ORIGINAL ARTICLE

Different brain and cardiac NF-kappaB/nitric oxide pathway in hypertensive and obese rats

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Abstract

OBJECTIVE: Recently, differences in brain and cardiac nuclear factor kappaB (NF-κB) expression and nitric oxide (NO) generation were documented in rats treated with NO synthase inhibitor – NG-nitro-L-arginine methyl ester (L-NAME). We aimed to analyse whether changes in brain and cardiac NF-κB expression may be associated with nitric oxide synthase (NOS) activity in spontaneously hypertensive rats (SHR) and rats with metabolic syndrome (MS).

METHODS: Normotensive 9 weeks old male Wistar Kyoto rats (WKY), age-matched spontaneously hypertensive rats and obese rats with MS were analysed. Blood pressure was measure by tail-cuff plethysmography. Protein expression of NF-κB (p65 subunit), endothelial NOS (eNOS), and inducible NOS (iNOS) as well as NOS activity were determined in the brain and heart.

RESULTS: In the brain of SHR, despite no changes in NOS activity, increased expression of NF-κB (p65) was associated with eNOS and iNOS up-regulation. There were no significant changes in the heart in both NF-κB (p65) and NOS isoforms expressions. In MS rats, increased NF-κB (p65) expression in the brain was associated with down-regulation of eNOS and iNOS leading to decreased NOS activity. On the other hand, increased NF-κB (p65) expression in the heart did not develop any significant changes in NOS isoforms expressions. Blood pressure of SHR and MS rats was increased similarly in comparison with normotensive WKY rats.

CONCLUSIONS: Down-regulated NOS isoforms and decreased NOS activity in the brain of rats with metabolic syndrome may contribute to blood pressure increase in this respective strain of rats.

Introduction

The nuclear factor kappaB (NF-κB) seems to represents one of the key systems mediating both neurohormonal and proinflammatory signals leading finally to pathophysiological changes in different tissues including brain and heart. NF-κB is critical for initiating the coordinated transcription and expression of classical components of the inflammatory response, including proinflammatory cytokines, chemokines, cell adhesion molecules and growth factors (Sekiguchi *et al* 2004; Zhou *et al* 2010; Orr *et al* 2005). Several studies, however, point to the fact that the NF-κB pathway may also mediate protective responses (Karin & Lin 2002) by upregulating anti-inflammatory genes and inducing leukocyte apoptosis.

Moreover, NF-κB has been suggested as one of the mechanisms responsible not only for inducible nitric oxide synthase (iNOS) but also for endothelial nitric oxide synthase (eNOS) upregulation (Grumbach *et al* 2005). Several analyses indicate that NF-κB mediates expression of iNOS and vice versa NO can regulate transcriptional activity of NF-κB (Parohová *et al* 2009). In general, nitric oxide can regulate DNA-binding and transcriptional activity of NF-κB either by directly interacting with the factor itself, or with its endogenous inhibitors represented by inhibitory IκB proteins or by activating upstream mechanisms that indirectly modulate the transcriptional activity in both cGMP-dependent or independent way (Contestabile 2008).

Numerous models of experimental hypertension, including spontaneous hypertension, and metabolic syndrome are characterized by increased levels of reactive oxygen species that lead finally to rapid nitric oxide degradation and NF-κB activation (Sander *et al* 1995; Kitamoto at al 2000; Pechanova & Simko 2009). Decreased nitric oxide availability belongs among the major factors responsible for blood increase in different forms of experimental and also human hypertension (Pechanova & Simko 2006; Pechanova & Simko 2010). NF-κB activation may however upregulate the genes for eNOS and iNOS and by that way compensate NO degradation. Protecting NO bioavailability is important not only due to blood pressure maintenance but also due to regulation of locomotion (Pechanova *et al* 2006; Barta *et al* 2012) and different nerve activities (Jagla *et al* 2009; Kovacsova *et al* 2010; Pechanova *et al* 2009).

It has been however suggested that NO can activate DNA binding activity of NF-κB in some cell types while exerting an inhibitory effect in others. And vice-versa, NF-κB may differentially activate the genes of NOS isoforms according to the tissue species (Lander *et al* 1993; Zhen *et al* 2008; Simpson & Morris 1999). Thus the aim of our study was to analyse whether changes in brain and cardiac NF-κB (p65 subunit) expression may be associated with upregulation of eNOS and iNOS and total nitric oxide synthase activity in spontaneously hypertensive rats (SHR) and rats with metabolic syndrome (MS).

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, age-matched spontaneously hypertensive rats (SHR) and age-matched rats with metabolic syndrome (MS) 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. Total NOS activity, nuclear factor NF-κB (p65) and eNOS and iNOS protein expressions were determined in the cerebellum of the brain and left ventricle of the heart.

NF-κB (p65), eNOS, and iNOS protein expressions

Samples of the brain and heart 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) – the antibody which recognizes the 65 kDa RelA (p65) protein (Biolegend, UK). Proteins were also probed with a polyclonal rabbit anti-iNOS and anti-eNOS antibody (Santa Cruz Biotechnology, CA). Bound antibody was detected using a secondary peroxidase-conjugated anti-rabbit antibody (Biolegend, UK; Santa Cruz Biotechnology, CA). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK) and analyzed densitometrically using Photo-Capt V.99 software.

Total NOS activity

Total NOS activity was determined in crude homogenates (Potter, teflon homogenizer) of the cerebellum and left ventricle by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine as previously described by 16: Bredt and Snyder (1990) with minor modifications (Pechanova *et al* 1997). Briefly, 50 μl of crude homogenate (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 mono-

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nucleotide 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 NOS 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 body weight

Systolic blood pressure in the SHR and MS rats was increased significantly to 167±5 and 176±2 mmHg, respectively *vs.* WKY (107±3 mmHg). The weight of MS rats increased significantly as well: 315±52 g *vs.* WKY (249±20 g) (Table 1).

NF-κB (p65) and eNOS and iNOS protein expressions

In the brain of SHR, despite no changes in NOS activity, increased expression of NF-κB (p65) was associated with significant eNOS and iNOS up-regulation (Figure 1). There were no significant changes in the heart in both NF-κB (p65) and NOS isoforms expressions (Figure 2).

In MS rats, increased NF-κB (p65) expression in the brain was associated with down-regulation of eNOS and iNOS leading to decreased NOS activity (Figure 1). On the other hand, increased NF-κB (p65) expression in the heart did not develop any significant changes in NOS isoforms expressions (Figure 2).

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

	WKY	SHR	MS
Blood pressure	107±3	167±5*	176±2*
Body weight	249±20	211±16	315±52*

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

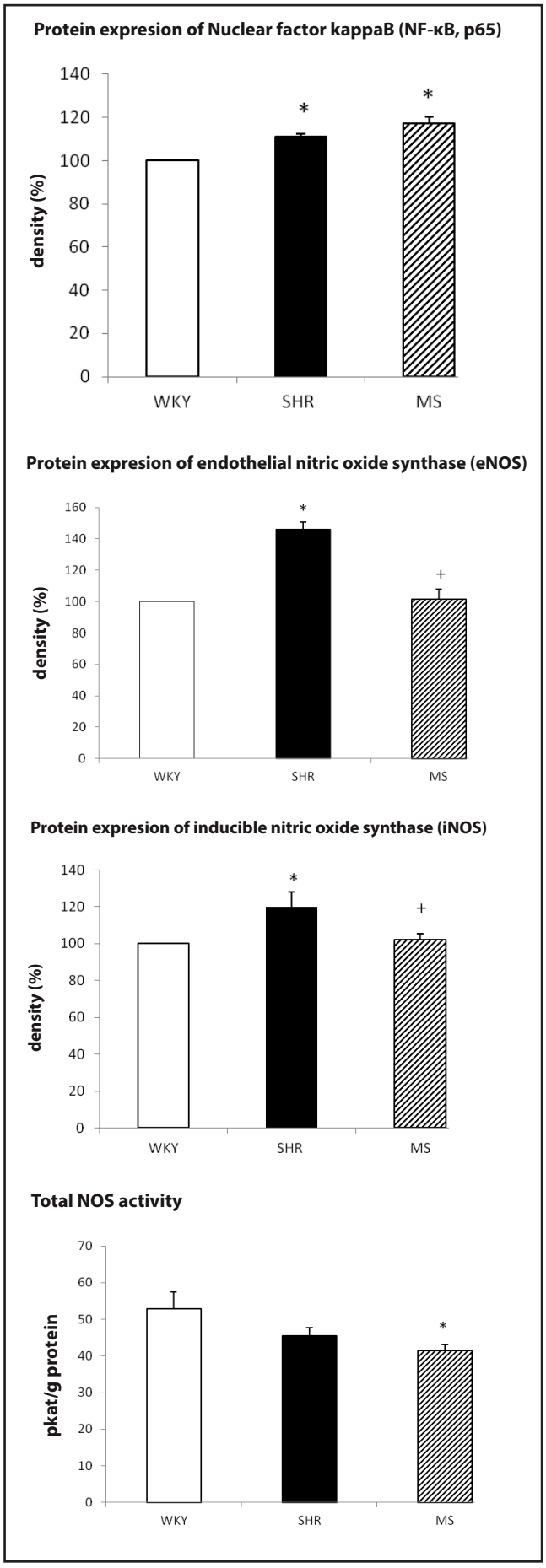
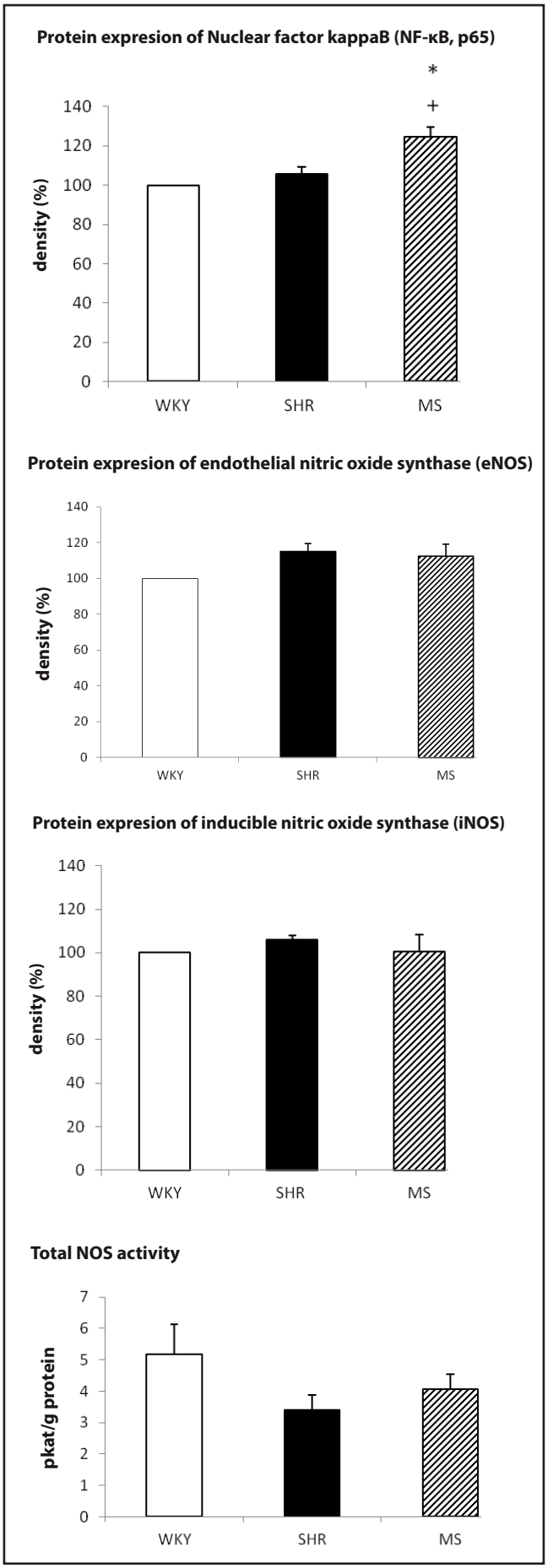


Fig. 1. Nuclear factor kappaB (NF-κB, p65), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) protein expressions, and total NOS activity of Wistar Kyoto (WKY), spontaneously hypertensive rats (SHR), and rats with metabolic syndrome (MS) in the brain. The results are expressed as mean ± SEM, *p<0.05 vs. WKY, + p<0.05 vs. SHR



Total NOS activity

Total NOS activity in the brain of SHR was on the control level, while it decreased significantly in MS rats (Figure 1). There were no significant changes in cardiac NOS activity in both SHR and MS rats (Figure 2).

Discussion

In the present study we have demonstrated that in the brain of SHR, despite no changes in NOS activity, increased expression of NF-κB (p65) was associated with eNOS and iNOS up-regulation. There were no significant changes in the heart in both NF-κB (p65) and NOS isoforms expressions. Activation of NF-κB has been suggested by Grumbach *et al* (2005) as one of the mechanisms responsible for eNOS upregulation. Numerous models of experimental hypertension, including L-NAME-induced hypertension, are characterized by increased levels of reactive oxygen species and NF-κB activation (Sander *et al* 1995; Kitamoto *et al* 2000). In our experimental conditions increased expression of NF-κB (p65) was associated with eNOS up-regulation in the brain only. The increased eNOS expression was not however sufficient to increase total NOS activity and to prevent blood pressure elevation. Nava *et al* (1995) documented increased eNOS and nNOS expressions also in cardiac endothelial cells of spontaneously hypertensive rats as a result of NO deficiency that accompanies spontaneous hypertension. Moreover, Llorens *et al* (2007) reported that the NO pathway was upregulated in the cardiovascular system and kidney both in aged normotensive and spontaneously hypertensive rats. The discrepancies may be caused by different leading pathways which could change according to the age of the animals. Our study demonstrated that 9 weeks old SHR did not express the alteration in cardiac NF-κB/NO pathway, however, the similar pathway was activated in the brain. Increased eNOS expression helped probably to keep brain NOS activity on the control level. Increased production of ROS in SHR led however to rapid NO degradation (Bouloumie *et al* 1997; Pechanova 2010) and thus blood pressure was increased.

In the rats with metabolic syndrome, increased NF-κB (p65) expression in the brain was associated with down-regulation of eNOS and iNOS leading to decreased NOS activity. On the other hand, increased NF-κB (p65) expression in the heart did not develop any significant changes in NOS isoforms expressions.

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

In general, metabolic risk factors associated alterations do not involve a decrease in eNOS expression. Indeed eNOS expression was either reported not to be affected by hypertension, obesity, and insulin resistance (Parohova *et al* 2009; Naoum *et al* 2004; Nisoli *et al* 2003; Huang 2009) or to be increased in pathological states associated with oxidative stress (Pechanova & Simko 2009; Pechanova & Simko 2010).

Upregulation of eNOS expression by increased ROS activity was shown *in vitro* in human coronary artery endothelial cells, and also *in vivo* in hypertensive animals. This effect may be partly mediated by limiting the availability of NO, thereby exerting a negative feedback on NOS expression through activation of nuclear factor NF-κB (Zhen *et al* 2008). It seems that both increased ROS formation and removal of endogenous NO per se are able to enhance eNOS gene and protein expression by NF-κB-activated mechanisms (Pechanova & Simko 2009; Kojsova *et al* 2006). Removal of NO prevents NF-κB subunit nitrosylation and association with the inhibitory factor IkB, thus enabling translocation of NF-κB subunits to the nucleus, which results in increased eNOS mRNA expression (Rockman *et al* 2002). A similar mechanism may be operative in human hypertension and metabolic syndrome as a response to increased ROS production and endogenous NOS inhibitors. In that case, NF-κB may represent an adaptive mechanism providing increased NO production during hypertensive and hypertrophic conditions when increased production of ROS and decreased NO generation can be assumed.

In our experimental conditions, increased NF-κB (p65) expression in the brain was associated with down-regulation of eNOS and iNOS leading to decreased NOS activity in rats with metabolic syndrome. This may be explain by the fact that all studies concerning NF-κB/NO patway in metabolic syndrome were done within cardiovascular system and not the brain. In the brain particularly, different NF-κB-dependent regulation may lead also to decrease NOS isoforms expressions.

In conclusion, down-regulated NOS isoforms and decreased NOS activity in the brain of rats with metabolic syndrome may contribute to blood pressure increase in this respective strain of rats.

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