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Tumour necrosis factor and interleukin 10 in blood pressure regulation in spontaneously hypertensive and normotensive rats

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ABSTRACT

Introduction: A growing body of evidence indicates that brain cytokines are involved in the control of the cardiovascular system. Tumour necrosis factor (TNF) is an archetypal cytokine, which exerts its proinflammatory actions via type 1 receptor (TNFR1). Interleukin 10 (IL-10) plays a critical anti-inflammatory role by binding to its receptor (IL-10Ra). The orchestrated inflammatory response is largely dependent on an intricate balance between proinflammatory and anti-inflammatory cytokines and expression of their receptors.

Aim: In the study we evaluated the expression of the cytokines and their receptors in the brains of spontaneously hypertensive (SH) and normotensive Wistar-Kyoto (WKY) rats, and how the cytokines affect arterial blood pressure.

Methods: In SH and WKY rats we recorded systolic blood pressure with tail cuff method and measured concentration of TNF, IL-10, TNFR1, and IL-10Ra in the serum, the brainstem, and the hypothalamus; we also measured serum concentrations of copeptin, a surrogate of vasopressin release, angiotensin II and norepinephrine. We immunostained brainstem sections for TNFR1, IL-10Ra, neurons, astrocytes and microglia for confocal imaging. In urethane anaesthetized SH and WKY rats, we invasively recorded blood pressure response to intracerebroventricular (IVC) infusion of TNF or IL-10. We also pharmacologically evaluated baroreflex with phenylephrine and chemoreflex with cyanide in SH and WKY rats.

Results: Compared to WKY rats, SH rats had: (1) higher blood pressure; (2) blunted baroreflex and augmented peripheral chemoreflex; (3) greater pressor response to ICV infused TNF and greater hypotensive response to ICV infused IL-10; (4) higher concentration of TNF in the ventral and dorsal aspects of the medulla oblongata; (5) higher expression of TNFR1 in the dorsal medulla; (6) higher concentration of IL-10 in both aspects of the medulla; (7) lower expression of IL-10Ra in the dorsal medulla. Confocal imaging showed co-localization of TNFR1 and IL-10Ra with neurons, astrocytes and microglia in both SH and WKY rats. The concentration of the cytokines and their receptors were significantly higher in the brain than in the serum. There were no significant differences in the concentration of the cytokines and their receptors in the hypothalamic region and in the serum between SH and WKY rats. Serum concentrations of norepinephrine, angiotensin II and copeptin were similar between SH and WKY rats.

Conclusions: Taken together, these findings suggest the presence of a potent milieu for effective TNF signalling in the brainstem, which is associated with the hypertensive phenotype and enhanced hemodynamic response to intrabrain administration of the cytokines. In addition, we hypothesize that the increased IL-10 concentration in the brainstem is a compensatory mechanism for the upregulated TNF system.

1. Introduction

Primary hypertension is one of the leading causes of morbidity and mortality in the developed countries [1]. It is estimated that up to 10% of hypertensive population is resistant to standard pharmacotherapy [2]. These patients often present with elevated sympathetic tone, altered cardiovascular reflexes, and worse cardiovascular outcomes, such as stroke, heart failure and renal insufficiency [2], which necessitates a better understanding of the multifactorial pathomechanism behind the elevated arterial blood pressure. One of the common findings in hypertensive patients is a low-grade inflammation detected in the peripheral tissues and blood [3–6]. Moreover, accumulating body of evidence indicates that it is not only the peripheral organs that present with inflammation, but also the cardiovascular centres of the brain that

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show signs of neuroinflammation in the experimental models of hypertension [7–9].

It has been shown that elevated blood pressure is accompanied by blunted baroreflex and increased sensitivity and tonicity of the arterial chemoreflex in the spontaneously hypertensive (SH) rats, the experimental model of essential hypertension in humans [10,11]. Neural circuitry of the both reflexes includes the nucleus of the solitary tract (NTS) within the dorsal medulla (DM), which receives sensory input from baro- and chemoreceptors, and the rostral ventrolateral medulla (RVLM) within the ventral medulla (VM), which is the key pressor centre of the brainstem involved in sympathoexcitation and elevation of the arterial blood pressure [12–14]. Furthermore, the activity of brainstem cardiovascular centres is modulated by the paraventricular nucleus (PVN) of the hypothalamus (HTH) [12]. Several studies indicate that hypertensive rats have higher expression of proinflammatory cytokines (PICs) in the PVN than normotensive animals [8]. Moreover, mRNAs of some proinflammatory mediators are upregulated in the NTS of SH rats in comparison to normotensive controls [15].

Tumour necrosis factor (TNF) is an archetypal proinflammatory cytokine of multifaceted functions. The proinflammatory action of TNF is mostly mediated by TNF type 1 receptor (TNFR1) [16]. A growing body of evidence indicates that in comparison to normotensive controls, increased expression of TNF is present in the PVN in angiotensin II (Ang II) induced hypertension in rats and mice [9,17] as well as in SH rats [18]. Moreover, TNF expression is increased in the brainstem in rats with 2 kidney-1-clip renovascular hypertension [7]. Furthermore, our previous studies in conscious normotensive rats and in rats with myocardial infarction have shown that intracerebroventricular administration of the cytokine increases arterial blood pressure and decreases baroreflex sensitivity [19,20]. Additionally, we showed that conscious rats with post-infarction heart failure have abnormally high activity of central angiotensin type 1 receptors, which is normalized by chronic inhibition of TNF in the brain [21]. In this line, several recent studies showed that TNF administered into the PVN, the subfornical organ or the carotid artery exerts sympathoexcitatory and pressor effects in anaesthetized rats [8,22–24].

Interleukin 10 (IL-10) is a key anti-inflammatory cytokine (AIC), which binds to the alpha subunit of its receptor, IL-10Ra. A growing body of evidence indicates that the cytokine has some protective effects in the cardiovascular system. Namely, the systemic upregulation of IL-10 in stroke-prone SH rats resulted in long-term decrease in arterial blood pressure and attenuated remodeling of the cardiovascular system and kidneys [25]. Moreover, the upregulation of IL-10 expression in the PVN by means of viral vectors blunts elevation of arterial blood pressure in rats with Ang II induced hypertension [26].

The effects of cytokines are dependent on the availability of their receptors and on the activation of PICs and AICs networks [16,27]. It is established that in microglia, a key source of the cytokines in the central nervous system, TNF stimulates release of IL-10 [28] and that exposure of microglia to the anti-inflammatory IL-10 inhibits TNF release [28,29]. These findings indicate that there is a negative feedback loop between TNF and IL-10, which controls the levels of TNF. It is plausible to hypothesize that the balance between the PICs and AICs combined with the expression of their receptors should determine the effects of the cytokines on the regulation of the cardiovascular system. Therefore, we wanted to determine whether the concentrations of TNF and IL-10 and their receptors TNFR1 and IL-10Ra in the cardiovascular centres of the brain differ between hypertensive SH rats and their normotensive Wistar-Kyoto (WKY) counterparts and to find out if intracerebroventricular administration of the cytokines leads to different hemodynamic responses depending on the normotensive or hypertensive phenotype.

2. Methods

2.1. Animals

The experiments were done on adult male SH and WKY rats obtained from the Central Animal Laboratory of the Medical University of Warsaw. The rats were 16 weeks old and weighted between 320 and 380 g. Before experiments, the rats were kept in groups of 2-3 animals in their home cages at room temperature with 12/12-hour light-dark cycle and free access to regular rat chow and water.

The experiments were carried out in accordance with domestic regulations and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The study protocol was approved by the Second Local Ethical Committee on Animal Experiments at the Medical University of Warsaw.

2.2. Expression of the cytokines and neurohormones

2.2.1. Blood pressure measurements

Systolic blood pressure (SBP) was noninvasively measured in SH (n = 12) and WKY (n = 12) rats by volumetric tail-cuff method (CODA, Kent Scientific, Torrington, CT, USA) according to AHA guidelines on blood pressure measurements in experimental animals [30]. Each rat was placed in a restrainer and warmed up on a heating plate till obtaining 34 – 35 °C measured at the base of the tail. Then cuffs were placed on the tail to occlude tail arteries and to record changes in the tail volume. Two cycles of 10 consecutive blood pressure recordings were carried out in each measurement session. The blood pressure was recorded on four separate days every third day over ten-day period to acclimatise the animals to the restrain and cuff inflation. This time period has been shown to be sufficient for obtaining reliable blood pressure measurements and at the same time it limits the exposure to repeated stress related to immobilisation during SBP recording [31]. Recordings of SBP from the last day of measurements were used for further evaluation and analysis. After discarding all technically invalid recordings, the mean value of SBP was averaged from at least five technically viable measurements from each individual rat for further statistical analysis. Blood pressure recordings were done in all animals on the same day.

2.2.2. Blood collection

The next day after the last SBP measurements, rats were deeply anaesthetized with an intraperitoneally administered overdose of ketamine (200 mg/kg body weight) and xylazine (20 mg/kg body weight), and blood samples were collected into glass vials by cardiac puncture with 20 gauge needle inserted via the fifth intercostal space. The vials were centrifuged for 15 min at 4000 r.p.m. in 4 °C. The serum was collected into cryovials and stored at −80 °C for further analysis.

2.2.3. Brain harvesting and preparation

After collection of the blood samples, rats were decapitated and the entire brains were removed from the cranium, snap-frozen in liquid nitrogen and stored at −80 °C till further preparation.

For obtaining the brain areas involved in the cardiovascular regulation, the coronal sections of the brain were cut in the brain matrix slicer (TedPela Inc., Redding, CA, USA). The coronal slice was made between −1.0 mm and −3.0 mm caudal from the Bregma and the hypothalamus (HTH) was isolated. The medulla oblongata was isolated between −11.0 mm and −14.0 mm caudal from the Bregma and dissected into two aspects, the ventral medulla (VM) and the dorsal medulla (DM), which contain the rostral ventrolateral medulla (RVLM) and the nucleus of the solitary tract (NTS), respectively [32] (Fig. 1A). The sectioning and isolation of the brain structures were carried out at −20 °C.

In addition, brains for immunostaining and confocal imaging were

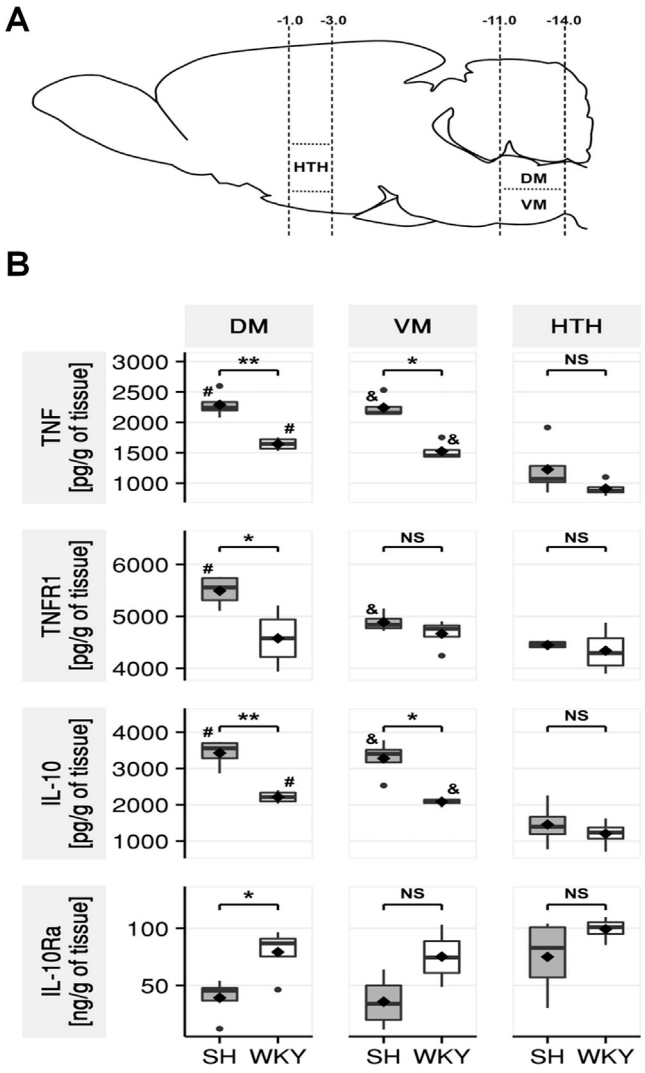

Fig. 1. A. Sagittal view of the brain with coronal planes rostral from the bregma indicating margins of regions used in the study: DM – dorsal medulla, VM – ventral medulla, HTH - hypothalamus. B. Concentrations of TNF, TNFR1, IL-10 and IL-10Ra in DM, VM and HTH of SH and WKY rats. * $p < 0.05$ and ** $p < 0.01$ SH vs WKY in the given brain region; # $p < 0.05$ DM vs HTH in SH or WKY rats; & $p < 0.05$ VM vs HTH in SH or WKY rats.

collected from WKY (n = 2) and SH (n = 2) rats. After anaesthesia with ketamine (200 mg/kg body weight) and xylazine (20 mg/kg body weight), rats were decapitated and brains were removed from the skull, snap-frozen in the liquid nitrogen and stored at −80 °C till sectioning on a cryostat.

2.2.4. Cytokines and their receptors

Frozen brain samples were homogenized in the lysis buffer (100 mg of tissue/ml lysis buffer). The lysis buffer contained 50 mM Tris, 2 mM EDTA, 10 mM EGTA and 1% Triton X-100 and 1 tablet of Complete Mini Protease Inhibitor Cocktail Tablet/10 ml of the solution (all substances from Sigma-Aldrich, Europe) [33]. Homogenates were centrifuged at 6000 r.p.m. TNF and IL-10 concentrations were evaluated in the supernatants, while TNFR1 and IL-10Ra were measured in the sediments. The concentrations of the cytokines and their receptors are expressed per gram of tissue weight. Before measurements, sera were centrifuged. ELISA assays for TNF (catalogue no. CSB-E11987r), IL-10 (catalogue no. CSB-E04595r), TNFR1 (catalogue no. CSB-E07381r) were obtained from Cusabio Biotech and for IL-10Ra (catalogue no. DL-IL10Ra-Ra) from Wuxi Donglin Science and Technology Development Co., Ltd. All tests were carried out according to the manufacturers' instructions and measurements were done in duplicates.

2.2.5. Norepinephrine, copeptin and angiotensin II in the serum

Serum concentration of norepinephrine was evaluated with the ELISA (catalogue no. BA E-5200, LDN, Nordhorn, Germany) according to the manufacturer's instructions. In order to prevent breakdown of norepinephrine, 1 mM EDTA and 4 mM sodium metabisulfite were added to blood samples collected for norepinephrine measurement. Serum concentration of copeptin was measured with ELISA kit (catalogue no. CSB-E16335r, Cusabio Biotech). Angiotensin II (Ang II) was measured with EIA kit along the manufacturer's instructions (catalogue no. EKE-002-12 Phoenix Pharmaceutical Inc., Burlingame, CA, USA). To stabilize the peptides in the serum, 150 µl of Protease Inhibitor Cocktail solution per 1 ml of blood (stock solution: 10 ml water added to P2714, SigmaAldrich, Europe) and 1 mM EDTA were added to blood samples used for Ang II measurements. All measurements were done in duplicates.

2.2.6. Immunostaining and confocal imaging

The brainstem sections were cut on a cryostat (Leica CM1850, Leica, Germany) at 30 µm thickness between −11 and −14 mm caudal from bregma (Fig. 2M). The sections were placed on silanized glass slides (A3648, Sigma Aldrich, Europe) and fixed in 4% PFA for 20 min. After fixation, the bainstem sections were immunostained with mouse primary monoclonal antibody against beta-tubullin to visualise neurons (1: 200; catalogue no. MA516308, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA); against OX-42 (CD11b) to detect microglia (1:200; catalogue no. MA181606, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) and against GFAP to show astrocytes (1:200; catalogue no. MA512023, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). Primary polyclonal rabbit antibodies were used to visualise TNFR1 (1:200; catalogue no. SAB4502988, Sigma Aldrich, Europe) and IL-10Ra (1:200; catalogue no. bs-2459R, Bioss Antibodies Inc., Woburn, MA USA). The incubation with primary antibodies was carried out at 4 °C overnight and 10% goat serum was used for blocking the secondary antibodies (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). This was followed by a two-hour incubation at 24 °C with the goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody with Alexa Fluor 488 (1:1000; catalogue no. A11001, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) and the goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody with Alexa Fluor 546 (1:1000; catalogue no. A11010, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). The cell nuclei were stained with Hoechst 33342 (1 µg/ml; catalogue no. 14533 Sigma Aldrich, Europe) during 20 min incubation before covering the sections with ProLong Gold Antifade Mountant (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). Negative controls were done according the above staining protocol except for omitting the primary antibodies during the first incubation to confirm that non-specific binding of secondary antibodies to the tissue sections was not present. Samples were visualised both in the VM and DM medially in regions containing RVLM and NTS and laterally (Fig. 2N) in the confocal scanning laser microscope (Zeiss LSM 710, Carl Zeiss, Oberkochen, Germany) and ZEN Black software.

2.3. Hemodynamic study

We evaluated changes of the mean arterial blood pressure (MABP) in response to intracerebroventricular (ICV) infusions of either sterile phosphate buffered saline (PBS), TNF or IL-10 in urethane-anaesthetized SH and WKY rats. Additionally, we evaluated cardiovascular reflexes in SH and WKY rats.

2.3.1. Surgical procedures

All surgical procedures were done under terminal anaesthesia with urethane administered intraperitoneally (1.5 g/kg body weight; Sigma-

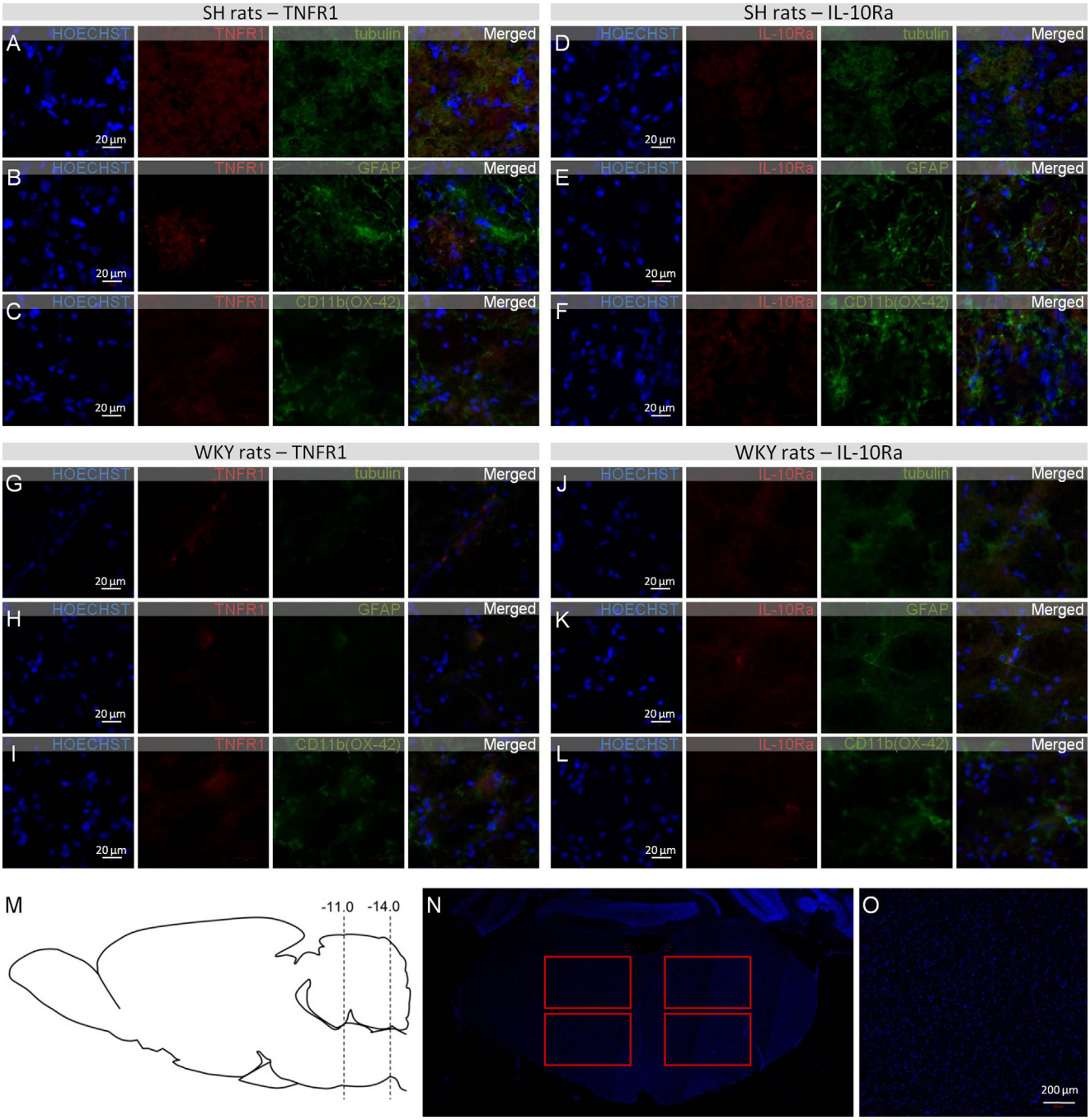

Fig. 2. Immunostaining of the brainstem sections from SH and WKY rats. Both ventral and dorsal aspects of the brainstem were analyzed in the regions containing RVLM and NTS as indicated in panel N. The analyzed sections were collected between −11 and −14 mm caudal from bregma as indicated in panel M. The panels A-L show representative images of immunostaining for: **A.** cell nuclei (Hoechst), TNFR1, neurons (beta-tubulin) in SH rat; **B.** cell nuclei (Hoechst), TNFR1, astrocytes (GFAP in SH rat; **C.** cell nuclei (Hoechst), TNFR1, microglia (CD11b (OX-42)) in SH rat; **D.** cell nuclei (Hoechst), IL-10Ra, neurons (beta-tubulin) in SH rat; **E.** cell nuclei (Hoechst), IL-10Ra, astrocytes (GFAP), in SH rat; **F.** cell nuclei (Hoechst), IL-10Ra, microglia (CD11b (OX-42)) in SH rat; **G.** cell nuclei (Hoechst), TNFR1, neurons (beta-tubulin) in WKY rat; **H.** cell nuclei (Hoechst), TNFR1, astrocytes (GFAP) in WKY rat; **I.** cell nuclei (Hoechst), TNFR1, microglia (CD11b (OX-42)) in WKY rat; **J.** cell nuclei (Hoechst), IL-10Ra, neurons (beta-tubulin) in WKY rat; **K.** cell nuclei (Hoechst), IL-10Ra, astrocytes (GFAP) in WKY rat; **L.** cell nuclei (Hoechst), IL-10Ra, microglia (CD11b (OX-42)) in WKY rat.

Aldrich, Europe). Degree of anaesthesia was confirmed during the experiments by regular checking of pawn pain reflex and responses of arterial blood pressure to pain stimulus. If needed, additional doses of urethane (0.1 g/kg body weight) were administered intravenously.

2.3.1.1. Implantation of vascular catheters. The femoral neurovascular bundle on the left side was exposed by a 1.5 cm cut in the skin and blunt dissection of the subcutaneous tissue in the femoral triangle. Both the femoral artery and vein were isolated from the bundle and closed with a suture on the distal end. Polyurethane catheters (Scientific Commodities, Inc., Lake Havasu City, AZ, USA) were filled with heparinised saline (unfractioned heparin 500 I.U./ml saline; Polfa Warszawa SA, Poland) and inserted through the femoral artery and vein into the aorta below the branching of the renal arteries and into the vena cava inferior, respectively. The catheters were secured to the vessels with a suture and cyanoacrylic glue. The venous catheter was

used for administration of substances. The arterial catheter was used for recording of pulsatile blood pressure.

2.3.1.2. Implantation of the intracerebroventricular cannula. Immediately after implantation of the catheters, the rat's head was placed in the stereotactic apparatus (ASI Instruments, Inc., Warren, MI, USA) and horizontally levelled. After cutting the skin and the epicranial aponeurosis, the skull was exposed and a hole was drilled in the parietal bone according to the following coordinates: 1.8 mm lateral from the sagittal suture, 1.4 mm caudal from the coronal suture. A stainless steel cannula (20 gauge) was inserted into the lateral cerebral ventricle with its tip placed at depth of 3.5–4.0 mm from the surface of the skull till obtaining outflow of the cerebrospinal fluid, which confirmed placement of the cannula in the cerebral ventricle. The cannula was secured to the skull's surface with dental cement (Duracryl Plus, SpofaDental, Poland).

2.3.2. Hemodynamic measurements

2.3.2.1. Blood pressure response to intrabrain infusion of the cytokines. After completion of surgeries, the arterial catheter was connected to the blood pressure transducer, amplifier and analog-digital converter and the blood pressure was continuously recorded (Biopac MP100 unit, Biopac Systems, Goleta, CA, USA). After stabilization of hemodynamic parameters, the baseline recording of arterial blood pressure was carried out for 15 min. This was followed by ICV bolus of either PBS (10 µl/30 sec), recombinant rat TNF (200 ng/10 µl/30 sec; catalogue no. 400-14, PeproTech House, London, UK) or rat recombinant IL-10 (200 ng/10 µl/30 sec; catalogue no. 400-19, PeproTech House, London, UK) in both SH and WKY rats. The recording of blood pressure continued for two hours after ICV administration of the cytokines or PBS. Altogether, there were three groups of SH rats (n = 6, each) ICV infused with PBS, TNF or IL-10 and three groups of WKY rats (n = 6, each) ICV infused with PBS, TNF or IL-10.

2.3.2.2. Baroreflex and chemoreflex evaluation. In the instrumented SH (n = 6) and WKY rats (n = 6), a bolus of phenylephrine, a selective adrenergic α1 receptor agonist, was administered intravenously (10 µg/100 µl; Sigma-Aldrich, Europe) to rise arterial blood pressure. After return of hemodynamic parameters to the pre-phenylephrine values, a bolus of potassium cyanide (KCN), an inhibitor of mitochondrial respiration, was administered intravenously (30 µg/100 µl; Sigma-Aldrich, Europe) to activate peripheral chemoreceptors. For evaluation of the baroreflex, the ratio of the change of heart rate (HR) to change of MABP at maximum increase of MABP from the pre-phenylephrine values was used. The chemoreflex sensitivity was evaluated as a maximum change of MABP and change of HR in response to KCN from the pre-KCN values. The MABP was integrated from the pulsatile pressure curve. The HR was derived from consecutive systolic peaks of blood pressure tracing.

All data acquisition and processing were done in AcqKnowledgle 3.7.3, software (Biopac Systems, Goleta, CA, USA). Obtained values of pulsatile blood pressure, MABP and HR were exported to text file for further statistical analysis.

2.4. Statistics

The distribution of continuous variables were first assessed with the Shapiro-Wilk test of normality, then, in a case of normally distributed variables, mean and standard deviation were reported in descriptive statistics, otherwise median and the 25th and 75th percentile were provided. Normally distributed variables were compared with Student's *t*-test or ANOVA test, if more than 2 variables were compared, otherwise Mann-Whitney test or Kruskal-Wallis test were used, respectively. Bonferroni correction was used for multiple comparisons.

Analysis of MABP during 120 min following ICV infusions was performed using generalized additive mixed model. The built model is a random intercept model with fixed effects for rat strain and a non-parametric smooth function of time estimated separately for each group. The details of the model are provided in the Supplementary material. All numerical values and plots obtained in the model are reported with 95% confidence intervals. Additionally, changes of MABP in the second hour of observation (MABP averaged over 60–120 min of measurement) from the baseline value (MABP averaged over 0–5 min of measurement) were analysed descriptively.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.07.003.

The significance level for all statistical analyses in the study was set at 0.05. Statistical analysis was performed in R ver. 3.4.0 [34].

3. Results

3.1. Blood pressure and serum neurohormones

Systolic blood pressure was significantly higher in the SH rats in comparison to the WKY ones (185.8 ± 14.1 mmHg vs 144.2 ± 17.6 mmHg, $p < 0.001$). Serum NE concentration was similar in the SH and WKY rats. There were no differences in the copeptin and Ang II levels between the SH and WKY rats (Table 1).

3.2. Serum, hypothalamic and brainstem cytokines and their receptors

3.2.1. Serum cytokines

There were no significant differences in serum concentrations of TNF, TNFR1, IL-10, IL-10Ra between SH and WKY rats (Table 1). Concentrations of the cytokines and their receptors were significantly lower in the serum than in the central nervous system in both SH and WKY rats ($p < 0.001$).

3.2.2. Brain TNF and TNFR1

There were no significant differences in the expression of TNF in the HTH of hypertensive and normotensive rats. Expression of TNF was higher in the VM and DM in the SH rats than in the normotensive WKY ones (VM: 2157.6 [2145.4, 2255.0] vs 1455.8 [1435.7, 1543.3] [pg/g tissue], $p = 0.029$; DM: 2290.0 ± 219.0 vs 1643.3 ± 104.5 [pg/g tissue], $p = 0.005$). In SH rats expression of TNFR1 was significantly higher in the DM region than in the VM ($p = 0.021$). Furthermore, concentration of TNFR1 in the DM was significantly higher in SH rats than in WKY rats (5493.8 ± 311.3 vs 4577.1 ± 564.7 [pg/g tissue], $p = 0.039$) (Fig. 1B).

In SH rats, both TNF and TNFR1 levels were significantly higher in the DM than in the HTH ($p = 0.012$ and $p = 0.042$, respectively). Similarly, TNF and TNFR1 concentrations in the VM were significantly

Table 1
Arterial blood pressure, hormones and cytokines in the serum.

	SH	WKY	p-value
n	12	12	
SBP [mmHg]	185.8 (14.1)	144.2 (17.6)	< 0.001
n	6	6	
NE [ng/ml]	0.53 (0.32)	0.61 (0.22)	0.619
Copeptin [pg/ml]	329.5 (161.3)	257.1 (213.6)	0.524
Ang II [ng/ml]	0.73 (0.078)	0.84 (0.13)	0.108
n	4	4	
TNF [pg/ml]	< LOQ	< LOQ	–
TNFR1 [pg/ml]	< LOQ	< LOQ	–
IL-10 [pg/ml]	0.028 [0.00, 1.37]	1.67 [0.42, 6.84]	0.460
IL-10Ra [ng/ml]	4.43 [2.23, 6.15]	2.32 [2.04, 3.84]	0.886

Data are expressed as mean (standard deviation) or median [interquartile range].
SBP – systolic blood pressure; NE – norepinephrin; Ang II – angiotensin II; LOQ – limit of quantitation.

higher than in the HTH (p = 0.042 and p = 0.010, respectively) in SH rats. In WKY rats, TNF levels in the DM and in the VM were significantly higher than in the HTH (p = 0.002 and p = 0.042, respectively). However, TNFR1 did not differ significantly between the hypothalamus and the brainstem regions in WKY rats (Fig. 1B).

3.2.3. Brain IL-10 and IL-10Ra

There were no significant differences in the expression of IL-10 in the HTH of hypertensive and normotensive rats. Expression of IL-10 was higher in the VM and DM in the hypertensive rats than in the normotensive ones (VM: 3281.0 ± 531.9 vs 2086.4 ± 118.3 [pg/g tissue], p = 0.018; DM: 3430.0 ± 399.1 vs 2215.7 ± 170.7 [pg/g tissue], p = 0.005). In SH rats expression of IL-10Ra was significantly lower in the DM region than in WKY rats (39.2 ± 18.6 vs 79.3 ± 22.5 [ng/g tissue], p = 0.035), and lower in the VM region (35.81 ± 23.51 vs 75.29 ± 23.65 [ng/g tissue], p = 0.056), however without reaching the pre-specified significance level of 0.05 (Fig. 1B).

In SH rats, IL-10 concentrations in the DM and the VM were significantly higher than in the HTH (p = 0.004 and p = 0.008, respectively). Similarly in WKY rats, IL-10 levels in the DM and the VM were significantly higher than in the HTH (p = 0.006 and p = 0.008, respectively). The levels of IL-10Ra did not differ significantly between the hypothalamic and brainstem regions in either SH or WKY rats (Fig. 1B).

3.2.4. Brainstem TNFR1 and IL-10Ra

Immunostaining of the brainstem sections from SH rats revealed that antibody against TNFR1 bound both in the ventral and dorsal aspects of the medulla oblongata within regions corresponding to NTS and RVLM and also in the lateral fragments. In addition, co-staining for beta-tubulin III, a marker of neurons, GFAP, a marker of astrocytes, and CD11b (OX-42), a marker of microglia, showed that all three types of cell express TNFR1 (Fig. 2A-C). Similar pattern of staining was detected in normotensive WKY rats (Fig. 2G-I). Immunostaining of the brainstem section from SH and WKY rats for IL-10Ra showed a positive staining in the medial and lateral aspects of both DM and VM. Co-staining for beta-tubulin III, a marker of neurons, GFAP, a marker of astrocytes, and CD11b (OX-42), a marker of microglia, showed that all three types of cell express IL-10Ra both in SH (Fig. 2D-F) and WKY rats (Fig. 2J-L). Negative control for the secondary antibodies showed that they specifically bind to the primary antibodies without non-specific staining to cellular structures (Fig. 2O).

3.3. Blood pressure and intracerebroventricular infusions of TNF and IL-10

Baseline MABP of urethane-anaesthetized animals was significantly higher in the SH rats in comparison to the WKY ones (91.5 [82.2, 104.9] mmHg vs 66.0 [58.5, 74.3] mmHg, p < 0.001).

Analysis of the MABP over the course of 120 min following the ICV infusions was performed using additive mixed model. Estimated in model intercept was 65.5 mm Hg (95% CI 59.7–71.3) and the estimated fixed effect of the rat strain was 15.7 mm Hg (95% CI 7.0–23.9) for SH rats. Fig. 3A presents estimated time-dependent effects of ICV infusions on MABP changes for each group.

Further analysis of MABP revealed that ICV treatments had a significant effect on changes in MABP between baseline and the second hour of measurement, regardless of the rat strain (p = 0.022). Despite decreasing trends in MABP observed in the control groups (−3.1 mm Hg [−8.3, 0.3]), ICV infusion of TNF resulted in an increase or a stabilization of MAPB (−0.3 mm Hg [−5.3, 5.9]), while ICV infusion of IL-10 resulted in a decrease in MABP (−7.0 mm Hg [−12.0, −4.3]). However, the additive mixed model showed greater changes of MABP

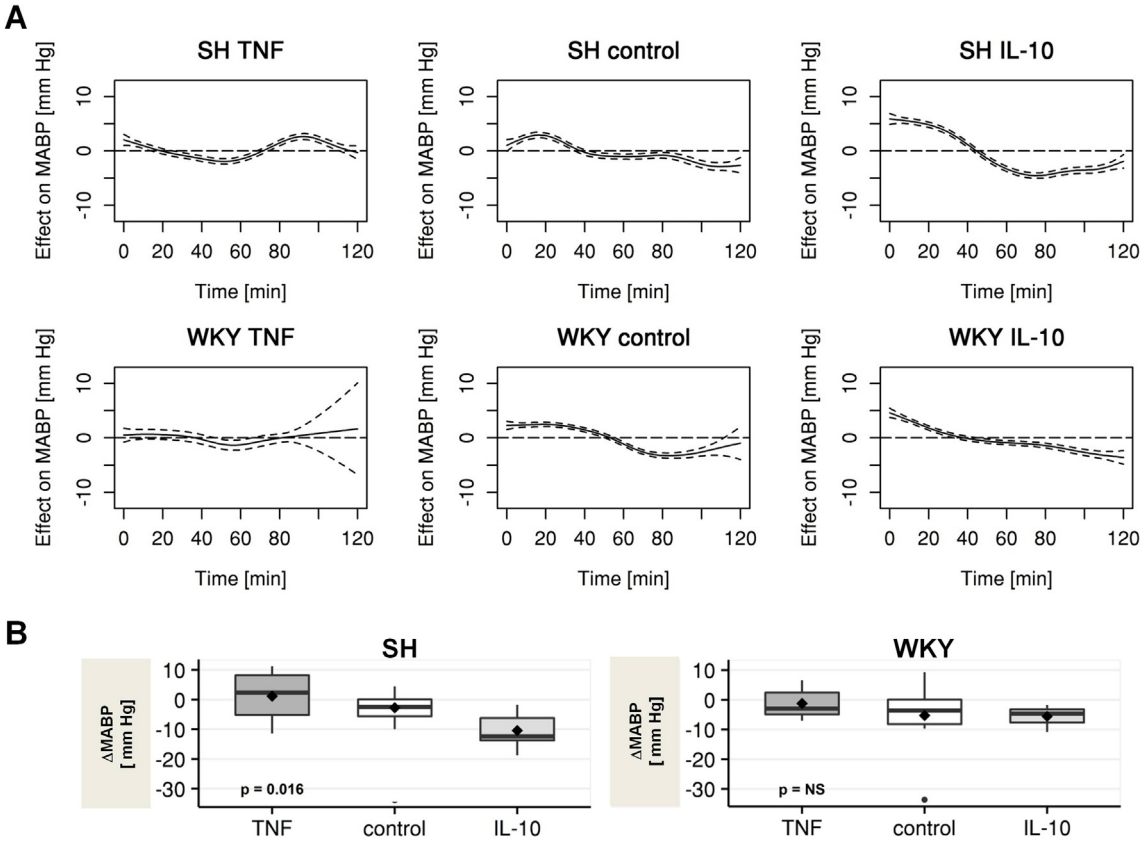

Fig. 3. A. Time-dependent effects of ICV infusions of TNF, PBS (control group) or IL-10 on MABP evaluated in additive mixed model. B. Change of MABP from baseline in the second hour of measurement in SH and WKY rats ICV infused with TNF, PBS (control group) or IL-10. ΔMABP – change of mean arterial blood pressure from baseline in response to ICV infusions of TNF, PBS (control group) or IL-10.

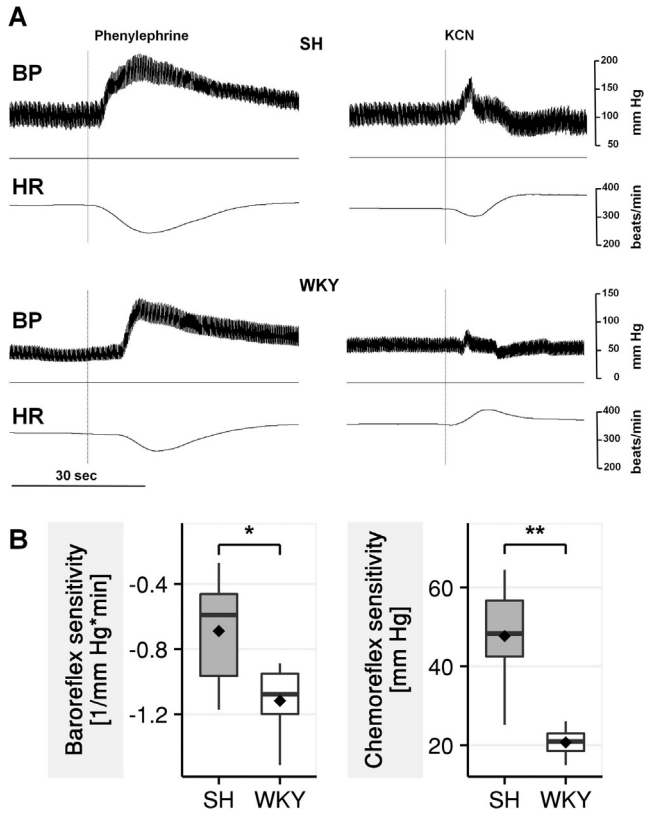

Fig. 4. A. Recordings of pulsatile blood pressure and heart rate in response to intravenous infusions of phenylephrine (10 μg/100 μl) and KCN (30 μg/100 μl). B. Baroreflex sensitivity was evaluated as change of HR to change of MABP at maximum increase of MABP from the pre-phenylephrine values. The chemoreflex sensitivity was evaluated as a maximum KCN-induced increase of MABP from the pre-KCN values. The KCN-induced changes in HR were not significant. BP – blood pressure, HR – heart rate, KCN – potassium cyanide. * $p < 0.05$; ** $p < 0.01$ SH vs WKY rats.

in response to ICV infusion of TNF and IL-10 in the SH rats than in WKY ones. The SH rats responded with an increase in MABP to ICV infusion of TNF and with a decrease in MABP to IL-10 infusion (changes of MABP from baseline in TNF, control and IL-10 groups were 2.4 mm Hg [−5.1, 8.2], −2.5 mm Hg [−5.6, 0.1] and −12.3 mmHg [−13.7, −6.2], respectively; $p = 0.016$). In WKY rats, the effects of ICV infusion of TNF and IL-10 on MABP were less pronounced (changes of MABP from baseline in TNF, control and IL-10 groups were −2.9 mm Hg [−4.9, 2.4], −3.6 mm Hg [−8.2, 0.07] and −4.6 mm Hg [−7.6, −3.2], respectively; $p = 0.471$) (Fig. 3B).

3.4. Cardiovascular reflexes

The baroreflex sensitivity, estimated as a ratio of a change in HR to a change in MABP after phenylephrine infusion was significantly decreased in the SH rats in comparison to the WKY ones (−0.70 ± 0.36 vs −1.12 ± 0.23 1/mmHg*min, $p = 0.039$) (Fig. 4B). The pressor response triggered by activation of the arterial chemoreflex with the infusion of KCN was greater in SH rats than in the WKY rats (47.7 ± 14.0 vs 20.7 ± 3.9 mm Hg, $p = 0.004$), without significant changes in HR in both SH and WKY rats (18.8 ± 38.1 vs 12.4 ± 23.3 1/min, $p = 0.736$) (Fig. 4B).

4. Discussion

The key finding of our study is that spontaneously hypertensive rats have higher expression of TNF, TNFR1, IL-10 and lower expression of IL-10Ra in the brainstem in comparison to the normotensive rats. To the best of our knowledge our results show for the first time that hypertensive animals have higher expression of TNFR1 and lower expression of IL-10Ra in the brainstem. In addition, in both hypertensive and normotensive rats TNFR1 and IL-10Ra are expressed in the brainstem by neurons, astrocytes and microglia. We also show that in comparison to WKY rats, SH rats have higher arterial blood pressure, blunted baroreflex sensitivity and augmented arterial chemoreflex. Furthermore, the SH rats show greater pressor and hypotensive response to intrabrain infusions of TNF and IL-10, respectively, which points to a functional role of the cytokines in the blood pressure control. Collectively, these results indicate that the altered expression of TNF, IL-10 and their receptors in the brainstem of the SH rats is linked to hypertension.

Spontaneously hypertensive rats have genetically determined high blood pressure and serve as an animal model of essential hypertension in humans [11]. In our study, the hypertensive phenotype of the SH rats is manifested by high systolic blood pressure, blunted baroreflex sensitivity and augmented chemoreflex, which is in line with previous findings [10,35]. Both the impaired baroreflex sensitivity and elevated tonic activity and sensitivity of the arterial chemoreflex promote increase in the sympathetic outflow to the cardiovascular system and have been postulated as important factors involved in the development and maintenance of hypertension [12,13]. It is well documented that the increased sensitivity of the arterial chemoreflex precedes development of hypertension in SH rats [36]. Furthermore, denervation or removal of carotid bodies in SH rats prevents development of hypertension, reduces already established high blood pressure, reduces sympathetic activity and resets the baroreflex function in SH rats [10,37,38]. In fact, increased sensitivity of the arterial chemoreceptor has been reported in young mild hypertensive men [39] and increased tonic activity of the arterial chemoreceptors has been also reported in hypertensive patients [40,41]. Moreover, unilateral removal of the carotid body effectively reduces arterial blood pressure in patients with resistant hypertension [42]. This large body of evidence points to the causative role of the augmented peripheral chemoreflex in the pathogenesis of arterial hypertension. Furthermore, the SH and WKY rats have similar serum concentrations of norepinephrine, Ang II and copeptin, a biomarker of vasopressin release, which is in agreement with other studies [43,44].

The brainstem and hypothalamic regions play a critical role in the nervous regulation of the cardiovascular system. Specifically, RVLM in the ventral medulla is a key pressor centre of the brainstem and its activation increases the sympathetic outflow to the cardiovascular system [12,14]. The NTS in the dorsal medulla integrates the sensory information from the peripheral receptors, such as baro- and chemoreceptors, and relays it to the RVLM and the hypothalamus directly or via the caudal ventro-lateral medulla (CVLM) [12–14]. The excitation or inhibition of presympathetic neurons of the RVLM depends on the source of the sensory input. Thus, activation of the carotid baroreceptors by an increase in arterial blood pressure leads to inhibition of RVLM neurons' activity and resultant withdrawal of sympathetic outflow, whereas stimulation of the arterial chemoreceptors results in excitation of the RVLM with subsequent sympathoexcitation and an increase in arterial pressure [13]. The activity of the brainstem centres is modulated and integrated with higher levels of the central nervous system by the hypothalamic PVN, which provides excitatory input to the RVLM and promotes sympathoexcitation and elevation of the arterial blood pressure [12,13]. In our study, the hypertensive phenotype is associated with altered expression of the proinflammatory TNF and anti-inflammatory IL-10 and their respective receptors, TNFR1 and IL-10Ra, in both the ventral aspect of the medulla oblongata containing RVLM region and the dorsal medulla encompassing NTS.

The outcome of the cytokine signalling depends on the balance within the intricate network of the cytokines and activation of their receptors. There is evidence that TNF induces TNF production in microglia by activating TNFR1, which provides basis for a positive feed-

back loop leading to increased release of the cytokine [45]. One of the key mediators, which limits this process, is IL-10. It was shown that incubation of microglia with TNF stimulates release of IL-10 [28] and that exposure of microglia to the anti-inflammatory IL-10 inhibits TNF release [28,29]. These findings indicate that there is a negative feedback loop between TNF and IL-10, which controls the levels of TNF. In our study the localization of the immunostaining for TNFR1 and IL-10Ra on microglial cells suggests that similar feedback mechanisms may be present in the brainstem of hypertensive and normotensive rats.

We found that TNF and TNFR1 levels in the hypothalamic region were significantly lower than in the brainstem in SH rats. However, there were no significant differences in the hypothalamic levels of TNF and TNFR1 between SH and WKY rats. Our finding that TNF concentration in the hypothalamus is similar in both normo- and hypertensive rats is different from observations, which indicate that SH rats have higher expression of TNF in the hypothalamic PVN than normotensive rats [8,18]. Similarly, we found that IL-10 concentration in the hypothalamus was significantly lower than in the brainstem of the SH rats, but there were no significant differences in the hypothalamic concentration of IL-10 between hypertensive and normotensive rats, different from observations of Agarwal and colleagues [18]. One of the possible explanations for these discrepancies in the cytokines' concentration may arise from the fact that we used the entire hypothalamus for evaluation of the cytokine concentration. Furthermore, the rats in the study by Agarwal et al. were exposed to high level of restraint stress during repeated recordings of blood pressure with tail-cuff method over a period of 16 weeks [18]. It has been shown that restraint during tail-cuff measurements triggers a robust stress response manifested by increase in arterial blood pressure and changes in the expression of cytokines in the brain [31,46].

We found that in the ventral medulla of SH rats concentration of TNF is increased in comparison to normotensive rats, which is in agreement with other studies [18]. Additionally, we show that increased levels of TNF are accompanied by expression of TNFR1 in the VM region, which was similar between SH and WKY rats. Furthermore, we found that IL-10 concentration in the VM is higher in SH rats than in WKY rats, different from previous observations [18]. A likely explanation of increased IL-10 concentration is the stimulatory effect of high levels of TNF on IL-10 expression [28]. Furthermore, we found that increased expression of IL-10 is accompanied by low concentration of IL-10Ra in the VM of the SH rats, however, the difference in IL-10Ra concentration between SH and WKY rats did not meet the pre-specified significance level of 0.05 with p value being 0.056.

In the dorsal medulla, we detected significantly higher concentration of TNF in SH rats than in WKY ones. Previous studies showed that expression of TNF mRNA in the NTS located in the DM is lower in SH rats [47] or does not differ between SH and WKY rats [15]. However, TNF concentration depends on the mRNA translation and protein expression of TNF, which may be independently regulated from mRNA expression [48]. Furthermore, TNF availability also depends on activity of TNF converting enzyme TACE and clearance of the cytokine in the tissues [16]. Along the increased concentration of TNF, we found that expression of TNFR1 is significantly higher in the DM of SH rats than in WKY rats. Moreover, the increased concentration of TNF is accompanied by significantly higher levels of IL-10 in the SH rats than in WKY ones. Nonetheless, this elevated concentration of IL-10 is accompanied by decreased levels of IL-10Ra, suggestive of less effective anti-inflammatory activity of the cytokine in the DM and a weaker negative feedback with TNF expression. Concentrations of the cytokines in all investigated regions of the brain of both the SH and WKY rats are significantly higher than in the serum, suggesting that they do not diffuse from the circulation, but are produced locally.

The role of the cytokines in the regulation of activity of the cardiovascular centres of the brain may depend on their indirect interaction with neurons via release of other transmitters, such as prostaglandins, activation of local renin-angiotensin system or changes in redox signalling [5,49]. However, cytokines may also directly affect function of neurons by changing expression of voltage gated ion channels [50–52], thus they can change the excitability of neural circuits in the cardiovascular centres of the brain. In fact, this has been recently shown in neurons of the subfornical organ (SFO), one of the key circumventricular organs of the brain involved in the central control of the cardiovascular system. Namely, Simpson and Ferguson showed that exposing SFO neurons to TNF leads to the decrease in excitation threshold, the recruitment of firing neurons and to the increase in action potential frequency and that the TNF-induced changes are dependent on modulation of voltage gated sodium channels [53], which may explain the sympathoexcitatory and pressor effects of TNF acting on the SFO [23,24]. Contrary to the TNF effects, IL-10 was shown to down-regulate expression of voltage gated channels and to reverse TNF-induced increase of sodium currents in the dorsal root ganglia [52]. The immunostaining for TNFR1 and IL-10Ra on neuronal cells detected in our study together with increased concentration of TNFR1 and decreased concentration of IL-10Ra in the brainstem of SH rats suggests that the above mechanisms regulating excitability of neurons may be also involved in the regulation of arterial blood pressure in hypertension.

A growing body of evidence shows that high blood pressure present in the arterial hypertension induced by chronic administration of Ang II depends on upregulation of PICs in the hypothalamus. Namely, in rats with Ang II induced hypertension, arterial blood pressure is lowered by chronic intrabrain infusion of etanercept, a TNF inhibitor [9]. Moreover, targeted depletion of microglia decreased levels of PICs in the PVN and effectively limited Ang II induced hypertension in mice [17], indicating that microglia are the key source of cytokines in this experimental model of hypertension. However, a recent study by Takesue et al. suggests that activation of microglia in the PVN is not critically involved in maintaining high blood pressure in already established hypertension in the SH rats [54]. In this light, the question arises whether the changes in TNF, IL-10 and their receptors are a bystander of hypertension or if they are causatively linked to increased arterial blood pressure in SH rats.

To answer this question we also investigated how ICV administration of TNF and IL-10 affects arterial blood pressure in normotensive and hypertensive rats. We found that ICV infusion of TNF in the SH rats triggers prolonged pressor response. This is in line with our previous findings in the awake rats, in which ICV infusion of TNF at similar doses increased arterial blood pressure [19,20]. Similarly, several studies have recently shown that TNF administered into the PVN, the subfornical organ or the carotid artery exerts sympathoexcitatory and pressor effects in anaesthetized rats [8,22–24]. Furthermore, chronic intrabrain infusion of etanercept, a selective TNF inhibitor, was shown to reduce hypertension in SH rats [55] and to normalize activity of central angiotensin type 1 receptors without effects on resting arterial blood pressure in rats with post-infarction heart failure [21]. This body of evidence strongly suggests that centrally acting TNF exerts a pressor response, possibly through the TNFR1 receptors, which we detected in key cardiovascular centres. Moreover, we found that ICV infusion of IL-10 results in a gradual decrease of arterial blood pressure, both in SH and WKY rats, however, the effect is greater in the hypertensive animals. This is in line with findings of the study by Shi et al., who showed that up-regulation of IL-10 in the brain attenuates Ang II-induced hypertension in rats [26].

Collectively, our results point to enhanced hemodynamic response to the intrabrain infusion of the cytokines in hypertensive rats, which further supports the notion that altered expression of TNF and IL-10 and their receptors is causatively associated with hypertension and regulation of the arterial blood pressure. However, further studies are needed to elucidate whether alterations in the expression of the cytokines and their receptors in the cardiovascular centres of the brain precede the development of high arterial blood pressure or if they occur secondarily to hypertension.

Taken together, our findings suggest the presence of a potent milieu for effective TNF signalling in the brainstem, which is associated with the hypertensive phenotype. In addition, we hypothesize that the increased IL-10 concentration in the brainstem is an expression of compensatory mechanism for the up-regulated TNF system.

5. Conclusions

The results of our study show that adult spontaneously hypertensive rats with already established high blood pressure express higher levels of proinflammatory cytokine TNF and its receptor TNFR1 in the brainstem than the normotensive animals. This is accompanied by increased expression of anti-inflammatory cytokine IL-10 and significantly lower expression of its receptor IL-10Ra in the brainstem of SH rats. Furthermore, the changes are associated with higher arterial blood pressure, blunted baroreflex and enhanced chemoreflex, and accentuated hemodynamic response to intrabrain infusions of TNF and IL-10 in SHR rats. Our findings support the hypothesis of involvement of the cytokines and neuroinflammation in the pathogenesis of essential hypertension.

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AS – recorded tail-cuff BP, collected brains and blood, did surgeries, hemodynamic experiments, ELISA tests and immunostaining, analysed data, did statistical analysis, prepared Figs, reviewed the manuscript; **PS** – recorded tail-cuff BP, collected brains and blood, did surgeries, hemodynamic experiments, ELISA tests and immunostaining, analysed data, reviewed the manuscript; **PK** – recorded tail-cuff BP, collected brains and blood, reviewed the manuscript; **TZ** – conceived and designed the study and supervised its execution, did immunostaining and confocal imaging, analysed data, did statistical analysis and interpreted results, prepared Figs and wrote and edited the manuscript.

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