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Immune stress up regulates *TLR4* and *Tollip* gene expression in the hypothalamus of ewes

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ABSTRACT. Bacterial endotoxin, LPS, is recognized by Toll-like receptor-4 (TLR4) and induces a signaling cascade leading to synthesis of proinflammatory cytokines and induction of sickness behavior in animals. Transduction of the TLR4 signal is controlled by a potent negative regulator—Toll-interacting protein. The presented study concerns the effect of intravenously injected LPS on the level of expression of *TLR4* and *Tollip* genes in the hypothalamus of ewes. Endotoxin increased ($P < 0.01$) cortisol release and expression of *TLR4* and *Tollip* genes in the preoptic area (1.87 ± 0.42 and 1.31 ± 0.15), anterior hypothalamus (1.77 ± 0.22 and 1.27 ± 0.13), medial basal hypothalamus (2.53 0.65 and 1.43 ± 0.15), and median eminence (2.93 ± 0.46 and 1.73 ± 0.10), respectively, in comparison with non-treated animals. Our results show that immune stress increases *TLR4* gene expression in the hypothalamus. Increased transcription of Tollip may be an attempt to reduce the effect of TLR4 stimulation.

Introduction

A bacterial endotoxin, lipopolysaccharide (LPS), is a pathogenic membrane component of virtually all Gram-negative bacteria. It is thought to play a major role in the pathophysiology of septic shock (Coleman et al., 1993). Antibiotic-induced release of lipopolysaccharide is an important cause of septic shock in animals and humans treated for severe infections by Gram-negative bacteria. LPS triggers the release of many inflammatory cytokines, in particular tumor necrosis factor α (TNFα), interleukin (IL) 1 beta (IL-1β), and IL-6 (Tsuji et al., 2003). After combining with LPS-binding protein, the endotoxin binds to Toll-like the endotoxin binds to Toll-like receptor-4 (TLR4), which activates complex intracellular signaling pathways leading to activation of transcription factors such as NF-κB, JNK, and mitogen-activated protein kinases p38 (Zhang and Ghosh, 2002; Leon et al., 2008). One of the potent negative regulators of TLR4 is Tollip (Zhang and Ghosh, 2002). This protein was found to associate with the cytoplasmic domain of TLRs and to inhibit the receptor signaling cascade by blocking IL-1R-associated kinase phosphorylation (Zhang and Ghosh, 2002). The typical symptoms of sickness, including weakness, fewer, malaise, listlessness, anorexia, sleeplessness, and inability to concentrate, which commonly accompany bacterial infections, have to a large part been attributed to the action of cytokines and other acute phase reactants (Dantzer, 2004; Chakravarty and Herkenham, 2005). The majority of these "sickness behaviors" are induced directly by centrally acting pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, especially at the hypothalamic level. It was found that neuroendocrine cells synthesize and release various cytokines and their corresponding receptors

(Besedovsky and del Rey, 1996). Some sickness symptoms, however, could be the result of hypothalamic–pituitary–adrenal axis (HPA axis) activation. Endotoxin is known as a profound stressor (Dadoun et al., 1998). It was previously reported that peripheral administration of LPS stimulates cortisol release in a variety of animal species, including sheep (Coleman et al., 1993). TLR4 expression was also found in the central nervous system (CNS) not only in microglia cells, but even in neurons (Chakravarty and Herkenham, 2005). This suggests that TLR4 and its ligand could be involved in the induction of sickness behavior at the level of the hypothalamus. It is still unclear if LPS is able to cross the BBB and act directly in the CNS during peripheral immune challenge. The result of experiments performed on rats are equivocal. Singh and Jiang (2004) suggest that LPS modulates the functioning and permeability of the BBB, but does not cross it. On the other hand, in vivo studies performed on rats showed that peripherally injected LPS previously labeled with radioactive iodine 125 crossed the BBB. The brain uptake of circulating LPS was found to be low but measurable (Banks and Robinson, 2010).

The presented study was designed to test the hypothesis that peripheral administration of LPS affects TLR4 and Tollip gene expression in the hypothalamus.

Material and methods

Animals

The studies were performed on adult, 3-year-old Blackhead ewes (n = 12). The animals were in good condition, maintained indoors in individual pens, and exposed to natural daylight. The ewes were well adapted to the experimental conditions; they always had visual contact with their neighbors, even during the experimental period, to prevent the stress of social isolation. The animals were fed a constant diet of commercial concentrates with hay and water available *ad libitum*.

All procedures on animals were performed with the consent of the Local Ethics Committee of the Warsaw Agriculture University.

Experimental procedure

In treated animals, immune stress was induced by iv. injection of LPS from *E. Coli* 055:B5 (Sigma, USA) dissolved in saline (0.9% w/v NaCl) (Baxter, USA) at a concentration of 10 mg · l^{-1} into the jugular vein. Control animals received an equivalent volume of saline.

The ewes (n = 12) were randomly divided into two experimental groups: controls (n = 6) and LPS-treated (n = 6). Jugular blood samples were taken for measurement of cortisol at 15-min intervals, beginning 2 h before injection of endotoxin (400 ng . kg^{-1}, iv., n = 6) or an equivalent volume of saline (iv., n = 6); sampling continued 3 h after the injections. Next, the animals received the ending of injection the animals were sacrificed by decapitation, the brains were rapidly removed from the skulls and the hypothalamic structures, i.e. the preoptic area (POA), anterior hypothalamus (AHA), medial basal hypothalamus (MBH), and median eminence (ME), from anestrous ewes were dissected. All tissues were frozen immediately after collection in liquid nitrogen and stored at –80°C.

Radioimmunoassay for cortisol

The cortisol concentrations were assayed by radioimmunoassay (RIA) according to a previously described protocol (Kokot and Stupnicki, 1985), using rabbit anti-cortisol antisera (R/75) and HPLC-grade cortisol standard (SIGMA, USA). The assay sensitivity was 0.95 ng · ml^{-1} and the intra- and inter-assay coefficients of variation for cortisol were 10% and 12%, respectively.

Determining relative gene expression

Total RNA from the hypothalamic tissues was isolated using the NucleoSpin® RNA II kit (MACHEREY-NAGEL GmbH & Co; Germany). The amount and purity of total RNA were determined using a NanoDrop® ND-1000 spectrometer (Thermo Scientific, USA) by measuring the optical density at 230 nm, 260 nm, and 280 nm. RNA integrity was verified by 1.5% agarose gel electrophoresis. cDNA synthesis was performed using the components of DyNAmoTM SYBR® Green 2-Step qRT-PCR Kit (Finnzymes; Finland) according to the manufacturer's instructions. For the PCR-assay, 1 µg total RNA was used as the starting material for cDNA synthesis. Specific primer pairs for determining the expression of genes were designed using Primer3 software and synthesized by Genomed (Poland). The sequences of TLR4 primers were: 5'-GGTTCCCAGAACTGCAAGTG-3' (forward) and 5'-GGATAGGGTTTCCCGTCAGT-3', (reverse) (Genebank accession no. AY957615), generated product size: 117 bp; Tollip primers: 5'-CTGGTGCTGTCCTACACGTC-3' (forward) and 5'-ACAGTGGGCATTCCTGTGAT-3' (reverse) (Genebank accession no. NM_001039961), generated product size: 122 bp; β-actin (ACTB) primers: 5'-CTTCCTTCCTGGGCATGG-3' (for-

ward) and 5'-GGGCAGTGATCTCTTTCTGC-3' (reverse) (Genebank accession no. U39357), generated product size: 168 bp; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers: 5'-AGAAGGCTGGGGCTCACT-3' (forward) and 5'-GGCATTGCTGACAATCTTGA-3' (reverse) (Genebank accession no. NM-001034034), generated product size: 134 bp; cyclophilin C (PPIC) primers: 5'-ACGGCCAAGGTCTTCTTTG-3' (forward) and 5'-TATCCTTTCTCTCCCGTTGC-3' (reverse) (Genebank accession no. NM-001076910), generated product size: 131 bp. Real-Time RT-PCR was carried out using SYBR® Green 2-Step qRT-PCR Kit (Finnzymes; Finland) components in a volume of 20 µl. One tube contained: 10 µl PCR Master Mix (2x), 7 µl RNase-free water, 2 µl primers (1 µl each, working concentration was 0.5 µM) and 1 µl cDNA template. The tubes were run on a Rotor-Gene 6000 (Corbett Research, Australia). The following protocol was used: 15 min at 95°C for Hot Star *Thermus brockianus* DNA polymerase and PCR for 30 cycles of 5 sec at 94°C for denaturation, 15 sec at 56°C for annealing, and 10 sec at 72°C for extension. After the cycles, a final melting curve analysis under continuous fluorescence measurement was performed to evaluate specific amplification. Additionally, representative samples of PCR products for all amplified genes were sequenced (Oligo IBB PAN; Poland) to confirm their specificity.

Data analysis

Plasma cortisol concentrations. The results are presented as a median ± S.E.M. of cortisol concentrations. The significance of differences between saline control and LPS-treated animals was assessed by one-way ANOVA followed by the Mann-Whitney U test using STATISTICA software.

PCR. All data were analyzed using Rotor Gene 6000 software 1.7. Relative gene expression was determined using the Relative Expression Software Tool 2008 published by Pfaffl et al., 2002. To compensate variation in cDNA concentrations and PCR efficiency between tubes, an endogenous control gene was included in each sample and used for normalization. Initially, three housekeeping genes (GAPDH, β-actin (ACTB), cyclophilin C (PPIC)) were tested. To determine the most stable housekeeping gene for normalizing the expression of genes of interest, BestKeeper software was used. This program uses pair-wise correlation analysis of all pairs of candidate genes (Pfaffl et al., 2004) and calculates variations of all reference genes (SD [± Ct]). A low SD is expected for useful reference genes. In a second analysis, BestKeeper estimates the correlations in the expression levels among all of the possible candidates. In the presented assay, GAPDH was chosen as the best endogenous control gene. It had the lowest SD [± Ct] value and good correlation coefficients with the remaining analyzed housekeeping genes (data not shown).

The results are presented as the relative gene expression of the target gene vs. housekeeping gene (GAPDH), relative expression value, and median ± S.E.M. The average relative quantity of gene expression in control groups was set to 1.0. The significance of differences between the results of the saline control and LPS treatment was assessed by the Mann-Whitney U test using STATISTICA 9 software.

Results

Effect of LPS injection on cortisol release

There were no significant differences between the median cortisol concentrations before saline (18.4 ± 4.2 ng · ml^{-1}) and LPS (25.3 ± 2.9 ng · ml^{-1}) injections. The plasma cortisol concentration was significantly ($P < 0.01$) increased compared with the control group from 30 min after endotoxin treatment to the end of the experiment. The median concentration of cortisol assayed at the end of blood collection was still increased (150 ± 24 ng · ml^{-1}) (Figure 1).

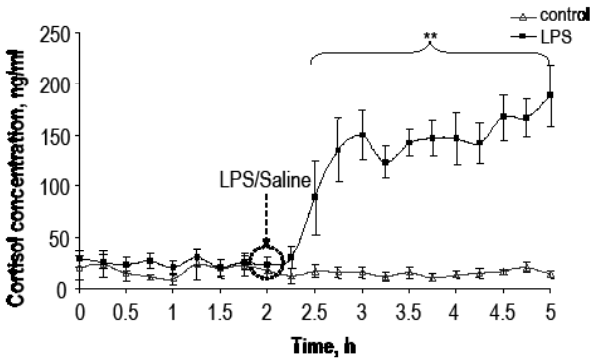

Figure 1. The effect of LPS on the plasma cortisol concentration. Data are presented as a median value ± S.E.M. (n = 12); ** P<0.01 (asterisks indicate values that differ significantly from the control group animals according to Mann–Whitney U test)

Effect of LPS injection on *TLR4* and *Tollip* gene expression in the hypothalamus

Intravenous injection of endotoxin significantly ($P < 0.01$) increased TLR4 gene expression in the POA, AHA, MBH, and ME (median exp. 1.87 ± 0.42, 1.77 ± 0.22, 2.53 ± 0.65, 2.93 ± 0.46, respectively) compared with the control group (Table 1).

Table 1. The effect of LPS on the relative TLR4 and Tollip mRNA level in the hypothalamus

Structure	TLR4 vs. GAPDH	
	control	LPS
POA	1 ± 0.13	1.87 ± 0.42**
AHA	1 ± 0.17	1.77 ± 0.22**
MBH	1 ± 0.14	2.53 ± 0.65**
ME	1 ± 0.32	2.93 ± 0.46**
	Tollip vs. GAPDH	
	control	LPS
POA	1 ± 0.15	1.31 ± 0.15**
AHA	1 ± 0.10	1.27 ± 0.13**
MBH	1 ± 0.08	1.43 ± 0.15**
ME	1 ± 0.12	1.73 ± 0.10**

POA – the preoptic area; AHA – the anterior hypothalamus; MBH – the medial basal hypothalamus; ME – the median eminence; control – group injected with saline; LPS – group received the endotoxin injection (400 ng · kg^{-1}); Data are presented as a median value ± S.E.M; ** P<0.01 (asterisks indicate values that differ significantly from the control group).

LPS also significantly increased the Tollip mRNA level in these structures (median exp. 1.31 ± 0.15, 1.27 ± 0.13, 1.43 ± 0.15, 1.73 ± 0.10 respectively) (Table 1).

Discussion

The presented study showed that peripheral administration of LPS increased the amount of mRNA encoding TLR4 and Tollip in all of the analyzed hypothalamic structures. The results pertaining to *TLR4* gene expression are interesting because previous studies with peripheral administration of LPS have demonstrated that LPS does not enhance TLR4 transcripts in the rat brain (Laflamme and Rivest, 2001; Nguyen et al., 2002). Reports on TLR4 expression during endotoxemia are contradictory, however. It was previously found that endotoxin increases the TLR4 mRNA level in human monocytes and polymorphonuclear leucocytes, as well as in murine macrophages (Muzio et al., 2000; Pedchenko et al., 2005). The activation of TLR4 transcription after LPS treatment could be mediated via transcription factor PU.1, which binds to the *TLR4* promoter and induces *TLR4* gene expression (Pedchenko et al., 2005). An *in vitro* study on mice macrophages suggests that LPS is a downregulator of TLR4 transcription (Nomura et al., 2000). Nonetheless, the elevation of *TLR4* gene expression in the hypothalamus could be not only an effect of centrally acting LPS molecules, but also the result of the stress induced by iv. endotoxin injection. In our study, we found a strong increase in the cortisol level in all animals after LPS administration. Endotoxin is a known stressor that activates the HPA axis. A study performed on sheep showed that peripheral immune challenge induced by LPS injection leads to elevation of the plasma cortisol level in sheep (Coleman et al., 1993). Immune stress stimulates not only the release of cortisol, but also of other HPA axis components, increases the synthesis and release of AVP and corticotropin-releasing factor (CRF) in the hypothalamus, stimulates secretion of ACTH from the pituitary and of corticosterone or cortisone from the adrenal cortex (Dadoun et al., 1998; Melmed, 2001). The activation of the stress axis may directly affect the level of *TLR4* gene expression in the hypothalamus. The *in vitro* study performed on a murine macrophage cell line showed that CRF, which initiates the HPA axis response to stress at the hypothalamic level, strongly increases *TLR4* gene transcription through the CRF2 receptor, via activation of transcription factors PU.1 and AP-1 (Tsuji et al., 2003; Tsatsanis et al., 2006). This suggests that a similar mechanism may exist in the region of the hypothalamus where CRF2 receptors are present as well. Better understanding of the immune response mechanisms in hypothalamic tissue and the contribution of TLR4 in this processes require future detailed research, however.

The changes in TLR4 gene expression in the hypothalamus after peripheral LPS administration are interesting because a study performed on rats suggests that LPS does not cross the BBB, but rather interferes with brain endothelial cells containing LPS binding sites (Singh and Jiang, 2004). On the other hand, a new study carried out on rats injected with radioactive-iodine-labeled LPS (I-LPS) suggests that some measurable amount of LPS crosses the BBB (Banks and Robinson, 2010). The results of these studies could have been affected by insufficiently sensitive LPS detection methods. It is worth mentioning, however, that BBB permeability is not constant, but depends on the physiological state of animals. It was previously found that BBB permeability could be affected by the level of gonadal steroids (Wilson et al., 2008), melatonin (Turgut et al., 2007), and stress (Esposito et al., 2001). So it can be speculated that the varied results on the ability of LPS to cross the BBB could be, at least partially, caused by the different physiological states of the animals used in those studies. Moreover, it has been conclusively proven that peripheral inflammation, especially through the action of numerous inflammatory mediators, significantly increases BBB permeability (Zhou et al., 2011). Nevertheless, LPS itself can affect BBB function and cause the endothelial cells of brain microvessels to express TLR4. It was previously found that TLR4 could be involved in the regulation of BBB

function and permeability (Singh and Jiang 2004; Feng et al., 2007). The transduction of the signal of this receptor leads to the synthesis of pro-inflammatory cytokines, nitric oxide (NO), and other cytotoxic compounds (Kaisho and Akira, 2006). It was shown that the stimulation of brain endothelial cells by LPS leads to the release of IL-1β and NO into the cerebrospinal fluid, initiating an inflammatory response in the brain. This leads to activation of microglia cells and secretion of pro-inflammatory cytokines IL-1β, IL-6, and TNFα (Feng et al., 2007). In turn, the elevated levels of these cytokines increase the permeability of the BBB (Feng et al., 2007; Zhou et al., 2011). It is worth stressing that the increase in *TLR4* gene expression observed in our study occurred concomitantly with the enhancement of *Tollip* gene expression. A stimulating effect of LPS on the Tollip protein level has been found previously *in vitro* in THP-1 cells, as well as in human primary blood mononuclear cells. Increased protein stability upon LPS challenge was likely the cause for the Tollip protein increase (Li et al., 2004). Another *in vitro* study also showed the stimulatory effect of LPS on Tollip gene and protein expression levels in intestinal epithelial cells (Otte et al., 2004). The upregulation of *Tollip* gene expression could be an attempt of the organism to prevent excessive development of the inflammatory response in the central nervous system through modulation of TLR4-mediated cellular responses by suppression of phosphorylation and kinase activity of IRAK.

Other data suggesting that LPS and its receptor system could play a role in the central regulation of processes during inflammation come from a study performed on rats. Central injections of LPS have been shown to induce brain expression of pro-inflammatory cytokines and to depress social behavior in rats, increase the duration of immobility, and induce body weight loss (Bluthé et al., 1999). Moreover, endotoxin given experimentally to the brain region affected the functioning of the central nervous system and led to strong suppression of the peripheral LH level (Ebisui et al., 1992). A study performed on chimeric mice showed that TLR4 is crucial for sustained inflammation in the brain after peripheral administration of LPS and that its function in CNS-resident cells is independent of systemic cytokine effects (Chakravarty and Herkenham, 2005).

Conclusions

The results of our study show that peripheral immune challenge affects *TLR4* gene expression in the hypothalamic area, suggesting that this receptor is involved in the inflammatory response in the CNS. On the other hand, the concomitant elevation of Tollip gene expression seems to be one of the mechanisms through which the organism regulates the intensity of this response. The mechanism by which peripheral administration of LPS affects TLR4 and Tollip transcription levels in the CNS still requires detailed research, however.

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