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Chemical sympathectomy attenuates lipopolysaccharide-induced increase of plasma cytokine levels in rats pretreated by ACTH

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ABSTRACT

The sympathetic nervous system participates significantly in the regulation of immune functions. In support of this, data indicate that besides vagal afferent and efferent pathway, sympathetic nerves represent crucial component of inflammatory reflex. In addition, it was shown that efferent arm of this reflex might be activated by ACTH. Therefore, we investigated the effect of chemical sympathectomy on lipopolysaccharide (LPS)-induced increases in plasma IL-1β, IL-6, and TNF-α levels in rats. Plasma IL-10 and corticosterone levels were also evaluated. We also investigated the effect of sympathectomy in rats pretreated with ACTH (1–24). We found that sympathectomy significantly attenuated LPS-induced increases of plasma IL-1β levels. Administration of ACTH (1–24) reduced LPS-induced increases of plasma IL-1β and IL-6 and exaggerated the rise of IL-10. In animals treated with ACTH (1–24) sympathectomy attenuated LPS-induced increases of IL-1β, IL-6, and IL-10 plasma levels. Plasma levels of TNF-α and corticosterone were not affected by any interventions. These data indicate that during acute immune challenge, sympathetic nerves stimulate the immune response. In addition, our data indicate that sympathetic nerves are not significantly involved in the anti-inflammatory effect of ACTH (1–24) and that the anti-inflammatory effect of ACTH (1–24) is independent of plasma corticosterone levels.

1. Introduction

The sympathetic nervous system (SNS) plays an important role in maintaining immune homeostasis (Elenkov et al., 2000; Wrona, 2006). This function of the SNS depends on the level of regulation (local, regional, systemic) and whether the activation of SNS is acute or chronic (Sternberg, 2006). It is thought that acute activation of SNS is adaptive as it potentiates immune cell activity, whereas chronic activation is maladaptive as it dysregulates immunity (Black, 2002; Dhabhar, 2014; Marsland et al., 2017). Importantly, immune challenge itself represents a factor (stressor) that activates the SNS (Collier et al., 2011).

In 2000, Borovikova et al. described a new neuronal mechanism regulating immune function. They found that electrical stimulation of vagal efferent pathways significantly reduced inflammatory responses at the periphery (Borovikova et al., 2000). Later studies showed that this effect is mediated *via* acetylcholine binding on α7ACh receptor localized at immune cells (Wang et al., 2003). It was also shown in laboratory animals that vagal anti-inflammatory pathway might be activated not only by electrical stimulation (Guarini et al., 2003; Meregnani et al., 2011), but also by mechanical stimulation (Huston et al., 2007), feeding animals by fat (de Haan et al., 2013; Lubbers et al., 2010), or by administration of chemical compounds, including ACTH (1–24) (Guarini et al., 2004). Even though initial studies indicated that the efferent pathways of the vagus nerve were able directly suppress immune cell activity (Tracey, 2002), data from later experiments questioned the exclusive role of vagal pathways in cholinergic anti-inflammatory mechanisms (Bratton et al., 2012). For example, the study of Vida et al. (2011) showed that in α7ACh receptor knock-out mouse, the anti-inflammatory effect of splenic sympathetic nerve stimulation on serum TNF-α is independent of the vagal cholinergic anti-inflammatory pathway. Based on recent data, it is suggested that efferent vagal fibers, component of cholinergic anti-inflammatory pathway, plays no exclusive role in the response to immune challenge, rather it is the splanchnic sympathetic nerves that mediate the anti-inflammatory effect of inflammatory reflex (Martelli et al., 2016; Martelli et al., 2014b).

Based on above-mentioned data showing crucial role of sympathetic nerves in inflammatory reflex and based on findings showing central activation of anti-inflammatory pathways by ACTH, we investigated the effect of sympathectomy on LPS-induced changes in plasma IL-1β, IL-6, TNF-α, IL-10, and corticosterone levels in rats treated by ACTH (1–24). We determined plasma cytokine and corticosterone levels over the first

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24 h after LPS administration.

2. Materials and methods

2.1. Experimental animals

A total of 24 adult male Sprague Dawley rats (275–300 g) were purchased from the AnLab **s.r.o.** (Prague, Czech Republic). Rats were housed 4 per cage under standard laboratory conditions using a 12/12 h light/dark cycle with free access to food and water. All experimental procedures were approved in accordance with the institutional guidelines of the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic and in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.2. Experimental design

After an acclimatization period of 7 days, a sympathectomy or sham intervention was performed in two randomly divided subgroups of animals (Fig. 1A). Then, 14 days after sympathectomy, a cannula was inserted into the jugular vein (Fig. 1B). The following day, after collection of baseline blood samples, rats were injected by saline or ACTH, exposed to LPS challenge, after which blood samples were collected for analysis (Fig. 1C).

2.3. Experimental procedures

2.3.1. Chemical sympathectomy

A chemical sympathectomy was performed in conscious animals (6-OHDA group) by intraperitoneal injection of 6-OHDA (100 mg/kg body weight, Sigma-Aldrich, Germany) over three consecutive days. The 6-OHDA was dissolved in sterile saline containing 0.1% of the antioxidant ascorbic acid (Sigma-Aldrich, Germany). This treatment has been shown to induce the destruction of peripheral sympathetic nerve endings after 3–5 days and this effect lasts for at least 21 days (Vo and Tomlinson, 1999). Efficiency of the sympathectomy was confirmed by the presence of ptosis in the sympathectomized rats (Claude Bernard-Horner's syndrome) as well as by the presence of blood in urine immediately after 6-OHDA application, indicating the destruction of sympathetic nerve endings in the urinary tract. Sham sympathectomy was performed in the control group by injection of an equivalent volume of saline.

2.3.2. Jugular vein cannulation

One day prior to the exposure of rats to immune challenge (Fig. 1B), the jugular vein was cannulated as previously described (Thrivikraman et al., 2002). The cannula allowed for repeated blood sampling and administration of saline or ACTH (1–24) with minimal stress to the animal.

Briefly, animals were anesthetized with a mixture of ketamine-xylazine as described above. The rats were then fixed in a supine position to an acrylic surgery platform. The area of incision (15 mm long) was on the right shoulder close to the base of the neck. To reach the jugular vein it was necessary to separate the surrounding muscle and membranous tissue. The exposed jugular vein was then cut with spring-scissors on the upper surface and a polyethylene tube (silicon tubing, PE 50; Becton-Dickinson, Parsippany, NJ) filled with heparinized saline (300 IU/mL) was carefully placed into the right jugular vein. About 0.1 mL of the heparinized saline was then infused through the catheter and a slow withdrawal of blood was attempted to confirm correct cannulation procedure. The cannula was then tied to the vein with rostral and caudal ligatures to avoid occluding the cannula. Following this, the rat was removed from the platform and a small incision was made in the center of the rat's nape with a trocar. The end of the cannula was advanced into the trocar, exteriorized at the other end of the ventral incision, and then attached with medical thread. After cannulation, the rats were housed individually. Exposure to drugs and blood sampling started on the next day after an overnight recovery. The duration of the one day recovery was selected according to our previous experiments and findings showing that jugular vein cannulation does not increase baseline cytokines and corticosterone levels or alter stress responses in animals exposed to stressors one or more days after the procedure (Ling and Jamali, 2003).

2.3.3. Drug administration and blood sampling

On the final day of the experiment (24th day, Fig. 1B), the first blood samples (baseline) were collected *via* the jugular cannula from freely moving animals while housed in their home cages. As replacement for the lost volume (0.6 mL) of blood, an equal volume of heparinized saline (50 IU/mL) was administered by cannula. After collection of baseline blood samples, saline (2 mL of saline/kg bw) or ACTH (1–24) (Sigma-Aldrich; 160 μg of ACTH dissolved in 2 mL saline/kg bw) was administered *via* jugular cannula (1st injection, Fig. 1A, C). 5 min later all rats were intraperitoneally injected with LPS (*Escherichia coli* serotype 055:B5, Sigma-Aldrich; 50 μg dissolved in 2 mL/kg bw) (2nd injection, Fig. 1A,C). One hour after LPS administration, the second blood sample was collected. Two and half hours after LPS administration, animals were injected intravenously by saline or ACTH (1–24) (3rd injection, Fig. 1A,C). Furthermore, additional blood samples were collected 3, 6, 9, and 24 h after LPS injection (Fig. 1C).

The doses of both ACTH (1–24) (160 μg/kg bw) and LPS (50 μg/kg bw) used in this experiment were chosen according to published data (Altavilla et al., 1998; Benicky et al., 2009; Guarini et al., 2004; Sanchez-Lemus et al., 2009; Sanchez-Lemus et al., 2008) and all substances were freshly prepared.

2.3.4. Determination of plasma cytokine and corticosterone levels

Following sample collection, the blood vials were immediately placed on ice and centrifuged at 10,000 $\times g$ for 10 min at 4 °C to separate the plasma, which was then stored at −20 °C until analyzed. Plasma samples were assayed for IL-1β, IL-6, TNF-α, and IL-10 using commercial rat IL-1β, IL-6, TNF-α, and IL-10 Rat MultiAnalyte Profiling Base Kit Fluorokine MAP (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions using a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA, USA). Assays were sensitive to 5 pg/mL of IL-1β, 21 pg/mL of IL-6, < 5 pg/mL of TNF-α, and < 10 pg/mL of IL-10 with inter- and intra-assay coefficients of variation < 10%. Plasma corticosterone concentration was determined by a commercially available radioimmunoassay kit (Corticosterone rat/mouse RIA kit, DRG Diagnostic, Germany) with a minimum detection limit for corticosterone of 7.7 ng/mL.

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego CA, USA). Statistical differences among the groups were determined by one- or two-way analyses of variance (ANOVA), followed by *post hoc* pair wise comparisons using Bonferroni's correction. The total amount plasma levels of cytokines and corticosterone were calculated as the area under the curve (AUC). Differences were considered statistically significant at $P < .05$. Data are expressed as mean ± SEM and represent the mean for 4–6 rats.

3. Results

3.1. Effect of sympathectomy on lipopolysaccharide-induced changes of plasma cytokine levels

The first goal of our experiment was to investigate the effect of sympathectomy on the dynamics of plasma cytokine levels in rats

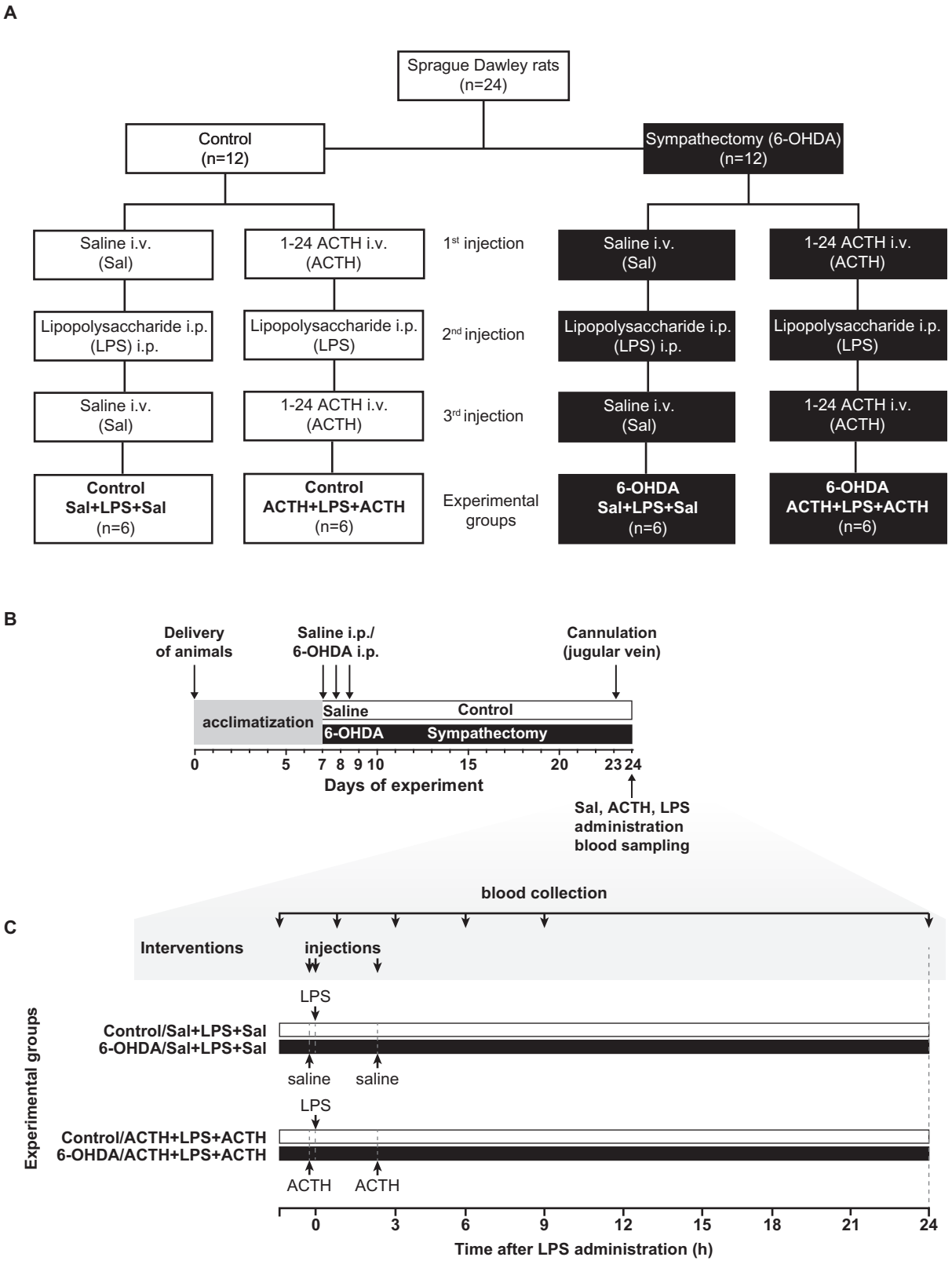

Fig. 1. Schematic illustration of our experimental design. Animals with intact sympathetic nerves (Control; □) and sympathectomized rats (6-OHDA, ■) were used in this experiment (A). At the 7th, 8th and 9th day of the experiment, animals from the 6-OHDA group were injected with 6-hydroxydopamine, while control (sham) animals received an equivalent injection of saline. Two weeks later, a jugular cannulation was performed on control and sympathectomized animals (B). The next day, animals were pretreated by saline or ACTH and then exposed to an immune challenge (LPS). Blood sampling was performed before the administration of drugs in addition to up to 24 h following LPS injection (C).

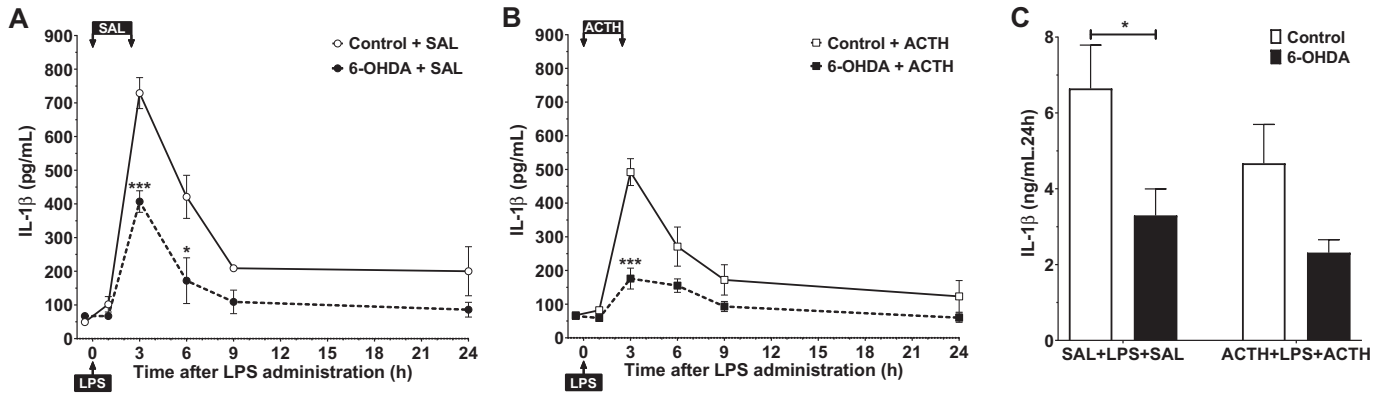

Fig. 2. Effect of an intraperitoneal injection (i.p.) of lipopolysaccharide (LPS) on plasma levels of IL-1β in animals with intact sympathetic nerves (Control; ○/□) and sympathectomized rats (6-OHDA; ●/■) pretreated by saline (A) or ACTH (B). The total amount of IL-1β in plasma was calculated as the area under the curve (AUC) (C). Data are expressed as mean ± SEM and represents an average of 6 rats. Significance is given as: * differences between Control and 6-OHDA animals undergone the same treatment, $^{*}P < .05$; $^{***}P < .001$.

exposed to LPS within first 24 h after immune challenge.

In animals with intact sympathetic nerves, LPS administration induced a significant increase of IL-1β, IL-6, TNF-α, IL-10, and corticosterone plasma levels, with the peak at 3 h after immune challenge. After 3 h, plasma levels slowly declined and with the exception of IL-1β, reached almost baseline levels 24 h after LPS injection (Fig. 2A - 6A).

Similarly, administration of LPS to sympathectomized animals induced a significant increase in plasma levels of the investigated cytokines and corticosterone (Fig. 2A - 6A). However, when compared to animals with an intact SNS, sympathectomized rats showed a significantly reduced rise in plasma IL-1β at 3 ($t_7 = 5.731$, $P < .0001$) and 6 h ($t_7 = 4.432$, $P < .05$) after LPS administration, which was also documented by comparing the time-courses of changes of plasma IL-1β levels (Control *vs.* 6-OHDA: $F_{1,42} = 33.96$, $P < .0001$; Fig. 2A) and based on calculation of AUC ($t_7 = 2.612$, $P = .0348$; Fig. 2C).

3.2. Effect of sympathectomy on lipopolysaccharide-induced changes of plasma cytokine levels in rats pretreated by ACTH (1–24)

The second goal of our experiment was to determine the role of SNS in the anti-inflammatory effects of ACTH (1–24). Published data indicate that sympathetic nerves play crucial role in inflammatory reflex (Martelli et al., 2016) and that efferent pathways of this reflex might be activated by ACTH (1–24) (Guarini et al., 2004). Therefore, we administered ACTH (1–24) to animals before and after immune challenge.

When comparing control animals with intact sympathetic nerves (Control SAL + LPS + SAL) and rats administered ACTH (1–24) (Control ACTH + LPS + ACTH), there were reduced increases of plasma IL-1β (Saline 729 ± 46 pg/mL *vs.* ACTH 492 ± 40; Fig. 2A,B) and IL-6 (Saline 56.04 ± 12.09 pg/mL *vs.* ACTH 39.91 ± 9.16; Fig. 3A,B) 3 h after LPS injection while there was an potentiation of the increase in IL-10 (Saline 53 ± 12 pg/mL *vs.* ACTH 354 ± 183; Fig. 5A,B) 1 h after LPS injection. When comparing the total amount of plasma cytokine levels as calculated as AUC, no differences were found between animals with intact sympathetic nerves treated by saline or ACTH (1–24) when analyzing IL-1β ($t_6 = 1.284$, $P = .2465$; Fig. 2C) and IL-6 ($t_6 = 0.3613$, $P = .7302$; Fig. 3C). However, in animals with intact sympathetic nerves total plasma levels of IL-10 were significantly increased when compared to animals injected by saline (Control SAL + LPS + SAL *vs.* Control ACTH + LPS + ACTH: $t_6 = 0.8.6941$, $P < .005$; Fig. 5C).

Sympathectomy significantly reduced plasma IL-1β ($t_7 = 7.871$, $P < .001$; Fig. 2B) and IL-6 ($t_7 = 7.513$, $P < .001$; Fig. 3B) 3 h after LPS administration and IL-10 levels ($t_7 = 3.925$, $P = .0019$; Fig. 5B) were reduced 1 h after LPS administration. Similarly, a significant reduction was also found in the total amount of plasma IL-6 (AUC Control *vs.* 6-OHDA: $t_7 = 3.210$, $P = .0148$; Fig. 3C) and IL-10 in sympathectomized rats (AUC Control *vs.* 6-OHDA: $t_7 = 3.4515$, $P = .0107$; Fig. 5C), whereas the total amount of IL-1β was only slightly reduced in sympathectomized rats (AUC Control *vs.* 6-OHDA: $t_7 = 2.1794$, $P = .0721$; Fig. 2C). Plasma levels of TNF-α, and corticosterone in animals injected by ACTH did not differ between sympathectomized rats and those with an intact SNS (Fig. 4B, 6B).

4. Discussion

The sympathetic nervous system exerts a complex effect on immune functions, which depends on several factors including the duration and intensity of SNS activation (Sternberg, 2006). Published data indicate that during acute stress, SNS increases the activity of immune cells enabling them to migrate to the site of injury and destroy pathogens (Dhabhar, 2009; Sanders and Straub, 2002). To characterize the role of SNS during exposure of an organism to an acute immune stressor, we determined the dynamics of plasma cytokines levels in sympathectomized rats exposed to a single intraperitoneal injection of LPS. We found significantly attenuated increases of plasma IL-1β levels in sympathectomized animals, indicating that SNS plays an important role in the rise of IL-1β, but not IL-6 and TNF-α in response to immune challenge (LPS). In addition, we also characterized the dynamics of increased of plasma cytokine levels within 24 h after immune challenge in animals with intact SNS and those with destroyed sympathetic nerve endings. Our data indicate that the main increase of plasma cytokine levels is found during the first 6 h after immune challenge and after that, cytokine plasma levels decline to baseline. Our observed temporal profile of cytokine levels is similar to those reported in other published studies (Kakizaki et al., 1999; Somann et al., 2019; Webel et al., 1997).

Published data indicate that sympathetic nerves are part of the neuronal circuity that mediates the effects of cholinergic anti-inflammatory pathway (Martelli et al., 2016; Martelli et al., 2014a). However, the precise neuroanatomical and functional characterization of SNS involvement in these effects is still questionable. Therefore, using ACTH (1–24) as a stimulator of the cholinergic anti-inflammatory pathway, we investigated the effect of sympathectomy on plasma cytokine levels in animals exposed to LPS. We found that administration of ACTH (1–24) reduced the peak plasma levels of the pro-inflammatory cytokines IL-1β and IL-6 and increased the peak plasma levels of the anti-inflammatory cytokine IL-10. These findings are in accordance with data from an *ex vivo* experiment on human blood samples stimulated by LPS and pretreated with ACTH (1–24) (Catania et al., 1998) and an *in vitro* experiment on murine microglia exposed to LPS and ACTH (1–24) (Delgado et al., 1998). Therefore, our data and published findings clearly indicate that ACTH (1–24) exerts an inhibitory effect on immunity. However, this effect might be mediated by

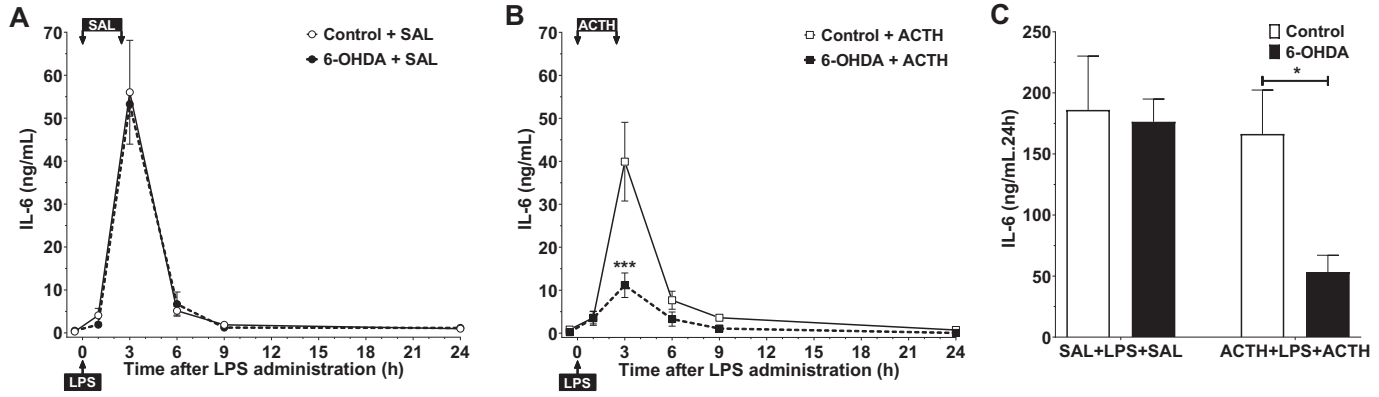

Fig. 3. Effect of an intraperitoneal injection (i.p.) of lipopolysaccharide (LPS) on plasma levels of IL-6 in animals with intact sympathetic nerves (Control; ○/□) and sympathectomized rats (6-OHDA; ●/■) pretreated by saline (A) or ACTH (B). The total amount of IL-6 in plasma was calculated as the area under the curve (AUC) (C). Data are expressed as mean ± SEM and represents an average of 6 rats. Significance is given as: * differences between Control and 6-OHDA animals undergone the same treatment, $^{*}P < .05$; $^{***}P < .001$.

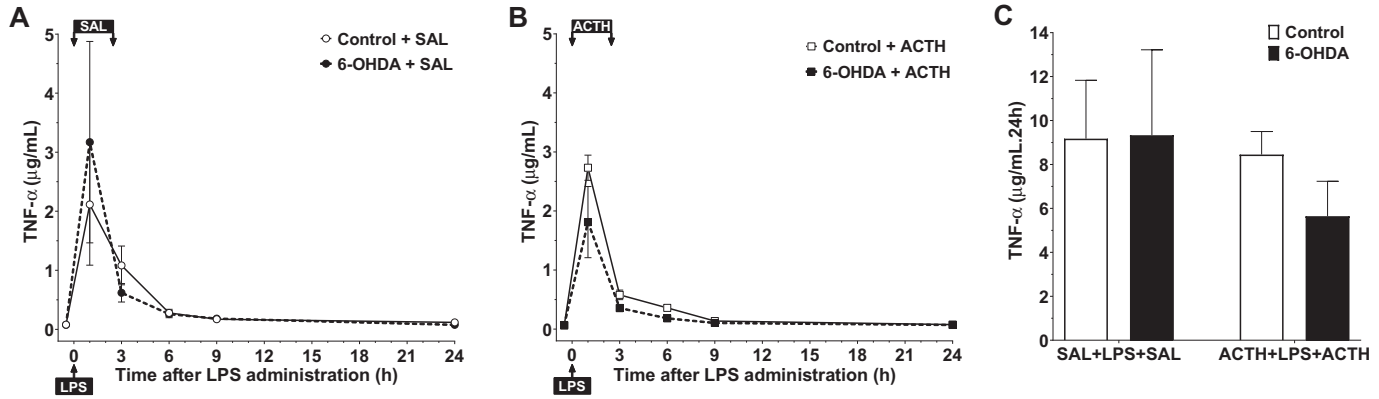

Fig. 4. Effect of an intraperitoneal injection (i.p.) of lipopolysaccharide (LPS) on plasma levels of TNF-α in animals with intact sympathetic nerves (Control; ○/□) and sympathectomized rats (6-OHDA; ●/■) pretreated by saline (A) or ACTH (B). The total of TNF-α in plasma was calculated as the area under the curve (AUC) (C). Data are expressed as mean ± SEM and represents an average of 6 rats.

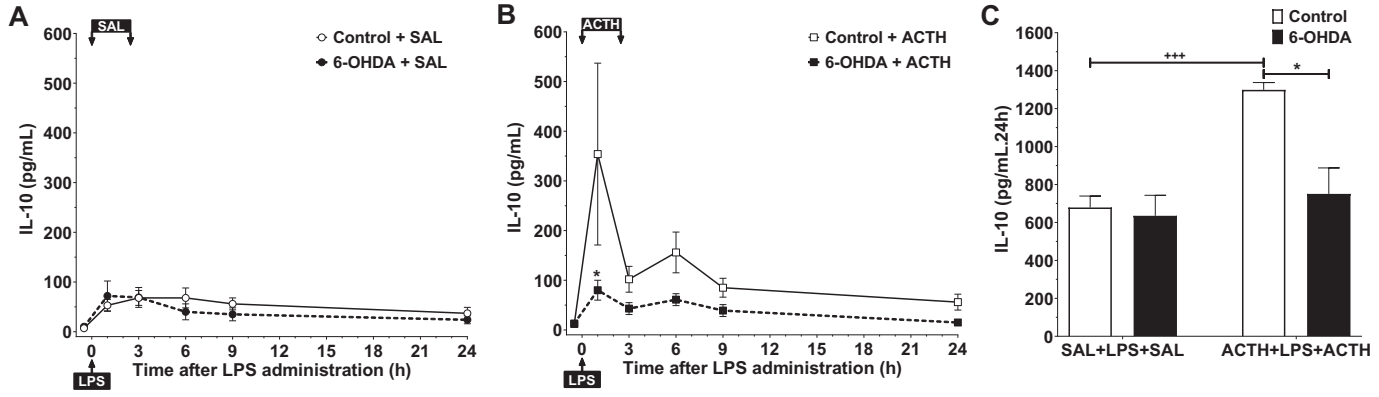

Fig. 5. Effect of an intraperitoneal injection (i.p.) of lipopolysaccharide (LPS) on plasma levels of IL-10 in animals with intact sympathetic nerves (Control; ○/□) and sympathectomized rats (6-OHDA; ●/■) pretreated by saline (A) or ACTH (B). The total of IL-10 in plasma was calculated as the area under the curve (AUC) (C). Data are expressed as mean ± SEM and represents an average of 6 rats. Significance is given as: * differences between Control and 6-OHDA animals undergone the same treatment, $^{*}P < .05$; $^{+}$ differences between animals with intact sympathetic nerves, $^{+++}P < .005$.

several mechanisms and pathways. Therefore, we investigated the effect of sympathectomy on LPS-induced changes in plasma cytokines and corticosterone in rats treated by ACTH (1–24). We found that plasma corticosterone levels were not affected by ACTH (1–24) administration, indicating that the anti-inflammatory effect of ACTH (1–24) is not mediated by an increase of plasma corticosterone. It has also been suggested that the anti-inflammatory effect of ACTH (1–24) is mediated by cholinergic anti-inflammatory pathway (Guarini et al., 2004). However, we found that elimination of sympathetic nerve endings reduced the LPS-induced rise of plasma IL-1β and IL-6 levels in rats treated by ACTH (1–24). These data indicate that sympathetic nerves do not participate in ACTH-induced anti-inflammatory effects. Based on the above-mentioned papers published by Catania et al. (1998) and Delgado et al. (1998) it is most likely that ACTH exerts its anti-

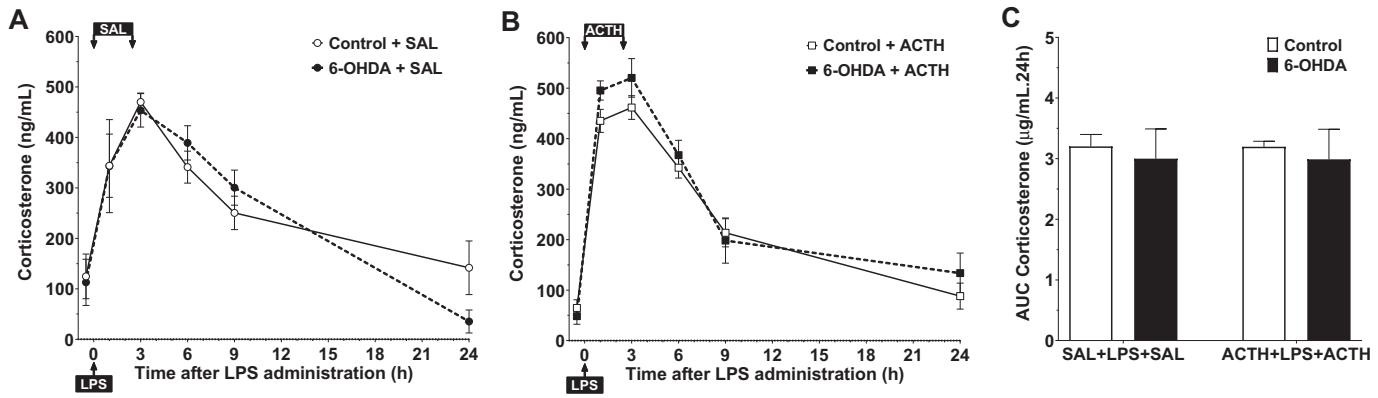

Fig. 6. Effect of an intraperitoneal injection (i.p.) of lipopolysaccharide (LPS) on plasma levels of corticosterone in animals with intact sympathetic nerves (Control; ○/□) and sympathectomized rats (6-OHDA; ●/■) pretreated by saline (A) or ACTH (B). The total of corticosterone in plasma was calculated as the area under the curve (AUC) (C). Data are expressed as mean ± SEM and represents an average of 6 rats.

inflammatory effect by binding on corresponding receptors on immune cells. Interestingly, in animals treated by ACTH (1–24) our data indicate that sympathetic nerves participate in the stimulation of immune functions. However, the mechanism responsible for the stimulatory effect of ACTH (1–24) on immune function mediated by SNS needs further investigation.

Importantly, our previously published data showed that subdiaphragmatic vagotomy significantly reduced the LPS-induced rise in plasma TNF-α levels but does not affect plasma levels of IL-1βb and IL-6. In addition, we found that subdiaphragmatic vagotomy did not affect gene expression of TNF-α, IL-1βb, and IL-6 in rats exposed to LPS (data not shown) (Ondicova et al., 2019). Moreover, subdiaphragmatic vagotomy did not affect baseline nor LPS-induced increase of plasma corticosterone levels (data not shown). These data are in contrast to experiments showing an exaggerated immune response in vagotomized animals (O'Mahony et al., 2009). These contradictory findings questioned the involvement of the subdiaphragmatic portion of the vagus nerve in immunosuppressive mechanisms mediated by cholinergic anti-inflammatory pathway. Based on the above-mentioned data related to the effect of sympathectomy on ACTH (1–24) and its anti-inflammatory influence, along with our previously published data related to the effect of vagotomy on the LPS-induced rise in plasma and spleen cytokine levels, we suggest that a more detailed study of the neuronal circuits mediating efferent cholinergic anti-inflammatory pathway is necessary.

5. Limitations of the study

Even if 6-OHDA-induced sympathectomy is frequently used for investigation of the role of SNS in regulation of immune functions as documented by many papers published in the *Journal* (*e.g.* Bellinger et al., 2005; Leo and Bonneau, 2000; Madden et al., 1994)), it is necessary to note several limitations related to use of this compound.

Firstly, 6-OHDA injection while destroying sympathetic nerve endings does not destroy chromaffin cells in the adrenal medulla. In fact, activity of tyrosine hydroxylase, the rate limiting enzyme of catecholamine biosynthesis, doubles in adrenal medulla and the epinephrine content of adrenal medulla is little changed in rats treated with 6-OHDA (Mueller et al., 1969; Thoenen et al., 1969). Therefore, LPS-induced epinephrine release in 6-OHDA treated rats might confound the interpretation of results because circulating epinephrine may have an anti-inflammatory action and suppress cytokine release (Pettipher et al., 1996). However, as demonstrated by Nardocci et al. (2015), even if pretreatment of rats by 15 mg/kg bw LPS increased plasma epinephrine levels, this increase was not so prominent comparing to another stressors (Pacak et al., 1998) and therefore it cannot explain our findings.

While sympathetic nerve terminals will be destroyed by 6-OHDA, so too will catecholamine-containing immune cells in the spleen and lymph nodes (Capellino et al., 2012). In addition, generation of cytotoxic T cells will be affected, as well (Livnat et al., 1987). Thus, it is difficult to distinguish effects of sympathectomy from loss of immune cells. However, we found that 6-OHDA attenuated only plasma IL-1β response to LPS. Therefore, we suggest that our results reflect the effect of elimination of sympathetic nerve endings on immune functions.

Lastly, 6-OHDA treatment will destroy many different sympathetic nerves that may affect a variety of bodily functions (Jänig, 2006). Because many physiological systems are affected, there is limited specificity provided by 6-OHDA use as an experimental tool.

6. Conclusion

Our data indicate that SNS plays an important role in the LPS-induced rise of plasma IL-1β that is in accordance with the view that acute activation of SNS stimulates immune function. We also demonstrated that ACTH (1–24) exerts an anti-inflammatory effect that is not mediated by corticosterone. In addition, our findings show that SNS does not play an important role in the anti-inflammatory effect of ACTH (1–24) and further questioned the anatomical composition of cholinergic anti-inflammatory pathway. Therefore, further investigation will be necessary to characterize anatomical and functional components of cholinergic anti-inflammatory pathway and mechanisms that participate in the observed ACTH-induced anti-inflammatory effect.

Declaration of Competing Interest

The author declare that there is no conflict of interest in the publication of this study.

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