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Upregulation of GAD65 mRNA in the Medulla of the Rat Model of Metabolic Syndrome

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Abstract

Metabolic syndrome is characterized by obesity, elevated blood pressure (BP), insulin resistance, and hypercholesterolemia. Recently an animal model of this disorder has been proposed in rats selectively bred based on their performance on a treadmill-running task. Accordingly, low capacity runner (LCR) rats exhibited all of the diagnostic criteria for metabolic syndrome, including elevated BP, as compared to their high capacity runner (HCR) counterparts [23]. Previous studies have highlighted the importance of GABAergic neurotransmission in the medullary cardiovascular-regulatory areas in the central control of BP. Thus, we hypothesized a dysregulation in GABAergic transmission in the medullary cardiovascular-regulatory nuclei of LCR rats. To begin testing this hypothesis we carried out experiments examining expression of the GABA synthetic enzymes, GAD65 and GAD67, mRNAs in the two rat strains via radioactive *in-situ* hybridization. Our results showed GAD65 and GAD67 mRNAs were widely expressed throughout the brainstem; quantification revealed increased GAD65 mRNA expression in LCR animals in the caudal nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (VLM) as compared to HCR rats. Conversely, no differences in the expression of GAD67 were detected in these regions. These data are consistent with the notion of altered GABAergic neurotransmission in the NTS and VLM in metabolic syndrome, and point to the importance of these regions in cardiovascular regulation.

Keywords

γ -amino-butyric acid; blood pressure; brainstem; ventrolateral medulla; nucleus tractus solitarius

Metabolic syndrome is a disorder characterized by a “deadly quartet” of hyperlipidemia, obesity, elevated blood pressure (BP) and elevated blood glucose [1,24]. Despite the high prevalence and increased risk of cardiovascular disease conferred by metabolic syndrome [24], studies have not revealed the mechanistic etiology of this complicated disorder. Recently Wisløff and colleagues generated an animal model of metabolic syndrome [23]. In this model, rats were selectively bred based on their ability to perform on a treadmill endurance running task. Accordingly, rats that have a high intrinsic aerobic capacity and are capable of running comparatively long distances are classified as high capacity runner (HCR) rats and are bred

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together. On the other hand, rats with a low intrinsic aerobic capacity that are only capable of running relatively short distances are classified as low capacity runner (LCR) rats and are bred with each other. Eleven generations of selective breeding resulted in elevated BP and the other signs of metabolic syndrome in LCR rats as compared to HCR rats [23].

One advantage of this model is that elevated BP in the LCR rats occurs together with the other hallmarks of metabolic syndrome. This is in contrast to animal models of hypertension such as the spontaneously hypertensive rat (SHR) in which elevated BP occurs in isolation of most other metabolic symptoms [2]. The LCR-HCR rats, on the other hand, provide a more representative model of human disease, since LCR rats' elevated BP occurs with other physiological alterations commonly found to be co-morbid in human disease states.

Several distinct nuclei within the medulla regulate resting BP, with the key region being the rostral ventrolateral medulla (VLM). This nucleus provides tonic excitatory drive to sympathetic vasoconstrictor nerves which function to maintain resting BP levels [5]. The caudal nucleus tractus solitarius (NTS) is another medullary site that plays a critical role in the regulation of BP levels by inhibiting the rostral VLM via interneurons located within the caudal VLM [13,15].

GABAergic neurotransmission is critical to the normal functioning of this cardiovascular-regulatory circuit. For example, blockade of GABA re-uptake at the synapse along with infusions of GABA receptor agonists into the NTS increase BP, heart rate and sympathetic nerve discharge [19-22]. These GABA-mediated pressor responses are potentiated in animal models of chronic hypertension, such as SHR [21]. Likewise, manipulating GABAergic transmission within the VLM has profound effects on resting BP levels. For example, infusions of muscimol, a GABA_A receptor agonist, into rostral VLM lower arterial BP to levels that are equivalent to those achieved with ganglionic blockade [17]. A major source of GABAergic innervation in the rostral VLM are neurons located within the caudal VLM [15], thus it seems feasible that alterations in the GABAergic neurotransmission in the VLM may also lead to hypertension.

Taken together, these data point to the importance of GABAergic neurotransmission in the regulation of resting BP levels. The current evidence also suggests that dysregulation of GABAergic transmission in multiple medullary areas can contribute to BP elevations such as those observed in LCR rats relative to HCR rats. We hypothesized dysregulations in the expression of mRNAs coding for the GABA synthetic enzyme – glutamic acid decarboxylase (GAD) – in two medullary structures of LCR rats. Using radioactive *in situ* hybridization we evaluated expression of mRNAs encoding for GAD65 and GAD67, the two isoforms of GAD, in the NTS and the VLM.

The use of experimental animals was carried out in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The development of the LCR and HCR rats through generations 6 and 11 has been described previously [23]. The protocol for estimation of endurance capacity required 2 weeks and was started when the rats were 10 weeks old. In the first week, rats were taught to run by placing them on the treadmill (model Exer-4, Columbus Instruments, Columbus, OH) for increasing duration each day, until the animals were able to run 5 min at 10 m/min on a 15° slope. During the second week, each rat underwent a daily endurance trial on five consecutive days at a constant slope of 15° and an initial velocity of 10 m/min. Treadmill velocity was increased by 1 m/min every 2 min until the third time a rat could no longer keep pace with the speed of the treadmill. For each of the five trials, the total distance run (in m) was used as the estimate of endurance capacity. The single best daily run of five trials for each rat was considered the trial most closely associated with the heritable component of exercise endurance.

The current study was carried out in 7 adult male HCR rats and 6 adult male LCR rats from the 18th generation. The animals were tested for their aerobic capacity as described above between 10 to 12 weeks of age and were sacrificed between approximately 24 and 26 weeks of age (Table 1). Prior to sacrifice animals were housed in pairs for one week – each cage containing a pair of either HCR or LCR male rats. Food and water were available *ad libitum*, and animals were kept in a 12 hour light, 12 hour dark environment.

Rats were sacrificed via rapid decapitation using a guillotine. The brains were extracted, flash frozen in 2-methylbutane at -30°C , and stored at -80°C . Brains from each animal were cryostat sectioned coronally to a thickness of 10 μm at -20°C from the pyramidal decussation to the level of the 7th nerve nucleus and thaw-mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA). For the purposes of anatomical alignment every tenth slide was stained with cresyl violet. All of the brains were aligned relative to the obex, defined as the opening of the fourth ventricle.

Slides were removed from -80°C and fixed in 4% paraformaldehyde at room temperature for 1 hour and processed for radioactive *in situ* hybridization as previously described [4,10]. Radioactive GAD65 and GAD67 probes were prepared from E. coli containing pBluescript SK⁺ cloning vectors (Stratagene, San Diego, CA). The GAD65 probe was designed to be 640 nucleotides in length and was based on a publicly-available sequence (accession number: M72422). The GAD67 probe was designed to be 900 nucleotides in length and was based on a publicly-available sequence (accession number: M34445). To verify that the inserts were of predicted lengths, the products were separated by gel electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Probes were also sequenced at the University of Michigan's DNA Sequencing Core and showed perfect alignment with predicted sequences.

For radioactive cRNA probe synthesis DNA was extracted and linearized with EcoR1, while BamH1 was used for linearization in sense control experiments. Probes were prepared with ^{35}S -UTP, ^{35}S -CTP, and linearized plasmid DNA in transcription buffer mix containing T3 RNA polymerase for antisense, and T7 for sense probes.

To determine the distribution of radioactive cRNA *in situ*, slides were apposed to radiosensitive film (Kodak Biomax; Eastman Kodak, Rochester, NY) and were developed 70-72-hours later. In negative control experiments the same exposure procedure was carried out for slides hybridized to sense probes.

Autoradiographs were digitized using a Sony XC-ST70 video camera connected to a computer running MCID Basic software (Imaging Research, St. Catharines, ON). For each brainstem level, two sections per animal were digitized and later analyzed with Scion Image Beta 4.02 software (Scion Corp., Frederick, MA) using an in-house macro designed to quantify signal 3.5 standard deviations above average background (defined as signal within the white matter). For each section, signal was averaged between the left and right side of the section, and total signal from two sections was averaged to get the final averaged value.

Statistical analyses were carried out using SAS software (v 9.1.3, SAS Institute., Cary, NC). For each region of interest, data were analyzed using a two-way ANOVA with rostrocaudal level and phenotype as independent variables and signal as a dependent variable. Significance was set as $p < 0.05$. In cases when a significant main effect was detected, post-hoc analysis was conducted using Tukey's Honestly Significant Difference post-hoc test. Data are presented as mean $\pm$ SEM.

HCR and LCR rats used in the current study significantly differed in both body weight and aerobic capacity (Table 1). Compared to LCR rats, HCR rats' best time to exhaustion was over three times greater; their best distance run was over five times greater; and their best speed was

two-fold greater (Table 1). There was no difference in age at the time of sacrifice between the two groups (Table 1).

To verify the specificity of our GAD65 and GAD67 *in situ* hybridization probes, we conducted experiments with both sense and anti-sense cRNA probes in tissue from an unhandled adult male Sprague-Dawley rat. The results revealed that hybridization of the tissue with an anti-sense GAD65 probe produced relatively heavy pattern of labeling in the tectum and a laminated pattern of labeling in the cortex (data not shown). This pattern of labeling was specific to the anti-sense probe since no labeling was observed when tissue was hybridized with a sense probe (data not shown). The GAD67 anti-sense probe produced a pattern of labeling similar to that observed for the GAD65 anti-sense probe; this labeling was also specific as hybridization with a GAD67 sense probe produced no detectable signal (data not shown).

Our initial analysis focused on the distribution of GAD65 and GAD67 mRNA in the medulla of HCR and LCR rats (Fig. 1). Labeling for GAD65 and GAD67 was present throughout the brainstem and included VLM, ventromedial medulla, NTS, area postrema, raphe nuclei, vestibular nuclei, and spinal trigeminal nucleus. Labeling was absent in motor nuclei, such as the facial nerve nucleus and the hypoglossal nerve nucleus (Fig. 1). The general patterns of GAD65 and GAD67 mRNA distributions were very similar.

The expression of GAD65 mRNA in LCR and HCR rats was quantified within NTS and VLM. Significant main effects of level and phenotype were observed within both regions ($p < 0.05$), indicating that LCR rats had higher levels of GAD65 mRNA compared to HCR rats. GAD65 expression in the rostral VLM was greater within LCR animals compared to HCR ($p < 0.05$, Fig. 2). Higher levels of GAD65 mRNA expression were also found in the NTS of LCR, with post-hoc analysis indicating a difference at -400 m relative to the obex (Fig. 2).

In the case of GAD67 mRNA, no significant differences in its expression were detected between HCR and LCR rats in either NTS or VLM (Fig. 3). Similarly, no significant differences in the expression of GAD65 and GAD67 mRNAs were observed either in nucleus raphe obscurus or ventral gigantocellular nucleus (data not shown).

The current study examined the distribution of GAD65 and GAD67 in the rat brainstem in two strains of rats that were bred based on their ability to perform on a treadmill running task. As described previously, the LCR rats exhibited dramatically poorer exercise capacity compared to their HCR counterparts. LCR rats became exhausted more quickly, ran for a shorter distance, and at a slower pace compared to HCR rats. LCR animals were also significantly heavier than HCR rats, and previous work has demonstrated HCR-LCR differences in mean BP levels with LCR rats exhibiting mean arterial pressure levels of about 13 mmHg higher than HCR rats [23]. These findings together with prior observations of increased visceral adiposity and increased insulin resistance suggest that LCR animals may represent an animal model of metabolic syndrome [23].

The overall pattern of distribution of GAD65 and GAD67 within the medulla described here is consistent with that described previously [18]. Our findings demonstrate higher levels of expression of GAD65 mRNA in LCR rats as compared to HCR rats within the caudal NTS and rostral VLM without a difference in the expression of GAD67 mRNA in these regions. It is generally thought that the two GAD isoforms are localized within different cellular compartments. GAD67 is associated predominantly with cytoplasmic pools of GABA, while GAD65 is associated with vesicular pools of GABA [16]. It is likely that GAD67 regulates GABA synthesis for metabolic functions of the cell, while GAD65 regulates GABA synthesis for synaptic release [16]. These observations together with our data suggest increased levels of GABAergic neurotransmission within medullary cardiovascular-regulatory regions of LCR rats.

Increased GABA neurotransmission within the caudal NTS as a potential cause of elevated BP in metabolic syndrome fits well with the current notions of central control of cardiovascular function. Caudal NTS is the first relay in the baroreceptor reflex loop; excitatory neurons project from this region to the inhibitory interneurons within the caudal VLM to inhibit the firing of the cardiovascular pre-sympathetic neurons of the rostral VLM [6,15]. Thus, increased GABAergic tone in the caudal NTS may explain differences in resting BP between these two strains of rats. Accordingly, increased GABAergic tone within the NTS produces higher BP, an effect that is mediated by both GABA_A and GABA_B receptors [19]. Hypersensitivity of GABA_B receptors has been implicated in the pathophysiology of hypertension in SHR, since injections of baclofen, a GABA_B receptor agonist, into the NTS lead to exaggerated BP increases compared to normotensive control rats [3]. In contrast, exercise-induced suppression of the baroreceptor reflex is mediated by the activation of GABA_A receptors in the NTS [13]. Thus, activation of either GABA_A or GABA_B receptor systems in the NTS can lead to BP increases. Our data suggest that such an increase in GABAergic tone in the NTS of LCR rats may contribute to their elevated BP as compared to the HCR rats.

In addition to alterations in GAD65 expression in the caudal NTS, we also observed an upregulation in the expression of GAD65 mRNA in the rostral VLM in the LCR rats. Unlike the finding in the caudal NTS, this observation does not fit neatly into the currently accepted model on central control of cardiovascular function. According to the classical model, increased GABAergic neurotransmission within the rostral VLM would lead to lower and not higher BP as we observed in the LCR rats [6]. Though this finding is difficult to resolve with existing literature, there may be other possibilities that may explain this observation. One option is that this alteration in GAD65 mRNA expression occurs in non-cardiovascular neurons. Rostral VLM is a heterogeneous region that also contains neurons with respiratory functions [14]. Thus, the upregulation in the expression of GAD65 mRNA in the rostral VLM of LCR rats may represent potential alterations in respiratory function.

Another possibility is that presumed increase in GABAergic neurotransmission activates the “accessory” vasomotor pathway originating from the rostral VLM [11]. This pathway is thought to activate “accessory” sympathetic preganglionic neurons, which are characterized by their unmyelinated axons and which drive hexamethonium-resistant transmission in sympathetic ganglia [11]. Unlike “regular” sympathetic preganglionic neurons which are inhibited by administration of GABA in the rostral VLM, the “accessory” sympathetic preganglionic neurons are activated by this manipulation [11]. Thus, it may be that activation of this “accessory” pathway contributes to resting BP differences between LCR and HCR rats.

Yet another possibility is that increased GAD65 mRNA expression in the rostral VLM of LCR rats leads to increased GABA production and secretion within projection neurons, rather than local interneurons. Data from anatomical and pharmacological experiments suggest that neurons within the rostral VLM project to the NTS where they inhibit the baroreceptor reflex [7-9]. This notion is supported by the observation that administration of glutamate into the rostral VLM, but not into surrounding regions, leads to the release of GABA in the caudal NTS [7]. This glutamate-induced increase in GABA secretion in the NTS is associated with the suppression of the baroreceptor reflex, an effect that is blocked by administration GABA_A and GABA_B receptor antagonists in the NTS [7]. Such an increase in baroreflex inhibition would lead to higher resting BP levels, which may contribute to increased BP levels in LCR rats. Future studies will be required to test these hypotheses.

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Fig 1.

Distribution of GAD65 (left column) and GAD67 (right column) mRNA in the rat medulla as determined by radioactive *in situ* hybridization technique (data are from a representative LCR animal). Drawings on the right are adapted from the atlas of Paxinos and Watson [12]. Numbers on the right indicate distances from obex and bregma (in parenthesis) in m. Scale bar – 3 mm. Abbreviations: 4v – fourth ventricle, 7 – facial nerve nucleus, GiA – gigantocellular nucleus pars α , GiV – ventral gigantocellular nucleus, LPGi – lateral paragigantocellular nucleus, NTS – nucleus tractus solitarius, py – pyramidal tract, pyx – pyramidal decussation, RMg – raphe magnus, ROb – raphe obscurus, RPa – raphe pallidus, VLM – ventrolateral medulla

Fig 2.

Expression of GAD65 mRNA within nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM). GAD65 mRNA expression was quantified at different rostro-caudal levels and signal intensity was plotted as a function of distance relative to obex for the different brain regions.

* – $p < 0.05$, LCR and HCR comparison at the same brainstem level.

Fig 3.

Expression of GAD67 mRNA within nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM). GAD67 mRNA expression was quantified at different rostro-caudal levels and signal intensity was plotted as a function of distance relative to obex for the different brain regions.

Characterization of high capacity (HCR) and low capacity (LCR) runner rats used in the present study.

Table 1

	N	Body Weight (g)	Best Time to Exhaustion (min)	Best Distance (m)	Best Speed (m/min)	Age at Sacrifice (weeks)
HCR	7	230.5 ± 10.8 *	62.0 ± 1.1 *	1551.9 ± 43.6 *	40.6 ± 0.5 *	24.6 ± 2.0
LCR	6	315.9 ± 19.7	20.5 ± 1.0	301.2 ± 19.7	20.0 ± 0.5	26.0 ± 2.9

* p < 0.05 as compared to LCR.