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Gene expression profiles of major cytokines in the nucleus tractus solitarii of the spontaneously hypertensive rat

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ABSTRACT

Since the nucleus of the solitary tract (NTS) is a pivotal region for regulating the set-point of arterial pressure, we proposed a role for it in the development of neurogenic hypertension. Recent studies have suggested that pro-inflammatory molecules are highly expressed in the NTS of an animal model of human essential hypertension — the spontaneously hypertensive rat (SHR), compared to normotensive Wistar–Kyoto rat (WKY). Based on this evidence, we hypothesized that inflammatory mediators such as cytokines are up-regulated in the hypertensive NTS. In the present study, we have assessed the level of gene expression of some cytokines in the NTS of SHR compared to WKY. In addition, for further confirmation of abnormal inflammatory condition within the NTS of SHR, we identified gene expression levels of an inflammatory marker, glycoprotein-39 (gp39) precursor, which is homologous to chitinase 3-like protein 1, human cartilage-gp39 or YKL40. The NTS was micro-dissected from 15-week-old male SHR and WKY rats. Total RNA was extracted and quantitative RT-PCR was performed. Gene expression of gp39 precursor and monocyte chemoattractant protein-1 were higher in the NTS of SHR while inter-leukin-6 was lower in the NTS of SHR compared to the WKY. In contrast, there were no significant differences in the expression of other cytokines including: inter-leukin-1 beta, tumor necrosis factor-alpha and transforming growth factor beta 1. These data together with our previous published finding of an over expression of junctional adhesion molecule-1 suggest that the NTS of the SHR exhibits a specific inflammatory state.

1. Introduction

Human essential hypertension is a complex polygenic trait with underlying genetic components that remain unknown. As described originally by Doba and Reis (1973), destruction of the nucleus tractus solitarii (NTS) leads to fulminating hypertension. This is highly suggestive that the NTS is a central brain stem structure that plays a vital role in maintaining the set point of arterial pressure. Equally, because baroreceptor afferents terminate in this nucleus (Ciriello, 1983), it is also one of the most effective central sites for modulating baroreceptor reflex function, a process that is critically important for blood pressure homeostasis (Frigero et al., 2000; Machado, 2001; Paton, 1999; Talman et al., 1981). Based on this evidence, we proposed a role for it in the development of neurogenic hypertension.

Recently, we identified that junctional adhesion molecule-1 (JAM-1) was over expressed in endothelial cells in the NTS of an animal model of human essential hypertension — the spontaneously hypertensive rat (SHR) compared to normotensive Wistar–Kyoto rat (WKY; Waki et al., 2007). Moreover, over expression of JAM-1 in NTS of WKY rats produced hypertension (Waki et al., 2007). JAM-1 is known to be expressed in endothelial cells and has numerous functions including binding to both leukocytes and platelets enhancing cell transmigration between tight junctions (Del Maschio et al., 1999; Ebnet et al., 2004; Fraemohs et al., 2004) and activating platelet aggregation (Ebnet et al., 2004; Naik et al., 1995). With the heightened mRNA of JAM-1 in the NTS of the SHR, we have also shown endogenous leukocyte accumulation inside capillaries within the NTS of SHR (but not WKY rats; Waki et al., 2007), suggesting an specific inflammatory condition. Although we do not have any data indicating a breakdown of the blood-brain barrier or influx of leukocytes into the brain parenchyma of the NTS of SHR (Waki et al., 2007), subtle immunological alterations including specific cytokine release could occur. For example, tumor necrosis factor-alpha (TNF-α) and inter-leukin-1 beta (IL-1β) are known to have central actions causing excessive sympathoactivation (Zhang et al., 2003; Kannan et al., 1996), indicating that some types of cytokines may have a crucial role in contributing to neurogenic hypertension. Based on this evidence, we hypothesize that an inflammatory response characterized by abnormal gene expression of inflammatory cytokines and chemokines is present in the brainstem of the SHR.

In the present study, we have assessed the level of gene expression of major cytokines in the NTS of SHR relative to WKY. IL-1β, inter-

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leukin-6 (IL-6), TNF-α, transforming growth factor beta 1 (TGF-β) and one of the chemokines, monocyte chemoattractant protein-1 (MCP-1) were selected because they are all known to be expressed in the brain and affect neuronal function (Banisadr et al., 2005a,b; D'Arcangelo et al., 2000; Gadient and Otten, 1994; Gosselin and Rivest, 2007; Mo et al., 1996; Nakashima et al., 1999; Tilg et al., 1997; Unsicker and Krieglstein, 2002). In addition, for further confirmation of an inflammatory condition within the NTS of SHR, we identified gene expression levels of an inflammatory marker, glycoprotein-39 (gp39) precursor, which is homologous to chitinase 3-like protein 1 (Chi3L1), human cartilage-gp39 or YKL40 (Ober et al., 2008; Rathcke and Vestergaard, 2006; Colton et al., 2006; Nigro et al., 2005; Peltomaa et al., 2001; Vos et al., 2000; Johansen et al., 2000). Here, we show that the NTS of the SHR exhibits a specific inflammatory state indicating potential novel mechanisms underlying the developmental and maintenance of hypertension in the SHR.

2. Methods

Procedures were carried out according to the UK Home Office guidelines on animals (Scientific Procedures) Act 1986. Some of the experimental protocols were performed in Japan and approved by Animal Ethics Review committee in Wakayama Medical University. The animals were housed individually, allowed normal rat chow and drinking water *ad libitum*, and kept on a 12 h light/12 h dark cycle.

2.1. Gene expression profiles of major inflammatory molecules

2.1.1. Extraction of RNA

Both three (SHR, $n=6$; WKY, $n=6$) and 15-week-old (SHR, $n=6$; WKY, $n=6$) male rats were humanely killed by cervical dislocation. The NTS was dissected out rapidly from each animal and RNA was extracted by a conventional method using TRIzol reagent (Invitrogen, Carlsbad, CA). To avoid contamination with genomic DNA, the RNA samples were treated with RNase-free DNase I (Roche Diagnostics GmbH, Mannheim, Germany). RNA purity was verified by performing PCR on samples not treated with reverse transcriptase.

2.1.2. Quantitative RT-PCR:

β-actin, gp39 precursor, IL-1β, IL-6, IL-6 receptor, TNF-α, TGF-β and one of the chemokines, MCP-1 genes were tested in this study. Except for gp39 precursor, all primer sets we used QuantiTect Primer Assay from Qiagen, which provides highly specific and sensitive results in SYBR Green based real-time reverse transcription (RT)-PCR. For gp39 precursor (AF062038), the following primer sequences were used: forward (agcaacaacaagctcagcac), reverse (gcgagatccagtccatcaaag). The sizes of the PCR products amplified with the primers were 230 bp. In preliminary experiments, we confirmed that the efficiency of these primer pairs is comparable (data not shown). Real-time RT-PCR reactions were carried out using a DNA Engine Opticon 2 system (MJ Research). The QuantiTect SYBR Green RT-PCR kit (Qiagen) was used according to the manufacturer's protocol: Two microliters of diluted RNA as a template were mixed with 1 μmol/L of each primer, 10 μL of 2xQuantiTect SYBR Green RT-PCR Master Mix, and 0.2 μL of QuantiTect RT mix and brought to 20 μL of total reaction volume using RNase-free water. The reactions were incubated at 50 °C for 30 min for RT and then for 15 min at 95 °C to inactivate RT and activate the HotStarTaq DNA Polymerase, followed by 40 cycles with 15 s denaturation at 94 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C. After each extension step, the fluorescence signal was measured. After 40 cycles, a melting curve for each product was obtained for quality control. Expression of target genes was assessed in relation to a housekeeping gene (ß-actin) using the comparative ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001) in each sample. Fold differences against average values of WKY were calculated.

Table 1
Candidates housekeeping genes in the NTS of SHR and WKY rats ranked according to the expression stability calculated by GeNorm (a) and Normfinder (b)

Gene Name	Stability value M	Ranking order
(*a*) *GeNorm*		
Hprt/Hspcb	0.198	1/2
ß-actin	0.222	3
RGD1564921_predicted	0.243	4
Tfrc	0.253	5
Rplp1	0.262	6
Rpl13a	0.281	7
Gusb	0.299	8
B2m	0.313	9
Ldha	0.326	10
Pgk1	0.340	11
Sdha	0.358	12
(*b*) *Normfinder*		
Tfrc	0.050	1
Hprt	0.063	2
ß-actin	0.065	3
RGD1564921_predicted	0.067	4
Hspcb	0.072	5
Gusb	0.081	6
Rpl13a	0.084	7
Ldha	0.085	8
B2m	0.099	9
Rplp1	0.108	10
Sdha	0.120	11
Pgk1	0.123	12

2.1.3. Data analysis

Group data of gene expression levels in inflammatory cytokines and gp39 precursor were expressed as mean±SEM. Unpaired *t*-test was used for comparisons between two groups. Differences were considered significant if $p<0.05$.

2.2. Justification of using β-actin as an internal control

To justify whether β-actin can be used as an internal control/good normalization gene in this study, the following experiment was also performed. RT^2 Profiler™ PCR Array Rat Housekeeping genes (Superarray Bioscience Corporation) was used on cDNA from NTS of SHR and WKY rats to identify the expression stability of 12 commonly used housekeeping genes: ribosomal protein, large, P1 (Rplp1), hypoxanthine guanine phosphoribosyl transferase (Hprt), ribosomal protein L13A (Rpl13a), lactate dehydrogenase A (Ldha), β-actin, phosphoglycerate kinase 1 (Pgk1), β-2 microglobuin (B2m), transferrin receptor (Tfrc), glucuronidase, beta (Gusb), heat shock 90 kDa protein1, beta (Hspcb), similar to peptidyl prolyl isomerase H (predicted) (RGD1564921_predicted), succinate dehydrogenase complex, subunit A, flavoprotein (Sdha) (accession numbers available on the company website). The RNA from the NTS of male SHR and WKY rats (10-week-old, $n=4$ each) was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) and cleaned-up by RNeasy Mini kit (Qiagen) following the manufacturers protocol. Equal quantities of RNA from each NTS were submitted to Reverse Transcription using the RT^2 First Strand Kit (Superarray Bioscience Corporation). The cDNA was submitted to real-time quantitative PCR reactions on RT^2 Profiler™ PCR Array plates using the Superarray RT^2 SYBR Green/Fluorescein qPCR Master Mix (Superarray Bioscience Corporation) following the manufacturer instructions. Two publicly available Visual Basic Applications for Microsoft Excel, GeNorm (Vandesompele et al., 2002, available on http://medgn.ugent.be/~jvdesomp/genorm/) and Normfinder (Andersen et al., 2004, available on http://www.mdl.dk) were used to assess the expression stability of the 12 candidates housekeeping genes in the NTS of SHR and WKY. GeNorm is an algorithm which performs a pair wise comparison of candidate housekeeping genes and ranks the potential reference genes according to their expression stability 'M'