixed with the adjuvant solution few hours prior to immunizations. Three times at 14-day intervals, a total of 0.8ml/rat/timepoint was subcutaneously injected into the hind footpads and in different sites of the back skin. The wounds in the hind footpads were sealed with Histoacryl® tissue glue (Braun, Tuttlingen, Germany). The first two immunizations were followed by an intravenous injection (in the tail vein) of $10^{10}$ cells of inactivated Bordetella pertussis bacteria (DSMZ, Braunschweig, Germany) dispersed in 0.5ml isotonic saline, whilst the third was followed by intraperitoneal injection of $5x10^9$ cells in 0.5ml isotonic saline (Doncel et al., 1989). Control animals (n=16) received complete Freund's adjuvant containing Bordetella pertussis but no testis homogenate. Fifty and eighty days after the first immunization, the animals were sacrificed by overdose of Isofluran (FORENE®, Abbott, Wiesbaden, Germany). The testes were removed under sterile conditions, weighted and either snap frozen in liquid nitrogen or placed in Bouin's solution for 12hrs. The fixed tissue was subsequently embedded in paraffin following standard procedures.

### 3.2.2 $PAR_2$ activation *in vivo* by human ß tryptase

Rats (200g; n=5-10/group) were anesthetized by intraperitoneal administration of 100mg/kg b.w. Ketamine (Ketavet, Pharmacia GmbH, Erlangen, Germany) and 10mg/kg b.w. Xylazine (Rompun, Bayer Vital GmbH, Leverkusen, Germany). The scrotum was carefully opened by a small incision.

Rats received by injection directly under the testicular capsule in both testes (using a thin insulin needle connected to a 1ml syringe) 50µl of a) 100nM recombinant ß tryptase diluted in isotonic saline containing 30µg/ml heparin (used to stabilize the tryptase), b) 100nM inhibited recombinant ß tryptase (with the irreversible inhibitor Pefabloc SC; residual tryptase activity ≤0.1%) diluted in isotonic saline containing 30µg/ml heparin; c) isotonic saline containing 30µg/ml heparin and d) isotonic saline alone (sham control). The injection site was always right of the testicular artery and special care was taken to minimize any damage to the testicular tissue. Skin and the cremaster muscle were sewed with surgical wire. After 5hrs the animals were sacrificed and both testes removed. The testes were snap frozen for RNA extraction and subsequent real time RT-PCR analysis. Based on the findings of an experiment employing a colored test substance to determine the distribution of injection solutions in the testis, only tissue around the injection site was used for gene expression analyses.

### 3.2.3 $PAR_2$ activation *in vivo* using specific stable synthetic peptides

Because human tryptase, in addition to activating $PAR_2$, causes a wide variety of proteolytic effects (Sommerhoff, 2001, Brown et al., 2002), subsequent experiments utilized a specific $PAR_2$ activating peptide; a peptide corresponding to a $PAR_2$-activating peptide with an N-terminal furoyl group modification, 2-furoyl-LIGRLO-$NH_2$ was reported to be equally effective to and 10 to 25 times more potent than SLIGRL-$NH_2$ for increasing intracellular calcium in cultured human and rat $PAR_2$-expressing cells, respectively. $PAR_2$-f-AP ([2-furoyl]-LI-GRLO-$NH_2$ represents the most potent, metabolically stable and selective activator of $PAR_2$ in biological systems described to date (McGuire et al., 2004a).

Rats (200g; n=5-10/group) received 50µl of $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$; $10^{-4}$ M), the control peptide (trans-cinnamoyl-OLRGIL-$NH_2$; $10^{-4}$M) (Vergnolle et

al., 1998a) or a sham control (isotonic saline) under the testicular capsule as described above. To study the involvement of NO production and prostaglandin synthesis in $PAR_2$-stimulated cytokine expression, two groups of rats received a subcutaneous injection of either a NOS-inhibitor (L-NAME: L-nitro L-arginine methyl ester, 30mg/kg, Merck Biosciences GmbH, Schwallbach, Germany) or a COX-2 antagonist (Metacam®: Meloxicam; 0,3mg/kg; Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany) 20 min prior to the administration of [2-furoyl]-LIGRLO-$NH_2$ or the control peptide. After 5hrs, animals were killed, testes removed and one testis was used for RNA extraction and RT-PCR analysis and the other for histology as described above.

### 3.2.4 Myeloperoxydase (MPO) staining

MPO, an iron-containing protein, is found in the azurophilic granules of neutrophilic polymorphonuclear leukocytes (PMNs) and in the lysosomes of monocytes. MPO is most abundant in the granules of neutrophils. Monocytes contain only about a third of the MPO present in neutrophils.

We used a standard protocol (Hematology laboratory, Uniklinikum Giessen) for detection of MPO containing neutrophils. Testicular cryosections (12µm) from EAO and adjuvant control groups were fixed for 30 seconds in a solution containing ethanol:formalin (9:1, v:v) and immediately washed in tap water. Slides were further incubated for 15min in a “working solution” containing 5mg 3-amino-9-ethyl-carbazol (AEC, myeloperoxidase substrate), 3ml DMSO, 25ml 0.1M Michaelis’ buffer and 250µl 0.3% $H_2O_2$. After incubation, the slides were washed for 5min in tap water and stained for 10 min with Mayers-Hemalaun solution. Finally the slides were washed 20 min in flowing tap water, air-dried, mounted in glycerin-gelatin and photographed using a trans-luminescence microscope (Carl Zeiss AG, Göttingen, Germany). MPO containing neutrophils have a red stained cytoplasm Mayers Hemalaun stains all nuclei blue.

### 3.2.5 Staining and quantification of mast cells

Cryosections of testis were cut at a thickness of 12µm. The slides were fixed in ice-cold isopropanol for 10 min and washed 3 times, for 5 min each in PBS. Toluidin blue O (Waldeck, Münster, Germany) stock solution (1% toluidin blue in 70% ethanol) was diluted 1:10 in freshly prepared 1% NaCl and added to the specimens. Metachromasia, tissue elements displaying a different color from

the dye solution after staining, is dependent on the pH, dye concentration and temperature of the basic dye. Blue or violet dyes will show a red color shift, and red dyes will show a yellow color shift with metachromatic tissue elements. Slides were stained for 20sec or until metachromasia developed, washed in distilled water, dried on a heating plate (70°C) and mounted in LR white resin. For quantification, the slides were examined in a trans-luminescence microscope (Carl Zeiss AG, Göttingen, Germany) using the 20-fold objective. On each section both the metachromatically stained cells and the cross-sectioned seminiferous tubules were counted and results were expressed as a ratio mast cell/tubule cross section.

### 3.2.6 $PAR_2$ immunohistochemistry on testicular paraffin sections

Paraffin sections (7µm) were dewaxed in xylene and rehydrated in decreasing concentrations of alcohol. Specimens were incubated in 3% $H_2O_2$ for 30min in the dark to block endogenous peroxidases, followed by 10% normal donkey serum and 4% bovine serum albumin in PBS for 1hr to minimize unspecific protein-protein interactions, prior to being incubated overnight at 4°C with a goat anti-rat $PAR_2$ (C-17, Santa Cruz, CA, USA). Source and dilution of antibodies are presented in table 3.2.6 After washing in PBS/tween, the sections were incubated for an hour with a donkey anti-sheep/goat biotinylated secondary antibody followed by incubation with HRP-conjugated streptavidin for 30 min. The color reaction was developed with diaminobenzidine, the slides were coverslipped in aqueous mounting medium (Cristalmount, Sigma-Aldrich) and finally photographed.

Table 3.2.6:

| Primary antibody | Dilution | Secondary antibody | Dilution |
|---|---|---|---|
| Goat anti rat PAR2 (Santa Cruz Biotechnology) | 1:25 | Donkey anti sheep/goat biotinylated (Amersham) | 1:400 |
| ED1 mose anti rat CD68 (Serotec) | 1:40 | Donkey anti mouse FITC (Dianova) | 1:400 |
| ED2 mouse anti rat CD163 (Serotec) | 1:40 | Donkey anti mouse FITC (Dianova) | 1:400 |
| Mouse anti rat smooth muscle actin (Dako) | 1:400 | Donkey anti mouse FITC (Dianova) | 1:400 |
| Rabbit anti Ki67 (Novocastra Laboratories Ltd. UK) | 1:700 | Donkey anti rabbit Cy3 (Dianova) | 1:900 |
| Mouse anti phosphor-p44/42 MAP kinase (thr202/Tyr204) (Cell Signaling, Massachusetts, USA) | 1:2000 | Goat-anti-mouse horseradish peroxidase-labeled antibody (Perbio Science, Bonn) | 1:5000 |
| Rabbit antit total p44/42 (Cell Signaling, Massachusetts, USA) | 1:1000 | Goat anti rabbit horseradish peroxidase-coupled antibody (Perbio Science, Bonn) and ECL | 1:3000 |

### 3.2.7 $PAR_2$ immunohistochemistry on isolated peritubular cells

Peritubular cells were plated on glass coverslips in 6 well plates. Fixation was done in ice-cold isopropanol for 10 min. Unspecific protein interactions were blocked by incubation at room temperature for 1h with a blocking solution containing 10% normal donkey serum and 4% bovine serum albumin in PBS. The primary antibody goat anti-rat $PAR_2$ antibody (C-17, Santa Cruz, CA, USA) was diluted 1:25 in blocking solution and applied to slides overnight at 4°C. Than the slides were washed three times with PBS for at least 1h. The donkey anti-sheep/goat biotinylated antibody (Amersham, Buckinghamshire, UK; 1:400 in PBS) was incubated at room temperature for 1h. After a 1h washing step, slides were incubated for 1h in dark at room temperature with streptavidin Texas-Red conjugated 1:100 diluted in PBS. After a final washing step of 1h the slides were mounted in a DAPI containing mounting medium (Vector Laboratories, Burlingame, CA) and photographed using a fluorescence microscope (Carl Zeiss AG, Göttingen, Germany). Source and dilution of antibodies are presented in table 3.2.6.

### 3.2.8 Double immunofluorescence on isolated testicular macrophages

Testicular macrophages plated on glass coverslips in 24 well plates were fixed with ice-cold methanol for 10 min. Thereafter slides were washed 3 times with PBS. Unspecific protein interactions were blocked by incubation for 1h at room

temperature with a blocking solution containing 10% normal donkey serum and 4% bovine serum albumin in PBS. The primary antibodies goat anti-rat $PAR_2$ (C-17, Santa Cruz, CA, USA; 1:25), mouse anti rat CD68 (ED1, Serotec, Oxford, UK; 1:40) and mouse anti rat CD163 (ED2, Serotec, Oxford, UK; 1:40) were all diluted in blocking solution and applied together on the slides for 12-15h at 4°C. Than the slides were washed three times with PBS for at least 1h. The donkey anti-sheep/goat biotinylated antibody (Amersham, Buckinghamshire, UK; 1:400 in PBS) was applied for 1 h at room temperature. After a 1h washing step slides were incubated 1h in the dark at room temperature, with a pool of donkey anti mouse FITC (Dianova, Hamburg, Germany) 1:400 and streptavidin Texas-Red conjugated 1:100 diluted in PBS. After a final washing step of 1h the slides were mounted in a DAPI containing mounting medium (Vector Laboratories, Burlingame, CA) and photographed using a confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany). Source and dilution of antibodies are presented in table 3.2.6.

### 3.2.9 Double immunofluorescence on testicular cryosections

Cryosections of testis were cut at a thickness of 12µm. After drying for 20min at room temperature the slides were fixed in ice-cold methanol for 10 min. After that slides were washed 3 times with PBS. To minimize the unspecific protein binding the slides were incubated for 1h at room temperature with a blocking solution containing 10% normal donkey serum and 4% bovine serum albumin in PBS.

Thereafter specimens were treaded overnight at 4°C with pooled primary antibodies in the following combinations: 1) smooth muscle actin (SMA) and $PAR_2$; 2) SMA and Ki67; 2) ED1/ED2 and Ki67. Corresponding fluorochrome labeled secondary antibodies were co-applied in the dark for 1h at room temperature. Slides were mounted in a DAPI containing mounting medium (Vector Laboratories, Burlingame, CA) and photographed using a fluorescence microscope (Carl Zeiss AG, Göttingen, Germany). Source and dilution of antibodies are presented in table 3.2.6.

### 3.2.10 Cell culture experiments

## 3.2.10.1 Isolation and culture of peritubular cells (PTs)

Peritubular cells (PTC) were prepared from 19-day-old rats according to a modified version of the protocol described by (Hoeben et al., 1995). Rats were killed with $CO_2$ and testes were removed, rinsed once in 1% iodine alcohol, and then washed several times in PBS (Dulbecco's PBS without $Ca^{2+}$ and $Mg^{2+}$, PAA Laboratories, Cölbe, Germany). After decapsulation the tissue was minced in PBS followed by a 30 min incubation at 32°C in PBS containing 0.25% trypsin and 10µg/ml DNase I (Roche, Mannheim, Germany) under constant shaking. The enzymatic reaction was stopped by the addition of 5mg/ml trypsin inhibitor (Roche) in PBS and the tubule fragments were allowed to settle for 10-20 min. The supernatant was removed and the pellet was rinsed in 20ml PBS, then 2.5% trypsin inhibitor, followed by six to eight washes in 30ml PBS. Subsequently, the fragmented tubules were incubated for 10min at 32°C in PBS containing 1mg/ml collagenase, 1mg/ml hyaluronidase and 10µg DNase I (Roche) in a shaking water bath. Thereafter, 30ml of PBS were added and tubule fragments were allowed to settle for 10-20 min. The PTC containing supernatant was collected, supplemented with 20ml RPMI standard medium (PAA Laboratories) containing 10% fetal calf serum (FCS) and centrifuged at 500xg for 10min at room temperature. PTC from 20 immature animals were seeded into ten 75$cm^2$ cell culture flasks and placed at 37°C in a humidified incubator in 5% $CO_2$ atmosphere. For experiments, PTC were starved in a FCS-free RPMI medium and moved to a 32°C/5%$CO_2$ incubator one day prior to stimulation.

## 3.2.10.2 Stimulation of peritubular cells with $PAR_2$ synthetic agonists

As FCS contains factors of coagulation that could possibly activate $PAR_2$ the FCS concentration was gradually reduced before stimulating the cells with $PAR_2$ agonists. Technically, FCS concentration was diminished in gradual steps, by incubating the cells at 32°C every hour with medium containing decreasing FCS concentrations (5%, 2,5% and 1%).

PCs were stimulated after 24hrs of incubation in 1%FCS medium. Cells were treated with $10^{-4}$ M of either $PAR_2$-activating peptide (SLIGRL-$NH_2$, $PAR_2$-AP)

or the reverse peptide (LSIGRL-$NH_2$, $PAR_2$-RP) as control. Stimulation time was 5 and 10 min for the analysis of phosphorylation of MAP kinases and 3, 6 and 9hrs for the assessment of cytokine expression using real time PCR.

### 3.2.10.3 Isolation and culture of testicular and peritoneal macrophages

*Testicular macrophages*

For each experiment, four rat testes were decapsulated in 10ml ice-cold endotoxin free DMEM:F12 medium (Life Technologies, Karlsruhe, Germany). The seminiferous tubules were gently prized apart using curved forceps following the method described by (Hedger and Eddy, 1986). Enzymes were not used because these would activate the macrophages. Dissociated tubules were transferred in a new Falcon tube and the volume adjusted to 50ml. The tubule fragments were allowed to settle for 5min, and then the supernatant was transferred in a new clean tube, cells were counted without differentiating between cell types and centrifuged at 300g for 10min at 4°C to sediment the interstitial cells. The supernatant was removed and the pellet was resuspended in at $5*10^6$ cells/ml in DMEM:F12. Cells were plated onto small Petri-dishes (50mm diameter) for RNA isolation (3ml each well) or in 24 well plates containing each a small glass coverslip (for further immunofluorescence experiments or for assessment of $[Ca^{2+}]_i$) (500µl each well) and incubated at 32°C for 30min. Contaminating cells were removed by washing the wells four times with DMEM:F12 taking advantage of the rapid adherence of macrophages to plastic surfaces.

*Peritoneal macrophages*

Two rats were killed with an overdose of Isofluoran and 50ml DMEM:F12 was injected in the peritoneal cavity. After gentle massage of the abdomen the medium was collected from the peritoneal cavity by peritoneal puncture and centrifuged at 300g for 10min to sediment the macrophages. Supernatant was removed and the pellet was resuspended in 1ml DMEM:F12. Cells were counted and the concentration adjusted to $5x10^6$ cells/ml. Peritoneal macrophages were plated in Petri dishes (50mm diameter) $1.8x10^6$ cells/dish or in 24 well plates containing each a small glass coverslip (for further immunofluorescence experiments or for assessment of $[Ca^{2+}]_i$) and incubated at

37°C for 30min in BSA free medium prior to vigorously wash the dishes 5 times with DMEM:F12 in order to remove non adherent cells. Outcome was generally approximately 50% of total plated cells. For RNA isolation, 5 similar preparations were polled after stimulation.

### 3.2.10.4 Stimulation of macrophages with $PAR_2$ synthetic agonists

Cultured macrophages (peritoneal and testicular) were stimulated directly after the last washing step. Cells were treated with $10^{-4}$ M of either $PAR_2$-activating peptide (SLIGRL-$NH_2$, $PAR_2$-AP) or the reverse peptide (LSIGRL-$NH_2$, $PAR_2$-RP) as control. As negative control cells received PBS and as positive control they received 2µM LPS in PBS. All chemicals used for stimulation were pipetted in the medium in volumes of 10-30µl of the corresponding stock solutions. After 6h stimulation time, cells were washed two times with sterile PBS and lysed with 200µl RLT buffer (Rneasy® Mini Kit, Qiagen, Hilden, Germany). The lysates were passed through a 24G needle 10 times, up and down using a sterile 1ml syringe and than stored at -80°C. Lysates from 6 experiments were pooled prior to RNA isolation as described at 3.2.10.3.

### 3.2.11 Assessment of intracellular free $Ca^{2+}$ concentration

PTC and macrophages were grown as a monolayer on coverslips and incubated at 32°C for 30 min with 3 µM Fura-2-AM (1 mM stock in dimethylsulfoxide [DMSO]; Molecular Probes, Leiden, Netherlands). Extracellular free Fura-2-AM was removed by rinsing the cells three times with Hepes buffer salt solution (HBSS, pH 7.4). To allow complete hydrolysis of the intracellular Fura2-AM, the cells were incubated a further 30 min at 32°C with 5% $CO_2$. After an additional washing step repeated twice, the coverslips were placed into a HBSS containing measuring chamber (Delta T System, Olympus, Hamburg, Germany) and examined with a 40X water immersion objective and a 10X ocular in an Olympus microscope (Olympus BX50WI, Olympus, Hamburg, Germany). The cells were stimulated at 340 nm and 380 nm using a monochromator (T.I.L.L. Photonich GmbH, Martinsried, Germany). Emission intensities were recorded as a ratio (340nm/380nm) at 510 nm with a photomultiplier (T.I.L.L. Photonich GmbH, Martinsried, Germany). The

background-corrected ratiometric signal R was determined by applying the standard equation:

$[Ca^{2+}] = K_d\ (R - R_{min})/(R_{max}-R)$,

where $R_{min}$ and $R_{max}$ were derived from the experimental readings. All measurements were performed between 60 and 90 minutes after addition of the dye. Data was aquired and analyzed using the T.I.L.L.Vision-v.4.0 software (T.I.L.L. Photonich GmbH, Martinsried, Germany). Cells were initially incubated with the reverse peptide (LSIGRL-$NH_2$, $PAR_2$-RP; $10^{-4}$ M) followed by addition of $10^{-4}$ M $PAR_2$-activating peptide (SLIGRL-$NH_2$, $PAR_2$-AP). Trypsin (100nM) was used as natural $PAR_2$ agonist and thapsigargin ($10^{-7}$-$10^{-5}$ M) as positive control.

### 3.2.12 Western Blot analysis

PTCs were lysed in Laemmli buffer (5% bromophenol blue, 5% mercaptoethanol, 62.5 mM Tris-HCL, 20% glycerol, and 2% SDS). Samples were boiled, the proteins separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond™-ECL™; Amersham Biosciences, Braunschweig, Germany) using the PerfectBlue™ semidry electroblotter (PeqLab Biotechnologie, Germany). To minimize non-specific protein binding, the membrane was blocked for 1hr in PBS containing 0.1% Tween-20 and 5% dry milk powder. The presence of phosphorylated p44/42 (ERK1/2) was detected using a mouse monoclonal antibody raised against phospho-p44/42 MAP kinase (thr202/Tyr204, see table 1), followed by horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (1:5000; Perbio Science, Bonn) and enhanced chemiluminescence detection (ECL, Amersham Biosciences). After exposure, the nitrocellulose membrane was stripped, blocked again under the same conditions and total p44/42 detected using a rabbit polyclonal antibody directed against p44/42 (see table 1) and a goat anti-rabbit horseradish peroxidase-coupled secondary antibody (1:3000; Perbio Science, Bonn, Germany) followed by ECL visualization.

### 3.2.13 Working with RNA

Because of the ubiquitous presence RNases, working with RNA requires special care for assuring an RNase-free environment. The entire work with RNA samples involved the use of sterile autoclaved single-use instruments and hot-sterilized glassware, respectively. Alternatively, the instruments were treated for at least 30min with 3% $H_2O_2$ before using. At each experimental step single-use gloves were worn.

### 3.2.14 Isolation of total RNA from cultured cells and tissues

Cell culture flasks containing maximum $10^7$ peritubular cells were washed two times with PBS before adding 600µl RLT buffer (Rneasy® Mini Kit, Qiagen, Hilden, Germany) containing 1%(v/v) ß-mercapto-ethanol. Cells were scraped from the surface of the flask before aspiring the cell content in a 1ml syringe through a 24G needle. The cell suspension in RLT buffer was discarded in a clean 1.5ml reaction tube and passed another 10 times through the same 24G needle. Samples were loaded on a QIAshredder spin column placed in a 2ml collection tube and centrifuged at maximum speed (13000rpm) for 2 min. The flow through was collected for each sample and used for RNA isolation with Rneasy Mini Kit (Qiagen, Hilden, Germany).

Tissue samples (max 30mg) were transferred each in 1.5ml reaction tubes containing 600µl RLT buffer with 1%(v/v) ß-mercapto-ethanol and one tungsten carbide bead (Qiagen, Hilden, Germany). Tissue samples were homogenized for 2.5min in a Retsch® MM300 (Retsch GmbH & Co, Hann, Germany) mixer mill at 30 agitations/s. Following the homogenization step, samples were centrifuged for 1 min at 10000rpm and the supernatant loaded on a QIAshredder spin column placed in a 2ml collection tube and centrifuged at maximum speed (13000rpm) for 2 min. The flow through was collected for each sample and used for RNA isolation using the Rneasy Mini Kit (Qiagen, Hilden, Germany).

After 6h stimulation time, the testicular and peritoneal macrophages were washed two times with sterile PBS and lysed with 200µl RLT buffer. The lysates were passed 10 times through a 24G needle, up and down using a sterile 1ml syringe and than loaded on a QIAshredder spin column placed in a 2ml collection tube and centrifuged for 2 min at maximum speed (13000rpm). The

flow through was collected for each sample and stored at -80°C. Lysates from 6 experiments as described at 3.2.10.3 and 3.2.10.4 were pooled prior to RNA isolation.

To the cell or tissue lysates (obtained as described above) an equal volume (400-600µl) of 70% ethanol was added and mixed by pipetting. Samples were then loaded on RNeasy mini spin columns and centrifuged for 15s at 12,000rpm. The flow-through was discarded. The columns were washed by adding 700µl RW1 (Rneasy® Mini Ki) buffer and the flow-through was discarded. The columns were transferred in a new 2ml collection tube and washed 2 times with 500µl RPE buffer (Rneasy® Mini Kit). Each time the columns were centrifuged for 15s at 12,000rpm and the flow-through discarded. After the last washing step, the columns were centrifuged for 2min at 12000rpm to dry the silica membrane of the RNeasy mini spin column. The total RNA was diluted in 20-40µl of RNase-free water and the RNases inactivated by addition of 1% (v/v) RNase inhibitor (Promega, Manheim, Germany). RNA samples were stored at –80°C.

### 3.2.15 Assessment on RNA concentrations by spectrophotometric analysis

The spectrophotometer measures the light-absorption of different samples at defined wavelengths. The maximum absorption of diluted RNA is at λ = 260nm. Based on the Lambert-Beer law:

$$A = c*l*\varepsilon$$

the concentration (c) of a substance could be calculated if the absorption (A) measured through a well with the length (l) and the extinction coefficient (ε) are known.

The assessment of RNA concentration was performed using a spectrophotometer (Ultrospec2100pro, Pharmacia, Erlangen, Germany) at 260nm. At the same time the ratio $OD_{260}/OD_{280}$ was calculated and gave an indication about the purity of the samples. A clean RNA isolation is reflected by

$OD_{260}/OD_{280}$ values between 1.8 and 2.0. Values smaller than 1.8 are an indication for protein contamination (Ibelgaufts et al., 1982).

### 3.2.16 DNase Digestion

For each sample 2.5µg RNA were taken separately in a new reaction tube together with DNase buffer and 3U DNase/µgRNA, (DNase I RNase-free, 10U/µl, Roche, Manheim, Germany) for each sample. The reactions were brought to an end volume by adding appropriate volumes of water (see reaction table below) and samples were incubated for 35min at 37°C. The DNase was inactivated at 72°C for 10 min. After that samples were transferred on ice for 2 min, centrifuged and stored at –80°C.

**DNase digestion reaction table:**

| Volume | Component |
|---|---|
| Xµl | RNA (corresponding to 2.5 µg) |
| 0.9µl | DNase I RNase free (10U/µl, Roche diagnostics, Manheim) |
| 2.0µl | 10X DNase I buffer |
| Up to 20µl | RNase free water |
| **20µl** | **TOTAL** |

The efficiency of DNase digestion was assessed by PCR amplification of GAPDH housekeeping gene. Lack of GAPDH (glycerin-aldehyd-3-phosphate-dehydrogenase) gene amplification proved the complete digestion of genomic DNA. Samples were stored at -80°C.

### 3.2.17 Reverse transcription

After removing the genomic DNA, the total RNA samples were subject to reverse transcription (RT) into complementary DNA (cDNA).

The RT was performed (see reaction tables below) using Oligo(dT) (Promega) primers and the reverse transcriptase from mouse Moloney-Leukemia Virus (M-MLV, Promega).

DNase digested RNA samples (2.5µg in 20µl, see DNase reaction table) were mixed with 2µl Oligo dT $_{(12\text{-}18)}$ primer and denaturated for 10min at 70°C. After

denaturation, samples were immediately transferred on ice and kept for at least 2 min.

**Denaturation of RNA and primer sequences (step A):**

| Volume | Component |
|---|---|
| 20µl | 2.5 µg of DNase digested RNA |
| 2µl | Oligo $dT_{(12-18)}$ |
| **22µl** | **TOTAL** |

**Denaturation 10min at 70°C and followed by transfer on ice**

Next step is preparing an RT mix as described below. The RNA-primers mix and the RT-mix were preheated at 42°C and the RT mix was added to the RNA.

**Preparing the RT-mix (step B):**

| Volume | Component |
|---|---|
| 8µl | 5X M-MLV RT buffer (Promega) |
| 2µl | dNTP Mix (ACGT, each 10mM, Promega) |
| 1µl | RNAsin (RNase inhibitor, 40U/µl, Promega) |
| 6µl | RNase-free water |
| **17µl** | **TOTAL** |

**RT-mix was preheated at 42°C and added to the denaturated RNA**

In the last step 1µl of reverse transcriptase (M-MLV RNase H-minus, 200U/µl, Promega) was added to each sample. The samples were incubated for 90 min at 42°C and thereafter the reaction was stopped by incubation for 15 min at 70°C. In the end samples were centrifuged, transferred on ice and the efficiency of RT reaction was checked by GAPDH-PCR.

### 3.2.18 Polymerase chain reactions (PCR)

The polymerase chain reaction (PCR) is a method which enzymatically amplifies a specific DNA sequence. The specificity of amplification is dependent on the two oligonucleotide-primer sequences which are hybridizing with complementary DNA sequences leading to a time-dependent exponential multiplication of the 3'-5' limited sequence.

All PCR reactions except for the iNOS PCR, were performed using the HotStartTaq® DNA Polymerase provided in a ready to use PCR mix for quantitative real time PCR (QuantiTect™ SYBR® Green PCR Kit, Qiagen).

iNOS primers were best established with the HotMaster™ Taq DNA Polymerase (Eppendorf). Details about the amounts of different reaction components are presented in the table below.

| PCR reaction | MCP-1 | TGFß-2 | COX-2 | GAPDH |
|---|---|---|---|---|
| cDNA template | 1µl | 1µl | 1µl | 1µl |
| QuantiTaq PCR Mix | 12.5µl | 12.5µl | 12.5µl | 12.5µl |
| 3'-primer (100pM/µl) | 0.25µl | 0.6µl | 0.3µl | 0.5µl |
| 5'-primer (100pM/µl) | 0.25µl | 0.6µl | 0.3µl | 0.5µl |
| Millipore water | 10µl | 9.9µl | 9.9µl | 9.5µl |
| **TOTAL** | **25µl** | **25µl** | **25µl** | **25µl** |

| PCR reaction | iNOS |
|---|---|
| cDNA template | 1.5µl |
| 10X PCR buffer | 2.5µl |
| dNTP mix (ACGT, each 10mM, Promega) | 0.5µl |
| 3'-primer, (100pM/µl) | 0.6µl |
| 5'-primer, (100pM/µl) | 0.6µl |
| $MgCl_2$ (50mM) | 1µl |
| HotMaster Taq Polymerase (Eppendorf) | 0.4µl |
| Millipore water | 18.9µl |
| **TOTAL** | **25µl** |

### 3.2.19 DNA agarose gel electrophoresis

2% to 0.5% agarose gels were routinely used to separate DNA fragments in a size range of 100 to 10,000bp (Sambrook, 2001). The appropriate amount of agarose was dissolved in 1X TAE buffer (see 3.1.1) by boiling for a few minutes in a microwave oven. When the gel solution has cooled down to some 60°C ethidium bromide was added to a final concentration of 0.5µg/ml. The clear solution was than poured into a gel mold using a suitable comb for generating the sample wells and allowed to harden for some 30-45 min. The gel was mounted in the electrophoresis chamber, which was filled with 1X TAE running buffer until the gel was just submersed. The PCR products and a suitable size

standard were mixed with 0.1 volumes of 10X loading buffer and applied to the wells. A voltage of 2-10V/cm was applied until the bromophenol blue and xylene cyanol FF dyes had migrated an appropriate distance through the gel. After completion of electrophoresis the gel was examined on a 305nm UV transilluminator and photographed using a gel documentation system (Intas, Göttingen, Germany).

### 3.2.20 Optimization of PCR reactions for real-time PCR

Running a regular PCR for each primer pair assessed primer specificity. Only if the expected product was amplified as a single specific band after at least 35 cycles (for conditions see table 3.2.1) was quantitation undertaken. Another prerequisite for quantitative measurement of gene expression is establishing the proper concentration of the primers. Because Sybr Green binds to all double-stranded DNA fragments, excess of primers could increase the fluorescent background affecting the accuracy of the measurement. Special care was taken to use primers with comparable PCR efficiency to the GAPDH primer pair, used as internal control.

### 3.2.21 Quantitative real time PCR

Real time PCR is a method for detection and quantification the gene expression and is based on the property of a fluorescent dye to bind to double-stranded DNA. One of these dyes is Sybr Green. Unbounded Sybr Green is emitting very little fluorescence. During the elongation step of a PCR reaction, the Sybr Green will bind to the double stranded DNA, which is exponentially amplified from one cycle to another leading to an exponential increase in the fluorescence signal measured after each PCR cycle. In this way, at the end of each cycle, the amount of amplified DNA can be measured in “real time”. The bigger the amount of cDNA template taken in the PCR reaction is, the fewer reaction cycles are necessary until the fluorescence signal will increase and cross the threshold line giving the value of threshold cycle (Ct).

The compounds for real time PCR are similar to a normal PCR reaction except for the DNA-binding fluorescent dye (Sybr Green) and the fluorescein. The

fluorescein is used for the initial internal calibration of the fluorescence reader. In this study the QuantiTect™ SYBR® Green PCR kit (Qiagen) was used. It is supplied as a 2-fold reaction buffer containing a HotStartTaq® DNA polymerase, dNTP and dUTP mix, SYBR Green, 5mM MgCl in a normal PCR buffer.

**The real-time PCR reaction was prepared as follows:**

| Real-time PCR reaction | GAPDH | MCP-1 | TGFß-2 | COX-2 |
|---|---|---|---|---|
| Template (cDNA) | 1µl | 1µl | 1µl | 1µl |
| 2X Reaction buffer (QuantiTect™ SYBR® Green PCR mix) | 12.5µl | 12.5µl | 12.5µl | 12.5µl |
| Primer-3' (100pM/µl) | 0.4µl | 0.25µl | 0.6µl | 0.3µl |
| Primer-5' (100pM/µl) | 0.4µl | 0.25µl | 0.6µl | 0.3µl |
| Fluorescein | 1µl | 1µl | 1µl | 1µl |
| Millipore water | 9.7µl | 10µl | 9.3µl | 9.9µl |
| **TOTAL** | **25µl** | **25µl** | **25µl** | **25µl** |

RT-PCR specific methods in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. This is especially relevant when the samples were obtained from different animals and will result in misinterpretation in the expression profiles of the target genes. Consequently, the question of what constitutes an appropriate standard arises (Thellin et al., 1999) and is an important aspect of experimental design. The accepted method for minimizing these errors and correcting for sample to sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalized (Karge et al., 1998). The ideal internal standard should be expressed at a constant level among different tissues of an organism at all stages of development and should be unaffected by the experimental treatment. The most commonly used internal controls are the GAPDH, ß-actin and ribosomal RNAs (rRNA) (Bustin, 2000). We tested GAPDH and ß-actin as

internal controls. Both gave similar results and were unaffected by experimental procedures. GAPDH was finally selected as internal control for all experiments in the present study.

The row data is collected as Ct values for each sample for both the gene of interest and for the internal control (GAPDH). Data were presented as relative expression

$$RE=2^{-[\Delta CtC_0-\Delta CtSample]}$$

where $\Delta Ct=Ct_{gene\ of\ interest}-Ct_{GAPDH}$ and $C_0$ is the experimental control (eg.: untreated cells or untreated animals).

The accuracy of pipetting was assessed by a real time PCR of a dilution series for each cDNA template (1:1, 1:10, 1:100, 1:1000). The curves corresponding to higher template concentration should cross the threshold line earlier, resulting in a smaller Ct value. Constant difference between the Ct values indicates an accurate pipetting of the template's dilution series (see chapter 4.8).

### 3.2.22 Statistics

All bar diagrams are representing mean values ± SEM. To investigate whether the experimental groups are significantly different, the nonparametric Mann-Whitney unpaired test was applied ($p < 0.05$ was considered significant).

# 4. Results

## *4.1. Testicular atrophy and decreased testicular weight associated with EAO*

A statistical significant decrease in testicular weights was observed in EAO group compared to adjuvant controls at 50 and 80 days (Fig 4.1.b).

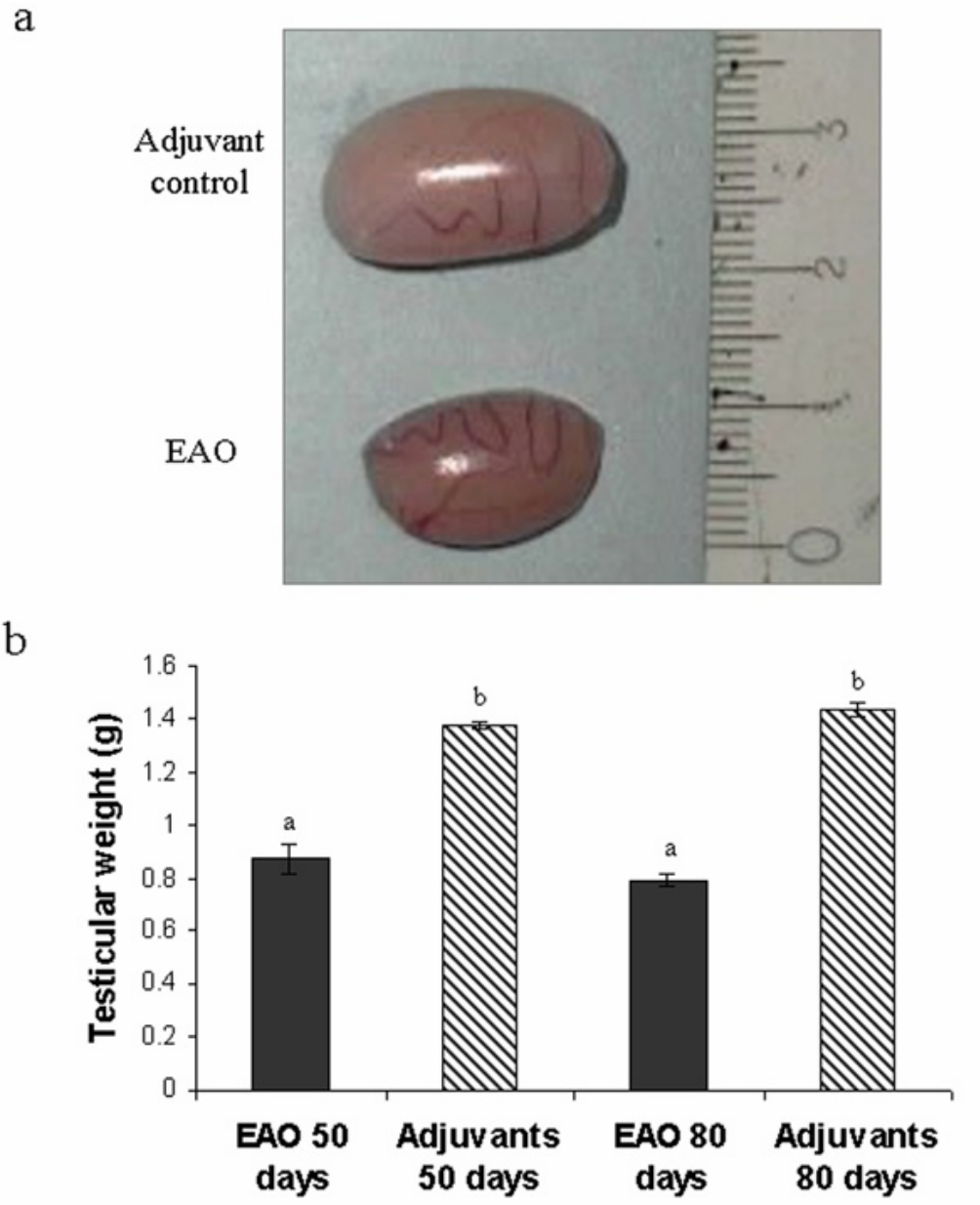



**Fig 4.1. a**): Photograph of EAO and adjuvant control testes at 50 days; **b**): Bar diagram of testicular weights. Highly significant decrease in testicular weights ($p<0.01$, impaired t-student test) in EAO animals as compared to adjuvant controls at 50 and 80 days. Values are mean ± SEM of *n*=10-28 rats per group. Groups sharing the same letter are not significantly different.

Macroscopically, the testes of EAO animals were atrophic, considerably smaller and had an increased subcapsular vascularization as compared to the testes of control animals (Fig 4.1.a).

Hematoxylin-eosin staining revealed that 50 days after the first immunization 79% (n=14) of the immunized rats had developed orchitis, whereas after 80 days all rats were affected. The pathological changes in testes of rats of the EAO group were characterized by germ cell loss, interstitial mononuclear cell infiltrates and granuloma formation, accompanies with a progression of histological damages from 50 to 80 days.

## *4.2. Increased numbers of neutrophils in EAO*

In normal rat testes neutrophils were present in very small numbers in the interstitial space and were never found within the seminiferous tubules (Fig. 4.2.a,b). Most neutrophils were detected in cross-sectioned testicular blood vessels, as circulating neutrophils, which have not entered the testicular interstitium.

In EAO large numbers of myeloperoxydase (MPO) containing polymorphonuclear cells (neutrophils) invaded the testis as shown in Fig 4.2. (c and d). They were distributed within the entire interstitial space and this pattern is similar at both time points investigated (50 and 80 days) after EAO induction.

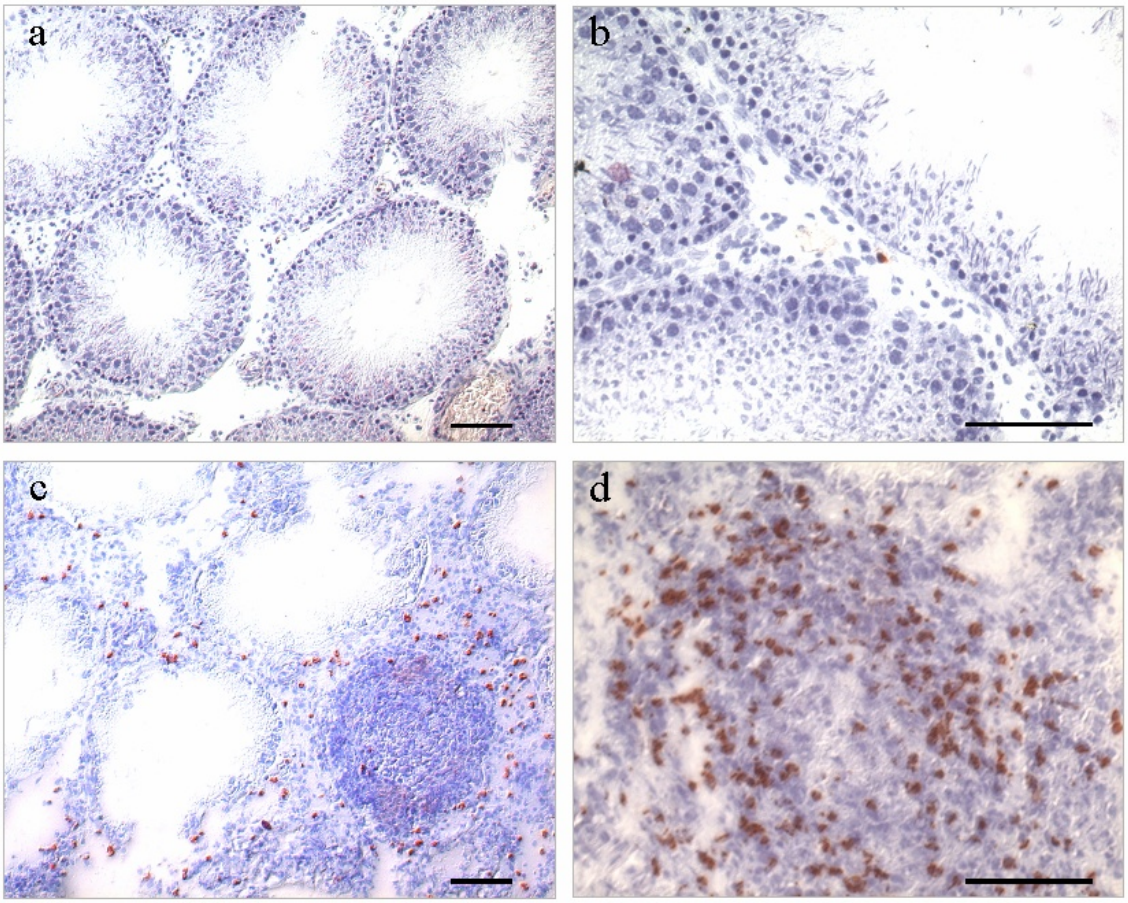



**Fig 4.2.** An increase in MPO positive neutrophils observed in cyosections of EAO. Adjuvant control testes at 50 days (**a**,**b**) EAO testis at 50 days (**c**) and 80 days (**d**). 10x magnification (**a**,**c**) and 20x magnification (**b**,**d**). Scale bars are indicating 100µm.

## *4.3. Strong increase in mast cell numbers in EAO*

Mast cells in normal rat testis were found only in small numbers, located exclusively directly under the capsule in the vicinity of blood vessels (Fig. 4.3.a). In testes collected 50 days after induction of EAO, however, the number of mast cells was markedly increased (10x) compared to those seen in the adjuvant control, and this increase was virtually unchanged even 80 days after induction (Fig. 4.3.d). Although the majority of mast cells were still localized to the periphery of the testis, in the EAO animals numerous cells were also found throughout the entire interstitial space (Fig. 4.3.b-c).

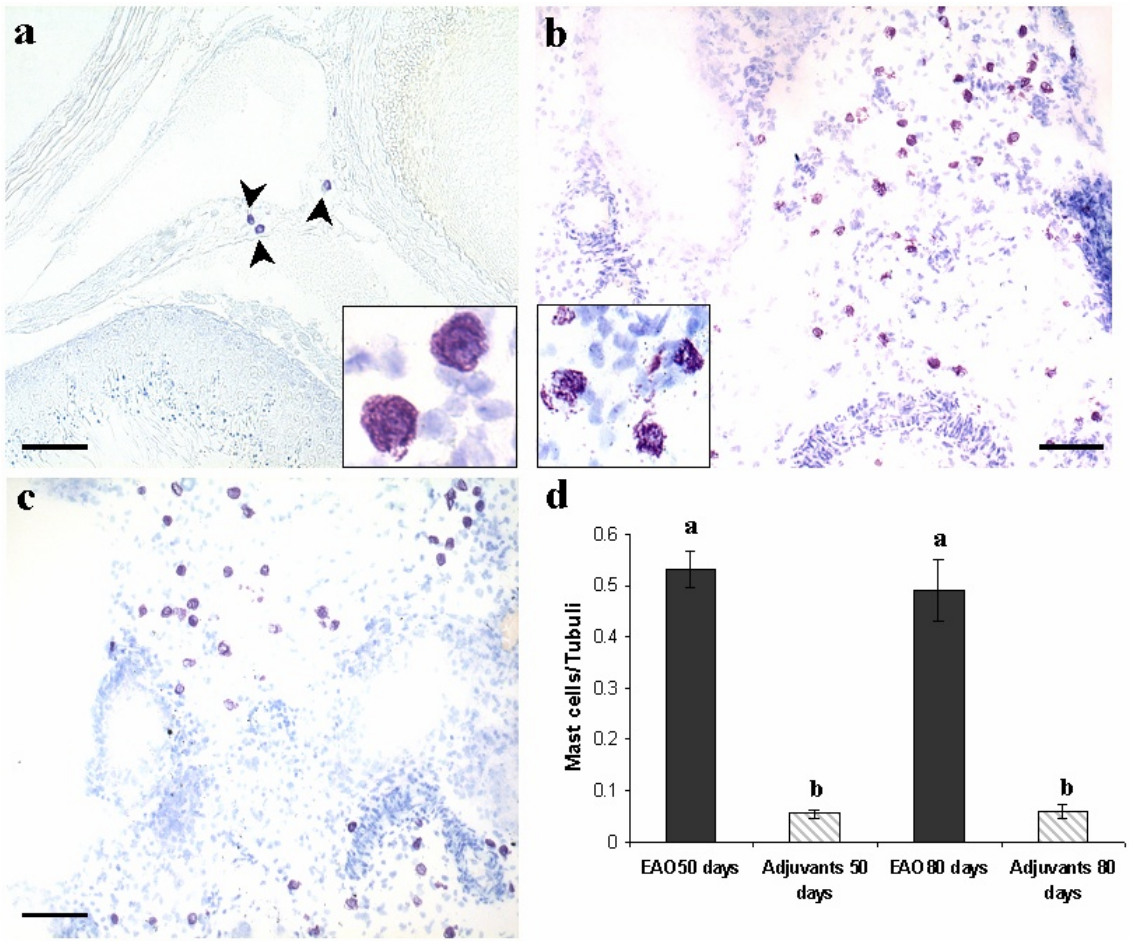



**Figure 4.3.** Changes in the number and localization of testicular mast cells during EAO. **a)** In adjuvant control testis mast cells (arrowheads) are found in small numbers almost exclusively under the testicular capsule in the vicinity of blood vessels. Degranulation of mast cells is not observed using toluidine blue staining (insert). **b-c**) 50 and 80 days after EAO induction a substantial increase of mast cell numbers is observed. In addition, mast cells are not restricted to the subcapsular space, but can also be seen throughout the entire interstitium. In EAO, but not in adjuvant controls, numerous mast cells had lost intracellular granules and metachromatically stained material was discharged diffusely into the surrounding interstitium as a sign of degranulation and tryptase release (insert). **d**) Quantification of mast cell numbers showed a 10-fold increase in EAO at 50 and 80 days after induction of disease ($p<0.01$, impaired t-student test). Results are expressed as ratio mast cells/tubule cross section. Values are mean ± SEM of *n*=6 per group. Groups sharing the same letter are not significantly different. Mast cells were stained with toluidine blue. Scale bars are indicating 100µm.

As a consequence, mast cells that contain abundant amounts of tryptase, one physiological $PAR_2$-agonist, are in a much closer anatomical proximity to $PAR_2$-positive cells (see below) in EAO than in the control testes, where mast cells were always restricted to the subcapsular space and never observed further centripetally in the testicular parenchyma. In EAO large numbers of mast cells were surrounded by diffusely dispersed metachromatically stained granules indicating that an increased degranulation rate is associated with chronic testicular inflammation (inserts in Fig. 4.3.a and b).

Thus, EAO is accompanied not only by increased mast cell numbers but apparently also by mast cell activation and degranulation, i.e. the release of the PAR2-agonist tryptase from intracellular secretory granules into the extracellular space.

## *4.4. $PAR_2$ immunoreactivity is elevated in chronically inflamed rat testis*

$PAR_2$ immunoreactivity in normal rat testis was detected in peritubular cells surrounding the seminiferous epithelium, in the acrosome of round and elongating spermatids and in a number of interstitial cells that resembled monocytes/macrophages in number, distribution and nuclear morphology (Fig. 4.4.).

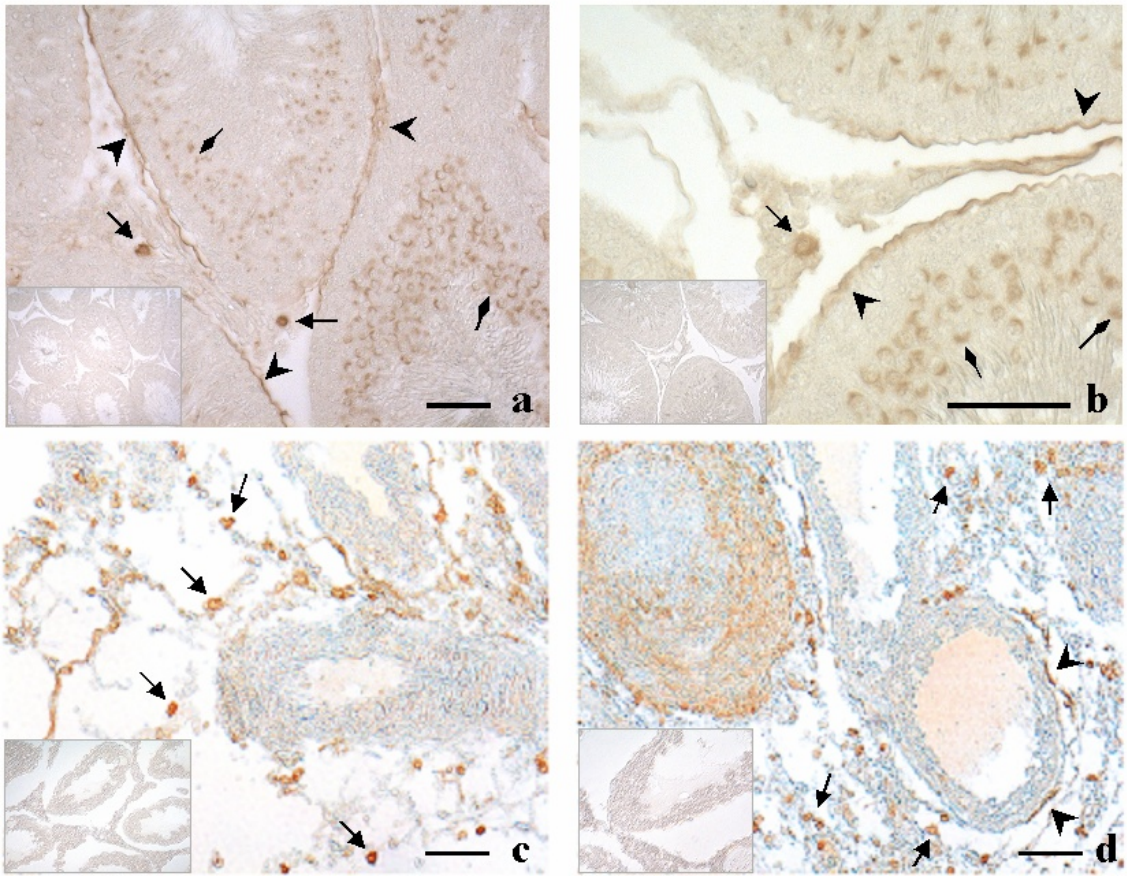



**Figure 4.4.** Immunohistochemical detection of $PAR_2$ in rat testis. **a-b**) untreated control rat testis: $PAR_2$ is localized to peritubular cells (arrowheads), the acrosome of spermatids (rhombic arrowheads) and to macrophages in the interstitium (arrows). **c-d)** EAO testis 80 days after induction of disease: a massive loss of germ cells with tubule sloughing is observed in parallel to leukocyte infiltration and granuloma formation. The number of $PAR_2$ positive macrophages (arrows) is strongly increased. $PAR_2$ positive, flat cells form multiple layers around granuloma. Inserts show negative controls (a-d). Scale bars are indicating 100µm.

The adjuvant control showed no difference to the untreated testis. Chronically inflamed testes, 50 and 80 days after induction of EAO, revealed a large increase in the number of interstitial $PAR_2$ positive cells (Fig. 4.4.c-d).

In addition, flat $PAR_2$ positive stained cells were found to form multiple concentric layers in the granuloma of EAO testes. Due to the almost complete loss of germ cells in EAO animals, the seminiferous tubules contained only Sertoli cells, if any cells at all (Fig. 4.4.c-d).

## *4.5 Detection of $PAR_2$ in isolated testicular macrophages*

In order to investigate whether the $PAR_2$ positive interstitial cells in normal testis (Fig. 4.4.) are indeed from the monocyte/macrophage lineage, testicular macrophages were isolated and cultured overnight. Monocytes/macrophages were characterized by combined staining for ED1 ("inflammatory" type monocytes) and ED2 (resident macrophages). Double immunofluorescence demonstrated that ED1/ED2 positive cells also express $PAR_2$ (Fig. 4.5.).

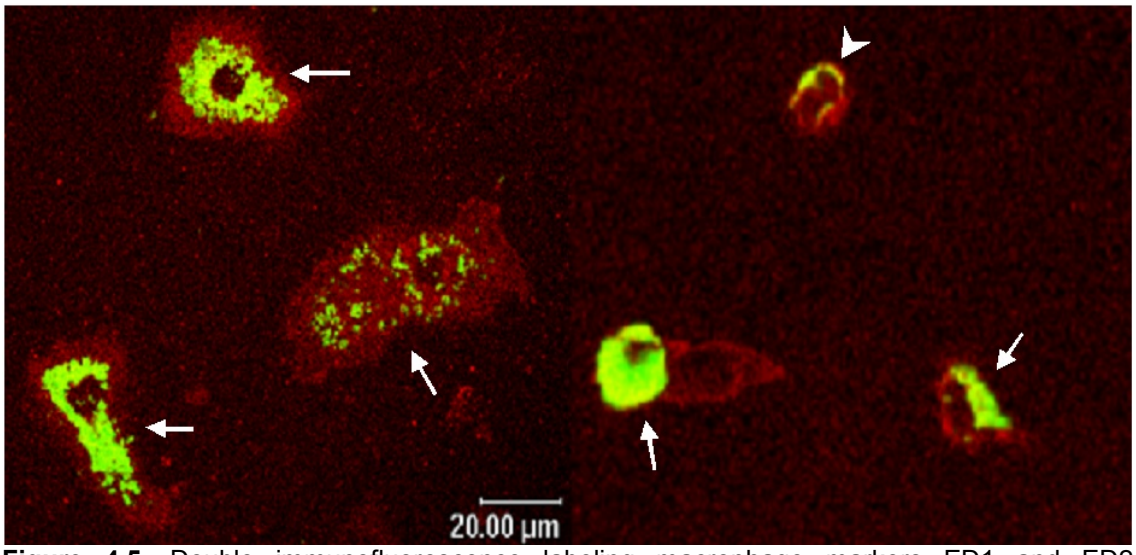



**Figure 4.5.** Double immunofluorescence labeling macrophage markers ED1 and ED2 (combined, FITC, green) and $PAR_2$ (Texas Red) visualized by confocal microscopy using isolated testicular macrophages. ED1 (arrows) is a cytoplasmic marker identifying the "inflammatory" subset of monocytes/macrophages, whereas ED2 (arrowheads) is a transmembrane protein characteristic for resident macrophages. Both subpopulation are positive for $PAR_2$.

## *4.6 Complete digestion of genomic DNA in total RNA samples*

Contamination of total RNA samples with genomic DNA can lead to misinterpretation of the later RT-PCR results. To avoid this problem the

following two steps should be performed: 1. Designing primer pairs that are spanning an intron (if genomic DNA has not been removed, the primers will amplify also a higher-size DNA fragment together with the target cDNA) and 2. Allocating special attention to completely digest the genomic DNA in total RNA samples.

The efficiency of DNase digestion was assessed by PCR amplification of the GAPDH housekeeping gene. Lack of GAPDH gene amplification after 35 cycles proved the complete digestion of genomic DNA in all 24 rat testes samples as shown in Fig 4.6.

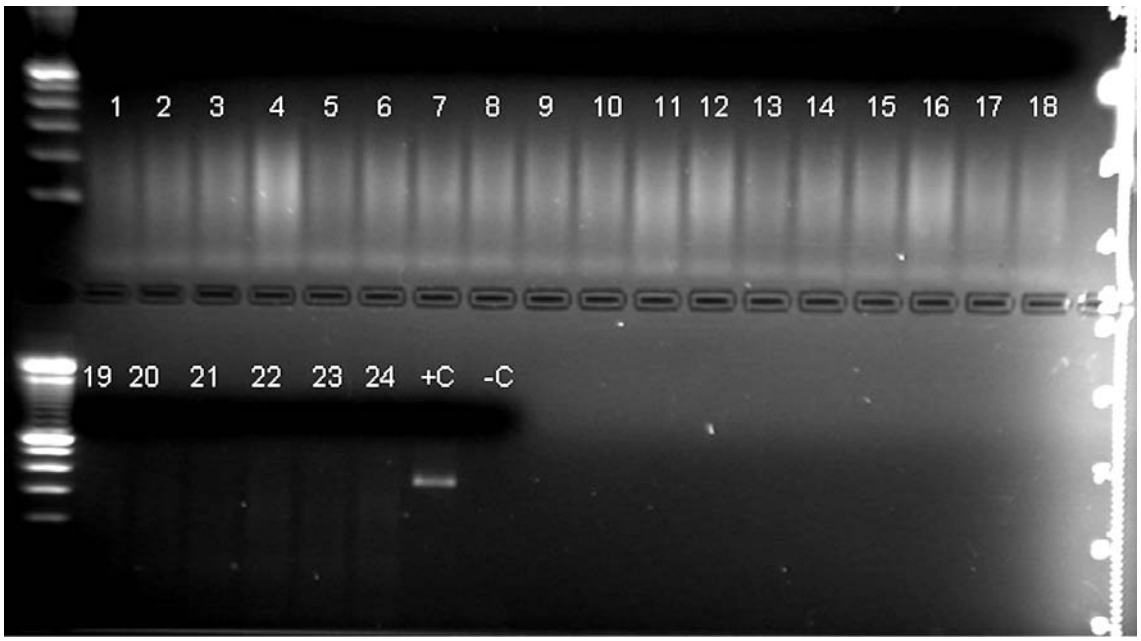



**Fig. 4.6: Complete digestion of genomic DNA in total RNA samples as shown by GAPDH-PCR.** All 24 samples were negative for GAPDH suggesting complete digestion of genomic DNA. (–C = negative control, template was omitted; +C = positive control, testis cDNA).

## *4.7 Successful reverse transcription was proved by GAPDH-PCR*

Real-time PCR quantification of mRNA transcripts is easily compounded by any variation in the amount of starting material between samples. This is especially relevant when the samples were obtained from different animals and will result in misinterpretation in the expression profiles of the target genes. Therefore, special care should be taken in order to transcribe equal amounts of mRNA with the same efficiency into complementary DNA. We tested for the expression of GAPDH after RT as shown in fig 4.7. All samples are GAPDH positive and

the intensity of the bands is similar in all 24 samples, indicating equal amounts of cDNA.

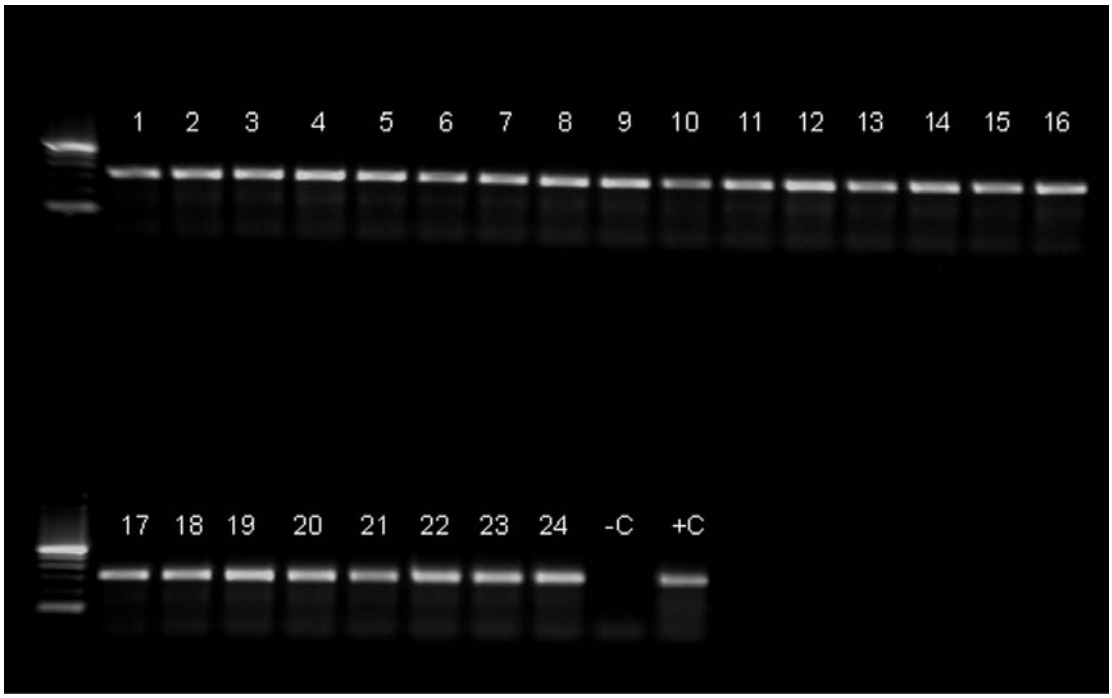



**Fig. 4.7: Success of reverse transcription proved by GAPDH-PCR.** The intensity of GAPDH bands is similar in all 24 samples. (–C = negative control, template was omitted; +C = positive control, rat testis cDNA template).

## *4.8 Quantitative real-time PCR reactions were accurately processed*

The accuracy of pipetting was assessed by real time PCR amplification of a dilution series (1:1, 1:10, 1:100, 1:1000) for each individual cDNA template (Fig. 4.8.1).

The fluorescence intensity is measured at the end of each cycle and values are plotted against the cycle number reflecting the amplification curve for each reaction. Each curve has a slowly increasing initial phase, an abrupt exponential phase and ends with a plateau phase due to the limitations of the PCR components. The threshold line is set just above the slow increasing phase (above the background) and is crossing each curve in the fast exponential phase. The curves corresponding to higher template concentration crossed earlier the threshold line, giving a smaller Ct value. The Ct values from consecutive dilutions were compared and there was a proportional correlation

between the amount of template taken into PCR reaction and the amount of the PCR product (Fig. 4.8.1).

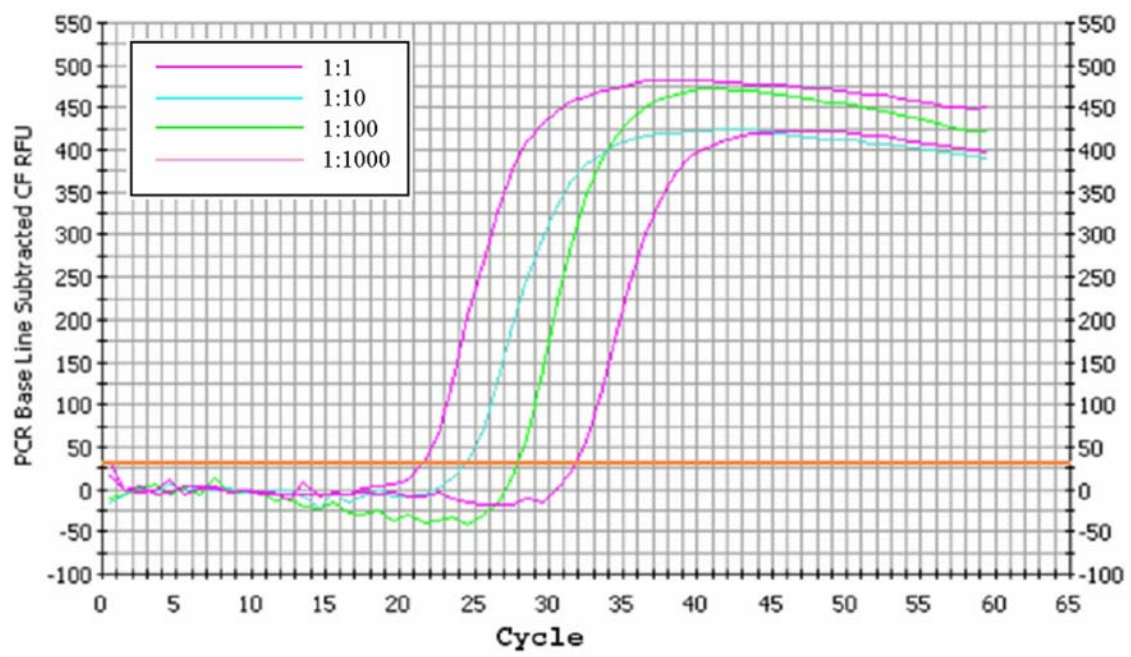



**Fig. 4.8.1: Progression of a PCR reaction plotted as fluorescence measured in time, after each reaction cycle**. Samples are a dilution series of the same cDNA template. Curves are showing a typical PCR amplification as an exponential function with a slow ascendant part, followed by an abrupt exponential amplification and ending (due to the limitation of the components in the PCR reaction) with a plateau phase. The curves corresponding to higher template concentration are crossing the threshold line earlier, giving a smaller Ct value. Constant differences between the Ct values are indicating accurate pipetting.

Figure 4.8.2 shows the same fluorescence curves in a logarithmic representation $\log_{10}$ (emitted fluorescence)/cycle. This representation is appropriate for setting the threshold line as low as possible, without interfering with the background fluorescence. For comparing different samples, it is required that the threshold is set at the same level and is crossing each curve only once, in the abrupt exponential phase.

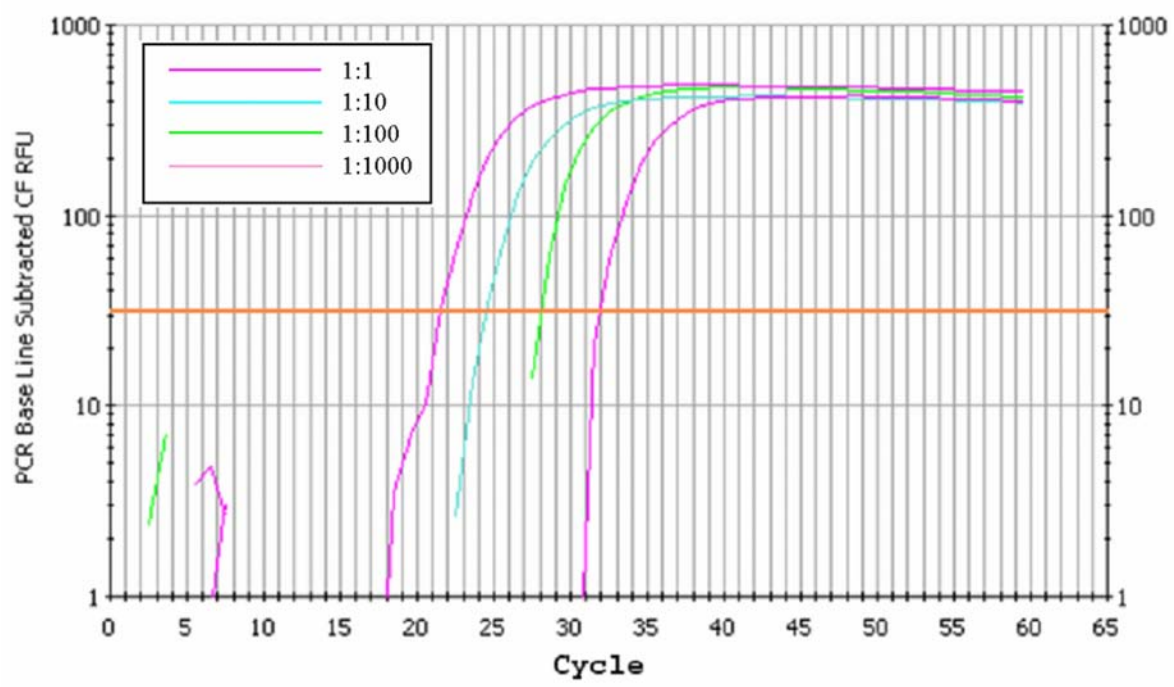



**Fig. 4.8.2: Progression of a PCR reaction in logarithmic representation ($log_{10}$fluorescence against the number of cycles).** Samples are a dilution series of the same cDNA template. The curves corresponding to higher template concentration are crossing the threshold line earlier, providing a smaller Ct value. Equal intervals between the Ct values are indicating accurate pipetting.

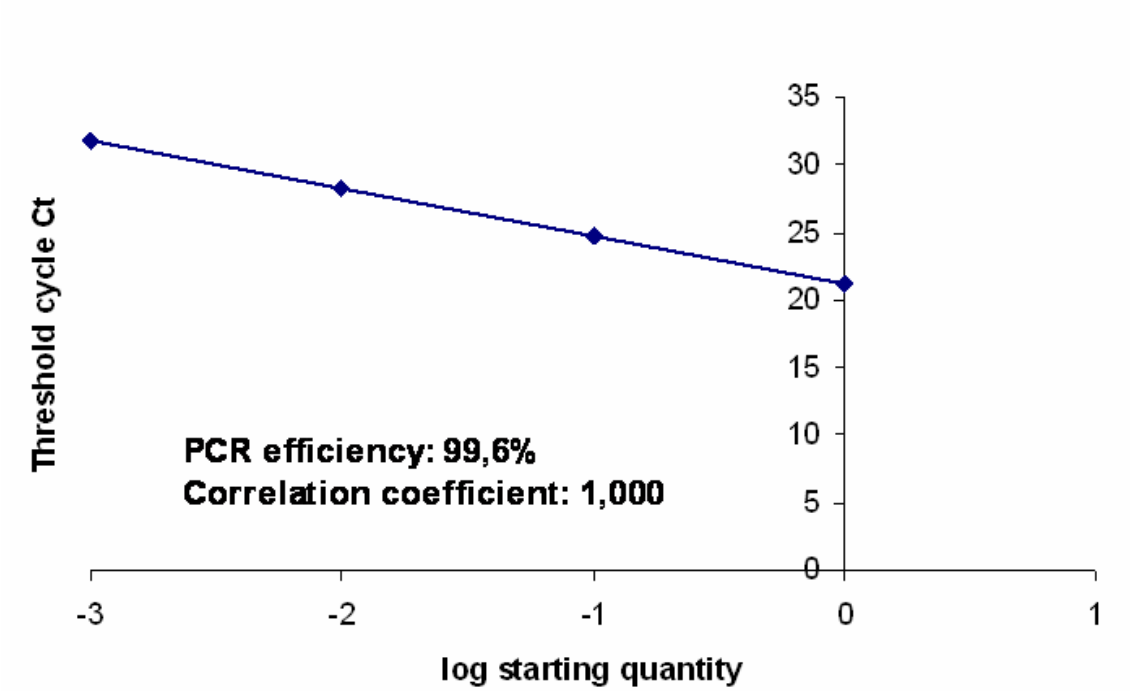



**Fig. 4.8.3 Standard curve generated for the dilution series.** Threshold cycle values are plotted against the $log_{10}$(cDNA dilution factor) All four plotted points are in a linear relationship. The coefficient of correlation with a linear relationship, a measure of the accuracy of pipetting, is 1,000 and the calculated PCR efficiency is 99.5%.

The raw data are collected as Ct values for each reaction and were plotted against the $\log_{10}$ (cDNA dilution factor). This plot is the standard curve and should give four points as close as possible to their linear fit. Indeed, as shown in Fig. 4.8.3 all four points are crossed by the linear fit with a correlation coefficient of 1 and a PCR efficiency of 99.6%. Optimally, the correlation coefficient should be as close as possible to 1 and the PCR efficiency as close as possible to 100%.

A melting curve is generated at the end of each PCR reaction by measuring the fluorescent signal (F) in all samples, at gradually increasing temperatures (T) from 50°C to 100°C. Plotting the ratio of derivatives –dF/dT against T, generates the melting curve (Fig.4.8.4).

Melting curve gives information about the specificity of the primers. If in one reaction more than one cDNA sequence is amplified, the melting curve for that reaction will show more than one peak. Superimposing the melting curves, one could conclude if in different samples the same PCR product was amplified.

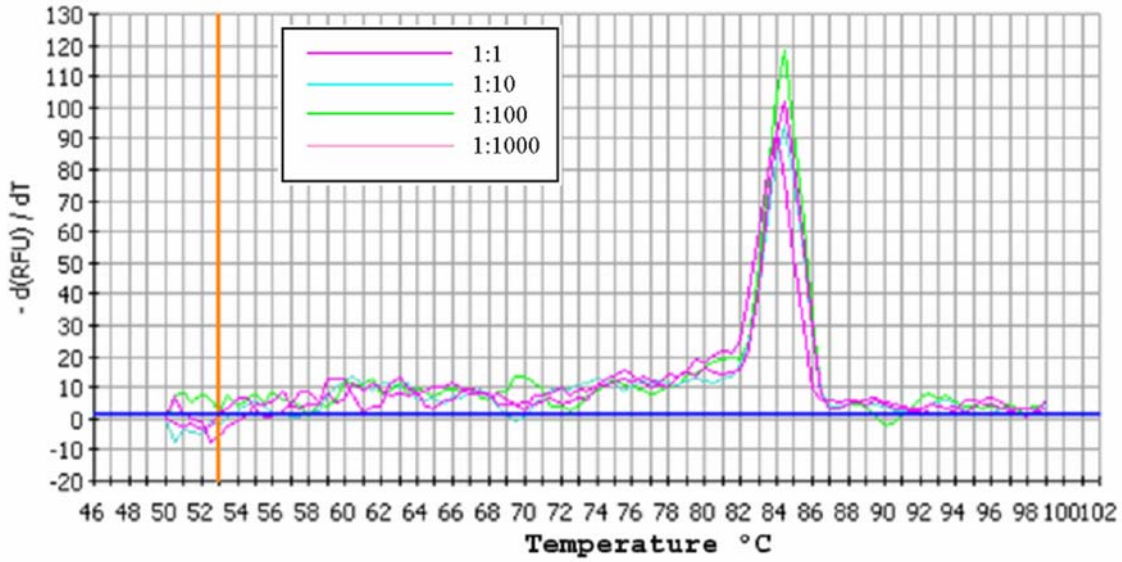



**Fig. 4.8.4 Melting curves of the PCR products.** All four curves are showing only one melting peak suggesting that in each reaction only one PCR product was amplified. The peaks of all curves are superimposing in the overlay suggesting that in all reactions it was amplified the same PCR product.

## *4.9 Expression profiles of inflammatory mediators in testicular and peritoneal macrophages stimulated with a $PAR_2$ agonist*

LPS is as a strong acute activator of macrophages (Chancellor-Freeland et al., 1995, Wu and Meydani, 2004) and has been used in models of acute systemic inflammation (O'Bryan et al., 2000, Gerdprasert et al., 2002a, Gerdprasert et al., 2002b). LPS strongly increased the MCP-1, TGFß2 and COX-2 mRNA expression levels in cultured testicular macrophages after 6h, whereas $PAR_2$ activating peptide ($PAR_2$-AP) had no influence (Fig 4.9.1.d-f).

LPS, but not $PAR_2$-AP, upregulated MCP-1 and COX-2 mRNA expression also in peritoneal macrophages (Fig. 4.9.1.a,c). Surprisingly, TGFß2 was found to be downregulated by LPS and $PAR_2$-AP, but not by $PAR_2$ reverse peptide ($PAR_2$-RP), suggesting a proinflammatory role of $PAR_2$ in peritoneal macrophages, by downregulating anti-inflammatory cytokines like TGFß-2 (Fig. 4.9.1.b).

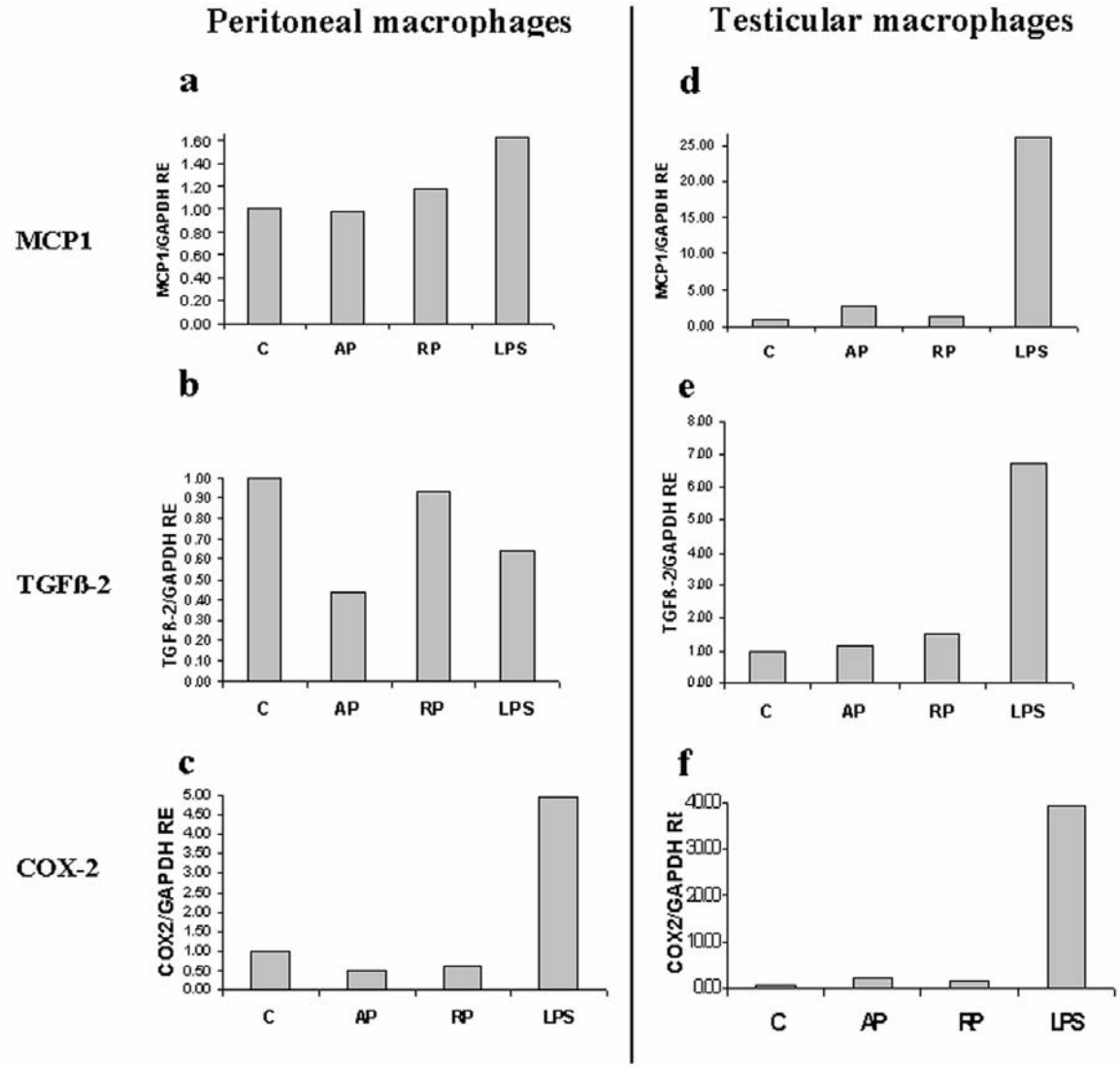



**Fig. 4.9.1.** Comparison of expression of key inflammatory mediators assesed by real time RT-PCR analysis in $PAR_2$ stimulated testicular (**a-c**) and peritoneal macrophages (**d-f**) after 6h of stimulation *in vitro*. LPS stimulation was used as control. (C=naive control, AP=$PAR_2$-AP, RP=$PAR_2$-RP, LPS=lipopolysacharide).

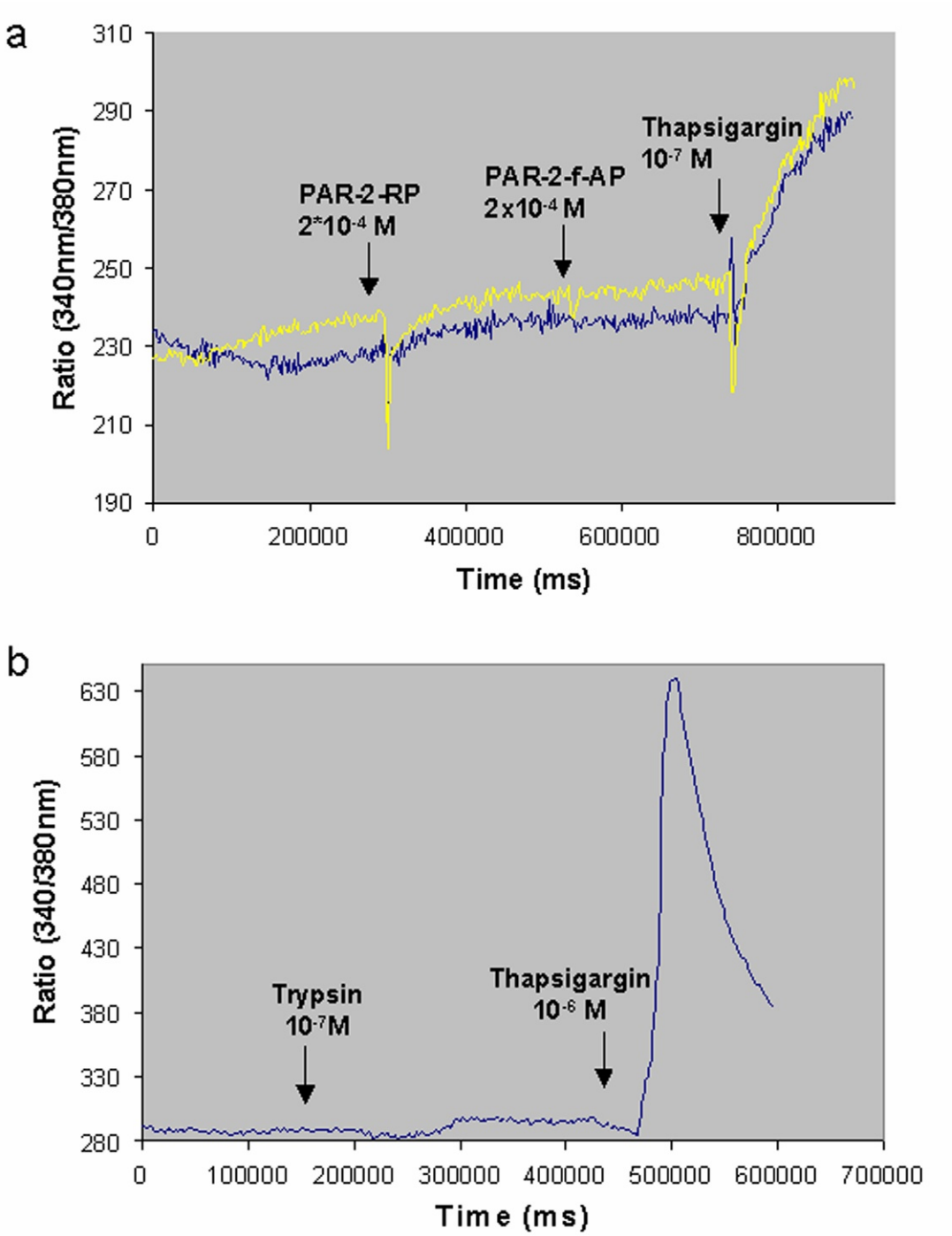



**Fig. 4.9.2.** $PAR_2$ activation did not change the free intracytoplasmic $[Ca^{2+}]$ in testicular macrophages. $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$; $10^{-4}$-$10^{-2}$M) **(a)** and trypsin (100nM) **(b)** have no influence on $[Ca^{2+}]_i$ of Fura-2 loaded testicular macrophages. Addition of the control peptide (LSIGRL-$NH_2$; $10^{-4}$M) prior to application of the $PAR_2$-f-AP caused no measurable change in intracytoplasmic $[Ca^{2+}]$ (**a**). Thapsigargin as positive control mediated a fast increase in free intracytoplasmic $[Ca^{2+}]$ immediately after exposure **(a,b)**.

$PAR_2$ was detected on testicular macrophages but $PAR_2$ activation did not change the MCP-1, TGFß-2 and COX-2 expression levels in these cells. As $PAR_2$ is a G-protein coupled receptor, it can functionally be assessed by the

characteristic increase in the free intracellular [$Ca^{2+}$] (Mari et al., 1996, Compton et al., 2000).

In our experiments the changes in intracytoplasmic [$Ca^{2+}$] induced by $PAR_2$-f-AP ([2-fuoryl]-LIGRLO-$NH_2$) and by trypsin were assesed in isolated testicular macrophages.

Trypsin (5-100nM) and $PAR_2$-f-AP ($10^{-4}$-$10^{-2}$M) did not change the $[Ca^{2+}]_i$, whereas thapsigargin ($10^{-7}$-$10^{-6}$M), which causes a rapid depletion of intracellular $Ca^{2+}$ stores from the endoplasmic reticulum, induced a fast increase in free $[Ca^{2+}]_i$ (Fig. 4.9.2).

Based on these results in can be concluded that either isolated testicular macrophages lost the $PAR_2$ functionality *in vitro*, or that special conditions are required to switch the $PAR_2$ receptor from a latent to a functional status in these cells.

## *4.10 Isolated peritubular cells express functional $PAR_2$ and activate second messengers*

$PAR_2$ was found in isolated testicular peritubular cells (PTC) on both the mRNA level using RT-PCR analysis (Fig. 4.10.1.a) and the protein level as detected by immunofluorescence (Fig. 4.10.1.b).

$PAR_2$ is a G-protein coupled receptor. Functionally it can be assessed by a characteristic increase in free intracytoplasmic $Ca^{2+}$ levels. Indeed an increase of free intracellular [$Ca^{2+}$] was demonstrated after stimulation of PTCs with the $PAR_2$ specific agonist peptide ($PAR_2$-AP, Fig. 4.10.2.). Incubation of the same cells with the control peptide ($PAR_2$-RP) prior to the addition of the agonist had no effect (Fig. 4.10.2.).

In addition, stimulation of PTC with $PAR_2$-AP resulted in the activation of protein kinase c (PKC) as evidenced by the recruitment of the PKC-EGFP fusion protein in transfected PTCs from the cytoplasm to the plasma membrane (Fig. 4.10.3.a) where PKC is activated by diacylglycerol. PKC-EGFP relocation was visible 1min after exposure to $PAR_2$-AP and continued at least until the 5min mark. Stimulation of PCs with control reverse peptide $PAR_2$-RP (LSIGRL-$NH_2$; $10^{-4}$M) did not change the cellular PKC-EGFP distribution (Fig. 4.10.3.b).

Furthermore, the incubation of PTCs with $PAR_2$-AP triggered phosphorylation of ERK1/2 MAP-kinases (p42/p44) after 5min and reached a maximum after 10min (Fig. 4.10.4). Reprobing of the stripped membranes revealed equal amounts of total p44/p42 in all samples excluding loading differences (Fig. 4.10.4).

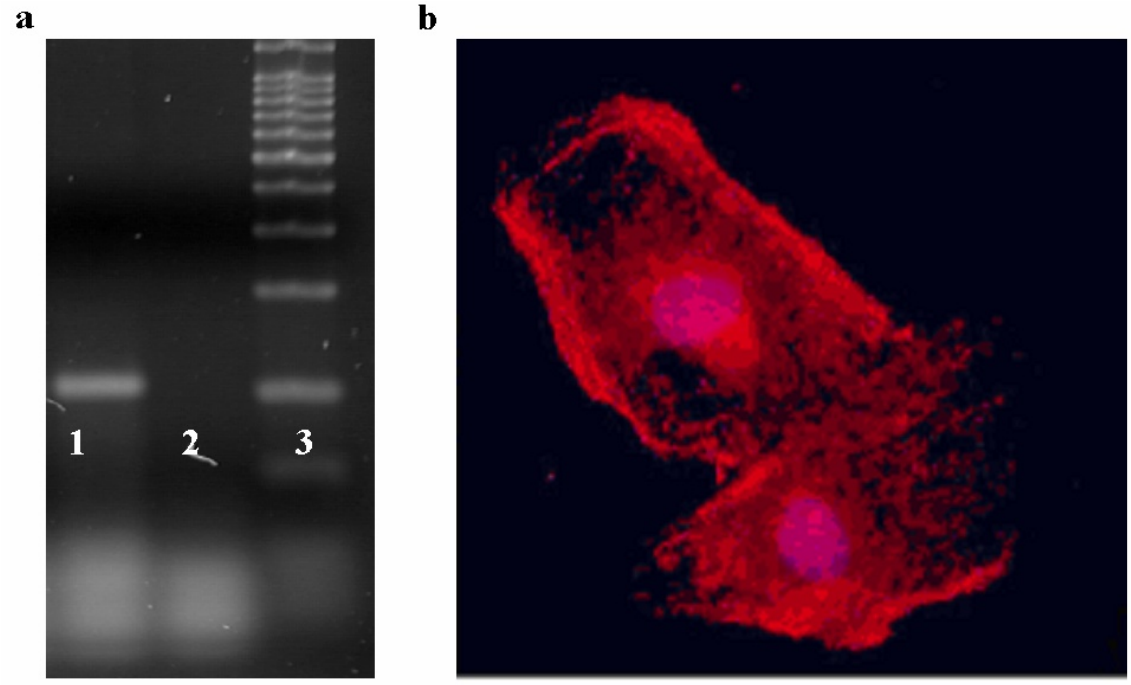



**Figure 4.10.1.** Isolated rat testicular peritubular cells express $PAR_2$. **a)** Lane 1: RT-PCR detection of $PAR_2$ in isolated peritubular cells. Lane 2: negative control; lane 3: 100 bp-marker. **b)** $PAR_2$ immunoreactivity in isolated peritubular cells detected by immunofluorescence. Red fluorescence (Texas red) shows the $PAR_2$ immunoreactivity; blue staining (DAPI) marks the nuclei of the cells. **b):** Courtesy of Katrin Bumbach (Diplomarbeit Humanbiologie Philipps Universität, Marburg, 2003)

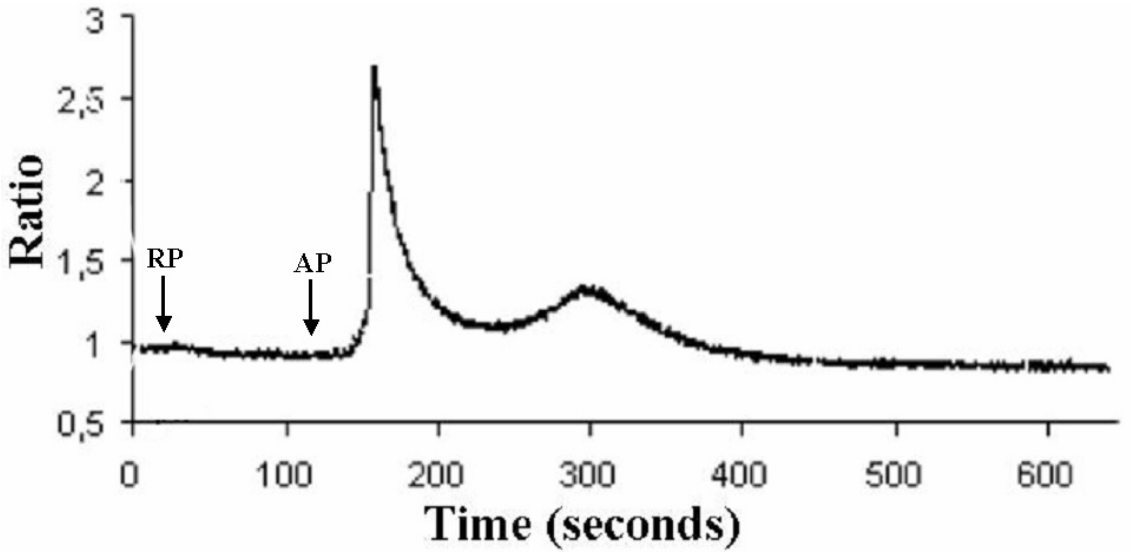


**Figure 4.10.2.** The $PAR_2$-AP (SLIGRL-NH2; $10^{-4}$M) mediated an instant increase in free intracytoplasmic [$Ca^{2+}$] immediately after exposure of Fura-2 loaded peritubular cells. Addition of the control peptide (LSIGRL-$NH_2$; $10^{-4}$M) prior to application of the $PAR_2$-AP caused no measurable change in intracytoplasmic [$Ca^{2+}$]. Courtesy of Katrin Bumbach (Diplomarbeit Humanbiologie Philipps Universität, Marburg, 2003)

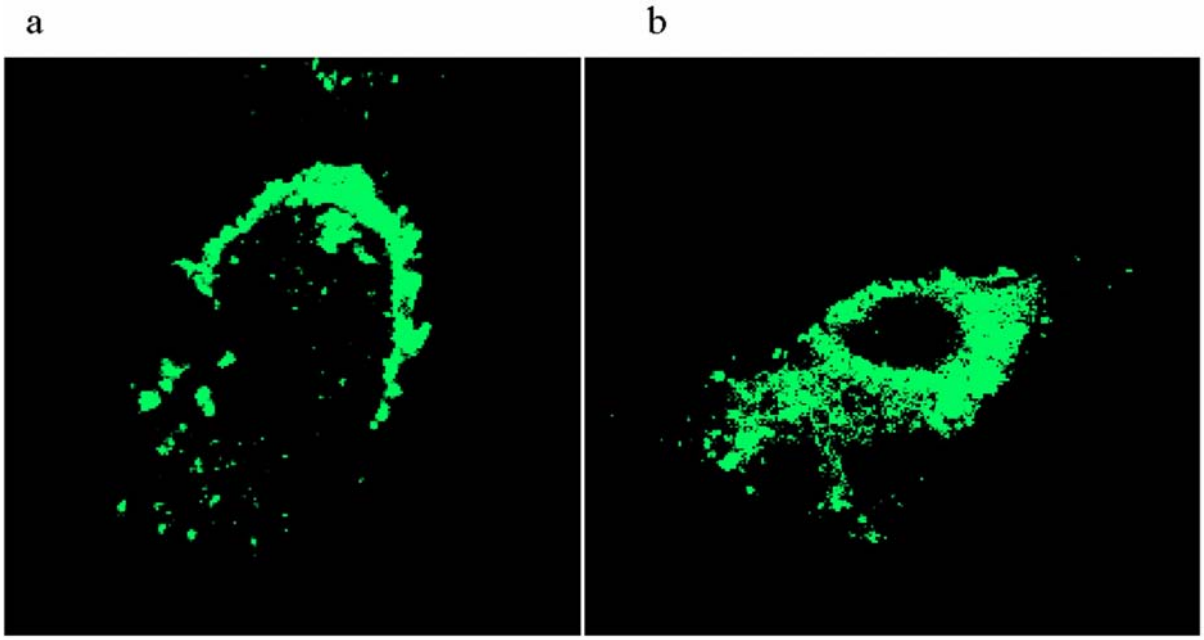
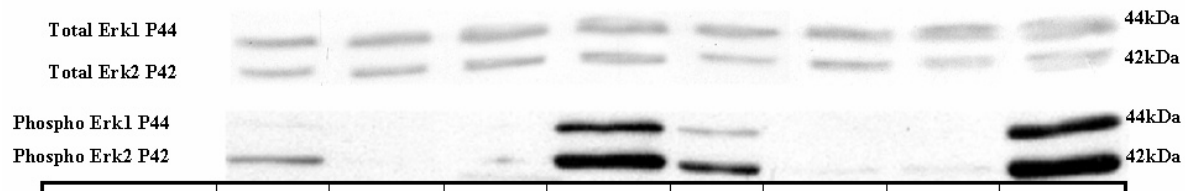



**Figure 4.10.3.** $PAR_2$-AP triggers activation of protein kinase c (PKC) in PKC-EGFP transfected peritubular cells demonstrated by recruitment of cytoplasmic PKC-EGFP to the plasma membrane in PCs stimulated for 1min with $PAR_2$-AP (SLIGRL-NH2; $10^{-4}$M) **(a)** as compared to PCs stimulated with control reverse peptide $PAR_2$-RP (LSIGRL-$NH_2$; $10^{-4}$M) **(b)**.




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **PAR-2 AP** | + | - | - | - | + | - | - | - |
| **PAR-2 RP** | - | + | - | - | - | + | - | - |
| **FCS 10%** | - | - | - | + | - | - | - | + |
| **Time** | **5 min** | | | | **10 min** | | | |

**Figure 4.10.4.** Western blot analysis shows phosphorylation of MAP kinases phospho-p44/p42 (ERK1/2), 5 and 10 min after $PAR_2$ activation of peritubular cells with $PAR_2$-AP (SLIGRL-NH2; $10^{-4}$M). Maximum effect is visible after 10min. The control peptide $PAR_2$-RP (LSIGRL-$NH_2$; $10^{-4}$M) had no effect. Addition of 10% FCS to serum free media served as positive control.

## *4.11 $PAR_2$ positive cells in the granuloma express smooth muscle actin and proliferate*

Double immunofluorescence was used to better characterize the $PAR_2$ positive cells identified within the granulomas in EAO testes.

The cells showing a circular distribution within the granuloma were found to express smooth muscle actin (SMA), a marker for the myoid peritubular cells (Fig. 4.11.a). SMA positive cells were identified to co-express $PAR_2$ (Fig. 4.11.a) as well as the nuclear proliferation marker Ki67 (Fig. 4.11.b).

Combined labeling of ED1/ED2 showed that monocytes/macrophages are localized to the exterior of the granuloma in the interstitial space of the testis, which demonstrates that sma-negative/$PAR_2$-positive cells found in concentric layers of the granulomas could not be macrophages (Fig. 4.11.c). In double-labeling experiments ED1/ED2-positive cells were negative for the Ki67 proliferation marker, indicating that the large increase in the number of monocytes/macrophages in EAO was mainly related to cell migration rather than proliferation (Fig. 4.11.c).

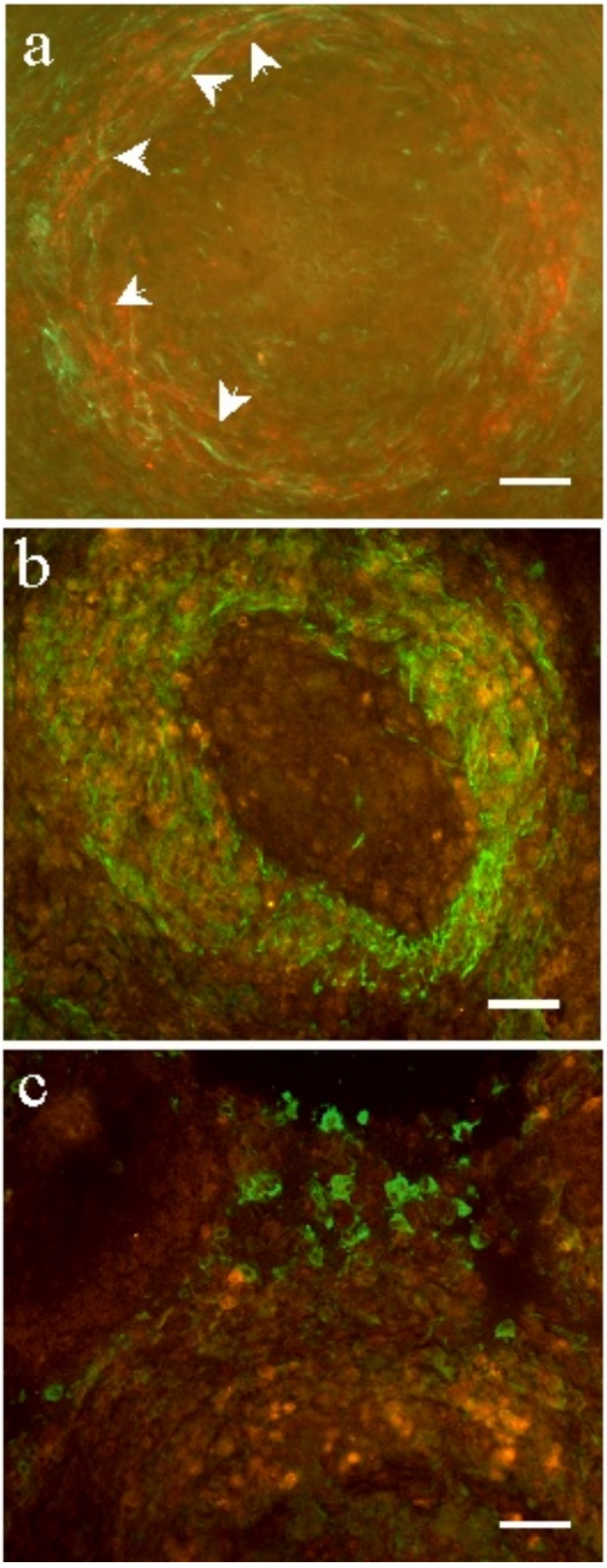



**Figure 4.11:** $PAR_2$ positive cells in granuloma express smooth muscle actin, proliferate and are not macrophages. **a)** Double immunofluorescence for $PAR_2$ (Texas Red) and smooth muscle actin (FITC) showed co-localization of both marker proteins at the periphery of granuloma (arrows). **b)** Co-localization of Ki67 (Cy3) provides evidence that smooth muscle actin positive cells (FITC) in the granuloma are proliferating. **c)** Double immunofluorescence labeling of

ED1/ED2 (combined, FITC) and Ki67 (Cy3) demonstrated that proliferating cells in the granuloma are not macrophages. Scale bars are indicating 50µm.

## *4.12 Expression profiles of inflammatory mediators in EAO and isolated peritubular cells stimulated with a $PAR_2$ agonist*

The expression of MCP-1 mRNA, the most potent monocyte and neutrophil chemoattractant molecule, was found to be highly upregulated (300 fold) 50 days after EAO induction compared to the levels seen in adjuvant controls (Fig. 4.12.1.a). Correspondingly, the expression of MCP-1 (at 3 and 6h) was statistically significant enhanced after the stimulation of cultured PTCs with $PAR_2$-AP compared to that seen after application of the control $PAR_2$-RP (Fig. 4.12.1.b).

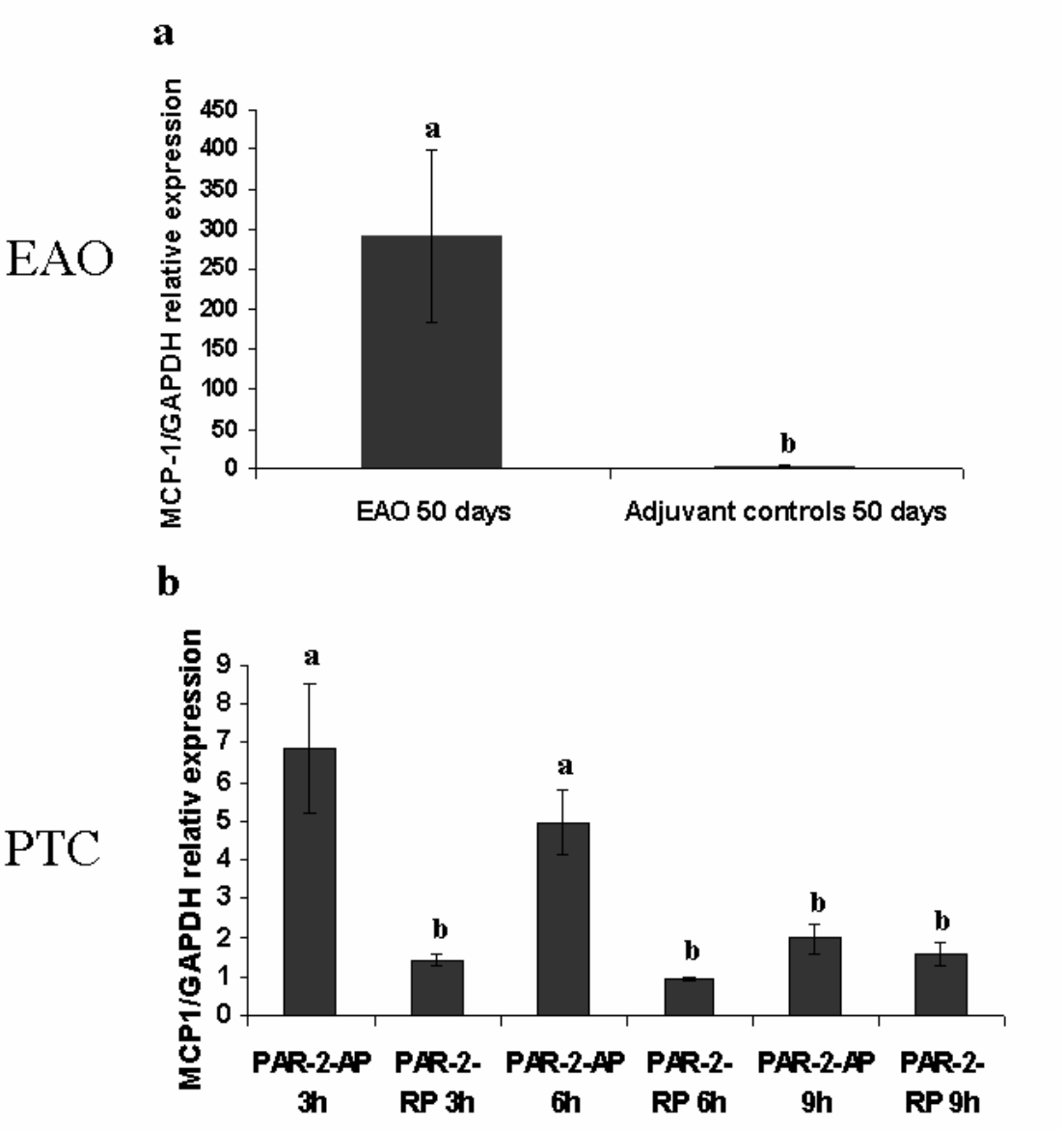


**Fig. 4.12.1.** Comparison of the expression of MCP-1 determined by real time RT-PCR analysis in EAO and in $PAR_2$ stimulated peritubular cells. **a)** MCP-1/GAPDH relative expression in EAO samples and **(b)** in peritubular cells stimulated with $PAR_2$-AP. $PAR_2$-RP served as negative control. GAPDH is used for normalization of expression data. Values are mean ± SEM of *n*=6 per group for EAO samples and *n*=3 per group for peritubular cells (from 3 different experiments). Values with different letters superscript differ significantly ($p<0.05$).

At 50 days after EAO induction, the TGFß-2 mRNA level was significantly 8 fold increased (Fig. 4.12.2.a).

$PAR_2$ activation in peritubular cells by $PAR_2$-AP significantly increased the expression of TGFß2 (at 3h and 6h) compared to the application of the control peptide $PAR_2$-RP (Fig. 4.12.2.b).

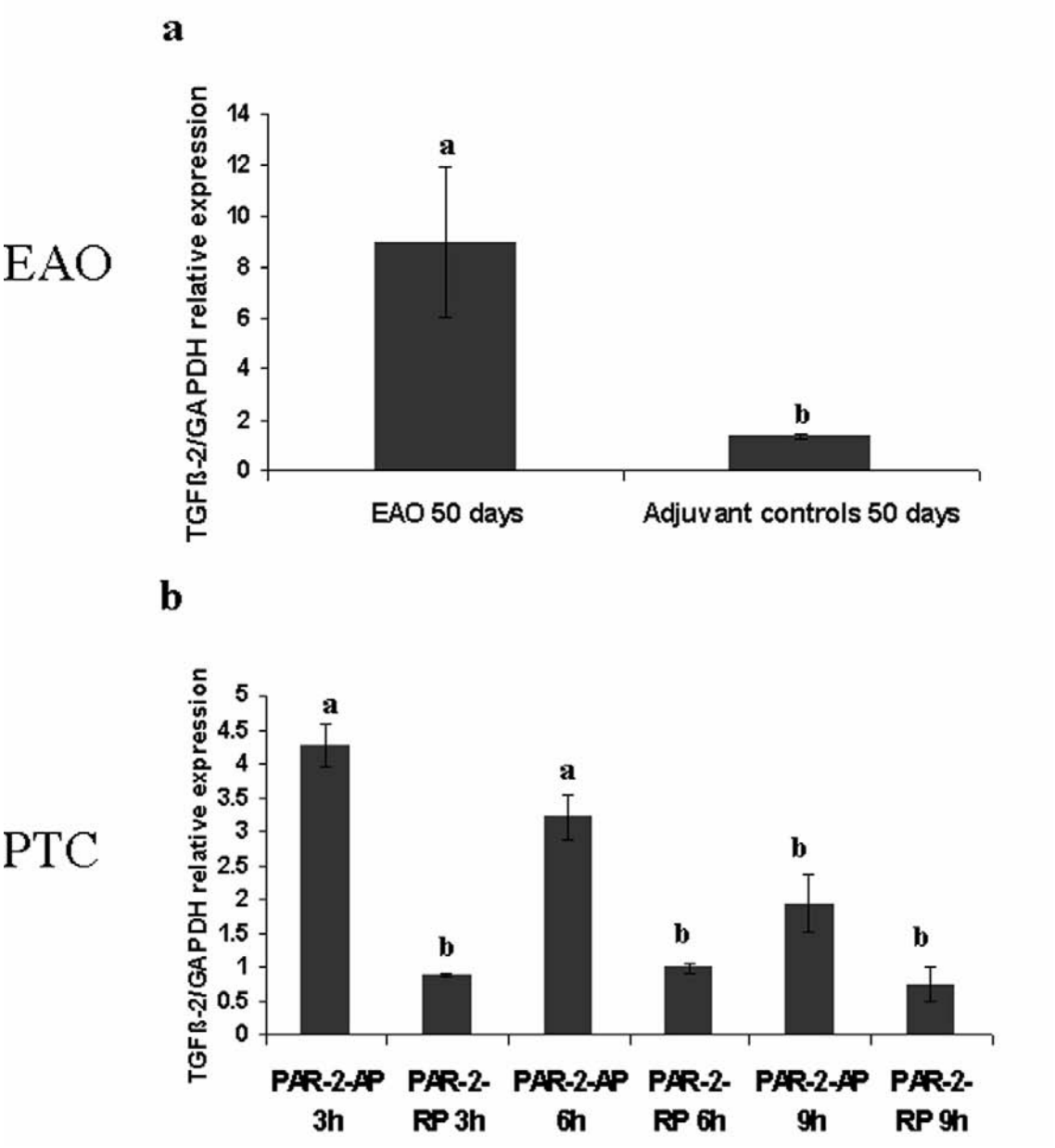


**Figure 4.12.2: Comparison of the expression of TGFß-2 determined by real time RT-PCR analysis in EAO and in $PAR_2$ stimulated peritubular cells. a)** TGFß-2/GAPDH relative expression in EAO samples and **(b)** in peritubular cells stimulated with $PAR_2$-AP. $PAR_2$-RP served as negative control. GAPDH is used for normalization of expression data. Values are mean ± SEM of *n*=6 per group for EAO samples and *n*=3 per group for peritubular cells (from 3 different experiments). Values with different letters superscript differ significantly ($p<0.05$).

The induction of COX-2 (Fig. 4.12.3.a) and iNOS (Fig. 4.12.4.a) expression in EAO was vastly increased already after gel electrophoretic analysis, which made real time PCR analysis redundant. This was particularly evident for iNOS where expression levels rose from undetectable levels (adjuvant control) to a clearly visible PCR product in 50-day EAO testes (Fig. 4.12.4.a).

The expression of COX-2 (6h and 9h) (Fig. 4.12.3.b) was significantly enhanced after the stimulation of cultured PTCs with $PAR_2$-AP compared to that seen after addition of the control $PAR_2$-RP. Expression of iNOS was not elevated by $PAR_2$-AP in PTCs (Fig. 4.12.4.b) suggesting either that the increase in iNOS mRNA synthesis observed in EAO was induced in testicular cells other than PTCs or that iNOS overexpression observed in EAO is not $PAR_2$ dependent.

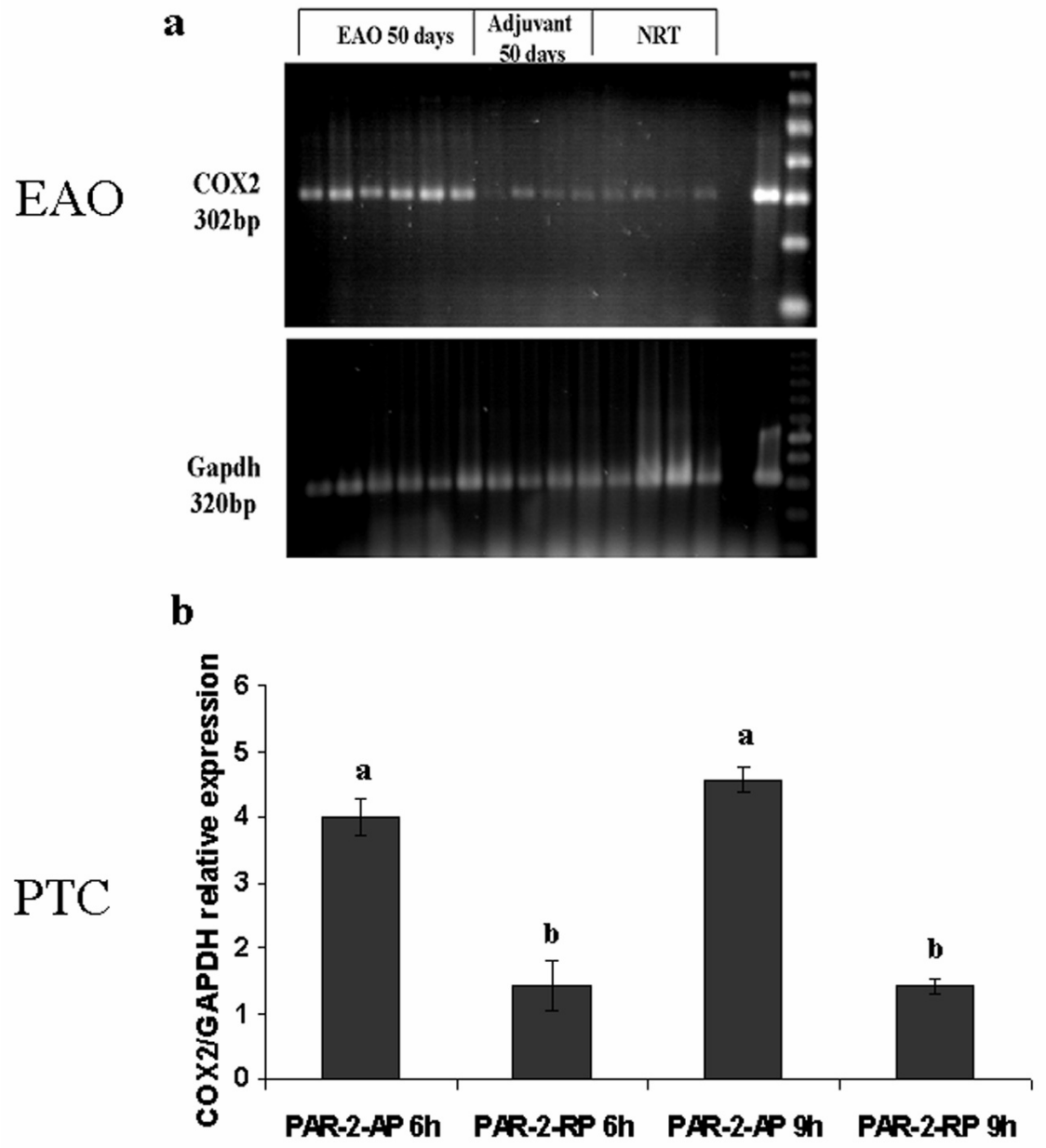



**Figure 4.12.3:** Comparison of COX-2 expression determined by agarose gel electrophoresis **(a)** or by real time RT-PCR analysis **(b)** in EAO and in $PAR_2$ stimulated peritubular cells. Real time PCR analysis was not performed for COX-2 in EAO **(a)** where agarose gel electrophoresis alone already revealed substantial differences. **a)** COX-2 expression shows a clear-cut upregulation in 50 days EAO samples. **b)** COX-2 expression in $PAR_2$ stimulated peritubular cells is increased at 6h and 9h. Values are mean ± SEM of *n*=3 per group for peritubular cells (from 3 different experiments). Values with different letters superscript differ significantly ($p<0.05$).

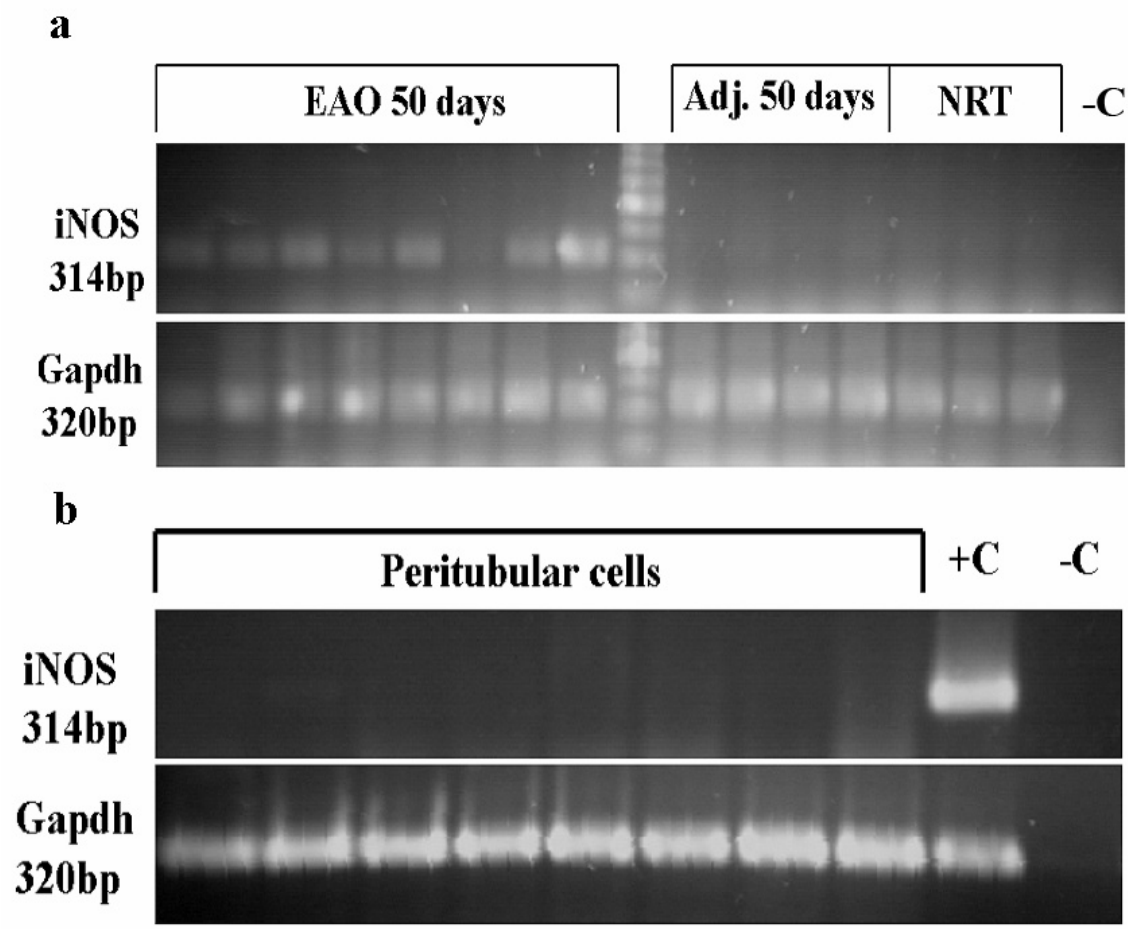



**Figure 4.12.4:** The expression of iNOS was evaluated using normal PCR followed by gel electrophoresis **a)** for 50 day EAO specimens and **b)** for isolated peritubular cells treated with $PAR_2$-AP and $PAR_2$-RP. No iNOS expression is observed in adjuvant and normal rat testis (NRT) control, but is clearly induced in EAO. Peritubular cells were negative for iNOS expression. +C: positive control. –C: negative control. GAPDH is used as loading control

## *4.13 In vivo activation of $PAR_2$ in rat testis*

Injection of recombinant human tryptase into the testis induced an increased expression of the MCP-1, TGFß2 and COX-2 mRNAs as compared to sham controls *in vivo* (Fig.4.13.1).

Heparin, which was used to stabilize tryptase, did not elicit an effect. Inactivated tryptase failed to evoke these effects when injected *in vivo*, proving that tryptase-dependent MCP-1, TGFß-2 and COX-2 upregulation is based on tryptase's proteolytic activity.

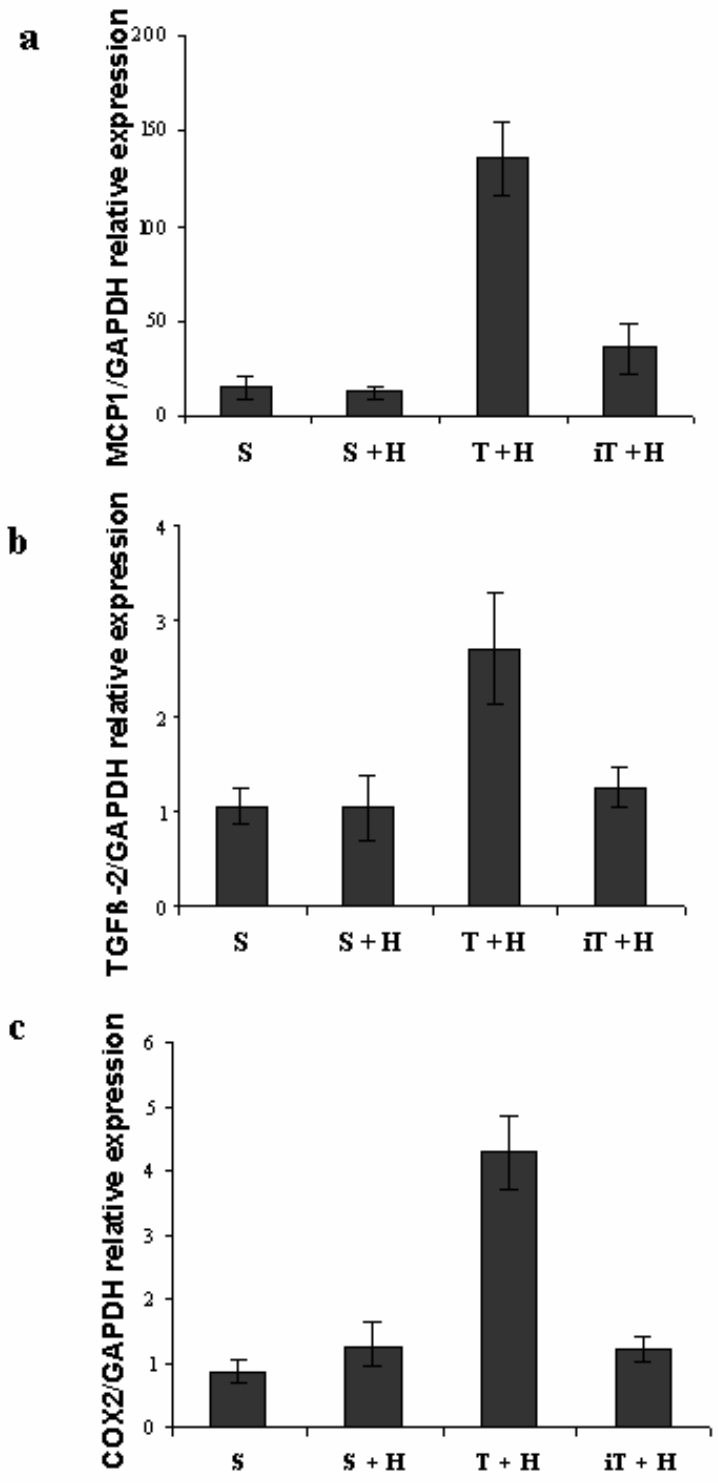



**Figure 4.13.1:** Application of recombinant human mast cell tryptase causes an increase of MCP-1, TGFβ2 and COX-2 mRNA synthesis in rat testes compared to saline control and to heparin after 5h (**a**, **b** and **c,** respectively). Enzymatically inactive tryptase fails to upregulate MCP-1, TGFß-2 and COX-2 expression. Expression levels were quantified using iCycler real time PCR. Bars are mean ± SEM, n=5-10 animals/group.

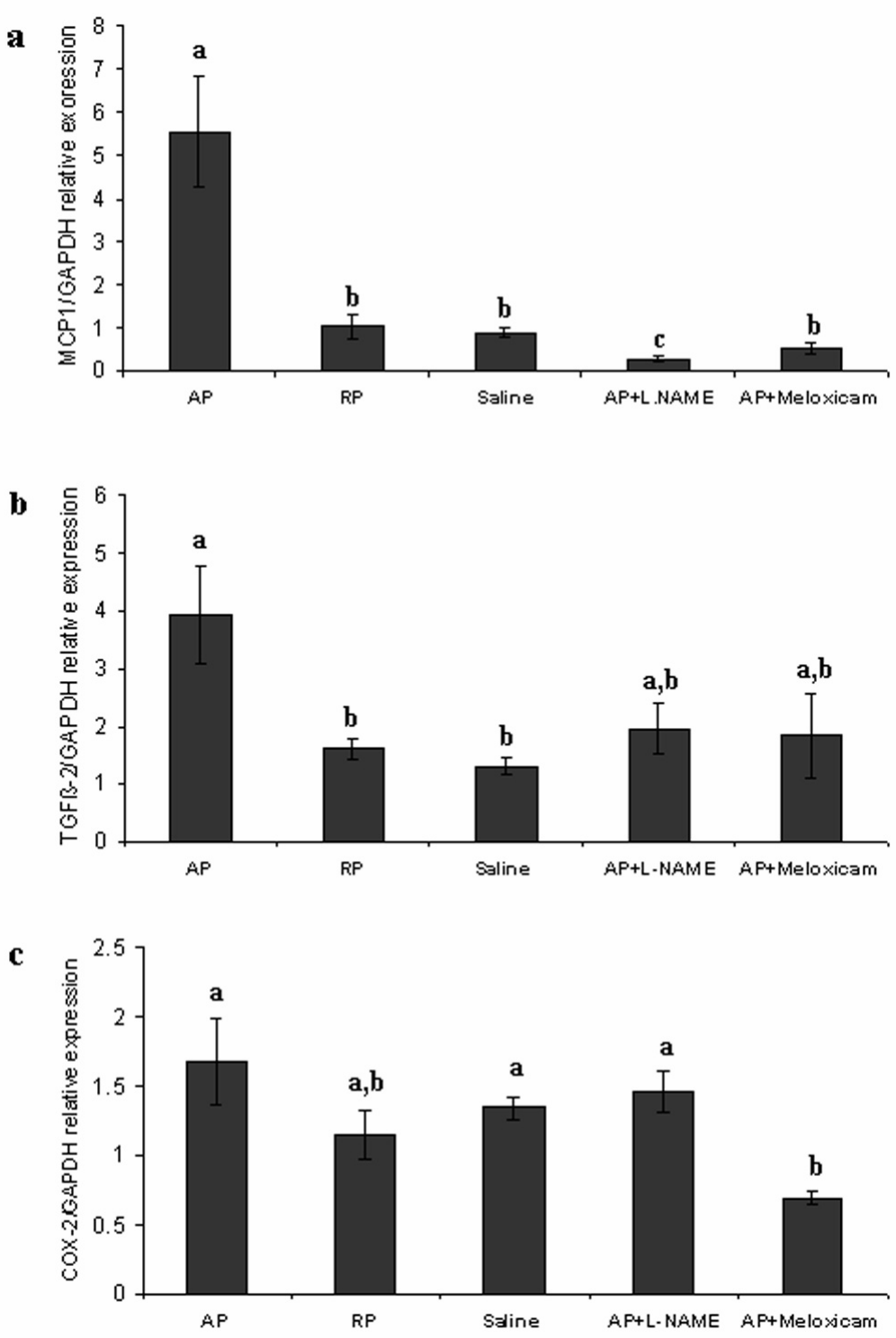



**Figure 4.13.2:** $PAR_2$-activating peptide $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$) *in vivo* stimulates the upregulation of MCP-1 and TGFβ2 mRNA expression in rat testes after 5h (**a**,**b**). Pretreatment for 20min with the specific COX-2 antagonist meloxicam significantly inhibits the stimulatory action of $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$) on MCP-1 and COX-2 synthesis. TGFβ2 mRNA levels were only partially suppressed. Pretreatment for 20min with the NOS-inhibitor L-NAME significantly blocks the stimulation of $PAR_2$-f-AP on MCP-1 expression levels, whereas only a gradual inhibition is observed for TGFβ-2. COX-2 expression was unaffected by L-NAME pretreatment. Injection of the control peptide was not different from sham-operated animals (saline injection). Expression levels were quantified using iCycler real time PCR. Bars

with same letter superscript are not significantly different ($p<0.05$; mean ± SEM, n=5-10 animals/group). Expression of iNOS was not affected in any treatment group (data not shown).

Because human tryptase, in addition to activating $PAR_2$, causes a wide variety of proteolytic effects (Sommerhoff, 2001, Brown et al., 2002), subsequent experiments utilized the specific $PAR_2$-agonist $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$) (McGuire et al., 2004a).

Application of this substituted peptide agonist, which due to the furoyl moiety has an improved tissue penetration and resistance to metabolic degradation than SLIGRL-$NH_2$, stimulated the upregulation of MCP-1 and TGFβ-2 mRNA expression in rat testes after 5h, whilst only marginally elevating the expression of COX-2 (Fig 4.13.2.a-c).

Pretreatment for 20 min with the COX-2 antagonist meloxicam significantly inhibited the stimulatory action of $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$) on MCP-1 and COX-2 synthesis while TGFβ-2 mRNA levels were only partially suppressed. Pretreatment with the general NOS-inhibitor L-NAME significantly blocked the stimulation of $PAR_2$-f-AP ([2-furoyl]-LIGRLO-$NH_2$) on MCP-1 expression levels (Fig. 4.13.2.a), whereas only a mild inhibition was observed for TGFβ-2 (Fig. 4.13.2.b) and COX-2 expression remained unaffected (Fig. 4.13.2.c). Consequently, it can be deduced that $PAR_2$ mediated MCP-1 mRNA synthesis is dependent upon the action of both NO and COX-2 (Fig. 4.13.2.a). Inducible NOS (iNOS) synthesis was not affected by $PAR_2$ stimulation *in vivo* at all (data not shown). The mRNA expression in animals injected with control peptide was found to be no different to that of the sham operated animals (saline injection).

## *4.14 In vivo siRNA gene silencing in rat testis*

In order to further prove the connection between $PAR_2$ activation and testicular inflammation the next step was antagonizing this pathway. As $PAR_2$ antagonists are not available and switching to a mouse $PAR^{-/-}$ knock out model would have been more technically complicated, we decided to silence $PAR_2$ gene expression *in vivo* in rats using small interference RNA technology.

The *in vivo* gene silencing using siRNA is described as a challenging and difficult target in tissues which are not intensively vascularized (de Fougerolles

et al., 2005). To date two main delivery techniques for siRNA are described *in vivo*: 1) Hydrodynamic delivery by intra venous injection of large volumes (10ml for rats) of siRNA containing isotonic saline (Lewis et al., 2002, McCaffrey et al., 2002, Giladi et al., 2003, Song et al., 2003) has been shown to be accompanied by gene silencing in well vascularized tissues like liver and kidney but reported to be ineffective in tissues which are not well vascularized; 2) Successful local siRNA (2-100µg) administration has been reported in brain, intranasal, intratumoral and subretinal (Makimura et al., 2002, Aharinejad et al., 2004, Tolentino et al., 2004, Zhang et al., 2004).

As the testis is less vascularized compared to organs like liver and kidney, siRNA was locally delivered by subcapsular injection.

In order to establish this new and challenging technique including the most effective dose and time point, a siRNA pool for lamin A/C (siCONTROL Lamin A/C siRNA, Cat. No. D-001050-01-20, Dharmacon RNA technologies, USA) was used as positive control.

SiRNA was injected unilaterally in the right testis under the testicular capsule in different doses (5, 50, 100µg). The left testis served as an internal control and received the buffer alone. Rats were killed after 24h and 72h. A small, but not significant silencing effect was observed 24h and 72h after injection of 50µg siRNA (Fig. 4.14.1.a). This effect could not be confirmed on the protein level by Western blot for lamin A/C (Fig. 4.14.1.b).

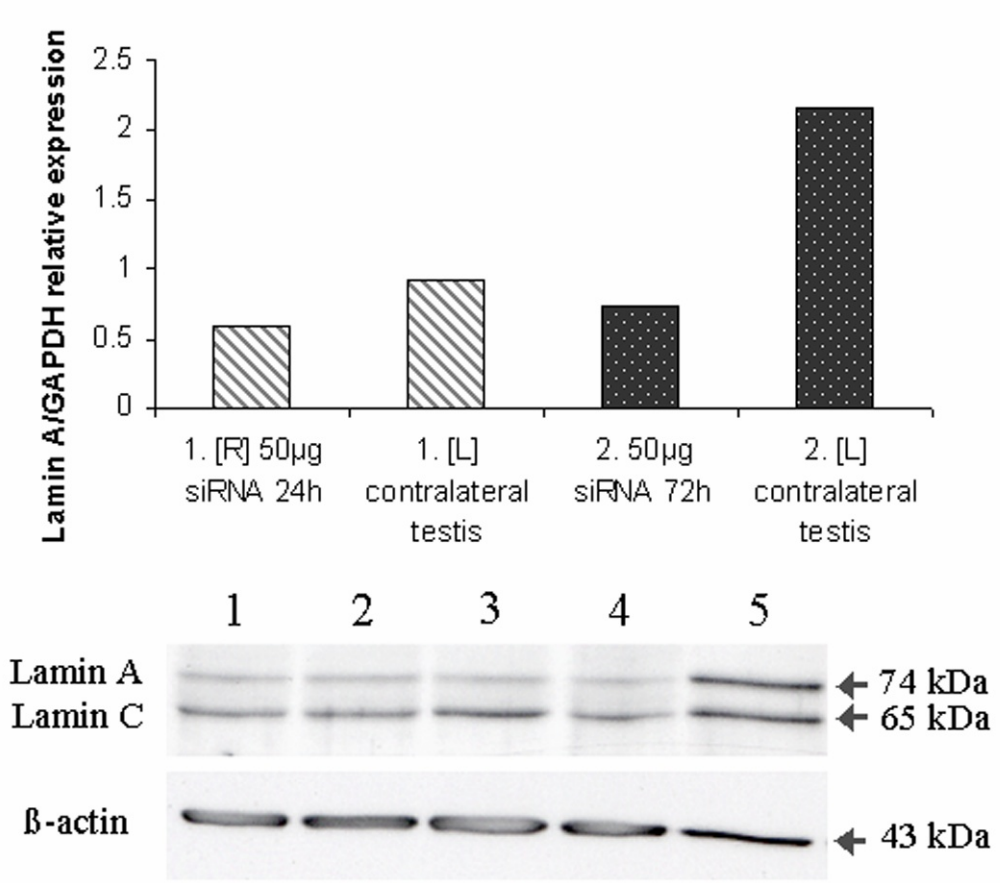



**Fig. 4.14.1: a**) Representative bar diagram showing the lamin A/GAPDH relative expression levels (measured by quantitative real-time PCR) in rat testes injected with 50µg lamin A/C siRNA by subcapsular injection compared to control testis (contralateral). A small decrease is observed 24h after injection and a more substantial effect after 72hrs. **b**) Western blot for Lamin A/C on rat testicular samples. Lines: **1**: 50µg lamin A/C siRNA 24h; **2**: control (contralateral) testis 24h; **3**: 50µg Lamin A/C siRNA 72h; **4**: control (contralateral) testis 72h; **5**: rat liver (positive control).

After careful evaluation of the variation of lamin A expression within buffer treated rat testes by quantitative real-time PCR we concluded that the small silencing effect observed at the mRNA level has to be regarded as experimental variation (Fig. 4.13.2).

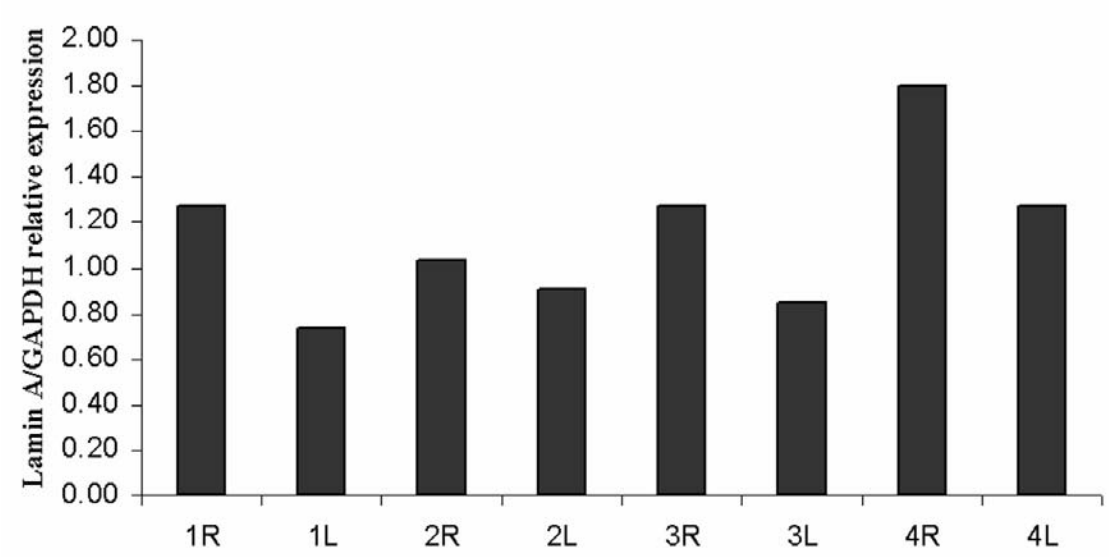



**Fig. 4.13.2.** Variation of lamin A mRNA levels within testicular samples from similarly treated rats. Bar diagram showing the lamin A/GAPDH relative expression (measured by quantitative real-time PCR) in testes from four rats bilaterally injected with isotonic saline. (R: right testis; L: left testis)

We concluded that gene silencing using siRNA delivered by subcapsular testicular injection was not an efficient method for our purposes.

# 5. Discussion

EAO is characterized by an increased number of immune cells in the testicular interstitium (Kohno et al., 1983, Doncel et al., 1989, Lustig et al., 1993). However, the mechanism by which immune cells migrate and extravasate in the testicular interstitium is poorly understood. Neutrophils are absent form the testicular interstitium in normal conditions, but present in inflamed or injured testis (Kohno et al., 1983, Gerdprasert et al., 2002a).
In EAO we detected a substantial increase in the number of neutrophils, macrophages and mast cells, enlarged interstitial space, severe reduction and sloughing of the seminiferous epithelium and granuloma formation.

Contrary to other organs where inflammation is accompanied by edema, testis suffers a decrease in total volume and weight with testicular atrophy as a characteristic of testicular inflammation. The later is mainly due to a massive loss of germ cells leading to the observed reduction of tubular compartment concomitant with a reduction of interstitial fluid (Hedger et al., 2005).

## *5.1 $PAR_2$ in the normal and inflamed testis*

$PAR_2$ expression in human testis was for the first time reported in cells of the testicular germinal epithelium (D'Andrea et al., 1998). Later on, $PAR_2$ was identified on ejaculated spermatozoa (Weidinger et al., 2003) and in the interstitium of the human testis. However, the precise nature of these $PAR_2$-positive interstitial cells in human testis was not specified (Frungieri et al., 2002). The present study describes the localization of $PAR_2$ in the rat testis, which appears to be similar to human. $PAR_2$ immunoreactivity in normal rat testis was detected on PTC, in a fraction of interstitial cells and on the acrosome of developing round and elongating spermatids. More specifically, the double immunofluorescence labeling showed unambiguously that isolated rat testicular macrophages positive for ED1 and ED2 also express $PAR_2$,

identifying the fraction of $PAR_2$-positive interstitial cells as testicular macrophages.

Many studies reported that proinflammatory stimuli induced an upregulation of $PAR_2$ both *in vitro* in different cell lines (Hamilton et al., 2001, Roche et al., 2003a) and *in vivo* in different models of chronic and acute inflammation (Olejar et al., 2001, Kim et al., 2003). In addition, more recent studies showed that $PAR_2$ is significantly involved in mediating both the short-term (Steinhoff et al., 2000, Macfarlane et al., 2001, Steinhoff et al., 2004) and the long-term inflammatory responses (Cocks and Moffatt, 2001, Roche et al., 2003b, Cederqvist et al., 2005, Holzhausen et al., 2005). Moreover $PAR_2$ has recently been found to play an essential role in a mouse model of chronic autoimmune arthritis (Ferrell et al., 2003). Also in our model of chronic testicular inflammation (EAO), $PAR_2$-expression was found to be highly upregulated in the interstitial space. Numerous $PAR_2$ positive macrophages were equally distributed in the interstitial space, always excluding the granulomas. On the other hand, strong $PAR_2$ immunoreactivity was detected within the granulomas, in flattened cells which were distributed in multiple circular layers displaying a structure similar to that of PTC. Our combined immunofluorescence studies identified these $PAR_2$ positive cells in the granulomas to co-express SMA and the nuclear proliferation marker Ki67. Based on these results we hypothesize that these cells of myoid origin (PTC) proliferate and form granulomas surrounding tubular lesions. Our assumption is also supported by other studies reporting that in testicular biopsies from infertile patients, seminiferous tubules are surrounded by many layers of proliferating peritubular or fibrocyte like cells (Meineke et al., 2000, Frungieri et al., 2002, Weidinger et al., 2003a).

In the normal rat testis the tubular compartment concentrates most of the $PAR_2$ immunoreactivity in the acrosomes, whereas in EAO testis the seminiferous tubules are almost depleted of germ cells. In contrast, the interstitial space is strongly enlarged in EAO due to cell infiltrates, among them numerous scattered $PAR_2$ positive macrophages. There is a shift of $PAR_2$ immunoreactivity in EAO from the tubular compartment to the testicular interstitial space, suggesting that under normal conditions $PAR_2$ may mediate

different functions as compared to chronic testicular inflammation. Indeed, in normal testis $PAR_2$, present on the acrosome of spermatids and spermatozoa, was reported to interact with tryptase released by mast cells in the fallopian tube, thus influencing the migration of spermatozoa through the female genital tract (Weidinger et al., 2003). On the other hand, in chronically inflamed testis $PAR_2$ is present on interstitial macrophages and their numbers are highly increased suggesting that $PAR_2$ could play a central role in mediating the inflammatory response during EAO.

In EAO testicular macrophages do not express the nuclear proliferation marker Ki67. Based on this result we conclude that the increase in numbers of $PAR_2$ positive macrophages in EAO is based on new recruitment of macrophages rather than on the proliferation of the existing ones. This is also supported by findings of a recent study reporting significantly elevated levels of MCP-1 in EAO testis as compared to controls (Guazzone et al., 2003), MCP-1 being a potent chemoattractant for mononuclear leukocytes.

## *5.2 Role of mast cells in testicular inflammation*

In the normal testis mast cells and $PAR_2$-positive cells are largely segregated; mast cells are restricted to blood vessels directly under the testicular capsule while the majority of $PAR_2$-positive cells are located in the tubular compartment. Mast cells have long been considered solely as effector cells, critical to allergic diseases and the immune response to parasites. The spark of interest in a role for mast cells in initiating or propagating autoimmune disease was prompted by studies on multiple sclerosis and its animal model, experimental allergic encephalomyelitis (EAE) (Rozniecki et al., 1995, Steinman, 2001). Increased amounts of proteolytic enzymes such as tryptase were measured in cerebrospinal fluid from patients with multiple sclerosis (Rozniecki et al., 1995). In addition, drugs considered to 'stabilize' mast cells have been shown to ameliorate the severity of EAE (Brosnan and Tansey, 1984, Dietsch and Hinrichs, 1989, Rozniecki et al., 1995). Mast cells accumulate in the swollen paws of mice suffering from collagen-induced arthritis, and they degranulate

during the disease process (Malfait et al., 1999) and vice versa mediators that prevent mast cell degranulation had a strong therapeutic effect on the progression of collagen-induced arthritis (Malfait et al., 1999).

Although mice that selectively lack only mast cells have not been reported, the c-kit mutant mice are almost completely devoid of them (Nakano et al., 1985, Kitamura, 1989, Galli et al., 1994, Blank and Rivera, 2004). The lack of mast cells in genetically mast cell−deficient c-Kit mutant mice can be selectively repaired by the adaptive transfer of genetically compatible mast cells, produced in vitro from stem cells (Nakano et al., 1985, Kitamura, 1989, Galli et al., 1994, Blank and Rivera, 2004). These mast cell knock-in mice can then be used to assess the extent to which differences in the expression of biological responses noted in mast cell−deficient and congenic wild-type mice reflect the absence of mast cells in the mast cell−deficient mice. Mast cell−deficient mice have also been used to examine the contributions of mast cells in models of an autoantibody-induced destructive arthritis (Lee et al., 2002), multiple sclerosis (Tanzola et al., 2003) and bullous pemphigoid, an autoimmune disease of the skin (Chen et al., 2001). In both instances, studies of mast cell knock-in mice indicated that much of the pathology was mast cell dependent.

The experimental autoimmune orchitis shows similarities to other autoimmune disorders; histologically it is accompanied by a strong increase in mast cell numbers and an increased degranulation rate. Mast cell numbers were not only 10-fold higher but they were widely distributed throughout the interstitial space, particularly around granulomas. This contrasts to what is seen in the normal rat testis where mast cells were exclusively found directly under the tunica albuginea. This suggests that mast cells could be key players in the pathophysiology of autoimmune orchitis and together with the increased $PAR_2$ immunoreactivity detected in EAO testis leads to the assumption that mast cell tryptase-$PAR_2$ pathway might play an important role during testicular chronic inflammation.

A role for mast cells has been previously indicated in male infertility (Meineke et al., 2000), and interestingly, mast cells are involved in fibrotic testicular disorders via a $PAR_2$-dependent mechanism (Frungieri et al., 2002). The numbers of tryptase containing mast cells are significantly increased in testes of patients with obstructive azoospermia, idiopathic azoospermia, varicocele (Yamanaka et al., 2000b) as well as in patients with mixed atrophy (Jezek et al., 1999, Meineke et al., 2000). Tranilast, a mast cell blocker, was found to be clinically useful for the treatment of severe idiopathic oligozoospermic men (Hibi et al., 2001).

The increase in numbers and the redistribution of mast cells to a location in the testicular parenchyma concomitant with the upregulation of $PAR_2$ immunoreactivity in the interstitial compartment, are important findings as it means that in EAO, mast cell tryptase and its receptor $PAR_2$ are brought into close proximity thereby facilitating an interaction between the two effectors. This is supported by the morphological observation that numerous mast cells, in contrast to control testes, were found to have degranulated in EAO gonads, thereby releasing tryptase in the surrounding interstitial tissue.

## *5.3 $PAR_2$ mediated action on PTC*

*In vitro*, the characteristic increase in intracellular calcium concentration following stimulation with the $PAR_2$ agonist SLIGRL-$NH_2$ indicated that the $PAR_2$ protein found morphologically on isolated PTC was indeed functional. The reverse peptide LSIGRL-$NH_2$ had no influence on the intracellular calcium concentration. The $PAR_2$ functionality was further confirmed by the activation of protein kinase C (PKC), which resulted from the addition of $PAR_2$ agonist to PKC-EGFP transfected PTC. PKC-EGFP was recruited to the plasma membrane already 1min after exposure to $PAR_2$-AP and this relocation continued at least 5min. Stimulation of PCs with control reverse peptide LSIGRL-$NH_2$ did not change the cellular PKC-EGFP distribution.

The initial mobilization of intracellular calcium levels by the $PAR_2$-agonist, may not only be required for PKC activation, but can also result in the observed phosphorylation of ERK1/2 in PTC following $PAR_2$ activation. Therefore, the PKC and MAPK pathways could play an important role in mediating mast cell-derived tryptase effects in acute testicular inflammation and potentially also in chronic orchitis. This could occur by two mechanisms. On one side, ERK1/2 activation coordinately regulates cell proliferation (Roux and Blenis, 2004) and indeed, the co-localization of the proliferation marker Ki67 and $PAR_2$ in the flattened, smooth muscle actin positive cells that are oriented in circular layers at the periphery of granulomas suggest that MAPK phosphorylation can mediate also proliferation of PTC. More evidence to strengthen this hypothesis has been recently brought by the study of Frungieri et. al (Frungieri et al., 2005) which demonstrates that tryptase-induced $PAR_2$-mediated cell proliferation in human fibroblasts was dependent of MAPK phosphorylation and ERK1/2 inhibitors completely abolished these effects.

On the other side, the observation that tryptase induced MAPK phosphorylation was preceding cytokine release from eosinophils (Temkin et al., 2002) led us to question whether PTC also contribute to the immune response in testicular inflammation, particularly as they are known sources of various cytokines like interferon-γ, activin, TGFß and MCP-1 (Hedger and Meinhardt, 2003).

## *5.4 Mast cell tryptase-$PAR_2$ pathway responsible for upregulation of key inflammatory mediators*

In EAO testes profiling of the key inflammatory mediators MCP-1, TGFβ, COX-2 and iNOS revealed markedly increased expression levels. This together with the above-mentioned findings prompted us to investigate whether the signaling pathway involving mast cell tryptase and its receptor $PAR_2$ can also be a regulator of the testicular immune response. This was subsequently studied in more detail *in vitro* using primary cultures of PTC preceding *in vivo* experiments

where tryptase and the $PAR_2$-agonist 2-furoyl-LIGRLO-$NH_2$ were directly injected in the testis to combine the lines of evidence.

*In vitro* $PAR_2$ activation of PTC induced ERK1/2 phosphorylation and an increase in MCP-1, TGFß-2 and COX-2 levels suggesting that PTC can be more actively involved in testicular inflammation than previously thought. Similarly, in smooth muscle cells MCP-1 production was dependent on MAPK phosphorylation (Kanellis et al., 2003). In addition, MAPK have been implicated in tryptase induced TGFß release in human air way smooth muscle cells, an action which in turn stimulated the accumulation of mast cells (Berger et al., 2003).

In an *in vivo* model of acute $PAR_2$ activation, human recombinant tryptase enhanced the expression levels of MCP-1, TGFß-2 and COX-2. The enzymatically inactive tryptase failed to elicit an effect suggesting that the tryptase-dependent upregulation of key inflammatory mediators is based on the proteolytic activity of tryptase. Because human tryptase, in addition to activating $PAR_2$, causes a wide variety of proteolytic effects (Sommerhoff, 2001, Brown et al., 2002), subsequent experiments utilized the specific $PAR_2$-agonist 2-furoyl-LIGRLO-$NH_2$ (McGuire et al., 2004b). Of note, application of this novel synthetic peptide in normal rat testis caused a significant elevation of MCP-1 and TGFβ-2 mRNA expression after 5hrs while COX-2 expression was not affected. Our *in vivo* data combined with those of other studies (Temkin et al., 2002, Shimizu et al., 2004, Shpacovitch et al., 2004) favor the hypothesis that MCP-1 and TGFβ expression is regulated by $PAR_2$, whereas the increase in COX-2 is based on the unspecific proteolytic action of tryptase independent of $PAR_2$-cleavage.

The role of TGFß-2 action in EAO is likely to be multifaceted. In addition to its well established anti-inflammatory role in controlling the outcome of an immune response (Elenkov and Chrousos, 2002), TGFß-2 may also recruit additional mast cells (Berger et al., 2003) as well as causing fibrosis and granuloma formation (Eickelberg, 2001). Granulomas were also regularly observed in EAO testis and high levels of TGFß-2 were measured at 50 days of EAO. This

together with the $PAR_2$ dependent TGFß-2 upregulation observed both *in vivo* and *in vitro* suggest that the mast cell tryptase-$PAR_2$ pathway can contribute to granuloma formation both indirectly by inducing TGFß expression in these cells and directly by activating ERK1/2 in PTCs within the granulomas (like previously shown). Moreover the observation that PTCs within granulomas were positive for Ki67 supports our hypothesis.

A number of recent *in vitro* studies have indicated that the inflammatory vasodilator nitric oxide (NO) is capable of inhibiting steroidogenesis by the Leydig cells, the granulosa luteal cells, and the adrenal cortex (Cameron and Hinson, 1993, Van Voorhis et al., 1994, Adams et al., 1996, Del Punta et al., 1996). Thus, NO appears to be an important factor mediating the effects of testicular inflammation. Production of NO occurs through the action of one of three nitric oxide synthase (NOS) enzymes. The inducible isoform of NOS (type II or iNOS) has been reported to induce germ cells apoptosis (Lue et al., 2003). Furthermore, it was shown that iNOS is upregulated in many tissues in response to an inflammatory episode (Xie et al., 1992). Also in EAO a clear induced *de novo* synthesis was recorded after 50 days. PTC *in vitro* do not express iNOS and *in vivo* $PAR_2$-stimulation did not induce an elevation of iNOS mRNA expression. In conclusion, iNOS upregulation in EAO could be responsible for the impairment of spermatogenesis and steroidogenesis, but the mechanism responsible for iNOS upregulation does not involve $PAR_2$ activation and still remains unclear.

## *5.5 $PAR_2$ dependent MCP-1 upregulation requires $COX_2$ and NO production.*

COX-2, the inducible COX isoform mediates the metabolism of arachidonic acid leading to the production of prostaglandins and it has been reported to be constitutively expressed in rat testis (Neeraja et al., 2003a). Of note, there are different effects of $PAR_2$ activation on COX-2 expression levels within the two experimental setups *in vivo* as compared to the data obtained *in vitro*. PTCs respond to $PAR_2$ activation by induction of COX-2 while *in vivo* $PAR_2$ specific activation with 2-furoyl-LIGRLO-$NH_2$ had no significant effect on COX-2 levels.

Similarly, other studies reported that COX-2 levels were enhanced after $PAR_2$ activation *in* vitro in human fibroblasts (Frungieri et al., 2002), but there is no data proving this link *in vivo.* Even if not influenced *in vivo* by $PAR_2$ activation, COX-2 seems to be required as a cofactor mediating $PAR_2$ effects on MCP-1 levels.

Interestingly, MCP-1 expression in $PAR_2$ stimulated testis was sensitive to both the general NOS-antagonist L-NAME and the COX-2 inhibitor meloxicam, as shown in the Fig. 5.5. For almost complete inhibition of MCP-1 production to be achieved, full suppression of COX-2 mRNA is not necessary, as meloxicam pretreatment resulted in only a 50% inhibition of COX-2 expression *in vivo*. Our data favors the hypothesis that MCP-1 expression is not directly regulated by $PAR_2$, but rather via an indirect pathway involving NO production, COX-2 and probably MAPK. This is akin with the findings in another myoid cell type, the vascular smooth muscle cell, where inhibition of p38, ERK1/2 or COX-2 each significantly suppressed uric acid-induced MCP-1 expression. Also, in femoral artery lesions COX-2 inhibition decreased MCP-1 expression (Wang et al., 2005).

NO has been reported to regulate the expression of MCP-1, but the data available to date varies from one cell type to another. In the renal cortex L-NAME induces MCP-1 upregulation indicating that NO inhibits MCP-1 production in these cells (Kashiwagi et al., 2002). In the ANA-1 murine macrophages L-NAME treatment leads to a decrease in MCP-1 levels supporting the hypothesis that NO increases MCP-1 expression (Guo et al., 2002). Similarly to COX-2, the inducible NOS isoform (iNOS) levels are strongly elevated in EAO, but $PAR_2$ activation had no effect on iNOS expression.
Our data indicate that $PAR_2$-dependent MCP-1 upregulation requires NO production and induction of COX-2 expression and provides a new insight into the regulation of CC-chemokine production during testicular acute inflammation.

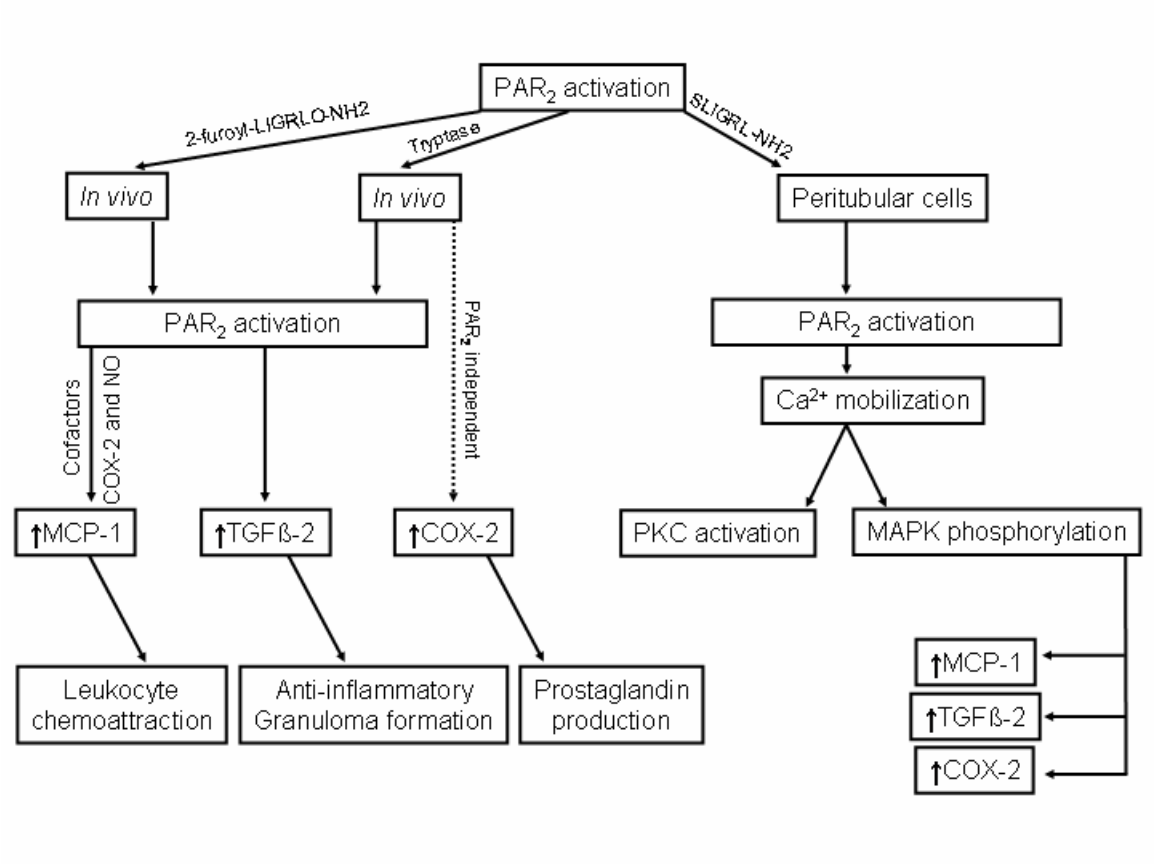



**Fig. 5.5 Diagram summarizing the effects of $PAR_2$ activation *in vivo* and on isolated PTC *in vitro*.**

## *5.6 Mast cell tryptase-$PAR_2$ pathway on testicular macrophages*

Macrophages are important mediators of testicular inflammation and their numbers are strongly elevated both in the acute testicular inflammation (Hedger, 2002) and in chronic inflammatory responses (EAO) (Guazzone et al., 2003). In addition, many subtypes of tissue macrophages as well as circulating monocytes were reported to express $PAR_2$ (Colognato et al., 2003, Ge et al., 2003, Roche et al., 2003b, Johansson et al., 2005) and increased numbers of $PAR_2$ expressing macrophages were associated with different pathological conditions like chronic smoking or asthma (Roche et al., 2003b). Our double immunofluorescence clearly shows that rat testicular macrophages express $PAR_2$ at the protein level. The numbers of $PAR_2$-positive macrophages within the testicular interstitium were highly upregulated after 50 days of EAO,

contrasting with normal testis and adjuvant controls where their numbers were considerably lower. As testicular macrophages are known to be the source of many key inflammatory mediators, we further investigated the effect of $PAR_2$ activation on the expression of MCP-1, TGFß-2 and COX-2 in rat testicular and peritoneal macrophages *in vitro*. 2-furoyl-LIGRLO-$NH_2$, the specific $PAR_2$ activating peptide had no influence on the MCP-1, TGFß-2 and COX-2 expression levels in these two populations of macrophages.

LPS was used as a positive control and induced an upregulation of COX-2 and MCP-1 in both subtypes of macrophages. TGFß-2 was increased by LPS stimulation in testicular macrophages while peritoneal macrophages showed a decrease in the levels of TGFß-2. Thus, based on the effect of LPS on TGFß-2 expression levels, we can conclude that there is a phenotypic difference between testicular and peritoneal macrophages. LPS-activated testicular macrophages respond by increased production of pro-inflammatory molecules like MCP-1 and COX-2 but also, of the potent anti-inflammatory molecule TGFß-2. This result supports previous findings that testicular macrophages have a "suppressed" phenotype compared to macrophages from other tissues (Hedger, 2002). Generally, the testis is described as an unique organ because it contains specific immunosuppressive factors like $\alpha$-melanocyte-stimulating hormone, $\beta$-endorphin, TGF$\beta$ and activin-A, that inhibit lymphocyte activation thus limiting the inflammatory responses and preventing graft rejection from this site (Maddocks and Setchell, 1990, Hedger, 2002).

An interesting aspect is that although $PAR_2$ expression has been shown to increase during differentiation of monocytes into tissue macrophages (Colognato et al., 2003, Johansson et al., 2005), our double immunofluorescence studies did not show any difference in the intensity of $PAR_2$ immunoreactivity between the two subtypes of testicular macrophages (ED1 and ED2). This result comes to support the findings that testicular macrophages behave different as compared to macrophages from other tissues.

Failure of 2-furoyl-LIGRLO-$NH_2$ to induce an effect on the expression levels of MCP-1, TGFß-2 and COX-2 raised the question if $PAR_2$ is functional in testicular macrophages. Assessment of changes in intracellular $Ca^{2+}$ concentration after $PAR_2$ activation did not show the characteristic increase associated with activation of G-protein coupled receptors. In summary our results are suggesting that $PAR_2$ is present, but not functional on testicular macrophages in culture.

## *5.7 In vivo gene silencing by local delivery of siRNA into the testis*

The roles of $PAR_2$ in different tissues were extensively investigated in the last years. Recently two synthetic peptides were identified that block the ability of trypsin to activate $PAR_2$ in kidney cells and in rat aorta rings (Al-Ani et al., 2002). A different study describes one of these two peptides to inhibit the response induced by FVIIa in bovine aortic endothelial cells (Sethi et al., 2005). However these peptides are not selectively blocking $PAR_2$ and were not shown to antagonize also tryptase dependent $PAR_2$ activation. As other specific $PAR_2$ antagonists are not available, we tried to establish a testis specific $PAR_2$ gene silencing approach, based on siRNA technology, with the aim to confirm the roles of $PAR_2$ in acute testicular inflammation.

RNA interference (RNAi) has become a powerful tool for the knock-down of target gene expression and subsequent phenotypic analysis of gene function both *in vitro* and *in vivo*. RNAi is facilitated by direct introduction of small inhibitory RNA into the cells. In the RNA-interference pathway, double-stranded RNA induces sequence-specific mRNA degradation through the action of the RNA-induced silencing complex (RISC). RISC complexes assemble on the dsRNA that triggers specific silencing. Base-pairing interactions between mRNAs and the processed products of the dsRNA silencing trigger guide RISC to its mRNA targets, which it then destroys. Success of siRNA mediated gene silencing depends on the design and efficient delivery of siRNA to target cells. In vivo the efficiency of the delivery differs among tissues. The siRNA

nucleotides are easily up-taken by well vascularized tissues when delivered hydrodynamically (Song et al., 2003) whereas in other organs local siRNA delivery appears to be more difficult and sometimes requires special conditions like electroporation (McCaffrey et al., 2002, de Fougerolles et al., 2005).

Lamin A/C siRNA was used as control for establishing the method. A small silencing effect was observed both at mRNA and protein level, but based on the analysis of natural variation of Lamin A/C within normal rat testes we conclude that local delivery of siRNA by subcapsular injection was not efficient. However, a very recent study reported the successful *in vivo* gene silencing by local application of siRNA into the rete testis followed by electroporation (Shoji et al., 2005), suggesting that the testis has a low uptake capacity for nucleic acids, and simple delivery of siRNA can not be successful if not accompanied by special conditions like electrical stimulation. On the other hand, electrical stimulation has been shown to affect the expression of different key inflammatory mediators (Chida et al., 2004) and can interfere with the pattern of cytokine expression, which we have used as a readout in our study. Thus it appears that the most appropriate way to confirm roles of $PAR_2$ in testicular inflammation would be the use of $PAR_2$-deficient mice in models of acute and chronic $PAR_2$ inflammation.

## *5.8 Conclusions*

The present study focused on the roles of mast cell tryptase and $PAR_2$ in acute and chronic testicular inflammation. Based on our results we conclude that mast cell tryptase, by activating $PAR_2$ on peritubular cells, plays an important role on testicular inflammation in mediating the expression of key inflammatory mediators. The following findings led to the conclusion that PTCs *in vivo*, despite being non-immune cells, secrete factors that are crucially involved in testicular inflammation: (I) the increase in the numbers of mast cells and enhanced expression of inflammatory molecules seen in EAO (MCP-1, $TGF\beta_2$ and COX-2); (II) in isolated PTCs, activation of $PAR_2$ resulted in a comparable increased expression of the same key inflammatory mediators; (III) connecting these lines of evidence, the *in vivo* application of recombinant mast cell tryptase was followed by a similar increase in testicular MCP-1, TGFβ-2 and COX-2 expression levels. In support, inactivated tryptase had no effect when injected *in vivo*, proving that the tryptase dependent effects are based on the proteolytic activity of the enzyme, thus on it's ability to cleave the N-terminal end of $PAR_2$.

In conclusion, based on the effects $PAR_2$ stimulation showed in our models of acute testicular inflammation and given the strong upregulation in EAO, we propose that its function may also extend to a more central role in autoimmune orchitis, where a large cohort of degranulating mast cells was seen, likely releasing its product tryptase which in turn can activate $PAR_2$ on PTC. This causes a strong increase in the levels of key inflammatory mediators and can also induce proliferation of peritubular-like cells leading to granuloma formation.

To date, EAO was best studied in rat. However, given the advantage mouse transgenic models provide, a feasible approach to continue this study is indeed the inclusion of transgenic mouse model for EAO. In particular, the use of genetically modified mice would provide a valuable tool to further elucidate the contribution of the mast cell tryptase-$PAR_2$ pathway in regulating testicular

inflammatory responses. We suggest to proceed for further experiments in the following two main directions: firstly inducing EAO in $PAR_2^{-/-}$ mice, to better characterize the roles of $PAR_2$ in EAO. Furthermore, the evaluation of the expression of key inflammatory mediators in this system will unambiguously distinguish between specific and unspecific effects of $PAR_2$ activation. Secondly studying EAO in a c-kit mutant mast cell deficient mice model will more accurately define the roles of mast cells in testicular inflammation. Ideally their role could be confirmed by repairing the mast cell deficient animals with genetically compatible mast cells, produced from stem cells *in vitro*.

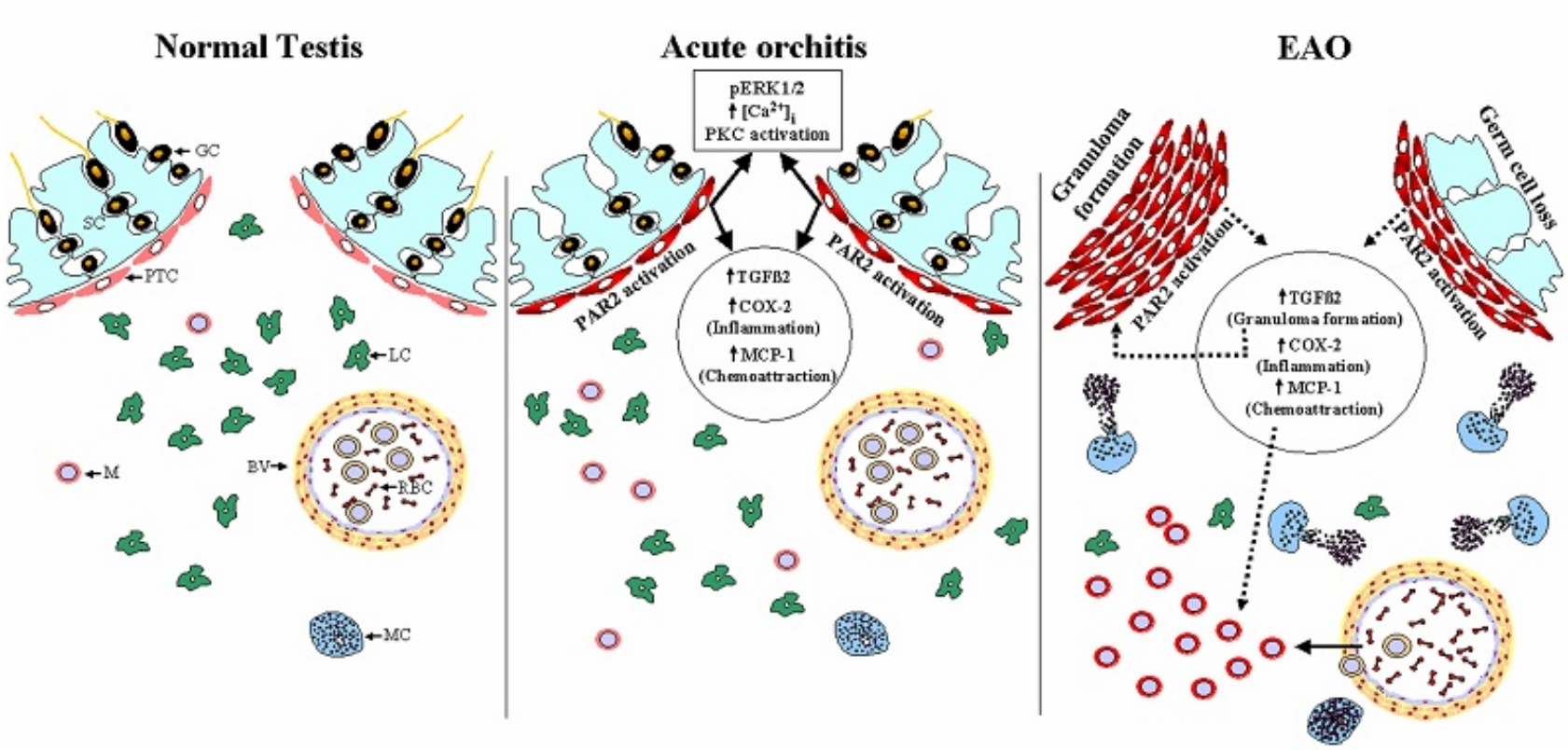



**Fig. 5.8 Pathomechanism of testicular inflammation involves PAR2 activation.**
Left side: normal rat testis. Middle: testis with acute inflammation. Right side: Testis with experimental autoimmune orchitis (EAO) show loss of germ cells and massive infiltration of monocytes/macrophages in the interstitial tissue as well as granuloma formation. In models of acute inflammation activation of PAR2 in PTC by tryptase or a PAR2 agonist resulted in phosphorylation of ERK1/2, protein kinase C activation, and increased intracellular [Ca2+] concentrations. In addition, expression of MCP-1, TGFß-2 and COX-2 was elevated in vitro and in vivo. In EAO testes, mast cell numbers increase substantially and frequent degranulation releasing mast cell tryptase in the interstitium is observed. PAR2 positive cells are peritubular cells (PTC) and monocytes/macrophages (M). Expression of MCP-1, TGFß-2, COX-2 and iNOS was found strongly elevated in EAO. Dotted lines in EAO are hypothetically derived from data obtained in the acute models. (GC=Germ cell; SC=Sertoli cell; LC=Leydig cell; BV=blood vessel; MC=mast cell; RBC=Red blood cell).

# 6 Summary

Mast cells are involved in early events crucial to inflammation and autoimmune disease and their numbers increase in infertile testis. Recently, proteinase-activated receptor-2 ($PAR_2$), a G-protein coupled receptor important to injury responses, was shown to be activated by mast cell tryptase. To investigate whether mast cells and $PAR_2$ are involved in the development and/or aggravation of testicular inflammation, we studied acute and chronic inflammatory models in the rat. Both testicular and systemic inflammatory responses can lead to impaired spermatogenesis, steroidogenesis and to male infertility. The rat experimental autoimmune orchitis (EAO) is thus far the best studied model for immunological male factor infertility. In EAO, we detected a substantial increase in the number of neutrophils, macrophages and mast cells, enlarged interstitial space, severe reduction and sloughing of the seminiferous epithelium and granuloma formation. Mast cells increased 10-fold in numbers, were more widely distributed and showed an increased degranulation rate.

In normal testes, $PAR_2$ was immunohistochemically detected in macrophages, in peritubular cells (PTCs) and spermatid acrosomes. In EAO, $PAR_2$ was strongly upregulated in macrophages and peritubular like cells, forming concentric layers around granulomas. $PAR_2$ was present on testicular macrophages, but not functional in culture, whereas isolated PTCs expressed functional $PAR_2$ and responded to $PAR_2$ activation by phosphorylating ERK1/2, activating protein kinase C, and increased intracellular $Ca^{2+}$ concentrations as well as MCP-1, $TGF\beta_2$ and COX-2 mRNA levels. Expression levels of these inflammatory mediators, together with iNOS, were also significantly increased in testes 50 days after EAO induction. *In vivo*, expression of cytokines and inflammatory mediators were upregulated after injection of recombinant tryptase (MCP-1, $TGF\beta_2$ and COX-2) and a specific $PAR_2$ peptide agonist (MCP-1, TGF$\beta$2) in the testis after 5hrs. A COX-2 inhibitor and a general NOS-antagonist suppressed $PAR_2$-stimulated MCP-1 expression, whereas TGF$\beta$2 levels were partially reduced.

In summary, our data imply that $PAR_2$ activation by mast cell tryptase or agonist orchestrates short-term testicular inflammation via the activation, expression and/or secretion of a key cytokines and this pathomechanism may play also an important role in autoimmune orchitis. Manipulation of this pathway could undermine the damage caused by inflammation on the testis and consequently may provide hope for the treatment of immunological male factor infertility.

# 7 Zusammenfassung

In der Frühentwicklung von entzündlichen und autoimmunologischen Erkrankungen spielen Mastzellen eine essentielle Rolle. Ihre Anzahl ist auch im Hoden infertiler Männer erhöht. Testikuläre und systemische Entzündungen können zu eine Beeinträchtigung der Spermatogenese und Steroidogenese und damit zu Infertilität bei Männern führen. Kürzlich konnte gezeigt werden, dass der G-Protein gekoppelte Proteinase aktivierte Rezeptor-2 ($PAR_2$), dem ein wichtige Funktion bei Wundheilungen zufällt, durch Mastzell-Tryptase aktiviert werden kann. In unserer Studie wurde eine mögliche Beteiligung von Mastzellen und $PAR_2$ bei der Entstehung und Progression testikulärer Entzündungen mit Hilfe von Modellen akuter und chronischer Entzündungen in Ratten untersucht. Die experimentelle Autoimmun-Orchitis (EAO) der Ratte stellt das am besten untersuchte Modell für immunologische Infertilität des Mannes dar. In EAO-Hoden konnten wir neben einer substanziellen Erhöhung der Anzahl von neutrophilen Granulozyten, Makrophagen und Mastzellen auch eine Ödembildung im interstitiellen Gewebe, eine deutliche Reduktion des Keimepithels sowie die Bildung von Granulomen dokumentieren. Ebenso ist die Anzahl der Mast-Zellen in EAO-Tieren um den Faktor 10 erhöht und die Zellen fanden sich häufig degranuliert in gesamten Interstitium verteilt. $PAR_2$ wurde immunhistochemisch auf Peritubulär-Zellen (PTC), dem Acrosom von runden und elongierten Spermatiden sowie testikulären Makrophagen lokalisiert. In EAO-Hoden findet sich eine starke Zunahme der $PAR_2$-Immunreaktivität in Monocyten/Makrophagen und in PTC, die in multilamellären Schichten am Rande von Granulomen zu finden sind und den Proliferationsmarker Ki67 co-exprimieren. $PAR_2$ konnte auf isolierten Makrophagen gefunden werden, war funktionell aber nicht aktiv. Im Gegensatz resultierte die Applikation eines $PAR_2$ Agonisten in PTC in einem raschen Anstieg der intrazellulären $Ca^{2+}$-Konzentration, der Phosphorylierung der Erk1/2 MAP-Kinasen und in einer deutlichen Hochregulation der mRNA-Expression der Entzündungsmediatoren TGFβ, MCP-1 und COX-2. Eine vergleichbar erhöhte Expression von TGFβ, MCP-1 und COX-2, zusammen mit iNOS, wurde auch im Hoden von EAO-Tieren gemessen. *In vivo* wurde die Expression durch intratestikuläre Injektion

von Mast-Zell-Tryptase (TGFβ, MCP-1, COX-2) und des $PAR_2$-Agonisten (TGFβ, MCP-1) ebenfalls hochreguliert, während enzymatisch inaktive Tryptase und das Kontrollpeptid wirkungslos blieben. Durch die Gabe von COX-2 und iNOS-Inhibitoren vor der Injektion von Tryptase und des $PAR_2$-Agonisten ließen sich diese Effekte für MCP-1 vollständig und für TGFß2 teilweise auf Kontrollniveau senken. Zusammenfassend kann festgestellt werden, dass Peritubulär-Zellen durch eine Mastzell-Tryptase ausgelöste Aktivierung von $PAR_2$ eine Rolle im Pathomechanismus von akuten und vermutlich auch chronischen Orchitiden spielen. Eingriffe in diesen Signalweg könnten entzündungsbedingte Gewebeschäden im Hoden verhindern und gegebenenfalls neue Behandlungsmöglichkeiten für immunologisch bedingte Infertilität bei Männern aufzeigen.

# 8. References


Adams, M. L., Meyer, E. R. and Cicero, T. J. (1996) *Biol Reprod,* **54,** 1128-34.

Aharinejad, S., Paulus, P., Sioud, M., Hofmann, M., Zins, K., Schafer, R., Stanley, E. R. and Abraham, D. (2004) *Cancer Res,* **64,** 5378-84.

Al-Ani, B., Saifeddine, M., Kawabata, A. and Hollenberg, M. D. (1999) **128,** 1105-1113.

Al-Ani, B., Saifeddine, M., Wijesuriya, S. J. and Hollenberg, M. D. (2002) *J Pharmacol Exp Ther,* **300,** 702-8.

Anton, F., Morales, C., Aguilar, R., Bellido, C., Aguilar, E. and Gaytan, F. (1998) *Zentralbl Veterinarmed A,* **45,** 209-18.

Aubry, F., Habasque, C., Satie, A. P., Jegou, B. and Samson, M. (2000) *Biol Reprod,* **62,** 1427-35.

Avallet, O., Vigier, M., Leduque, P., Dubois, P. M. and Saez, J. M. (1994) *Endocrinology,* **134,** 2079-87.

Baggiolini, M. and Dahinden, C. A. (1994) *Immunol Today,* **15,** 127-33.

Bambino, T. H. and Hsueh, A. J. (1981) *Endocrinology,* **108,** 2142-8.

Banchereau, J. and Steinman, R. M. (1998) *Nature,* **392,** 245-52.

Becker, S., Warren, M. K. and Haskill, S. (1987) *J Immunol,* **139,** 3703-9.

Benoist, C. and Mathis, D. (2002) *Nature,* **420,** 875-8.

Berger, P., Girodet, P. O., Begueret, H., Ousova, O., Perng, D. W., Marthan, R., Walls, A. F. and Tunon de Lara, J. M. (2003) *Faseb J,* **17,** 2139-41.

Blank, U. and Rivera, J. (2004) *Trends Immunol,* **25,** 266-73.

Bohm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G. and Bunnett, N. W. (1996) *J. Biol. Chem.,* **271,** 22003-22016.

Bohring, C. and Krause, W. (2003) *Hum Reprod,* **18,** 915-24.

Brosnan, C. F. and Tansey, F. A. (1984) *J Neuropathol Exp Neurol,* **43,** 84-93.

Brown, J. K., Jones, C. A., Rooney, L. A., Caughey, G. H. and Hall, I. P. (2002) *Am J Physiol Lung Cell Mol Physiol,* **282,** L197-206.

Bustin, S. A. (2000) *J Mol Endocrinol,* **25,** 169-93.

Camerer, E., Huang, W. and Coughlin, S. R. (2000) *PNAS,* **97,** 5255-5260.

Cameron, L. A. and Hinson, J. P. (1993) *J Endocrinol,* **139,** 415-23.

Caussanel, V., Tabone, E., Hendrick, J. C., Dacheux, F. and Benahmed, M. (1997) *Biol Reprod,* **56,** 357-67.

Cederqvist, K., Haglund, C., Heikkila, P., Hollenberg, M. D., Karikoski, R. and Andersson, S. (2005) *Pediatr Res,* **57,** 831-6.

Chancellor-Freeland, C., Zhu, G. F., Kage, R., Beller, D. I., Leeman, S. E. and Black, P. H. (1995) *Ann N Y Acad Sci,* **771,** 472-84.

Chen, R., Ning, G., Zhao, M.-L., Fleming, M. G., Diaz, L. A., Werb, Z. and Liu, Z. (2001) *J. Clin. Invest.,* **108,** 1151-1158.

Cheng, C. Y. and Mruk, D. D. (2002) *Physiol Rev,* **82,** 825-74.

Chida, Y., Sudo, N., Motomura, Y. and Kubo, C. (2004) *Neuroimmunomodulation,* **11,** 419-24.

Cocks, T. M., Fong, B., Chow, J. M., Anderson, G. P., Frauman, A. G., Goldie, R. G., Henry, P. J., Carr, M. J., Hamilton, J. R. and Moffatt, J. D. (1999) *Nature,* **398,** 156-160.

Cocks, T. M. and Moffatt, J. D. (2001) *Pulm Pharmacol Ther,* **14,** 183-91.

Colognato, R., Slupsky, J. R., Jendrach, M., Burysek, L., Syrovets, T. and Simmet, T. (2003) *Blood,* **102,** 2645-2652.

Compton, S. J., Cairns, J. A., Palmer, K.-J., Al-Ani, B., Hollenberg, M. D. and Walls, A. F. (2000) *J. Biol. Chem.,* **275,** 39207-39212.

Compton, S. J., Renaux, B., Wijesuriya, S. J. and Hollenberg, M. D. (2001) **134,** 705-718.

Coquelin, A. and Desjardins, C. (1982) *Am J Physiol,* **243,** E257-63.

D'Andrea, M. R., Derian, C. K., Leturcq, D., Baker, S. M., Brunmark, A., Ling, P., Darrow, A. L., Santulli, R. J., Brass, L. F. and Andrade-Gordon, P. (1998) *J Histochem Cytochem,* **46,** 157-64.

de Fougerolles, A., Manoharan, M., Meyers, R. and Vornlocher, H. P. (2005) *Methods Enzymol,* **392,** 278-96.

DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D. and Bunnett, N. W. (2000) *J. Cell Biol.,* **148,** 1267-1282.

Del Punta, K., Charreau, E. H. and Pignataro, O. P. (1996) *Endocrinology,* **137,** 5337-43.

Derrick, E. K., Barker, J. N., Khan, A., Price, M. L. and Macdonald, D. M. (1993) *Histopathology,* **22,** 157-62.

Dery, O., Thoma, M. S., Wong, H., Grady, E. F. and Bunnett, N. W. (1999) *J. Biol. Chem.,* **274,** 18524-18535.

Dietsch, G. and Hinrichs, D. (1989) *J Immunol,* **142,** 1476-1481.

Doncel, G. F., Di Paola, J. A. and Lustig, L. (1989) *Am J Reprod Immunol,* **20,** 44-51.

Dym, M. and Romrell, L. J. (1975) *J Reprod Fertil,* **42,** 1-7.

Eickelberg, O. (2001) *FEBS Lett,* **506,** 11-4.

Elenkov, I. J. and Chrousos, G. P. (2002) *Ann NY Acad Sci,* **966,** 290-303.

Ellis, G. B. and Desjardins, C. (1982) *Endocrinology,* **110,** 1618-27.

Fawcett, D. W., Neaves, W. B. and Flores, M. N. (1973) *Biol Reprod,* **9,** 500-32.

Ferrell, W. R., Lockhart, J. C., Kelso, E. B., Dunning, L., Plevin, R., Meek, S. E., Smith, A. J., Hunter, G. D., McLean, J. S., McGarry, F., Ramage, R., Jiang, L., Kanke, T. and Kawagoe, J. (2003) *J Clin Invest,* **111,** 35-41.

Fiorucci, S., Mencarelli, A., Palazzetti, B., Distrutti, E., Vergnolle, N., Hollenberg, M. D., Wallace, J. L., Morelli, A. and Cirino, G. (2001) *PNAS,* **98,** 13936-13941.

Frungieri, M. B., Albrecht, M., Raemsch, R. and Mayerhofer, A. (2005) *Cell Signal,* **17,** 525-33.

Frungieri, M. B., Weidinger, S., Meineke, V., Kohn, F. M. and Mayerhofer, A. (2002) *Proc Natl Acad Sci U S A,* **99,** 15072-7.

Galli, S. J., Zsebo, K. M. and Geissler, E. N. (1994) *Adv Immunol,* **55,** 1-96.

Gartner L. P., H. J. L. (2001) *Color Textbook of Histology,* Saunders (W.B.) Co Ltd.

Gaytan, F., Carrera, G., Pinilla, L., Aguilar, R. and Bellido, C. (1989) *J Androl,* **10,** 351-8.

Ge, L., Ly, Y., Hollenberg, M. and DeFea, K. (2003) *J. Biol. Chem.,* **278,** 34418-34426.

Gerdprasert, O., O'Bryan, M. K., Muir, J. A., Caldwell, A. M., Schlatt, S., de Kretser, D. M. and Hedger, M. P. (2002a) *Cell Tissue Res,* **308,** 277-85.

Gerdprasert, O., O'Bryan, M. K., Nikolic-Paterson, D. J., Sebire, K., de Kretser, D. M. and Hedger, M. P. (2002b) *Mol Hum Reprod,* **8,** 518-24.

Ghinea, N. and Milgrom, E. (1995) *J Endocrinol,* **145,** 1-9.

Giladi, H., Ketzinel-Gilad, M., Rivkin, L., Felig, Y., Nussbaum, O. and Galun, E. (2003) *Mol Ther,* **8,** 769-76.

Gow, R. M., O'Bryan, M. K., Canny, B. J., Ooi, G. T. and Hedger, M. P. (2001) *J Endocrinol,* **168,** 193-201.

Griffith, O. W. and Stuehr, D. J. (1995) *Annu Rev Physiol,* **57,** 707-36.

Guazzone, V. A., Rival, C., Denduchis, B. and Lustig, L. (2003) *J Reprod Immunol,* **60,** 143-57.

Guo, H. T., Cai, C. Q., Schroeder, R. A. and Kuo, P. C. (2002) *Immunol Lett,* **80,** 21-6.

Haider, S. G. (2004) *Int Rev Cytol,* **233,** 181-241.

Hales, D. B. (2002) *J Reprod Immunol,* **57,** 3-18.

Hales, D. B., Diemer, T. and Hales, K. H. (1999) *Endocrine,* **10,** 201-17.

Hamilton, J. R., Frauman, A. G. and Cocks, T. M. (2001) *Circ Res,* **89,** 92-98.

Head, J. R. and Billingham, R. E. (1985) *Perspect Biol Med,* **29,** 115-31.

Hedger, M., Klug, J., Frohlich, S., Muller, R. and Meinhardt, A. (2005) *J Androl,* **26,** 379-386.

Hedger, M. P. (2002) *J Reprod Immunol,* **57,** 19-34.

Hedger, M. P. (1997) *Rev Reprod,* **2,** 38-47.

Hedger, M. P. and Culler, M. D. (1997) *Reprod Fertil Dev,* **9,** 659-64.

Hedger, M. P. and Eddy, E. M. (1986) *Biol Reprod,* **35,** 1309-19.

Hedger, M. P. and Meinhardt, A. (2003) *J Reprod Immunol,* **58,** 1-26.

Hedger, M. P. and Meinhardt, A. (2000) *J Reprod Immunol,* **48,** 69-80.

Hibi, H., Kato, K., Mitsui, K., Taki, T., Yamada, Y., Honda, N., Fukatsu, H. and Yamamoto, M. (2001) *Arch Androl,* **47,** 107-11.

Hoeben, E., Briers, T., Vanderstichele, H., De Smet, W., Heyns, W., Deboel, L., Vanderhoydonck, F. and Verhoeven, G. (1995) *Endocrinology,* **136,** 2862-73.

Hoek, A., Allaerts, W., Leenen, P. J., Schoemaker, J. and Drexhage, H. A. (1997) *Eur J Endocrinol,* **136,** 8-24.

Holzhausen, M., Spolidorio, L. C. and Vergnolle, N. (2005) *Mem Inst Oswaldo Cruz,* **100 Suppl 1,** 177-80.

Huckins, C. and Clermont, Y. (1968) *Arch Anat Histol Embryol,* **51,** 341-54.

Huhtaniemi, I. (1995) *Reprod Fertil Dev,* **7,** 1025-35.

Hume, D. A., Halpin, D., Charlton, H. and Gordon, S. (1984) *Proc Natl Acad Sci U S A,* **81,** 4174-7.

Ibelgaufts, H., Jones, K. W., Maitland, N. and Shaw, J. F. (1982) *Acta Neuropathol (Berl),* **56,** 113-7.

Inoue, H., Ohshima, H., Kono, H., Yamanaka, M., Kubota, T., Aihara, M., Hiroi, T., Yago, N. and Ishida, H. (1997) *Biochem Pharmacol,* **53,** 1941-4.

Itoh, M., De Rooij, D. G., Jansen, A. and Drexhage, H. A. (1995) *J Reprod Immunol,* **28,** 217-32.

J. W. Heath, B. Y. (2000) *Wheater's Functional Histology: A Text and Colour Atlas,* Churchill Livingstone.

Jezek, D., Banek, L., Hittmair, A., Pezerovic-Panijan, R., Goluza, T. and Schulze, W. (1999) *Andrologia,* **31,** 203-210.

Johansson, U., Lawson, C., Dabare, M., Syndercombe-Court, D., Newland, A. C., Howells, G. L. and Macey, M. G. (2005) *J Leukoc Biol***,** jlb.0704422.

Kaneko, T., Itoh, M., Nakamura, Y., Iimura, A., Hayashi, S., Takahashi, K., Stivala, F., Bendtzen, K. and Nicoletti, F. (2003) *Cytokine,* **22,** 50-53.

Kanellis, J., Watanabe, S., Li, J. H., Kang, D. H., Li, P., Nakagawa, T., Wamsley, A., Sheikh-Hamad, D., Lan, H. Y., Feng, L. and Johnson, R. J. (2003) *Hypertension,* **41,** 1287-93.

Karge, W. H., 3rd, Schaefer, E. J. and Ordovas, J. M. (1998) *Methods Mol Biol,* **110,** 43-61.

Kashiwagi, M., Masutani, K., Shinozaki, M. and Hirakata, H. (2002) *Nephron,* **92,** 165-73.

Kawabata, A. (2002) *Expert Rev Mol Med,* **16,** 1-17.

Kawabata, A., Kinoshita, M., Nishikawa, H., Kuroda, R., Nishida, M., Araki, H., Arizono, N., Oda, Y. and Kakehi, K. (2001) *J. Clin. Invest.,* **107,** 1443-1450.

Kawabata, A., Kuroda, R., Minami, T., Kataoka, K. and Taneda, M. (1998) *Br J Pharmacol,* **125,** 419-22.

Kim, J. A., Choi, S. C., Yun, K. J., Kim, D. K., Han, M. K., Seo, G. S., Yeom, J. J., Kim, T. H., Nah, Y. H. and Lee, Y. M. (2003) *Inflamm Bowel Dis,* **9,** 224-9.

Kitamura, Y. (1989) *Annu Rev Immunol,* **7,** 59-76.

Kohno, S., Munoz, J. A., Williams, T. M., Teuscher, C., Bernard, C. C. and Tung, K. S. (1983) *J Immunol,* **130,** 2675-82.

Kunkel, E. J. and Butcher, E. C. (2002) *Immunity,* **16,** 1-4.

Le Magueresse-Battistoni, B., Morera, A. M., Goddard, I. and Benahmed, M. (1995) *Endocrinology,* **136,** 2788-91.

Lee, D. M., Friend, D. S., Gurish, M. F., Benoist, C., Mathis, D. and Brenner, M. B. (2002) *Science,* **297,** 1689-1692.

Lerner, D. J., Chen, M., Tram, T. and Coughlin, S. R. (1996) *J. Biol. Chem.,* **271,** 13943-13947.

Leung, P. C. and Steele, G. L. (1992) *Endocr Rev,* **13,** 476-98.

Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A. and Herweijer, H. (2002) *Nat Genet,* **32,** 107-8.

Lindner, J. R., Kahn, M. L., Coughlin, S. R., Sambrano, G. R., Schauble, E., Bernstein, D., Foy, D., Hafezi-Moghadam, A. and Ley, K. (2000) *J Immunol,* **165,** 6504-6510.

Lue, Y., Sinha Hikim, A. P., Wang, C., Leung, A. and Swerdloff, R. S. (2003) *Endocrinology,* **144,** 3092-3100.

Lustig, L., Lourtau, L., Perez, R. and Doncel, G. F. (1993) *Int J Androl,* **16,** 279-84.

Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D. and Plevin, R. (2001) *Pharmacol Rev,* **53,** 245-82.

Maddocks, S. and Setchell, B. P. (1990) *J Reprod Immunol,* **18,** 9-18.

Mahi-Brown, C. A., Yule, T. D. and Tung, K. S. (1987) *Cell Immunol,* **106,** 408-19.

Makimura, H., Mizuno, T. M., Mastaitis, J. W., Agami, R. and Mobbs, C. V. (2002) *BMC Neurosci,* **3,** 18.

Malfait, A.-M., Malik, A. S., Marinova-Mutafchieva, L., Butler, D. M., Maini, R. N. and Feldmann, M. (1999) *J Immunol,* **162,** 6278-6283.

Mari, B., Guerin, S., Far, D., Breitmayer, J., Belhacene, N., Peyron, J., Rossi, B. and Auberger, P. (1996) *FASEB J.,* **10,** 309-316.

McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J. and Kay, M. A. (2002) *Nature,* **418,** 38-9.

McDonald, D. M., Bowden, J. J., Baluk, P. and Bunnett, N. W. (1996) *Adv Exp Med Biol,* **410,** 453-62.

McGuire, J. J., Saifeddine, M., Triggle, C. R., Sun, K. and Hollenberg, M. D. (2004a) *J Pharmacol Exp Ther,* **309,** 1124-1131.

McGuire, J. J., Saifeddine, M., Triggle, C. R., Sun, K. and Hollenberg, M. D. (2004b) *J Pharmacol Exp Ther,* **309,** 1124-31.

Meineke, V., Frungieri, M. B., Jessberger, B., Vogt, H. and Mayerhofer, A. (2000) *Fertil Steril,* **74,** 239-44.

Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M. and Brass, L. F. (1997) *J. Biol. Chem.,* **272,** 4043-4049.

Morris, S. M., Jr. and Billiar, T. R. (1994) *Am J Physiol,* **266,** E829-39.

Mullaney, B. P. and Skinner, M. K. (1993) *Mol Endocrinol,* **7,** 67-76.

Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y. and Galli, S. (1985) *J. Exp. Med.,* **162,** 1025-1043.

Napoli, C., Cicala, C., Wallace, J. L., de Nigris, F., Santagada, V., Caliendo, G., Franconi, F., Ignarro, L. J. and Cirino, G. (2000) *PNAS,* **97,** 3678-3683.

Neeraja, S., Sreenath, A. S., Reddy, P. R. and Reddanna, P. (2003a) *Reprod Biomed Online,* **6,** 302-9.

Neeraja, S., Sreenath, A. S., Reddy, P. R. K. and Reddanna, P. (2003b) *Reprod Biomed Online,* **6,** 302-9.

Nistal, M., Santamaria, L. and Paniagua, R. (1984) *Acta Anat (Basel),* **119,** 155-60.

Nussler, A. K. and Billiar, T. R. (1993) *J Leukoc Biol,* **54,** 171-8.

Nystedt, S., Emilsson, K., Wahlestedt, C. and Sundelin, J. (1994) *PNAS,* **91,** 9208-9212.

O'Bryan, M. K., Schlatt, S., Gerdprasert, O., Phillips, D. J., de Kretser, D. M. and Hedger, M. P. (2000) *Biol Reprod,* **63,** 1285-93.

Olejar, T., Matej, R., Zadinova, M. and Pouckova, P. (2001) *Int J Gastrointest Cancer,* **30,** 113-21.

Ozturk, H., Buyukbayram, H., Ozdemir, E., Ketani, A. i. n., Gurel, A., Onen, A. and Otcu, S. (2003) *Journal of Pediatric Surgery,* **38,** 1621-1627.

Picker, L. J. and Butcher, E. C. (1992) *Annu Rev Immunol,* **10,** 561-91.

Pollanen, P. and Maddocks, S. (1988) *J Reprod Fertil,* **82,** 437-45.

Pollanen, P. and Niemi, M. (1987) *Int J Androl,* **10,** 37-42.

Pollanen, P., von Euler, M., Jahnukainen, K., Saari, T., Parvinen, M., Sainio-Pollanen, S. and Soder, O. (1993) *J Reprod Immunol,* **24,** 123-37.

Proost, P., Wuyts, A. and van Damme, J. (1996) *Int J Clin Lab Res,* **26,** 211-23.

Ricciardolo, L. M., Steinhoff, M., Amadesi, S., Guerrini, R., Tognetto, M., Trevisani, M., Creminon, C., Bertrand, C., Bunnett, N. W., Fabbri, L. M., Salvadori, S. and Geppetti, P. (2000) *Am. J. Respir. Crit. Care Med.,* **161,** 1672-1680.

Ritchie, A. W., Hargreave, T. B., James, K. and Chisholm, G. D. (1984) *Br J Urol,* **56,** 79-83.

Robbie-Ryan, M. and Brown, M. (2002) *Curr Opin Immunol,* **14,** 728-33.

Roche, N., Stirling, R. G., Lim, S., Oliver, B. G., Oates, T., Jazrawi, E., Caramori, G. and Chung, K. F. (2003a) *J Allergy Clin Immunol,* **111,** 367-73.

Roche, N., Stirling, R. G., Lim, S., Oliver, B. G., Oates, T., Jazrawi, E., Caramori, G. and Chung, K. F. (2003b) *J Allergy Clin Immunol,* **111,** 367-73.

Roux, P. P. and Blenis, J. (2004) *Microbiol Mol Biol Rev,* **68,** 320-44.

Rozniecki, J. J., Hauser, S. L., Stein, M., Lincoln, R. and Theoharides, T. C. (1995) *Ann Neurol,* **37,** 63-6.

Sambrook, J., Russell, D.W., (2001) *Molecular cloning: A Laboratory Manual,,* Cold Spring Harbor: Cold Spring Harbor Laboraory Press.

Sethi, A. S., Lees, D. M., Douthwaite, J. A. and Corder, R. (2005) *Clin Sci (Lond),* **108,** 255-63.

Sezer, C., Koksal, L. T., Usta, M. F., Gulkesen, K. H., Erdogru, T., Ciftcioglu, A. and Baykara, M. (2005) *Arch Androl,* **51,** 149-58.

Shimizu, T., Nishihira, J., Watanabe, H., Abe, R., Honda, A., Ishibashi, T. and Shimizu, H. (2004) *J Biol Chem,* **279,** 13729-37.

Shoji, M., Chuma, S., Yoshida, K., Morita, T. and Nakatsuji, N. (2005) *Dev Biol,* **282,** 524-34.

Shpacovitch, V. M., Varga, G., Strey, A., Gunzer, M., Mooren, F., Buddenkotte, J., Vergnolle, N., Sommerhoff, C. P., Grabbe, S., Gerke, V., Homey, B., Hollenberg, M., Luger, T. A. and Steinhoff, M. (2004) *J Leukoc Biol,* **76,** 388-98.

Sisk, C. L. and Desjardins, C. (1986) *Endocrinology,* **119,** 1195-203.

Smith, T. J. and Parikh, S. J. (1999) *Endocrinology,* **140,** 3518-3525.

Snyder, S. H. (1995) *Nature,* **377,** 196-7.

Sobey, C. G., Moffatt, J. D., Cocks, T. M. and Kontos, H. A. (1999) *Stroke,* **30,** 1933-1941.

Sommerhoff, C. P. (2001) *Am J Respir Crit Care Med,* **164,** S52-8.

Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P. and Lieberman, J. (2003) *Nat Med,* **9,** 347-51.

Steinhoff, M., Buddenkotte, J., Shpacovitch, V., Rattenholl, A., Moormann, C., Vergnolle, N., Luger, T. A. and Hollenberg, M. D. (2005) *Endocr Rev,* **26,** 1-43.

Steinhoff, M., Buddenkotte, J., Shpacovitch, V., Rattenholl, A., Moormann, C., Vergnolle, N., Luger, T. A. and Hollenberg, M. D. (2004) *Endocr Rev*.

Steinhoff, M., Vergnolle, N., Young, S. H., Tognetto, M., Amadesi, S., Ennes, H. S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Geppetti, P., Mayer, E. A. and Bunnett, N. W. (2000) *Nat Med,* **6,** 151-8.

Steinman, L. (2001) *Nat Immunol,* **2,** 762-4.

Suarez-Pinzon, W., Korbutt, G. S., Power, R., Hooton, J., Rajotte, R. V. and Rabinovitch, A. (2000) *Diabetes,* **49,** 1810-8.

Suescun, M. O., Calandra, R. S. and Lustig, L. (1994) *J Androl,* **15,** 442-8.

Suescun, M. O., Lustig, L., Calandra, R. S., Groome, N. P. and Campo, S. (2001) *J Endocrinol,* **170,** 113-20.

Tada, K., Murakami, M., Kambe, T. and Kudo, I. (1998) *J Immunol,* **161,** 5008-5015.

Tanzola, M. B., Robbie-Ryan, M., Gutekunst, C. A. and Brown, M. A. (2003) *J Immunol,* **171,** 4385-4391.

Temkin, V., Kantor, B., Weg, V., Hartman, M. L. and Levi-Schaffer, F. (2002) *J Immunol,* **169,** 2662-9.

Theas, S., Rival, C. and Lustig, L. (2003) *Am J Reprod Immunol,* **50,** 166-76.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. and Heinen, E. (1999) *J Biotechnol,* **75,** 291-5.

Tolentino, M. J., Brucker, A. J., Fosnot, J., Ying, G. S., Wu, I. H., Malik, G., Wan, S. and Reich, S. J. (2004) *Retina,* **24,** 660.

Tompkins, A. B., Hutchinson, P., de Kretser, D. M. and Hedger, M. P. (1998) *Biol Reprod,* **58,** 943-51.

Tung, K. and Teuscher, C. (1995) *Hum Reprod Update,* **1,** 35-50.

Van Voorhis, B. J., Dunn, M. S., Snyder, G. D. and Weiner, C. P. (1994) *Endocrinology,* **135,** 1799-806.

Vergnolle, N. (1999) *J Immunol,* **163,** 5064-5069.

Vergnolle, N., Hollenberg, M. D., Sharkey, K. A. and Wallace, J. L. (1999) *Br J Pharmacol,* **127,** 1083-90.

Vergnolle, N., Macnaughton, W. K., Al-Ani, B., Saifeddine, M., Wallace, J. L. and Hollenberg, M. D. (1998a) *Proc Natl Acad Sci U S A,* **95,** 7766-71.

Vergnolle, N., Macnaughton, W. K., Al-Ani, B., Saifeddine, M., Wallace, J. L. and Hollenberg, M. D. (1998b) *PNAS,* **95,** 7766-7771.

Vergnolle, N., Wallace, J. L., Bunnett, N. W. and Hollenberg, M. D. (2001) *Trends in Pharmacological Sciences,* **22,** 146-152.

Wang, J., Wreford, N. G., Lan, H. Y., Atkins, R. and Hedger, M. P. (1994) *Biol Reprod,* **51,** 551-61.

Wang, K., Tarakji, K., Zhou, Z., Zhang, M., Forudi, F., Zhou, X., Koki, A. T., Smith, M. E., Keller, B. T., Topol, E. J., Lincoff, A. M. and Penn, M. S. (2005) *J Cardiovasc Pharmacol,* **45,** 61-7.

Watanabe, M., Kashiwakura, Y., Kusumi, N., Tamayose, K., Nasu, Y., Nagai, A., Shimada, T., Daida, H. and Kumon, H. (2005).

Weidinger, S., Mayerhofer, A., Frungieri, M. B., Meineke, V., Ring, J. and Kohn, F. M. (2003a) *Hum. Reprod.,* **18,** 2519-2524.

Whitehead, S. S. N. S. A. (1999) *Endocrinology, An Integrated Approach,* BIOS Scientific Publishers Ltd.

Wu, D. and Meydani, S. N. (2004) *Brain Behav Immun,* **18,** 487-94.

Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. and Nathan, C. (1992) *Science,* **256,** 225-8.

Yamanaka, K., Fujisawa, M., Tanaka, H., Okada, H., Arakawa, S. and Kamidono, S. (2000a) *Hum Reprod,* **15,** 1543-7.

Yamanaka, K., Fujisawa, M., Tanaka, H., Okada, H., Arakawa, S. and Kamidono, S. (2000b) *Hum. Reprod.,* **15,** 1543-1547.

Zhang, X., Shan, P., Jiang, D., Noble, P. W., Abraham, N. G., Kappas, A. and Lee, P. J. (2004) *J Biol Chem,* **279,** 10677-84.

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- Fijak M., **Iosub R.**, Schneider E., Linder M., Respondek K., Klug J., Meinhardt A. (2005) Identification of Immunodominant Autoantigens in Rat Autoimmune Orchitis. *J. Pathology*, 207:127-38.
- **Iosub R**, Klug J, Fijak M, Schneider E, Fröhlich S, Blumbach K, Wennemuth G, Sommerhoff CP, Steinhoff M, Meinhardt A. (2005) Development of Testicular Inflammation in Rat Involves Activation of Proteinase Activated Receptor 2. *J. Pathology, in press*.

- Rival C, Lustig L, **Iosub R**, Guazzone V, Schneider E, Meinhardt A and Fijak M. (2005) Identification of a Dendritic Cell Popuation in Normal Testis and in Chronically Inflamed Testis of Rats with Autoimmune Orchitis. *Cell Tissue Res, in revision*.

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- **Iosub R**, Schneider E, Fijak M, Sommerhoff C and Meinhardt A. (2004). Role of Mast Cells and Proteinase-Activated Receptor-2 in Chronic Testicular Inflammation. 13th European Testis Workshop, Dunblane, Scotland, 24.-28. April 2004.
- Fijak M, **Iosub R**, Schneider E, Linder M, and Meinhardt A. (2004). Identification of Immunodominant Auto-Antigens in Rat Autoimmune Orchitis. 3rd European Conference of Andrology, Münster, Germany, 11.-14. September. Published in Andrologia 36, Vol 4, abstr. PO093
- **Iosub R**, Klug J, Fijak M, Schneider E, Wennemuth G, Steinhoff M, and Meinhardt A. (2004). The Role of Mast Cells and Proteinase-Activated Receptor-2 in Experimental Autoimmune Orchitis (EAO). 3rd European Conference of Andrology, Münster, Germany, 11.-14. September. Published in Andrologia 36, Vol 4, abstr. PO094
- **Iosub R**, Jörg Klug J, Fijak M, Schneider E, Wennemuth G, Steinhoff M, and Meinhardt A. (2004). Proteinase-Activated Receptor-2 Mediated Inflammatory Response in Experimental Autoimmune Orchitis: Role of Mast Cells. 21. Arbeitstagung der Anatomischen Gesellschaft, Würzburg 29.09.-1.10.2004.
- Meinhardt A, **Iosub R**, Schneider E, Linder M, Klug J, and Fijak M. Identification of Immunodominant Autoantigens in Rat Autoimmune Orchitis. Vortrag vor dem 38th Annual Meeting of the Society for the Study of Reproduction July 24?27, 2005, Quebec City, Quebec, Canada. Publiziert: Biol Reprod Special Issue 2005, abstract 153.
- Fijak M, **Iosub R**, Schneider E, Linder M, Respondek K, Klug J, and Meinhardt A. (2005). Identification of Immunodominant Autoantigens in Rat Autoimmune Orchitis. 22. Arbeitstagung der Anatomischen Gesellschaft, Würzburg 28.9.-30.9.2005. Annals Anat Suppl. 2005

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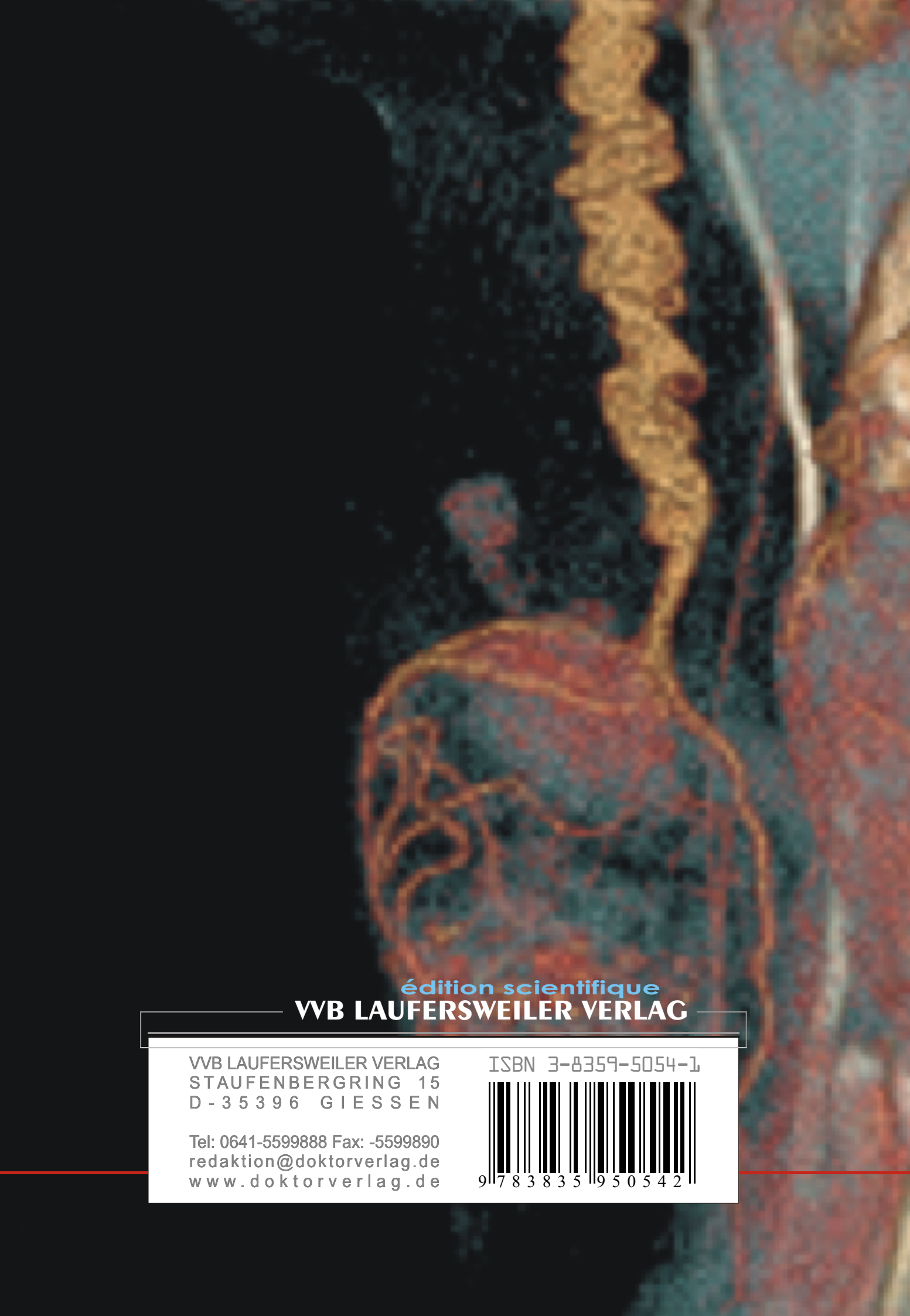

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