Original article

Tumor Necrosis Factor-alpha as a possible auto-/paracrine factor affecting estrous cycle in the cat uterus

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Abstract

Tumor Necrosis Factor-alpha (TNFα) is a pleiotrophic cytokine, affects either normal or tumor cells, and influences cellular differentiation. TNFα role in female reproduction has been proven to be mediated through an influence on prostaglandin (PGs) synthesis and output. To evaluate the possible role of TNFα in an auto-/paracrine regulation in the cat uterus, mRNA expression coding for TNFα and its receptors (TNFR1 and TNFR2), and TNFα protein content at different stages of the estrous cycle were investigated. Additionally, TNFα involvement in PG secretion at different stages of the estrous cycle was investigated by in vitro tissue culture. Gene expressions coding for TNFα and TNFR1 were the highest at diestrus ($P < 0.05$). TNFα protein expression was the lowest at interestrus ($P < 0.05$). Nevertheless, TNFR2 was not affected by the estrous stage. TNFα at a dose of 1 ng/ml significantly increased $PGF_{2\alpha}$ secretion at estrus ($P < 0.01$) and PGE_2 secretion at diestrus ($P < 0.001$) after 12h incubation. Overall findings indicate that TNFα locally produced in the cat's uterus, stimulates PG secretion in an estrous cycle-related manner.

Key words: Tumor Necrosis Factor-alpha, prostaglandin, uterus, cat

Introduction

TNFα controls growth of normal and tumor cells, affects the expression of genes associated with cell differentiation, and influences several cell functions (Hunt et al. 1996). TNFα is produced by uterine fibroblasts and macrophages (Vince et al. 1992), glandular epithelial cells (Hunt et al. 1992) and immune cells massively infiltrating the reproductive tract of humans and animals (Terranova et al. 1995, Pate and Keyes 2001). TNFα messenger RNA and protein has been shown in amniotic fluid, placenta and blastocysts, and thus TNFα participation in embryonic development and early pregnancy has been proven (Hunt et al. 1996). TNFα has been reported to augment PG secretion in the uterus of humans (Chen et al. 1995) and several other investigated animals (Skarzynski et al. 2000, Blitek and Ziecik 2006, Gamo et al. 2007). In the uterus, under pathologic conditions, contaminating bacteria interact with germ-line-en-

coded pattern recognition receptors (Toll-like receptors, TLRs) and thereby initiate a cascade of signaling events that include an early inflammatory response (Horne et al. 2008), such as a stimulation of TNFα production (Sheldon and Dobson 2004). In such case $PGF_{2\alpha}$ induced by TNFα can enforce a uterine immune defence by abrogating the immunosupressive effects of progesterone indirectly by causing luteolysis and directly in the uterus (Singh et al. 2008). Thus, the study of TNFα involvement in PG production would broaden our knowledge of the immune-endocrine process in the uterus under physiological as well as under pathological conditions. Although the modulating and supportive role of TNFα in the regulation of the reproductive process has been extensively studied in other species, the physiological action of TNFα in the reproductive tract of *Carnivora* species, including the cat, remains poorly understood.

Even though prostaglandins (PGs) and cytokines are involved in many physiological events that occur in the uterus of humans and the other investigated species (Kelly 1996), the role of both PGs and cytokines on the estrous cycle, early embryo development and immune response in the uterus in the cat remains uncertain. The paradigm of $PGF_{2\alpha}$ as a universally luteolitic hormone (McCracken et al. 1999, Okuda et al. 2002), however, is questionable in the case of cat reproduction. It has been demonstrated that hysterectomy does not prolong corpus luteum (CL) life span and luteal phase, excluding the role of endometrial $PGF_{2\alpha}$ in luteolysis initiation in this species (Wheeler et al., 1988). Nevertheless, mRNA for $PGF_{2\alpha}$ receptor (PTGFR) has been shown in a cat CL at early-, mid- and late-luteal stage (Siemieniuch, unpublished data). Furthermore $PGF_{2\alpha}$ administered in therapeutic doses did not cause pregnancy termination in the cat, when injected in the first half of pregnancy (Shille et al. 1979, Wildt et al. 1981). On the other hand $PGF_{2\alpha}$ administered in the female after mid-pregnancy as been reached, seems to be an efficient luteolytic and abortive agent (Henderson 1984). PGE_2 is thought to be either a luteotrophic or an anti-luteolytic agent in farm animals (Pratt et al. 1977, Magness et al. 1981). In contrast to data collected in humans and several investigated species, it is critical to recognize how little is known of the PGE_2 role either in cyclic and pregnant females or during bacterial contamination of the uterus in a cat. Even if the role of uterine originated PGs in luteolysis in the cat is doubtful, it's possible involvement as an auto-/paracrine factor in the cat uterus merits extensive study. In the present study we aimed at 1) comparing the pattern of TNFα, TNFR1 and TNFR2 expression throughout the feline estrous cycle and 2) evaluating TNFα-stimulated PG secretion in the cat uterus.

Materials and Methods

Animals

The study was approved by the Local Animal Care and Use Committee in Olsztyn, Poland (No. 41/2007/N). Uteri together with ovaries and oviducts were collected from 24 adult domestic cats (1-3 years old), after routine ovariohisterectomy procedures at the Department of Animal Reproduction, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, at the owner's request and transported in cold saline to the laboratory within 1.5 hours after surgery.

Tissue collection and culture

The phase of the estrous cycle was determined by the macroscopic observation of the ovaries and uterus as described previously (Karja et al. 2002). Each horn was slit. The uteri horns after being slit were cut into pieces (30-50 mg) and then washed three times in sterile saline. The tissue weight was used to standardize the results. Each piece was placed in an individual culture glass vial into which 1 ml of Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO, USA, D8900), supplemented with 0.1% bovine serum albumin (BSA; Sigma, A9056) and gentamycin (Sigma, G1397), was added. The tissue cultures were pre-incubated in a shaker water bath for 1 hour (h) at 38°C in a gas mixture (5% CO_2 in air). Tissue strips collected from other uteri were used for examination of gene and protein expression.

Experiment 1. Effect of estrous stage on TNFh, TNFR1, TNFR2 mRNA expression and TNFh protein expression.

To determine the expression of mRNA coding for TNFα, TNFR1 and TNFR2 tissue samples (estrus n = 3, diestrus n = 3, interestrus n = 3) were subjected to the semi-quantitative RT-PCR. β-actin (ACTB) was used as a reference gene. For TNFα protein expression measurement, tissue samples (estrus n = 3, diestrus n = 3, interestrus n = 3) were frozen, then analyzed by Western blotting. ACTB was used as a reference protein.

Experiment 2. Effect of TNFα on PG secretion in tissue explants from different stages of the estrous cycle.

Cyclic changes in uterine $PGF_{2\alpha}$ and PGE_2 secretion in response to TNFα were measured. The following estrous phases were measured at estrus (n = 5), diestrus (n = 5), interestrus (n = 5). Pre-incubated slices of uterus were next treated with different con-

centrations of TNFα. Incubation of the tissue was conducted in a shaker water bath at 38°C in a gas mixture bubbled through the medium during incubation as described previously (Woclawek-Potocka et al. 2005). The tissue slices, after 1 h of preincubation, were exposed to TNFα (human recombinant TNFα; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan, HF-13; 0, 1, 10 and 100 ng/ml) for 2, 4 and 12 h. After incubation, the conditioned media were collected, EDTA and aspirin were added to the culture medium, and frozen at -20°C until PG measurements.

Reverse transcription and mRNA expression

For the studies on TNFα and its receptors mRNA expression, small uteri fragments were kept overnight at 4°C in RNA*later* (AM7021, Ambion, Applied Biosystem) and then stored at -80°C until further analysis. Total RNA was obtained from the uteri tissue using TRIzol Reagent according to the manufacturer's instructions. 1 μg of each sample of total RNA was reverse transcribed using a PrimeScript™ First Strand cDNA Synthesis Kit for RT-PCR (6110 A, TaKaRa, Kyoto, Japan) as described previously (Korzekwa et al. 2008, Siemieniuch et al. 2010). ACTB served as an internal control. ACTB primer was added at the appropriate cycle number by the "primer-dropping method" according to Wong et al. (1994). The conditions for the PCRs were as follows: 21 (ACTB), 32 (TNFα), 28 (TNFR1) and 32 (TNFR2) cycles of reactions including denaturation for 30 sec at 95°C, annealing for 1 min at 60°C, and extension for 1 min at 70°C, followed by an additional extension for 5 min at 72°C. Details of the PCR reaction: primers used and length (bp) are given in Table 1. Aliquots of the PCR reaction products were electrophoresed on a 1.5% agarose gel containing ethidium bromide with a known standard (100 bp Ladder; D-1030; BIONEER Korea), and photographed under ultraviolet light. The band intensities were analyzed using a NIH computerized densitometry program (National Institute of Health,

Bethesda, MD, USA). Bands of the appropriate size were cut out of the gel and sent to the sequencing service (GENOMED, Warsaw, Poland). The sequence obtained was compared with the databases using BLAST analysis.

TNFα protein analysis

TNFα protein in feline uterine samples originating from three different estrous stages was detected using Western blotting. Tissue fragments were put in liquid nitrogen, crushed, and placed in ice-cold homogenization buffer (25 mM Tris-HCL, 300 mM sucrose, 2mM edetic acid [EDTA], Complete [protease inhibitor cocktail, No 1697498; Roche Diagnostic], pH 7.4) until TNFα protein analysis by Western blotting. Protein concentration in the samples was measured according to Osnes et al. (1993), using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (0.5 M Tris-HCL [pH 6.8], 10% SDS [2.0 ml], glycerol [2.5 ml], 2-mercaptoethanol [0.5 ml], 1% bromophenol blue [1.0 ml], Mili-Q water [2.8 ml]) and heated at 95°C for 10 min. Samples (50 μg of protein) were subjected to electrophoresis in 10% SDS-PAGE for 1 h at 200 V. The separated proteins were electrophoretically transblotted to a 0.2 μm nitrocellulose membrane (Amersham Biosciences Corp., Piscatanay, NY; No. RPN78D) at 250 mA for 3 h in a transfer buffer (25 mM Tris-HCL, 192 mM glycine, 20% methanol, pH 8.3). The membrane was then washed in TBS-T (0.1% Tween 20 in Tris buffered saline [TBS] [25 mM Tris-HCL, pH 7.5], 137 mM NaCL) and cut into two pieces. One piece was used for a target protein (TNFα, 14 kDa) and the other one was used for ACTB (42 kDa). Both pieces were incubated in blocking buffer (4% nonfat dry milk in TBS-T) overnight at 4°C. After the blocking incubation, the pieces of membrane were separately incubated with a primary antibody specific to each protein (hTNFα antibody, kindly donated by Dr D. Schams [1:1000 in TBS-T] and ACTB antibody [A2228; 1:4000 in TBS-T; Sigma Aldrich]) for 1 h at

Table 1. Forward (For) and reverse (Rev) primers sequences (5' → 3'), RT-PCR product length and reference list of investigated factors.

Primer	Sequence (5' → 3')	Length (bp)	References
ACTB	For GGGGTCACCCACACTGTGCCCATC Rev ACGTCACACTTCATGATGGAGTTG	588	Mizuno et al. 2001
TNF	For TGGCCTGCAACTAATCAACC Rev GTGTGGAAGGACATCCTTGG	251	Avery and Hoover 2004
TNFR1	For CGAAGTGCCACAAAGGGACCTAC Rev TGGTTCTTCCTGCAGCCACACAC	215	Mizuno et al. 2001
TNFR2	For CTCAGGCAGCACCGCAGACGG Rev GCCGGAGGAGCTGGCATCCACG	244	Mizuno et al. 2001

room temperature (RT). The original concentration of primary antibodies was 1 mg/1 ml for TNFα and 2 mg/ml for ACTB. After incubation, the membrane pieces were washed for 10 min once in blocking buffer and then twice in TBS-T at RT and subsequently incubated with a secondary antibody to TNFα (1:5000 in TBS-T) (anti-rabbit Ig, horseradish peroxidase [HRP]-linked whole antibody produced in donkey, NA934; Amersham Biosciences Corp., Piscataway, NJ) and to ACTB (1:40000 in TBS-T) (anti-mouse Ig, HRP-conjugated whole antibody produced in sheep, A931; Amersham Biosciences Corp.) for 1 h and washed for 10 min twice in TBS-T and once in TBS at RT. The signal was detected using an ELC Western blotting detection system (RPN2109; Amersham Biosciences Corp.). The intensity of immunological reaction was evaluated by measuring optical density in the defined area using a NIH computerized densitometry program (National Institute of Health, Bethesda, MD, USA).

PGF$_{2\alpha}$ and PGE$_2$ determination

The concentrations of PGF$_{2\alpha}$ and PGE$_2$ in conditioned media were determined with an enzyme immunoassay (EIA), as described previously (Woclawek-Potocka et al., 2005) using commercial anti-PGF$_{2\alpha}$ and anti-PGE$_2$ sera (Sigma; P5539 and P5164, respectively). The PGF$_{2\alpha}$ standard curve ranged from 0.19 to 50 ng/ml, and the ED50 of the assay was 2.4 ng/ml. The intra- and inter-assay coefficients of variation were 6.8% and 10.9%, respectively. The PGE$_2$ standard curve ranged from 0.078 ng/ml to 20 ng/ml and the ED50 of the assay was 1.25 ng/ml. The intra- and inter-assay coefficients of variation were on average 6.3% and 9.7%, respectively.

Statistical analyses

Data are shown as the mean ± SEM of values obtained in 3 or 5 separate experiments, each performed in triplicate. The statistical differences between controls and treated groups were assessed by one-way ANOVA followed by Newman-Kuels Multiple Comparison Test (GraphPad PRISM v 4; GraphPad Software Inc., San Diego, CA, USA) or Student's *t*-test.

Results

Experiment 1. Effect of estrous stage on TNFα, TNFR1, TNFR2 mRNA expression and TNFα protein expression.

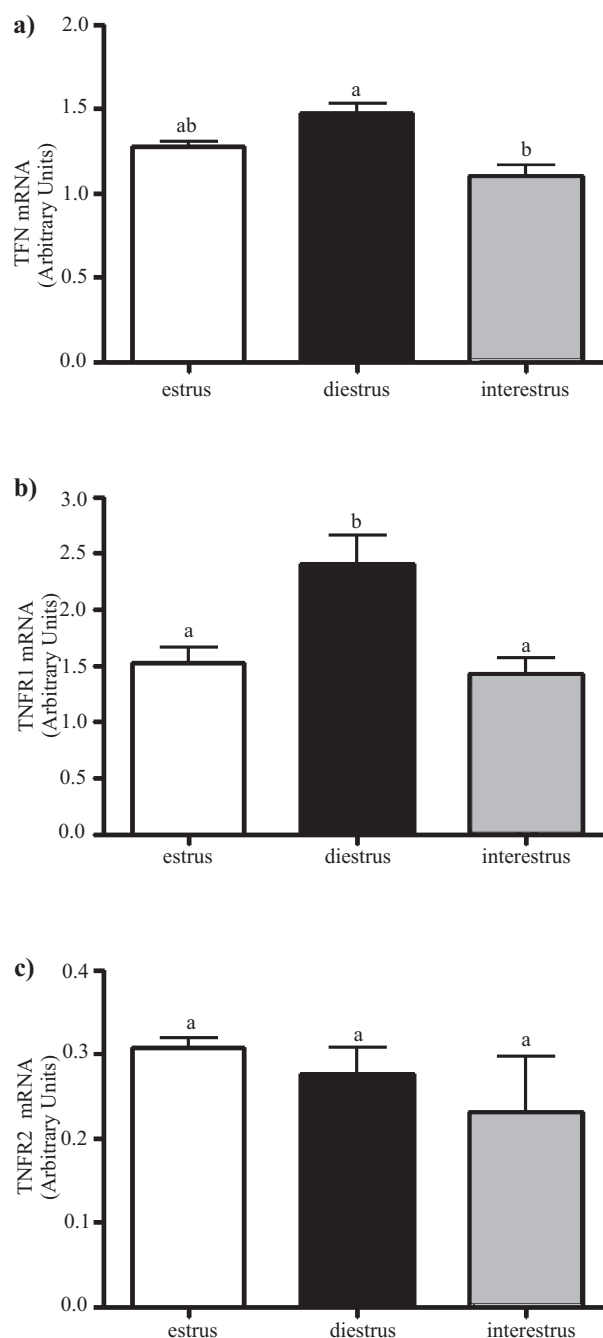The relative signal intensities of PCR products specific for TNFα, TNF1 and TNF2 were assessed

Fig. 1. Tumor necrosis factor-alpha (TNFα), TNF receptor type 1 and 2 (TNFR1 and TNFR2) mRNA expression in uterine tissue during different estrous stages. (**a**) Relative levels of TNF mRNA (RT-PCR-arbitrary units) in the tissue. All values are the mean ± sem of the densitometry values of mRNA levels in the tissue (relative to ACTB mRNA levels). Different letters indicate significant differences (P < 0.05). (**b**) Relative levels of TNFR1 mRNA (RT-PCR-arbitrary units) in the tissue. All values are the mean ± SEM of the densitometry values of mRNA levels in the tissue (relative to ACTB mRNA levels). Different letters indicate significant differences (P < 0.05). (**c**) Relative levels of TNFR2 mRNA (RT-PCR-arbitrary units) in the tissue. All values are the mean ± SEM of the densitometry values of mRNA levels in the tissue (relative to ACTB mRNA levels). Different letters indicate significant differences (P < 0.05).
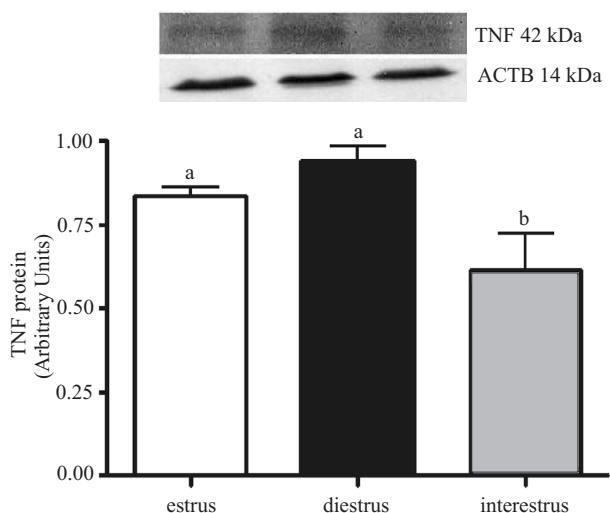
Fig. 2. TNF protein expression in the uterine tissue during different estrous stages. Upper panel: representative Western blot of TNF protein in estrus, diestrus and interestrus; lower panel: relative levels of TNF protein (Western blot-arbitrary units) in the tissue. All values are the mean ± SEM of the densitometry values of protein levels in the tissue (relative to ACTB protein levels). Different letters indicate significant differences ($P < 0.05$).

after correction based on the ACTB signal intensities and are shown on Fig. 1. mRNA expression levels for TNFα were the highest at diestrus and the lowest at interestrus ($P < 0.05$) (Fig. 1a). TNFR1 gene expression was the highest at diestrus ($P < 0.05$) (Fig. 1b). The expression levels of mRNA for TNFR2 were not affected by the stage of estrous cycle (Fig. 1c). A representative Western Blot showing the presence of a TNFα-specific band in the cat uterus is presented in Fig. 2. The expected bands with a molecular weight of 42 kDa (TNFα) have been shown with comparison to a housekeeping protein (ACTB) with a molecular weight of 14 kDa. The intensities of TNFα specific bands, after normalization to ACTB specific bands, were the lowest at interestrus ($P < 0.05$).

Experiment 2. Effect of TNFα on PG secretion in tissue explants from different stages of the estrous cycle.

PG secretion after TNFα treatment was dose- and time-dependent (Fig. 3a and b, respectively). TNFα at a dose of 1 ng/ml significantly increased PG output at estrus and at diestrus after 12h incubation. Effect of TNFα on PG secretion depended on the estrous stage
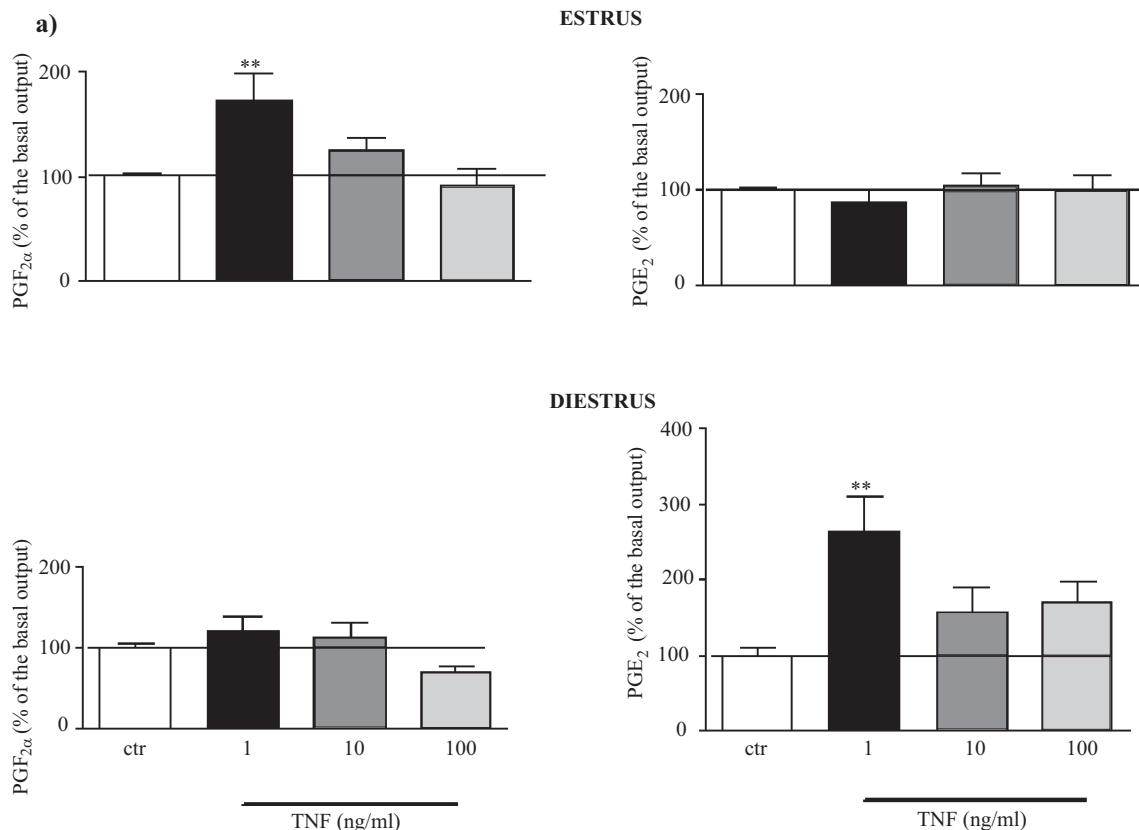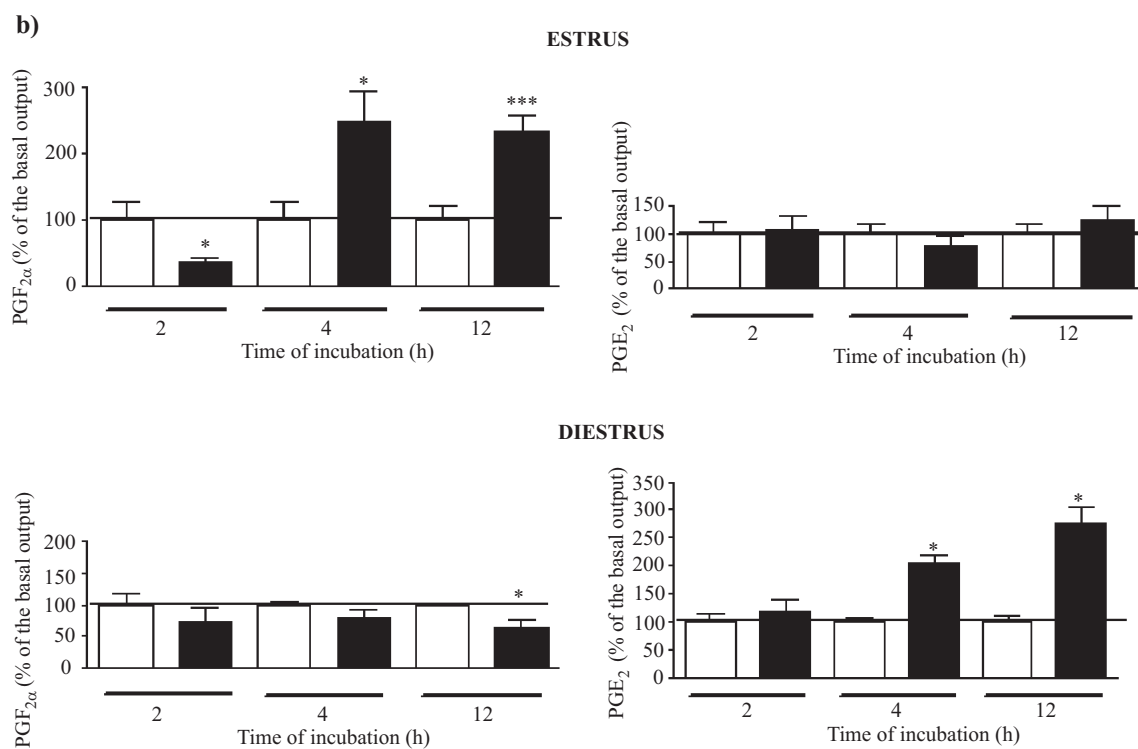

Fig. 3a

b)

Fig. 3. Effects of TNF at a dose of 0 (Ctr), 1, 10 and 100 ng/ml on the $PGF_{2\alpha}$ and PGE_2 output for 12 hours of culture in uterine slices at estrus and diestrus (Fig. 3a, n = 6). Effects of TNF at a dose of 1 ng/ml on the $PGF_{2\alpha}$ and PGE_2 output for 4, 8 and 12 hours of culture in uterine slices at estrus and diestrus (Fig. 3b, n = 6). Asterisk indicates significant differences (P < 0.05).

(Fig. 4). TNFα distinctly augmented $PGF_{2\alpha}$ output at estrus (P < 0.01) and reduced it at diestrus (P < 0.05). TNFα increased PGE_2 secretion at diestrus (P < 0.001). TNFh had no effect on PG secretion at interestrus compared with the control (P > 0.05).

Discussion

We clearly demonstrated that the TNFα gene is transcribed and translated in the cat uterus in a cycle related manner. In the current study, TNFα expression was the highest at diestrus and at estrus, but significantly lower at interestrus and TNFα protein expression was similar to that for mRNA. Expression of the TNFα gene and protein has been previously shown in the endometrium of humans (Hunt et al. 1992), rats (Yelavarthi et al. 1991) and mice (Roby and Hunt 1994). TNFα transcript and protein level increased in the human endometrium in the proliferative stage, subsequently declined in the early secretory stage and rose again in the mid-to-late luteal stage. These authors suggested that TNFα mRNA cyclic fluctuations correspond to the sex steroids level (Hunt et al. 1992). In the human and mouse, TNFα activity has been shown in estrogen-primed and progesterone-exposed endometrial cells (Terranova et al. 1995). Thus, it is also possible that TNFα gene

expression is up-regulated by sex steroids in the cat uterus, with the highest TNFα bioactivity at estrus and diestrus compared with interestrus, when the sex steroid hormones are at nadir. Messenger RNA coding for TNFα has been demonstrated in the canine corpus luteum (Hoffmann et al. 2004, Engel et al. 2005), early diestrus uterus and preimplantation embryos (Schäfer-Somi et al. 2008), suggesting a possible role of this cytokine in dog reproduction. Thus the likely auto-/paracrine role of TNFα should not be limited only to the regulation of the estrous cycle in the cat, it's possible involvement in early pregnancy merits further discussion. In addition, TNFα is involved in the innate response in the case of microbiological infection. Cats produce TNFα in response to lipopolisaccharide (LPS) in a fashion similar to other species (Otto and Rawlings 1995). The uterus is usually a sterile environment, but it is temporarily contaminated with bacteria during coitus or parturition. Thus, the role of TNFα under pathologic conditions, such as microbiological contamination, needs to be clarified.

The present study provided evidence that TNFα modulates PG uterine secretion in the cat, which appears to be dependent on the estrous stage. Previous data collected in humans (Chen et al. 1995), rats (Arslan and Zingg 1996, Gamo et al. 2007), pigs (Blitek and Ziecik 2006) and cows (Murakami et al. 2001,
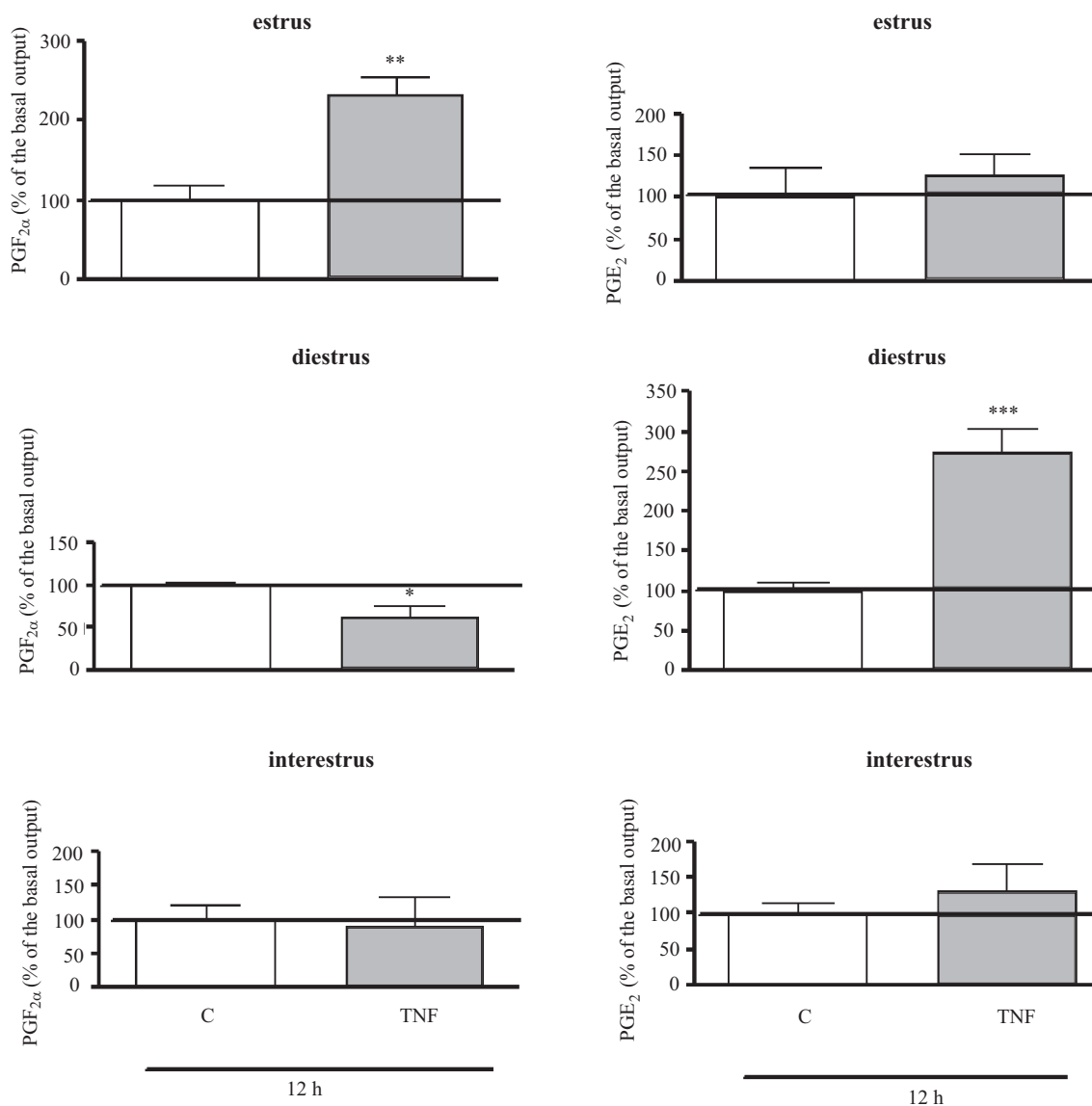
Fig. 4. Effects of TNF at a dose of 0 (Ctr) and 1 ng/ml on the $PGF_{2\alpha}$ and PGE_2 output for 12 hours of culture in uterine slices in different stages of the estrous cycle (n = 15). Asterisk indicates significant differences (P < 0.05).

Skarzynski et al. 2003) have proven that TNFα increased endometrial PG production, playing a role as an auto-/paracrine regulator of the endocrine events in the reproductive tract (von Wolff et al. 1999). TNFα/TNFR1 complex has been demonstrated to activate PLA2 and, hence, increase PG secretion (Clark et al. 1988). In the rat endometrium, TNFα affected both PGE_2 (Gamo et al. 2007), and $PGF_{2\alpha}$ (Arslann and Zingg 1996). Murakami et al. (2001) provided arguments that TNFα affects both $PGF_{2\alpha}$ and PGE_2 in the bovine endometrium and this action is strongly related to the stage of the estrous cycle. In the present study TNFα significantly stimulated PGE_2 output from the feline uterus at diestrus, but also augmented $PGF_{2\alpha}$ secretion at estrus, although TNFα did not alter PG concentration at interestrus. The previous observations that TNFα can be both a luteolytic or a luteotrophic factor (Skarzynski et al. 2003, 2007, 2009) support our present findings in cats in which TNFα stimulated both $PGF_{2\alpha}$ and PGE_2 production dependent on the estrous cycle. In particular, the favorable PGE_2 production at diestrus to support CL maintenance and/or induce vascularisation of the endometrium, and thus to play some significant roles as a luteotrophic factor, is conceivable in the cat.

Earlier data stated that TNFα-stimulated uterine PG synthesis occurred through TNFα binding to its receptor in the cow (Miyamoto et al. 2000). Thus, we undertook a subsequent investigation to check the cycle-related changes of both TNFR1 and TNFR2 mRNA expressions in the feline uterus. While TNFR1 expression was clearly estrous cycle-related, showing the highest mRNA level at diestrus, TNFR2 mRNA was not affected by the estrous cycle. The

present observations regarding TNFR1 are in agreement with the data collected in humans and mice (Roby and Hunt 1994, Hunt et al. 1996). In the human endometrium mRNA encoding TNFR1 peaked at the late secretory stage (Hunt et al. 1996). Similarly, in mice endo- and myometrium TNFR1 transcriptional activity fluctuated during the estrous cycle and was the highest at diestrus (Roby and Hunt 1994). TNFR2 mRNA expression fluctuated during the human menstrual cycle and peaked in both stromal and epithelial cells at the late proliferative and the late secretory stage (Hunt et al. 1996). However, results obtained by Tabizadeh et al. (1995) did not confirm this cycle-related pattern and demonstrated that TNFR2 was not affected by the menstrual cycle in humans, which is in accordance with the data collected here for the cat uterus. The overall findings suggest that TNFα exerts its auto-/paracrine function on uterine PG secretion under physiological conditions in cats mostly at diestrus and at estrus, but not at interestrus, which is in accordance with the highest mRNA expressions for TNFα and TNFR1.

In conclusion, TNFα is transcribed and translated in an estrous cycle-related fashion in the cat uterus, thus it is possible that TNFα is involved in modulation/regulation of the estrous cycle in cat. By showing the changes of PG concentration following TNFα treatment, we demonstrated for the first time that the cat uterus has the capacity to secrete PGs.

Acknowledgements

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