Acute hypothalamo-pituitary-adrenal axis response to LPS-induced endotoxemia: expression pattern of kinin type B1 and B2 receptors

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Abstract: Bradykinin (BK) and des-Arg^9-BK are pro-inflammatory mediators acting via B2 (B2R) and B1 (B1R) receptors, respectively. We investigated the role of B2R and B1R in lipopolysaccharide (LPS)-induced hypothalamo-pituitary-adrenal (HPA) axis activation in SD rats. LPS given intraperitoneally (ip) up-regulated B1R mRNA in the hypothalamus, both B1R and B2R were up-regulated in pituitary and adrenal glands. Receptor localization was performed using immunofluorescence staining. B1R was localized in the endothelial cells, nucleus supraopticus (SON), adenohypophysis and adrenal cortex. B2R was localized nucleus paraventricularis (PVN) and SON, pituitary and adrenal medulla. Blockade of B1R prior to LPS further increased ACTH release and blockade of B1R 1 h after LPS decreased its release. In addition, we evaluated if blockade of central kinin receptors influence the LPS-induced stimulation of hypothalamic neurons. Blockade of both B1R and B2R reduced the LPS-induced c-Fos immunoreactivity in the hypothalamus. Our data demonstrate that a single injection of LPS induced a differential expression pattern of kinin B1R and B2R in the HPA axis. The tissue specific cellular localization of these receptors indicates that they may play a crucial role in the maintenance of body homeostasis during endotoxemia.

Keywords: B1R; B2R; c-Fos; HPA-axis; receptor localization; sepsis.

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Introduction

Endotoxemia is a severe stress situation and demands necessary adaptive and defensive mechanisms directed against the stressor to maintain the body homeostasis. Bacterial lipopolysaccharide (LPS) applied intraperitoneally (ip) mimics sepsis and stimulates the hypothalamo-pituitary-adrenal axis (HPA axis) (Leshin and Malven, 1984; Beishuizen and Thijs, 2003). The HPA axis response to LPS is due to the release of cytokines from stimulated peripheral immune cells, which in turn stimulate a number of physiological systems at different levels of the HPA axis (Bertok, 1998). During sepsis, the major hypothalamic component involves corticotropin-releasing hormone (CRF), which is released into the pituitary portal circulation and drives the release of ACTH from the anterior pituitary. ACTH, in turn stimulates the synthesis and secretion of cortisol/corticosterone (CORT) from the adrenal cortex.

CORT is a pluripotent hormone acting on multiple tissues to regulate numerous aspects of metabolism, growth and physiological functions, which makes it essential for survival in critical illness, as in septic shock (Munck and Guyre, 1986). Systemic LPS injection also stimulates the synthesis and secretion of aldosterone (ALDO) from the adrenal glands (Sanchez-Lemus et al., 2008).

Bradykinin (BK) and des-Arg^9-BK are biologically active peptides that act on type 2 (B2R) and type 1 (B1R) receptors, respectively. They produce a variety of pathophysiological responses, including pain, hyperalgesia, increased vascular permeability and vasorelaxation, thus implicating them in inflammatory and cardiovascular diseases (Bhoola et al., 1992; Marceau et al., 1997; Couture et al., 2001; Marceau and Regoli, 2004). These receptors differ in their expression pattern: the B2R is constitutively expressed, whereas the B1R is weakly expressed in most tissues under physiological conditions but strongly up-regulated during tissue injury, inflammation and some pathological conditions (Marceau and Bachvarov, 1998; Couture and Lindsay, 2000; Leeb-Lundberg et al., 2005). Up-regulation of B1R during LPS-induced endotoxemia has been shown in different organs such as porcine arterial smooth muscle cells (Schremmer-Danninger et al., 1996), mouse heart (McLean et al., 1999) and mouse kidney (Seguin et al., 2008), indicating a crucial role in the modulation of organ function and ultimately in the maintenance of body homeostasis in endotoxemia. This was confirmed by studies performed in B1R transgenic rats (Merino et al., 2008) and B1R-KO mice (Pesquero et al., 2000) in our laboratory. In our previous studies, we demonstrated a differential regulation of B1R and B2R in the HPA axis in different animal disease models such as in spontaneously hypertensive rats (Qadri et al., 2002, 2003); and in rat models of type 1 and type 2 diabetes, such as streptozotocin-induced and Zucker diabetic fatty rats, respectively (Qadri et al., 2004).

To our knowledge, the expression patterns and potential functions of kinin B1R and B2R in the HPA axis during LPS-induced endotoxemia have previously not been shown. Therefore, the main aim of the present study was to investigate the role of kinins in LPS-induced HPA axis activation. First, we studied the effect of a single injection of LPS given ip on the expression pattern of B1R and B2R at mRNA level in the hypothalamus, pituitary and adrenal glands (neuroendocrine pathway), and also in dorsal and ventral medulla of the brainstem (autonomic/sympathetic pathway). Both pathways are important in the maintenance of body homeostasis. As alterations occur in immediate hormonal release and gene expression early in the course of sepsis, all analyses were carried out at 3 different time points, i.e. 1, 3 and 6 h after a single LPS ip administration. Second, we characterized the immunohistological and cellular localization of B1R and B2R in the HPA axis after LPS injection. In addition, the role of kinin receptors on LPS-induced hormonal secretion into the systemic blood was evaluated.

Results

Effect of LPS given ip on B1R and B2R mRNA levels in the HPA axis

Figure 1 shows LPS-induced B1R and B2R mRNA levels in the HPA axis. Compared to vehicle-treated rats, LPS induced a significant and time-dependent increase in B1R mRNA in the hypothalamus, without affecting the B2R mRNA levels [(A) and (B) left panel]. In the pituitary gland, LPS induced a marked increase in both B1R and B2R mRNA levels after 1 h and the expression remained up regulated all the time [(A) and (B) middle panel]. Moreover, there was a time-dependent and significant increase in B1R and B2R mRNA at 3 and 6 h after systemic LPS [(A) and (B) right panel]. An additional quantitative real-time PCR analysis was performed using cDNA from 3 h LPS (ip) animals in hypothalamus, pituitary and adrenal glands to give more credibility to the data. The seim-quantitative and quantitative real-time data was comparable (Supplemental Figure 1).

LPS-induced B1R and B2R mRNA levels were also measured in the brainstem. Only 6 h after LPS injection, a significant increase in the B1R mRNA level was found both in dorsal medulla (DM) and ventral medulla (VM) of the brainstem, but B2R mRNA levels remained unchanged (Supplemental Figure 2).

LPS-induced immunofluorescence localization of B1R and B2R in the HPA axis

Conformation of antibody specificity

Next we analyzed the protein expression of B1R and B2R in the HPA axis after LPS stimulation. To this purpose, we first verified the specificity of the antibodies. Very often, unspecific immunoreactivity of antibodies directed against G protein-coupled peptide receptors is found (Beck and Lock, 2014; Day, 2014). Therefore, we used B1R and B2R knock-out (KO) mouse tissue to verify the specificity of antibodies against B1R (developed by

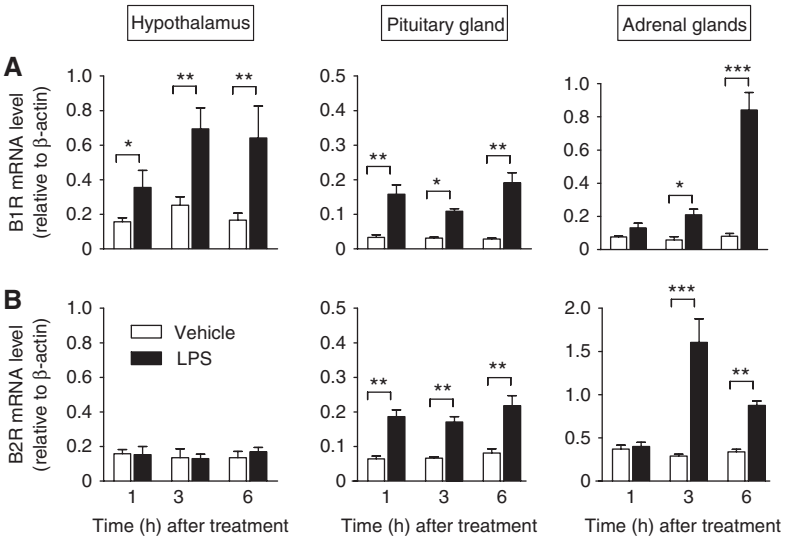

Figure 1: RT-PCR analysis of mRNA levels.
(A) Kinin B1R (upper panel) and (B) B2R (lower panel) mRNA levels normalized to β-actin mRNA in the hypothalamus, pituitary and adrenal glands 1, 3 and 6 h after a single injection of LPS (5 mg/kg, ip) or 0.9% saline which served as vehicle control in SD rats, as analyzed by RT-PCR. Values are mean±SEM (n=4 each group, treatment and each time point). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. vehicle. n=4/group/time point.

Dr. Leeb-Lundberg, Sweden) and B2R (commercially available from US Biologicals, USA), respectively. To verify the antibody against B1R, we used kidney tissue from adult male wild type (WT, C57BL/6 strain), B1R-KO, B2R-KO mice, both animals on C57BL/6 genetic background. From these mice, kidneys were removed 3 h after vehicle or LPS ip injection. To verify the specificity of B2R antibody, adrenal glands from untreated mice (described as above) were used. Five μm thick paraffin sections of kidney and adrenal glands were processed to stain B1R or B2R using immunofluorescence, respectively (Supplemental Figures 3 and 4). Before processing the tissue for immunofluorescence staining, real-time PCR was performed in snap frozen kidney or adrenal gland to confirm that B1R or B2R mRNA is absent in our KO mice, respectively (data not shown).

Localization of B1R and B2R in the hypothalamic SON

Figure 2 shows representative fluorescent microscopic images of SON in vehicle- and LPS-treated SD rats. Compared to vehicle treatment, LPS induced a strong up regulation of B1R in neurons located in the SON as well as in endothelial cells of the cerebral capillaries [(A) and (B), respectively]. The B2R immunopositive signal was detected in SON in both control and LPS-treated rats; however, there was no significant difference between them. Furthermore, there was no detectable B2R expression in the cerebral capillaries in vehicle- or LPS-treated rats [(C) and (D), respectively].

Localization of B1R and B2R in the hypothalamic PVN

Figure 3 shows representative fluorescent microscopic images of PVN in vehicle- and LPS-treated SD rats. There was no immunopositive signal for B1R in PVN, however, there was a weak signal in the basal region of the ependymal cell lining of the third ventricle in LPS-treated PVN [(A) and (B), respectively]. On the other hand, a B2R immunopositive signal was detected in PVN of both control and LPS-treated rats, however, there was no significant difference in B2R signal between them [(C) and (D), respectively].

Localization of B1R and B2R in the pituitary gland

Figure 4 shows representative fluorescent microscopic images of pituitary gland of vehicle- and LPS-treated SD rats. Compared to vehicle-treated rats, B1R in LPS-treated rats was highly up regulated in adenohypophysis, whereas a very weak signal was found in neurohypophysis and no signal was found in pars intermedia. Interestingly, B1R was also up regulated in the endothelial cells of the capillaries found in all parts of the gland (B).

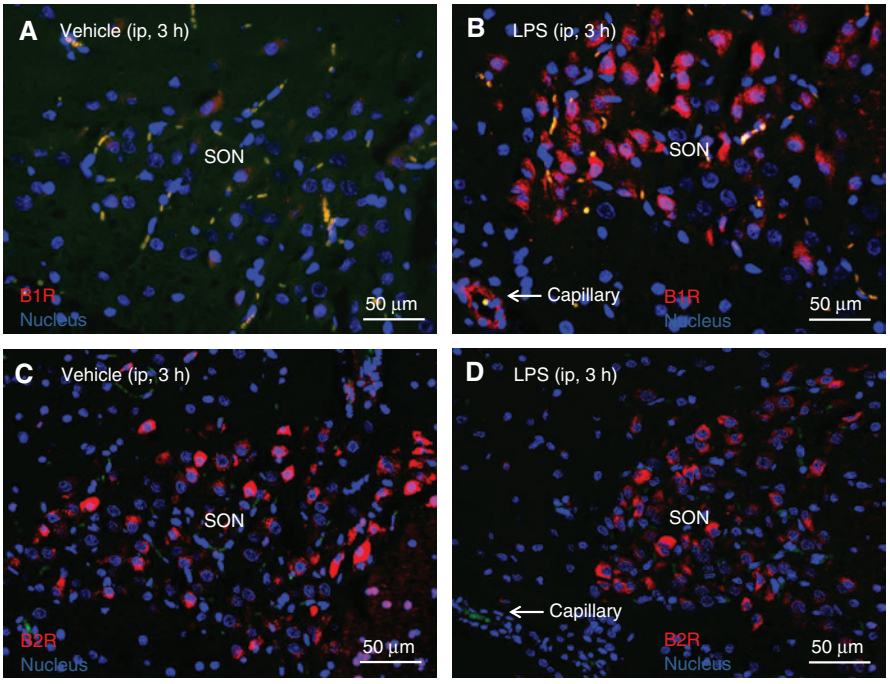

Figure 2: Immunofluorescence localization of kinin B1R and B2R in hypothalamic supraoptic nucleus (SON) from LPS (ip)-treated or vehicle-treated SD rats.
Hypothalamic 5 µm thick paraffin sections were labeled with primary antibodies against B1R (1:200) [upper panel (A) and (B)] or B2R (1:200, US Biologicals) [lower panel (C) and (D)], followed by anti-rabbit Cy3-conjugated secondary antibodies. DAPI stained nuclei (blue). Green/yellow dots are auto-fluorescent red blood cells. n=3/group and 4–5 Sections from each animal were stained for kinin receptors.

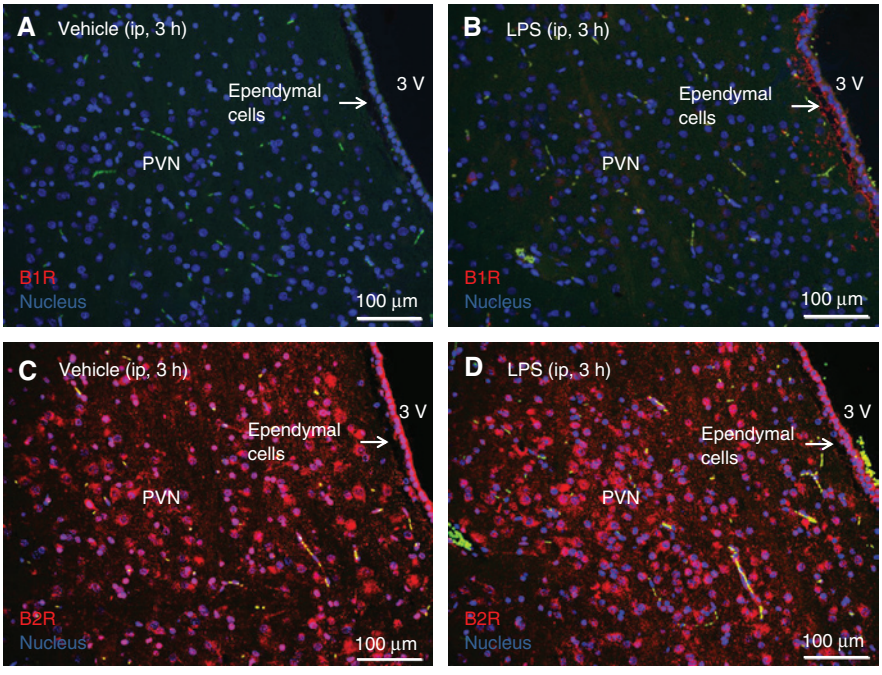

Figure 3: Immunofluorescence localization of kinin B1R and B2R in hypothalamic paraventricular nucleus (PVN) from LPS- or vehicle-treated (ip) SD rats.
Hypothalamic 5 µm thick paraffin sections were labeled with primary antibodies against B1R (1:200) [upper panel (A) and (B)] or B2R (1:200, US Biologicals) [lower panel (C) and (D)], followed by anti-rabbit Cy3-conjugated secondary antibodies. DAPI stained nuclei (blue). Green/yellow dots are auto-fluorescent red blood cells. n=3/group and 4–5 Sections from each animal were stained for kinin receptors. PVN, Nucleus paraventricularis; 3 V, brain third ventricle.

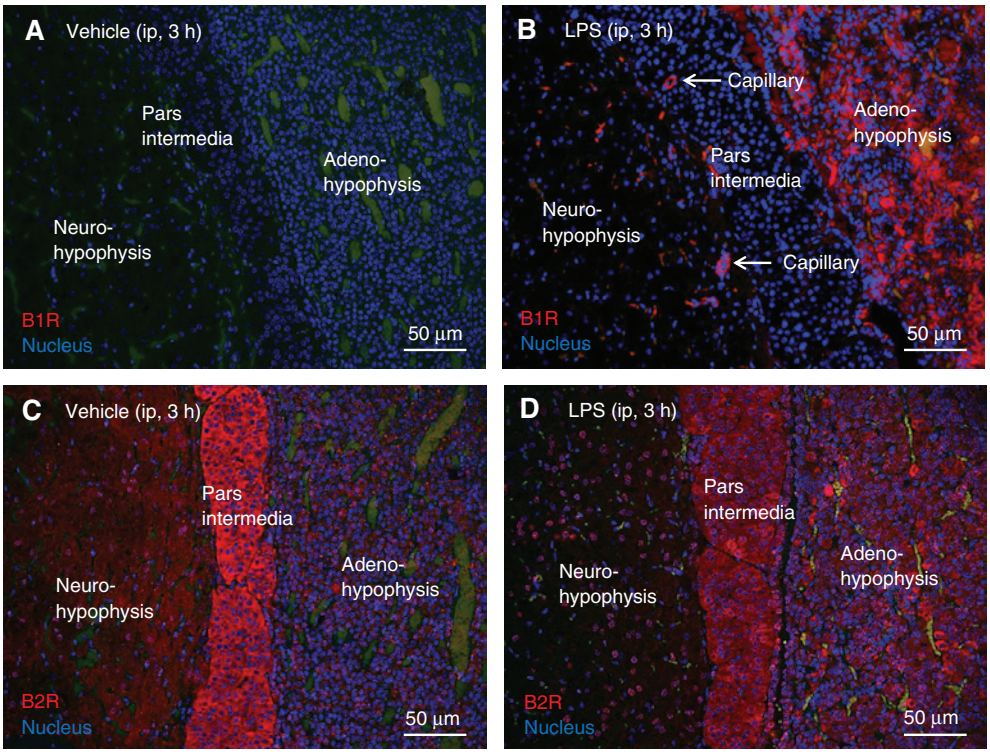

Figure 4: Immunofluorescence localization of kinin B1R and B2R in pituitary glands from LPS (ip)-treated or vehicle-treated SD rats. Three µm thick paraffin sections of pituitary were labeled with primary antibodies against B1R (1:200) [upper panel (A) and (B)] or B2R (1:200, US Biologicals) [lower panel (C) and (D)], followed by anti-rabbit Cy3-conjugated secondary antibodies. DAPI stained nuclei (blue). Green/yellow dots are auto-fluorescent red blood cells. n=3/group and 4–5 Sections from each animal were stained for kinin receptors.

Under physiological conditions, B2R is constitutively expressed in almost all tissue. In the pituitary, we observed that B2R was localized not only in the cytoplasm but also in the nucleus of the cells located in the adeno and neurohypophysis, whereas this receptor was localized primarily in the cytoplasm of pars intermedia cells (C). Following LPS-treatment, there was an increase in B2R expression both in adeno and neurohypophsis, where as a reduced expression occured in pars intermedia (D).

Localization of B1R and B2R in the adrenal gland

Figure 5 shows representative fluorescent microscopic images of adrenal glands from vehicle- and LPS-treated SD rats. There was clear and differential expression of B1R and B2R following LPS stimulation. LPS-treatment induced an increase in B1R in the adrenal cortex as compared to vehicle (A and B). Interestingly, there was a differential expression in the adrenal cortex: very high signal in zona glomerulosa, weak signal in zona fasciculata and no signal in zona reticularis. As we found in the pituitary gland, the B1R expression in the blood vessels around the adrenal gland was also up regulated. B2R were highly up regulated in adrenal medulla following LPS treatment vs. control (C and D).

Effect of B1R on LPS-induced plasma ACTH, CORT and ALDO levels

In order to evaluate the importance of the increased LPS-induced B1R expression, we inhibited the B1R centrally and assessed the effect on HPA activation. Compared to vehicle treated rats, LPS ip induced a significant and time-dependent increase in plasma ACTH, CORT and ALDO levels (Figure 6). The maximum plasma levels were observed at 1 h following LPS and decreased gradually with time. Pretreatment of rats with B1R antagonist R-715 (icv) 10 min prior to LPS ip administration further increased the plasma ACTH level without affecting either CORT or ALDO [(A) left panel]. On the other hand, icv application of the specific B1R antagonist R-715 for 1 h after LPS ip administration significantly decreased plasma ACTH levels without affecting CORT or ALDO plasma concentrations [(B) right panel].

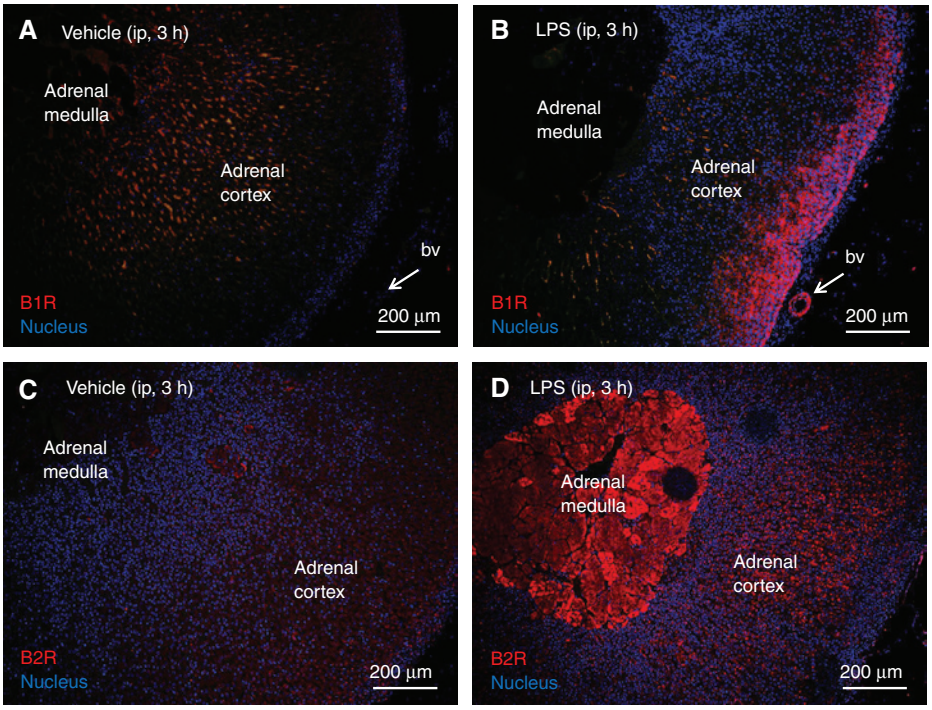

Figure 5: Immunofluorescence localization of kinin B1R and B2R in adrenal glands from LPS (ip)-treated or vehicle-treated SD rats. Adrenal 5 µm thick paraffin sections were labeled with primary antibodies against B1R (1:200) [upper panel (A) and (B)] or B2R (1:200, US Biologicals) [lower panel (C) and (D)], followed by anti-rabbit Cy3-conjugated secondary antibodies. DAPI stained nuclei (blue). Green/yellow dots are auto-fluorescent red blood cells. n=3/group and 4–5 Sections from each animal were stained for kinin receptors.

Effect of B1R and B2R on LPS-induced c-Fos expression in the subfornical organ and hypothalamus

As a second parameter of HPA axis activation, we studied the LPS-induced activation of neurons in the hypothalamus by c-Fos staining after inhibition of B1R and B2R. The expression of c-Fos protein in the subfornical organ (SFO) and hypothalamus was undetectable in rats treated with vehicle [Figure 7 (A)]. Ninety minutes following LPS injection, there was a marked increase in c-Fos immunoreactivity in SFO and within a set of hypothalamic structures. Very high density of c-Fos immunolabelled cells were found in the hypothalamic supraoptic nucleus (SON) and paraventricular nucleus (PVN) [Figure 7 (B)]. In posterior hypothalamic areas, LPS treatment had no effect on c-Fos expression (data not shown). Few and scattered c-Fos immunopositive cells were recognized in the lateral hypothalamus. When compared to LPS-treatment alone, pretreatment of rats with the B1R antagonist R-715 prior to LPS highly reduced the c-Fos expression in SFO and PVN and weakly in SON [Figure 7 (C)] middle panel). On the other hand, the specific B2R antagonist Hoe 140, also given icv prior to LPS, weakly reduced the c-Fos expression in SFO (mainly upper part of it), SON and PVN [Figure 7 (D)].

Discussion

Induction of kinin B1R and B2R in hypothalamus in response to systemic LPS challenge

We have shown an acute up regulation of B1R at mRNA and protein levels in the hypothalamus after a single injection of LPS applied systemically. The mRNA and protein expression of B1R was correlated with the induction of LPS-induced c-Fos expression in hypothalamic SON. The SON together with hypothalamic PVN are the key site for integrating neuroendocrine, autonomic and behavioral adjustments to diverse homeostatic challenges, including patho-physiological (e.g. inflammation) and emotional stressors (e.g. restraint) (Sawchenko, 1991). It has been shown by many authors that concomitant with an increase in plasma ACTH, LPS administration (ip or iv) resulted in an activation of neurons in the hypothalamic

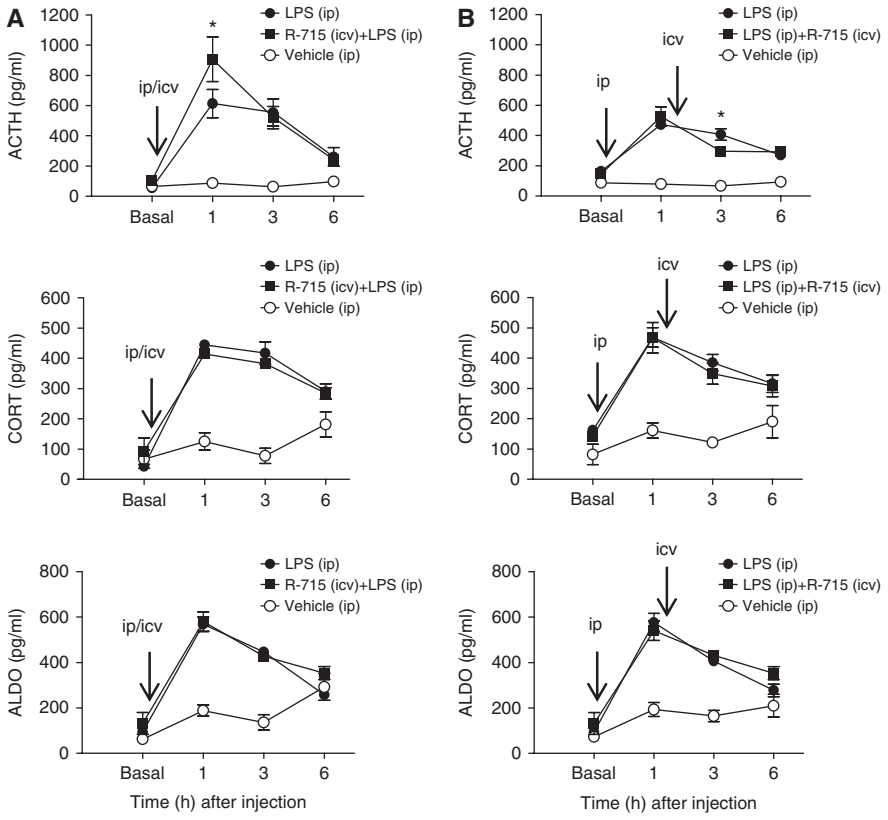

Figure 6: (A) Plasma ACTH, CORT and ALDO levels in basal, 1, 3 and 6 h after a single injection of LPS (5 mg/kg, ip) or vehicle (0.9% saline, ip) and pre-treatment with kinin B1R antagonist R-715 (1 μg/10 μl, icv) 10 min prior to LPS (ip). (B) Plasma ACTH, CORT and ALDO levels in basal, 1, 3 and 6 h after a single injection of LPS (5 mg/kg, ip) or vehicle (0.9% saline, ip) and treatment with kinin B1R antagonist R-715 (1 μg/10 μl, icv) 1 h after LPS (ip) injection. n=4–6/group. ACTH, Adrenocorticotropine releasing hormone; CORT, corticosterone; ALDO, aldosterone; LPS, bacterial lipopolysaccharide; ip, intraperitoneal; icv, intracerebroventricularly.

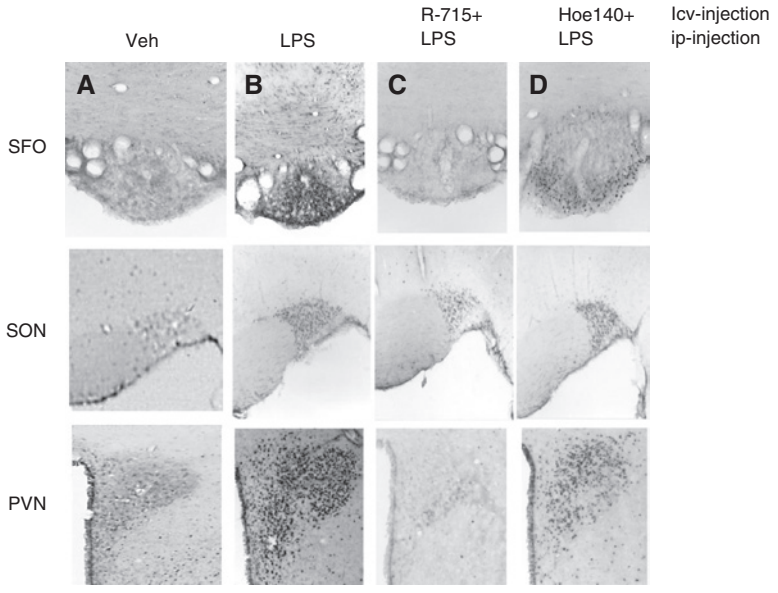

Figure 7: Immunohistochemical localization of c-Fos immunoreactivity in hypothalamic supraopticus and paraventricular nuclei. Analysis was performed at 90 min after a single LPS injection (5 mg/kg, ip) (B); effect of blockade of B1R with R-715 (1 μg/10 μl, icv) 10 min prior to LPS (ip) injection (C) and effect of blockade of B2R with Hoe 140 (1 μg/10 μl, icv) 10 min prior to LPS (ip) (D) and compared with vehicle-treated control rats (A). n=3/group. LPS, Bacterial lipopolysaccharide; SON, hypothalamic supraoptic nucleus; PVN, hypothalamic paraventricular nucleus; icv, intracerebroventricular; ip, intraperitoneal.

SON and PVN as indicated by the enhanced expression c-Fos (Hare et al., 1995; Takemura et al., 1997; Reyes et al., 2003; Xia and Krukoff, 2003).

In the current study, besides hypothalamic SON/PVN, we also observed LPS-induced c-Fos expression in the subfornical organ (SFO). SFO is a highly vascularized region and is devoid of blood-brain-barrier and it has direct connections to hypothalamic SON/PVN: It is established that SFO co-ordinates various physiological systems involved in the maintenance of body fluid homeostasis (Miselis 1981; Lind et al., 1982; Johnson et al., 1996). It 'senses' the changes occurring in systemic humoral factors induced by, for example, LPS and conducts these information to the hypothalamic SON and PVN. Accordingly, ablation of SFO significantly reduced LPS-induced c-Fos expression in SON and PVN, and also affected neuro-hormonal secretion (Borges and da Rocha, 2006). Due to its location in the brain, substances have direct access to SFO when injected into the lateral brain ventricle. In the present study, pretreatment of rats with kinin B1R or B2R antagonists given into the lateral brain ventricle reduced the LPS-induced c-Fos expression not only in SFO but also in hypothalamic SON and PVN. It is not fully clarified, whether LPS directly binds to its receptors in SFO or affects it via pro-inflammatory cytokines such as IL-1, IL-6 or TNFα or prostaglandins. There is, however, limited evidence that SFO possess receptors for cytokines and prostaglandins (Cartmell et al., 1999; Zhang and Rivest, 1999, 2000). Our present data allow us to suggest that during endotoxemia pro-inflammatory factors may bind to their receptors in SFO and activate 'kininergic' pathway(s) leading to the hypothalamus. The increase in B1R expression in the hypothalamus may function as a mediator between systemic endotoxin and the initial defense reaction of the CNS (activation of HPA axis) to release stress hormones into portal circulation of pituitary gland. Our present data is in line with the data published by Coelho et al. (1997), irrespective of the route of LPS application, who have demonstrated kinin B1R induction plays an important role in LPS-induced febrile response.

Besides hypothalamus, brain stem regions such as dorsal (DM) and ventral (VM) medulla play important roles in maintaining vascular homeostasis. There are ascending noradrenergic projections from DM and VM to hypothalamic PVN (Sawchenko and Swanson, 1981), which were activated upon systemic LPS challenge (Cunningham et al., 1994; Gaykema et al., 2007; Bienkowski and Rinaman, 2008). We found an increase in B1R mRNA levels both in DM and VM 6 h after LPS injection and no change in B2R mRNA levels. This shows a low susceptibility of the brainstem regions to acute LPS when compared to hypothalamus, where B1R was highly expressed directly after systemic LPS. This may affirm that 'kininergic' action of the hypothalamus is crucial to systemic LPS exposure and the medullary regions (DM and VM) play less important roles during acute endotoxemia.

Induction and differential localization of kinin B1R and B2R in pituitary in response to systemic LPS challenge

Systemic LPS can directly or indirectly (via cytokines) influence the pituitary gland, since part of the gland lacks a blood-brain barrier. In the present study, a high susceptibility of pituitary gland to systemic LPS was observed. A significant elevation of both B1R and B2R mRNA and protein levels were found at 1 h after LPS exposure. Our finding that systemic LPS induced a specific and differential expression of B1R and B2R in defined parts of this endocrine gland suggests a modulatory role of kinins in synthesis/release of neuro hormones.

The maximal increase in ACTH release was observed at 1 h following LPS ip. Interestingly, the pretreatment of rats with the B1R antagonist (icv) prior to LPS exposure resulted in a significant increase in ACTH release compared to systemic LPS. A possible explanation could be that the further increase in ACTH release could be due to the endogenous central kinin B2R. Madeddu et al. (1992) have demonstrated that BK when applied icv increased the concentration of plasma ACTH via B2R. On the other hand, blocking of 'induced B1R' centrally during endotoxemia inhibited the neuronal pathway leading to SON/PVN and hence decreased the release of ACTH from pituitary gland. This could be a possible explanation for the reduction in plasma ACTH concentration when B1R antagonist was applied (icv) 1 h after the LPS ip, as found in our current data. Interestingly, we observed a positive immunostaining for B2R not only in cytoplasm but also in the nuclei of pituitary cells found mainly in the neurohypophysis and it seems that systemic LPS increased the nuclear expression of B2R. Further investigations are necessary to elucidate the precise function(s) of nuclear B2R in the pituitary gland during endotoxemia.

Induction and differential localization of B1R and B2R in adrenal gland in response to systemic LPS challenge

In the present study, adrenal glands responded with a different time course to systemic LPS from hypothalamus

and pituitary gland. We observed the maximum increase of B1R mRNA at 6 h and of B2R at 3 h after LPS. Interestingly, there was differential cellular localization of B1R and B2R in adrenal gland indicating differential regulation of CORT, ALDO and/or monoamines. A higher B1R immunofluorescent signal was observed in zona glomerulosa compared to CORT secreting zona fasciculata. On the bases of cellular localization in the adrenal cortex, it can be postulated that LPS-induced up-regulation of B1R may influence the secretion of both ALDO and CORT independent of central influence, since B1R antagonist given icv did not change the levels of these hormones. The most striking and interesting finding was that the adrenal medulla did not show B1R immunostaining. On the contrary, when compared to control, systemic LPS induced an up-regulation of B2R significantly in adrenal medulla. This may confirm our previous work where we showed increased endogenous B2R in the adrenal medulla from spontaneously hypertensive rats (Qadri et al., 2003, 2004). LPS also induced low levels of B2R expression in adrenal cortex compared to medullary expression. This may suggest that during endotoxemia induced B2R in adrenal cortex may also affect ALDO and CORT secretion (Rosolowsky and Campbell, 1992), whereas medullary B2Rs are crucial for monoaminergic neurotransmitter release and/or synthesis. Previously, BK via B2R has been shown to induce an increase in norepinephrine release from chromaffin and PC12 cells *in vitro* (Dendorfer and Dominiak, 1995; Dendorfer et al., 1996). Endotoxemia is known to be associated with increased sympathetic nerve activity and depletion of norepinephrine and epinephrine contents in the adrenal gland and in sympathetically innervated tissues (Wang et al., 2000). LPS-induced pro-inflammatory cytokines or LPS may directly affect B2R expression in the adrenal medulla resulting in catecholamine release. Since systemic LPS resulted in hypotension, it is conceivable that catecholamine released by adrenal gland after B2R activation counter-regulate the hypotension during endotoxemia.

Induction of endothelial B1R in response to systemic LPS challenge

Activation of kinin B1R and B2R leads to vascular dilatation via release of endothelial factors and could provide beneficial effects in cardiovascular disease therapy. On the other hand, induction of B1R activates inducible nitric oxide and NADPH oxidase, which are associated with vascular inflammation, increased permeability and cause endothelial dysfunction. A number of *in vitro* and *in vivo* studies have been published demonstrating the significance of B1R induction after various manipulations which causes severe diseases in animal models (Calixto et al., 2004, Duchene and Ahluwalia, 2009; Viel and Buck, 2011; Girolami et al., 2014; Bhat et al., 2014). Overexpression of B1R in endothelial cells increased the susceptibility and its gene deletion attenuated the LPS-induced endotoxic shock in rodents (Pesquero et al., 2000; Cayla et al., 2007; Merino et al., 2008). In the current study, upon systemic LPS, we observed an up-regulation of B1R expression in the endothelial cells in brain capillaries and in pituitary and adrenal glands. Further investigations are needed to find out, whether the upregulaton of B1R in endothelium is beneficial or deleterious to the animals during endotoxemia.

In conclusion, our current data demonstrated that systemic injection of LPS induced an early induction/up-regulation of kinin B1R in the hypothalamus, pituitary and adrenal glands. B1R cellular expression was tissue-specific and could modulate the neuronal functions, since blockade of B1R centrally reduced the neuronal c-Fos expression in the hypothalamus and affected the LPS-induced ACTH release. The tissue-specific up-regulation of B2R in pituitary and adrenal glands indicate their significance in release of hormones. The specific cellular localization of B1R and B2R in HPA axis will help us to study the role played by these receptors and their significance during endotoxemia.

Materials and methods

Animals

Male Sprague-Dawley (SD) rats (10–12 weeks) were purchased from Charles River (Sulzfeld, Germany). They were kept under controlled conditions with a 12-h light/dark cycle and had free access to standard rat diet and water. The experiments were conducted in accordance with the NIH Guide for the care and use of laboratory animals. Experimental protocols were approved by the state Government of Germany and also by the local Ethics Committee of the University of Lübeck, Germany.

Experimental protocols

The following experimental protocols were designed:

Determination of B1R and B2R expression at the mRNA level following injection of LPS: Rats were divided into two groups. Each rat from group 1 received a single injection of vehicle (0.9% saline, ip) and served as control, rats of group 2 received a single injection

of LPS (5 mg/kg body weight, ip) (*Escherichia coli* serotype 055:B5; Sigma, Taufkirchen-München, Germany). Rats were sacrificed 1, 3 and 6 h following ip injection (n=4/group and time point). Pituitary and adrenal glands were removed and frozen in isopentane kept on dry ice (-40°C). Hypothalamic block consisting of nucleus paraventricularis (PVN) and supraoptic nucleus (SON), part of lateral hypothalamus and posterior hypothalamus was dissected according to the method described by Palkovits and Brownstein (1988). Brainstem was removed *en bloc* under the posterior half of the cerebellum. It was divided into dorsal (DM) and ventral medulla (VM). The DM consisted of area posterema (AP), nucleus of the solitary tract (NTS) and core of the vagus and the VM consisted of caudo-ventrolateral medulla (CVLM), rostro-ventrolateral medulla (RVLM) including reticular nuclei. All tissue samples were frozen immediately and stored at -80°C until further use.

RNA isolation, cDNA synthesis and semi-quantitative RT-PCR analysis

The techniques used here were described earlier in detail (Qadri et al., 2002; Häuser et al., 2005). In short, hypothalamus, DM, VM, pituitary and adrenal glands were homogenized in the presence of guanidinium isothiocyanate. Total RNA was isolated, first strand cDNA was synthesized from 1 µg of total RNA. Specific B1R, B2R and β-actin sense and antisense oligonucleotide primers were designed based on their published cDNA sequences. The following primers were used: fw-GCATCCCCACATTCCTTCTA and rev-AAGAAGTGGTAAGGGCACCA *for B1R* (acc. #AJ132230, amplicon size 301bp); fw-CTTGGGTGAGCTCAGTGTCA and rev-AG GGGCAGACATTTGAAGG *for B2R* (acc. #M59967, amplicon size 299bp) and fw-GTGGGTATGGGTCAGAAGGA and rev-AGCGCGTAACCCTCATAGAT *for β-actin* (acc. #NM-031144, amplicon size 308bp). Semi-quantitative RT-PCR was performed using 3 µl of undiluted cDNA for kinin receptors and 3 µl of diluted cDNA (1:100) for β-actin, which served as house keeping gene.

Because of the high sensitivity of the PCR, several control experiments were performed to exclude false positive results as described previously (Qadri et al., 2002; Häuser et al., 2005). In brief, to monitor possible contamination with genomic DNA, a control RT-PCR was performed without the reverse transcriptase. PCR cycle numbers for each gene were optimized by kinetic analysis to ensure that the amplification is within the exponential phase of the PCR. To verify the specificity of the PCR, restriction analysis of amplicons was performed by incubating the amplicons with specific restriction enzyme which digested the PCR product into two distinct fragments (data not shown).

Immunohistological localization of LPS-induced B1R and B2R receptors in the HPA axis: Rats were divided into two groups. Each rat from group 1 received a single injection of vehicle (0.9% saline, ip, n=3) and served as control and from group 2 received a single injection of LPS (5 mg/kg body weight, ip, n=3). Rats were sacrificed 3 h after LPS injection (n=3/group). Whole brain, pituitary and adrenal glands were removed and fixed in 4% phosphate buffered PFA, processed and embedded in paraffin blocks. Five µm sections were cut (Microm HM355 S, MICROM International GmbH, Walldorf, Germany), deparaffinized, rehydrated and processed for immunostaining using a polyclonal antibody raised against human B1R (kindly donated by Dr. L.M. Fredrik Leeb-Lundberg), which cross-react with mouse and rat tissue (1:200) (Sandén and Leeb-Lundberg, 2013) and commercial available antibody against B2R (1:200) (US Biologicals, Biomol GmbH, Hamburg, Germany; catalog #B2676-02 and Lot #L61017766). Both B1R and B2R antibodies were tested for their specificity using B1R and B2R knock-out mouse tissue, respectively (Boyce et al., 1996; Pesquero et al., 2000). Secondary anti-rabbit antibody was conjugated to Cy3 (1:300, Jackson Immunoresearch Europe Ltd, Suffolk, UK). Sections were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Axxora, Lörach, Germany). Microscopic images were taken using the fluorescence microscope 'Keyence, BZ-9000' (Keyence Deutschland GmbH, Neu-Isenburg, Germany).

We used 4–5 Sections from each brain, pituitary and adrenal glands from vehicle- or LPS-treated rats. Brain sections were used comprising of hypothalamic PVN and SON. Care was taken in choosing the sections from pituitary and adrenal glands, comprising adenohypophysis, neurohypophysis and pars intermedia in the pituitary, and cortex and medulla in adrenal glands.

Determination of the involvement of central B1R in LPS (ip)-induced ACTH, CORT and ALDO release into the blood circulation

Experimental design 1

Intracerebroventricular (icv), intraperitoneal (ip) and intrafemoral catheters were implanted chronically in rats under anesthesia as described previously in detail (Qadri et al., 1991 and 1993). Experiments were performed between 7 am and 1 pm in conscious and freely moving and unrestraint rats. Rats were randomly allocated to three groups. Group 1 received vehicle (0.9% saline, ip) and served as control. Group 2 (n=4) received a single injection of LPS (5 mg/kg body weight, ip). The animals of group 3 (n=4) received 10 min prior to LPS (ip), R-715, a specific B1R antagonist (1 µg/10 µl vehicle, icv) (R-715 was kindly donated by Dr. D. Regoli to Dr. A. Dendorfer). Blood samples each 1 ml were collected from the femoral artery 1, 3 and 6 h after LPS injection. The blood volume was substituted with 1 ml 0.9% saline.

Experimental design 2

All experiments in this protocol were done as described above except that the B1R antagonist (R-715, 1 µg/10 µl vehicle, icv) was given 1 h after the LPS (ip) injection. Blood samples were collected at 1, 3 and 6 h to measure plasma ACTH, CORT and ALDO (n=4). The blood volume was substituted with 1 ml 0.9% saline. The plasma levels of ACTH, CORT and ALDO were measured using commercially available RIA kits (Linco Research Inc., MO, USA).

Determination of the effect of B1R and B2R on LPS-induced c-Fos expression in the hypothalamus: Several studies have demonstrated that the 'immediate early gene', *c-fos* is expressed in neurons whose activity is strongly stimulated by different challenges including systemic LPS-induced inflammation. Using immunocytochemistry the protein c-Fos can be localized in defined brain regions (Wan et al., 1994; Sagar et al., 1995). We used this method to determine the effect of B1R and B2R receptor on LPS-induced c-Fos expression in hypothalamus. Anesthetized animals were placed in a stereotaxic frame for the implantation of a chronic icv cannula (Qadri et al., 1991). In brief, a polypropylene cannula was inserted into the left lateral brain ventricle with the following coordinates: 0.6 mm caudal to bregma 1.3 mm lateral to the left side of the midline and 5.0 mm ventral from the dural surface according to 'The Rat Brain in Stereotaxic Coordinates' by G. Paxinos and C. Watson (1986). A chronic catheter was inserted intraperitoneal for the administration of LPS

or vehicle. Rats were housed individually for a 7 day post-operative recovery period. To avoid unspecific stress-induced c-Fos expression, animals were handled by the same experimenter every day for 10–15 min before the implantation of chronic catheters and until the experiment was performed.

Experimental design

Experiments were performed between 7 am and 1 pm in conscious freely moving and unrestraint rats (n=4/group). Group 1 received LPS (5 mg/kg body weight, ip). Group 2 received vehicle (0.9% saline ip) and served as control. The animals of group 3 received 10 min prior to LPS (ip) R-715, the specific B1R antagonist (R-715, 1 µg/10 µl vehicle, icv). The animals of group 4 were pretreated with Hoe 140, the specific B2R antagonist (Tocris, Bristol, UK) (1 µg/10 µl vehicle, icv) prior to LPS (ip). Ninety minutes after LPS injection, rats were anesthetized with sodium pentobarbital and perfused intracardial with 0.9% saline containing heparin and then with 4% phosphate buffered PFA. Brains were removed, post fixed overnight in the same fixative and incubated for approximately 72 h in 30% sucrose at 4°C for cryoprotection. Coronal sections (50 µm) were cut at -20°C (Leica CM3050S, Wetzler, Germany) and transferred into PBS at room temperature. The free-floating brain sections were processed for immunocytochemistry using the conventional avidin-biotin complex peroxidase reaction as recommended by the manufacturer (Vector Laboratories, Axxora, Lörach, Germany, UK). The dilution of primary anti c-Fos antibody was 1:3000 (BD Transduction Laboratories, Lexington, USA). Sections were mounted onto Super-Frost® slides (Menzel-Gläser, Braunschweig, Germany), dehydrated in alcohol, cleared in xylene and cover-slipped with the mounting medium Eukitt (Sigma-Aldrich, Taufkirchen-Münschen, Germany).

Statistical analysis

Data are expressed as mean±SEM. Differences in the expression of kinin receptors in HPA axis between vehicle treated control and LPS treated rats at each time point were evaluated by Student's *t*-test and (where necessary) one-way ANOVA followed by Bonferroni post-hoc test using the computer program GraphPad Prism (GraphPad Prism Software Inc). A value of $p<0.05$ indicated significant differences.

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