ORIGINAL ARTICLE

Defferences in cerebellar nuclear factor kappaB pathways in hypertensive and obese rats: behavioural aspect

Andrej Barta, Stanislava Vrankova, Jana Parohova, Mária Kovácsová, Zuzana Matúšková, Kristína Bučková, Fedor Jagla, Ludovit Paulis, Olga Pechanova

Institute of Normal and Pathological Physiology and Centre of Excellence for Nitric Oxide Research, Slovak Academy of Sciences, Bratislava, Slovak Republic.

Correspondence to: Andrej Barta, DVM Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Sienkiewiczova 1, 813 71 Bratislava, Slovak Republic, tel.:+421-2-52926271, fax: +421-2-52968516, e-mail: olga.pechanova@savba.sk

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Abstract

Recently, alterations in cerebellar nuclear factor kappaB (NF-κB) expression and nitric oxide (NO) generation were documented to be associated with increased locomotor activity in spontaneously hypertensive rats (SHR). We aimed to analyze whether changes in cerebellar NF-κB pathway in hypertension associated with metabolic syndrome (MS) may affect locomotor activity of MS rats. Furthermore, the age-dependent changes were investigated as well. Male Wistar Kyoto rats (WKY, 9 weeks old), spontaneously hypertensive rats (SHR, 9 weeks old), and rats with MS aged 9 and 12 weeks were analyzed. Blood pressure was measure by tail-cuff plethysmography. Locomotor activity was tested by the open field method. Protein expression of NF-κB (p65 subunit), inducible NOS (iNOS) and NOS activity were determined in the cerebellum.

In SHR, despite no changes in NOS activity, increased NF-κB (p65) expression was associated with increased of both horizontal and vertical motor activities. Similarly, in young MS rats, increased NF-κB (p65) expression led to increased horizontal motor activity despite decreased NOS activity. On the other hand, no significant changes in NF-κB (p65) expression were demonstrated in adult MS rats. Horizontal and vertical motor activities as well as NOS activity were markedly reduced in this group of rats.

In conclusion, increased cerebellar NF-κB (p65) expression was associated with increased horizontal motor activity in both SHR and young MS rats, despite the obesity which accompanied also young rats with metabolic syndrome. Downregulated NF-κB pathway together with increased weight of rats may belong to the causes of decreased locomotor activity of adult MS rats.

Introduction

Number of human and animal data suggests an association between hypertension, metabolic syndrome and certain alterations in locomotor activity (Franco *et al* 2004; Hagg *et al* 2004; Prasad *et al* 2012). The spontaneously hypertensive rats (SHR) exhibit locomotor hyperactivity in comparison to its normotensive progenitor Wistar-Kyoto strain (Amini *et al* 2004; Whitehorn *et al* 1983). SHR receiving antihypertensive therapy exhibit a significant decrease in blood pressure, however without changes in locomotor activity.

Vice versa, normotensive WKY after unilateral renal clips develop increase in blood pressure also without alterations in locomotor activity (Whitehorn *et al* 1983). Moreover, obesity associated with hypertension within the cluster of metabolic syndrome may represent a significant contribution to the decrease of motor activity (Novak *et al* 2010).

Recently, we have demonstrated a correlation between alterations in brain nuclear factor kappaB (NF-κB) expression and locomotor activity in spontaneously hypertensive rats (Pechanova *et al* 2006). In general, NF-κB represents a protein complex that controls the transcription of DNA. NF-κB has been found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, different antigens, and free radicals particularly (Gilmore 2006; Perkins 2007). Several studies have shown that NF-κB is either constitutively active in neurons or activated by excitatory amino acid, neurotransmitters or both (Massa *et al* 2006). Although nitric oxide (NO) belongs to the untraditional neurotransmitters, the role of NO in the neuron NF-κB activation could be suggested. In neurons, nitric oxide may be synthesized by neuronal NO synthase (nNOS) and inducible NO synthase (iNOS) isoforms. Inducible NOS is independent of agonist stimulation and intracellular calcium levels and is regulated by cytokines primarily via the transcriptional factor NF-κB. Several analyses indicate that NF-κB mediates expression of iNOS and vice versa NO can regulate transcriptional activity of NF-κB (for review see Parohova *et al* 2009).

Nitric oxide was documented to be also involved in the many brain physiological and/or pathological processes, such as excitability and frequency of action potentials (Pehli and Schmid 1997), long-term potentiation (LTP) in hippocampus (Bohme *et al* 1991), long-term depression (LTD) in cerebellum (Linden and Connor 1992), neurotoxicity as well as neuroprotection (Buisson *et al* 1993; Castagnoli *et al* 1999; Pechanova 2010; Jagla *et al* 2009; Zenebe and Pechanova 2002). It was also reported that NO plays an important role in the control of behaviour (Reddy and Kulkarni 1998) as well as of locomotion (Pechánová *et al* 2003, 2004). Alterations in NO synthase activity and/or NO concentration were shown to have modulatory effect on the locomotor activity of the rats and mice (Del Bel *et al* 2005; Kayir and Uzbay 2003). Previously, it was shown that NO modulates locomotor behaviour, probably by interfering with dopaminergic, serotonergic, cholinergic and glutamate neurotransmission in the striatum (Del Bel *et al* 2005). Concerning spontaneously hypertensive rats, the findings indicated that genetic factors are responsible for the increased locomotion activity of this strain. Both, the genes responsible for the hypertensive trait and those responsible for the hyperactivity trait could be taken into account (Hendley *et al* 1983; Markel 1986).

Thus, it should be supposed that both NO level and alterations associated with hypertension and/or obesity participate in the changes of locomotor activity in rats. The aim of our study was to analyze whether changes in cerebellar NF-κB and NOS activity in hypertension associated with metabolic syndrome (MS) may affect locomotor activity of SHR and MS rats.

Materials and methods

Chemicals and drugs

All the chemicals used were purchased from Sigma Chemicals Co. (Germany) when not specified.

Animals

All procedures and experimental protocols were approved by the *Ethical Committee of the Institute of Normal and Pathological Physiology SAS*, and conform to the *European Convention on Animal Protection* and *Guidelines on Research Animal Use.*

Male Wistar Kyoto rats (WKY) 9-week-old, spontaneously hypertensive rats (SHR) 9-week-old and rats with metabolic syndrome, 9- and 12-week-old, were used for the investigation (n=8 in each group). All animals were housed at a temperature of 22–24 °C, drank a tap water and fed with a regular pellet diet *ad libitum*. Blood pressure (BP) was measured by the non-invasive method of tail-cuff-plethysmography. The horizontal and vertical locomotor activity of the animals was tested in an exploratory box by the open-field method. Thereafter, total NOS activity, nuclear factor NF-κB (p65) and iNOS protein expressions were determined in the cerebellum of the same animals which were used for the locomotor activity study.

Locomotor activity study

All animals were tested for locomotor activity in an exploratory box by the open-field method. The box was placed in a moderately sound proof room and equally illuminated from above. The rat was placed in the centre of the exploratory arena 80×60×40 cm in size with the floor divided into 8 squares. Number of crossings between squares and of rearing was recorded by video system. Horizontal and vertical motor activities were registered by QuickCamPro Logitech cameras with 640×480 resolution and video MGI software. The crossings between the squares on the floor as well as the squares on the translucent vertical wall of the experimental cage were counted automatically. The vertical and horizontal motor activities of the rats were registered in 20 minutes sessions in the same daytime period. The box was cleaned with a mild detergent between animals.

NF-κB and iNOS protein expression

Samples of the cerebellum were homogenized in 25 mmol/l Tris-HCl, pH 7.4, containing 5 mmol/l EDTA, 50 mmol/l NaCl, 1 μmol/l leupeptin, 0.3 μmol/l aprotinin, 0.1 mmol/l PMSF, 1 mmol/l gestating and 1% SDS. After the centrifugation (15,000×g, 20 min, twice)

supernatants were subjected to SDS-PAGE using 10% gels. Following the electrophoresis, proteins were transferred to nitrocellulose membranes and were probed with a polyclonal rabbit anti-nuclear factor-κB (NF-κB) antibody which recognizes the 65 kDa RelA (p65) protein (Santa Cruz Biotechnology, CA). Bound antibody was detected using a secondary peroxidase-conjugated anti-rabbit antibody (Alexis Biochemicals, Germany). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK) and analysed densitometrically using Photo-Capt V.99 software.

Total NO synthase activity

Total NO synthase activity was determined in crude homogenates (Potter, teflon homogenizer) of the cerebellum by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine as previously described by Bredt and Snyder (1990) with minor modifications (Pechánová *et al* 1997). Briefly, 50 µl of crude homogenate of the brain part (7.5 mg of wet tissue) was incubated in the presence of 50 mmol/l Tris/HCl, pH 7.4, containing 1 µmol/l [^{3}H]-L-arginine (specific activity 5 GBq/mmol, approx. 100,000 d.p.m.), 0.5 mg/ml calmodulin, 0.5 mmol/l β-NADPH, 250 µmol/l tetrahydrobiopterin, 4 µmol/l FAD, 4 µmol/l flavin mononucleotide and 1 mmol/l Ca^{2+}, in a total volume of 100 µl. After a 30-min incubation at 37 °C, the reaction was stopped (by adding 0.02 M Hepes containing 2 mM EDTA, 2 mM EGTA and 1 mM [^{3}H]-L-citrulline), the samples were centrifuged, and supernatants were applied to 1-ml Dowex 50WX-8 columns (Na^+ form). [^{3}H]-L-citrulline was eluted with 2 ml of water and radioactivity was determined by liquid scintillation counting. Total NO synthase activity was expressed as pkat/g of proteins.

Statistical Analysis

The results are expressed as mean ± SEM. Values were considered to differ significantly if the two-tailed probability value (p) was less than 0.05 (one-way ANOVA with Bonferroni post-test).

Results

Blood pressure and weight

Systolic blood pressure in the SHR and young and adult MS rats was increased significantly to 167±5, 173±2, and 179±2 mmHg *vs.* WKY (107±3 mmHg). The weight of young and adult MS rats increased significantly as well: 315±52 and 478±35g, respectively *vs.* WKY (249±20 g) (***Tab. 1***).

Horizontal and vertical motor activities

Horizontal motor activity (HMA) was increased significantly in both SHR and young MS rats. However, in adult MS rats it reached the control WKY level and even decreased in comparison to young MS group (***Fig. 1***). Vertical motor activity (VMA) increased significantly only in SHR. In young MS rats it was on the control level and in the adult group it even decreased in comparison to both control WKY and young MS rats (***Fig. 1***).

NF-κB (p65) and iNOS protein expressions

Similarly as HMA, cerebellar NF-κB (p65) protein expression was increased significantly in both SHR and young MS rats. However, in adult MS rats it reached the control WKY level (***Fig. 2***). Protein expression of iNOS increased significantly only in SHR. There were no significant changes in both groups of MS rats (***Fig. 2***).

Total NO synthase activity

NO synthase activity in the cerebellum of SHR was not changed in comparison with the normotensive WKY. It decreased significantly in both MS groups with markedly lower activity in adult than in young MS rats (***Fig. 3***).

Discussion

The present study demonstrates that in spontaneously hypertensive rats, increased NF-κB (p65) expression was associated with increased of both horizontal and

Tab. 1. Blood pressure and body weight of Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR) and young and adult rats with metabolic syndrome (MS).

	WKY	SHR	MS young	MS adult
Blood pressure	107±3	167±5*	176±2*	179±2*
Body weight	249±20	211±16	315±52*	478±35*+

The results are expressed as mean ± SEM, *p<0.05 vs. WKY, +p<0.05 vs. MS young

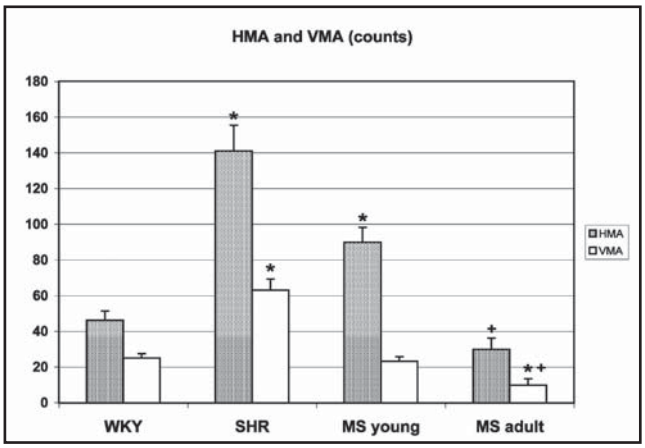

Fig. 1. Horizontal motor activity (HMA) and vertical motor activity of Wistar Kyoto (WKY), spontaneously hypertensive rats (SHR), and young and adult rats with metabolic syndrome (MS). The results are expressed as mean ± SEM, *p<0.05 vs. WKY, +p<0.05 vs. MS young.

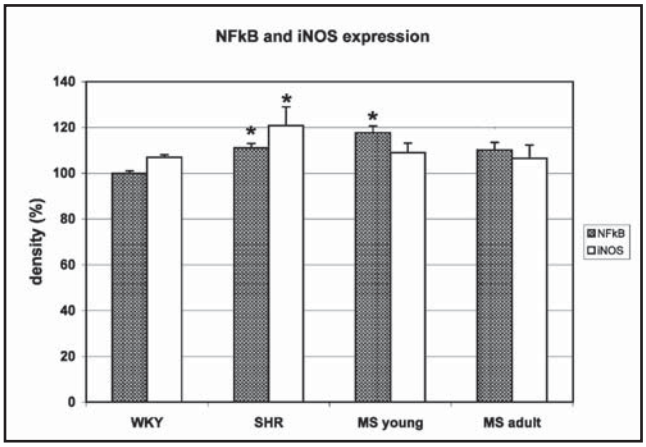

Fig. 2. Nuclear factor kappaB (NF-κB, p65) and inducible nitric oxide synthase (iNOS) protein expressions of Wistar Kyoto (WKY), spontaneously hypertensive rats (SHR), and young and adult rats with metabolic syndrome (MS). The results are expressed as mean ± SEM, *p<0.05 vs. WKY, +p<0.05 vs. MS young.

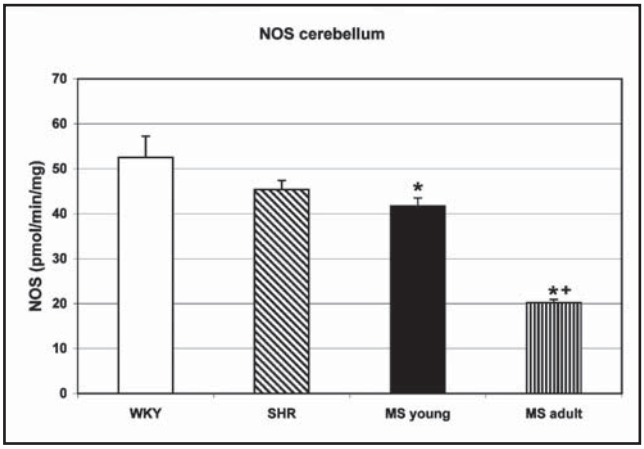

Fig. 3. NO synthase (NOS) activity of Wistar Kyoto (WKY), spontaneously hypertensive rats (SHR), and young and adult rats with metabolic syndrome (MS). The results are expressed as mean ± SEM, *p<0.05 vs. WKY, +p<0.05 vs. MS young

vertical motor activities, despite no changes in NO synthase activity. Similarly, in young rats with metabolic syndrome, increased NF-κB (p65) expression led to increased horizontal motor activity despite decreased NOS activity. On the other hand, no significant changes in NF-κB (p65) expression were demonstrated in adult rats with metabolic syndrome. Horizontal and vertical motor activities as well as NOS activity were markedly reduced in this group of rats.

It is supposed that activation of NF-κB in spontaneously hypertensive rats is accelerated by increased production of reactive oxygen species (ROS) in this type of rats. Increased ROS production likely accelerates the inactivation of NO and accounts for the apparent decrease in bioactive NO as it was documented also in several studies (Noll *et al* 1997; Bouloumie *et al* 1997; Kovacsova *et al* 2010; Pechanova 2010). Thus, the concentration of NO in the brain parts of SHR might be lower despite comparable NO synthase activity with normotensive rats. Qadri *et al* (2003) pointed to the fact that NOS activity was even impaired in the cerebral cortex and brainstem of prehypertensive SHR. Only at established hypertension, SHR showed an augmentation in NOS activity in hypothalamus and brainstem as a response on blood pressure increase. It seems, however, that this adaptive process is not able to restore NO concentration on the physiological level of normotensive rats. In our previous study we have shown that two weeks of NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME) treatment resulted in the decreased horizontal and vertical locomotor activity. There were no changes in the locomotor activity of rats after prolongation of L-NAME treatment to four weeks (Halcak *et al* 2000). Thus, it seems that NO availability in the brain may not be responsible for locomotor activity changes in the rats studied.

We have found however that increased expression of NF-κB (p65) in the cerebellum is associated with increased horizontal motor activity in both SHR and young rats with metabolic syndrome. Interestingly, this increase was monitored despite the fact that also young MS rat expressed obesity along with increased blood pressure and thus lower than higher locomotor activity would be expected in this type of rats. The cerebellum is important for movement control and plays a particularly crucial role in balance and locomotion. As such, one of the most characteristic signs of cerebellar damage is walking ataxia. It is not known how the cerebellum normally contributes to movement, although recent work suggests that it plays a role in the generation of appropriate patterns of limb movements, dynamic regulation of balance, and adaptation of posture and locomotion through practice (Morton and Bastian 2004). The programming of the locomotor activity as well as the rhythmic discharges connected with locomotion and with many stereotyped movements (e.g. rearing) are under the various intervening influences not only from the environment but also from the particular animal as well. The role of the cerebellum in programming and controlling the motor activity is well known and the biochemical pathways of these functions are intensively studied. Here we suggest the involvement of NF-κB activation in cerebellar movement regulation. Since NF-κB activation may lead also to the expression of iNOS, this parameter was evaluated as well. However, protein expression of iNOS increased significantly only in SHR. There were no significant changes in both groups of MS rats. Thus, during our experimental conditions, we suggest other than NO-dependent pathway which could be activated by NF-κB and affect the locomotor activity.

Activation of nuclear factor kappaB and mitogen-activated protein kinase pathways in skeletal muscle has been shown to enhance the gene expression of several enzymes that play an important role in maintaining oxidant-antioxidant homeostasis, such as mitochondrial superoxide dismutase (MnSOD). While an acute bout of exercise activates NF kappaB and upregulates MnSOD, administration of chemical agents that suppress reactive oxygen species production can cause attenuation of exercise-induced MnSOD expression. Thus, ROS generation during exercise may have duel effects: the infliction of oxidative stress and damage, and the stimulation of adaptive responses favouring long-term protection. This scenario explains why animals and humans involved in exercise training have demonstrated increased resistance to oxidative damage under a wide range of physiological and pathological stresses (Ji *et al* 2007). Thus it seems, that activation of NF-κB in our experimental conditions may activate antioxidant enzymes like MnSOD leading to the protection against decrease in locomotion despite appearing obesity. In the adult MS rats with developed obesity this adaptive process is however insufficient which was documented by decrease of both expression of NF-κB and locomotor activity.

In conclusion, increased cerebellar NF-κB (p65) expression was associated with increased horizontal motor activity in both SHR and young rats with metabolic syndrome. Rather than NO-dependent regulation, we suggest NF-κB/antioxidant enzymes pathway to be involved in this process. Downregulated NF-κB together with increased weight of rats may belong to the causes of decreased locomotor activity of adult rats with metabolic syndrome.

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