

NADPH Oxidase Activity and Reactive Oxygen Species Production in Brain and Kidney of Adult Male Hypertensive Ren-2 Transgenic Rats

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Received November 5, 2015

Accepted November 18, 2015

Summary

Hypothalamic paraventricular nucleus (PVN) and rostral ventrolateral medulla (RVLM) play an important role in brain control of blood pressure (BP). One of the important mechanisms involved in the pathogenesis of hypertension is the elevation of reactive oxygen species (ROS) production by nicotine adenine dinucleotide phosphate (NADPH) oxidase. The aim of our present study was to investigate NADPH oxidase-mediated superoxide ($O_2^{\cdot-}$) production and to search for the signs of lipid peroxidation in hypothalamus and medulla oblongata as well as in renal medulla and cortex of hypertensive male rats transgenic for the murine Ren-2 renin gene (Ren-2 TGR) and their age-matched normotensive controls – Hannover Sprague Dawley rats (HanSD). We found no difference in the activity of NADPH oxidase measured as a lucigenin-mediated $O_2^{\cdot-}$ production in the hypothalamus and medulla oblongata. However, we observed significantly elevated NADPH oxidase in both renal cortex and medulla of Ren-2 TGR compared with HanSD. Losartan (LOS) treatment (10 mg/kg body weight/day) for 2 months (Ren-2 TGR+LOS) did not change NADPH oxidase-dependent $O_2^{\cdot-}$ production in the kidney. We detected significantly elevated indirect markers of lipid peroxidation measured as thiobarbituric acid-reactive substances (TBARS) in Ren-2 TGR, while they were significantly decreased in Ren-2 TGR+LOS. In conclusion, the present study shows increased NADPH oxidase activities in renal cortex and medulla with significantly increased TBARS in renal cortex. No significant changes of NADPH oxidase and markers of lipid peroxidation were detected in the studied brain regions.

Key words

Paraventricular nucleus • Rostral ventrolateral medulla • Renal medulla • Renal cortex • Oxidative stress • Lipid peroxidation

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Introduction

Neurogenic basis of hypertension is accepted in both humans and various animal models. Two relevant centers of arterial blood pressure (BP) control in the brain are the hypothalamic paraventricular nucleus (PVN) and rostral ventrolateral medulla (RVLM) (Guyenet 2006, Gabor and Leenen 2012). Numerous studies described the elevation of reactive oxygen species (ROS) production as one of the important mechanisms involved in the pathophysiology of hypertension (Peterson *et al.* 2006, Harrison and Gongora 2009, Hirooka *et al.* 2011, Chan and Chan 2014). One of the major sources of cellular ROS is multicomponent plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Griendling *et al.* 2000). The augmentation of oxidative stress through increased NADPH oxidase-mediated $O_2^{\cdot-}$ production has also been reported in the pathogenesis of hypertension (Paravicini and Touyz 2008, Datla and Griendling 2010). Our previous study performed in adult salt hypertensive Dahl rats showed neither the increased activity of NADPH oxidase-mediated $O_2^{\cdot-}$ production nor the increased levels of markers of lipid peroxidation damage in hypothalamus or medulla oblongata (Vokurková *et al.* 2015). However, we found the enhanced NADPH oxidase-mediated $O_2^{\cdot-}$ production (without significant increase of lipid peroxidation) in the renal medulla of salt hypertensive Dahl sensitive rats.

The renin-angiotensin system is a powerful

biological modulator of mammalian BP. Its dysregulation causes hypertension, whereas its pharmacological blockade by direct renin inhibitors, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers lowers BP (Crowley and Coffman 2014). A well-defined monogenetic model of angiotensin II-dependent hypertension is adult male heterozygous Ren-2 transgenic rats (Ren-2 TGR). In this strain murine Ren-2 gene was inserted into the genome of normotensive Hannover Sprague Dawley rats (HanSD) and renin-angiotensin system (RAS) was inappropriately activated (Mullins *et al.* 1990). Angiotensin II is known to stimulate ROS production by NADPH oxidase (Nguyen Dinh Cat *et al.* 2013).

The aim of our present study was to investigate NADPH oxidase-mediated O_2^- production and to search for the enhancement of lipid peroxidation in hypothalamus and medulla oblongata as well as renal medulla and renal cortex of Ren-2 and HanSD rats. We also studied whether angiotensin II type 1 receptor antagonist, losartan (LOS), which normalizes BP in this model, could attenuate NADPH oxidase-mediated O_2^- production and lipid peroxidation in the above mentioned tissues.

Material and Methods

Reagents

Nicotinamide-adenine dinucleotide phosphate (NADPH), ethylene glycol-bis(2-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, 1,1,3,3-tetraethoxypropane, lucigenin (9, 9'-bis[N-methyl acridinium nitrate] and Folin reagent were purchased from Sigma-Aldrich Co (USA), Lubrol from Serva (Germany) and losartan (Lozap) from Zentiva (Czech Republic). All other reagents were of the purest grade commercially available.

Animals

Twenty 4-week-old male heterozygous Ren-2 transgenic rats and 10 age-matched transgene-negative normotensive Hannover Sprague Dawley rats were used for our experiments. All animals were housed under standard laboratory conditions (temperature $23\pm 1^\circ\text{C}$, 12-h light/dark cycle) in the facility of Institute of Physiology accredited by the Czech Association of Laboratory Animal Care and maintained on tap water and standard rodent chow (Altromin) *ad libitum*.

Antihypertensive regime

From the age of 4 weeks 10 randomly chosen Ren-2 TGR were given tap water containing 100 mg/l of losartan, i.e. 10 mg/kg/day (Ren-2 TGR+LOS) for 2 months similarly as in our previous study (Rakušan *et al.* 2010). All procedures and experimental protocols in the experimental animals, which were approved by the *Ethical Committee of the Institute of Physiology CAS*, conform to the *European Convention on Animal Protection and Guidelines on Research Animal Use*.

Monitoring of systolic blood pressure

Systolic blood pressure (SBP) was measured by automated tail-plethysmography system MC 4000; Hatteras, North Carolina, USA (ERDF, OPK Biomodels CZ.2.16/3.1.00/24017). To eliminate the influence of circadian SBP variation, the measurements were always done approximately at the same time of day (between 8:00 and 10:00 a.m.).

Sample preparation

At the end of the experiment, the animals subjected to ether anesthesia were euthanized by decapitation. The heart and kidney were removed and weighted. The brain was quickly removed from the skull on ice-cold platform. The macroscopic structures of the brainstem (without cerebellum) were manually separated in two parts according to a rat brain atlas (Paxinos and Watson 2005). Part A means hypothalamus including PVN and part B medulla oblongata with pons including RVLM (AP -9 to AP -15). The mean weights of the individual blocks of brain tissue (\pm SEM) were 61 ± 2.1 and 176 ± 2.3 mg for parts A and B, respectively. The dissected parts of the brain were homogenized in 10 % (wt/vol) ice-cold lysis buffer containing 20 mmol/l KH_2PO_4 , 1 mmol/l EGTA, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin, 1 mmol/l phenylmethylsulfonyl fluoride (pH 7.4) and placed on ice. Folin method was used for the determination of protein concentration in the homogenates using bovine serum albumin as standard (Lowry *et al.* 1951).

Measurement of lipid peroxidation

Lipid peroxidation in the frozen-thawed samples was measured by measuring thiobarbituric acid-reactive substances (TBARS) formation (Ohkawa *et al.* 1979). To determine TBARS, the brain homogenates were incubated with thiobarbituric and acetic acid at 95°C for 45 min. After cooling the developed fluorescent

substance was extracted with n-butanol. Fluorescence of the organic phase was measured at an excitation wavelength of 515 nm and an emission wavelength of 553 nm using Tecan Infinite M200 multimode microplate fluorometer. A calibration curve was prepared from 1,1,3,3-tetraethoxypropane. The results were expressed as nmol of TBARS per mg of protein.

Determination of conjugated dienes

The concentration of conjugated dienes (CD) was determined in freshly isolated lipid extracts of brain part B, renal cortex and medulla and heart homogenates. Briefly, homogenates were extracted in a chloroform-methanol (1:1) mixture. After organic solvent evaporation under nitrogen atmosphere, the lipid residues were dissolved in cyclohexane and determined spectrophotometrically at 233 nm according to previously described method (Kogure *et al.* 1982).

Samples of brain and kidney frozen-thawed homogenate (0.010-0.020 mg of protein) were added to 1 ml of 10 mmol/l phosphate buffer (pH 7.4) with 1 % (wt/vol) Lubrol. Formation of conjugated dienes was measured from the absorbance ratio A_{233}/A_{215} (oxidative index) using a spectrophotometer Beckman DU-7 (Klein 1970, Kaplan *et al.* 2000).

Measurement of NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase-mediated superoxide radical (O_2^-) production in the two parts of freshly isolated brain and renal medulla and cortex homogenates (Matsui *et al.* 2006). The reaction was started by the addition of NADPH (0.1 mmol/l) to the suspension (250 μ l final volume) containing assay phosphate buffer (50 mmol/l KH_2PO_4 , 1 mmol/l EGTA, 150 mmol/l sucrose, pH 7.4), sample and lucigenin (5 μ mol/l). The luminescence was measured using Tecan Infinite M200 multimode microplate fluorometer at 30 °C every 5 s for 10 and 3 min, in brain and kidney homogenates, respectively (Fig. 1). Buffer blank was subtracted from each reading. The activity of NADPH oxidase was expressed as counts per mg protein.

Determination of thiol concentrations

The intracellular content of reduced glutathione (GSH) in the renal cortex, heart and liver was determined according to the method described earlier (Ellman 1959). Briefly, the tissue samples were homogenized in 3 % sulfosalicylic acid and 10 % homogenates were

centrifuged for 10 min at 3 000 g. A portion of the supernatant was mixed with 0.02 M 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 8) and the absorbance of a colored product was read on a spectrophotometer at 412 nm. The concentration of GSH was calculated from a calibration curve prepared by serial dilution of 1 mM stock solution. The results were expressed as μ mol GSH/g tissue.

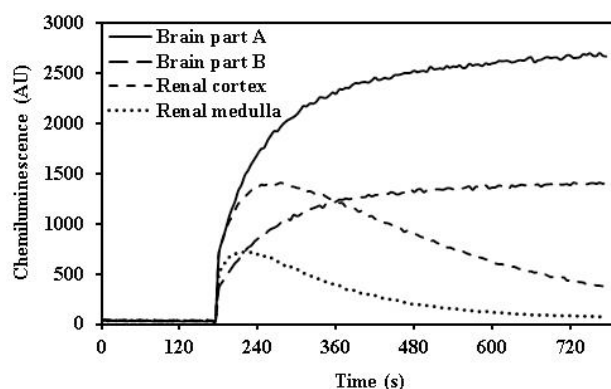


Fig. 1. Time course of NADPH oxidase-mediated O_2^- production in the brain part A and B, renal medulla and cortex.

Statistical analysis

Results are expressed as mean \pm SEM and the statistical differences between experimental groups were evaluated by Student's t-test. Values of $P \leq 0.05$ were considered to be statistically significant.

Results

Table 1 shows significantly higher SBP in three-month-old Ren-2 TGR than in normotensive HanSD, but the treatment with LOS significantly decreased SBP. Body weights were similar in HanSD and Ren-2 TGR but Ren-2 TGR+LOS were significantly heavier. Hypertension in Ren-2 TGR was accompanied by the increase in absolute and relative heart weight. Treatment with LOS significantly decreased heart weight. Significantly lower relative kidney weight was found in Ren-2 TGR+LOS as compared to untreated animals (Table 1).

Table 2 indicates that there were no significant differences between individual experimental groups in NADPH oxidase-dependent O_2^- production in brain part A (hypothalamus including PVN) or part B (medulla oblongata with pons including RVLM). However, we obtained enzyme activities, which were nearly twice as high in part A as in part B after 10 min of measurement. To evaluate the degree of brain damage by ROS we

measured TBARS, conjugated dienes and oxidative index as the indirect markers of lipid peroxidation.

Nevertheless, we did not find any significant differences between the studied groups (Table 2).

Table 1. Basic characteristic of the experimental groups.

	HanSD	TGR	TGR + LOS
<i>Number of rats</i>	10	10	10
<i>Systolic blood pressure (mm Hg)</i>	133 ± 5	204 ± 6 *	144 ± 7 #
<i>Body weight (BW, g)</i>	489 ± 10	504 ± 9	532 ± 6 *#
<i>Heart weight (HW, g)</i>	1.33 ± 0.06	1.55 ± 0.05 *	1.27 ± 0.02 #
<i>Kidney weight (KW, g)</i>	2.96 ± 0.11	3.15 ± 0.07	3.11 ± 0.06
<i>Relative heart weight (HW/BW, mg/g)</i>	2.72 ± 0.10	3.08 ± 0.10 *	2.39 ± 0.03 *#
<i>Relative kidney weight (KW/BW, mg/g)</i>	6.04 ± 0.16	6.32 ± 0.15	5.85 ± 0.11 #

Data are means ± SEM. Significantly different: *P<0.05 from HanSD rats, #P<0.05 TGR rats. HanSD – Hannover Sprague-Dawley control rats, TGR – transgenic rats (Ren-2), LOS – losartan.

Table 2. Oxidative stress in the brain of experimental groups.

	HanSD	TGR	TGR + LOS
<i>NADPH oxidase activity in the brain part A (counts/mg protein x 10⁵)</i>	12.32 ± 0.96	12.24 ± 0.82	12.98 ± 0.57
<i>NADPH oxidase activity in the brain part B (counts/mg protein x 10⁵)</i>	6.95 ± 0.80	6.09 ± 0.46	6.25 ± 0.47
<i>TBARS in the brain part A (nmol/mg protein)</i>	1.22 ± 0.06	1.24 ± 0.05	1.29 ± 0.07
<i>TBARS in the brain part B (nmol/mg protein)</i>	1.78 ± 0.22	1.68 ± 0.29	1.31 ± 0.11
<i>Conjugated dienes in the brain part B (μmol/g tissue)</i>	1.45 ± 0.19	1.96 ± 0.34	1.95 ± 0.34
<i>Oxidative index in the brain part A (A₂₃₃/A₂₁₅ ratio)</i>	0.46 ± 0.01	0.49 ± 0.01	0.45 ± 0.01
<i>Oxidative index in the brain part B (A₂₃₃/A₂₁₅ ratio)</i>	0.40 ± 0.01	0.41 ± 0.01	0.41 ± 0.01

Data are means ± SEM. HanSD – Hannover Sprague-Dawley control rats, TGR – transgenic rats (Ren-2), LOS – losartan.

Due to a quite different shape of the recorded curves of ROS production in brain and kidney, we evaluated ROS production in renal cortex and medulla for 3 min only (Fig. 1). Contrary to the brain tissue we found significantly elevated NADPH oxidase-dependent O₂⁻ production in both renal cortex and medulla of Ren-2 TGR compared with HanSD (Table 3). The treatment with LOS did not change NADPH oxidase-dependent O₂⁻ production in renal cortex and medulla of Ren-2 TGR. TBARS in renal cortex were significantly elevated in Ren-2 TGR and significantly decreased in Ren-2

TGR+LOS but conjugated dienes and oxidative index did not differ significantly among studied groups. In the renal medulla we did not find any significant differences in TBARS, CD and oxidative index among experimental groups (Table 3).

Concentrations of reduced glutathione (GSH) in renal cortex and medulla (Table 3) did not show any significant difference among experimental groups. Similarly, concentrations of GSH in heart and liver were not significantly different among studied groups of rats (Table 4).

Table 3. Oxidative stress in the kidneys of experimental groups.

	HanSD	TGR	TGR + LOS
<i>NADPH oxidase activity in renal cortex (counts/mg protein x 10⁴)</i>	15.85 ± 1.65	22.38 ± 1.56*	22.02 ± 2.37*
<i>NADPH oxidase activity in renal medulla (counts/mg protein x 10⁴)</i>	9.45 ± 0.74	12.64 ± 0.91*	11.43 ± 1.66
<i>TBARS in renal cortex (nmol/mg protein)</i>	2.16 ± 0.21	2.97 ± 0.25*	2.34 ± 0.16 [#]
<i>TBARS in renal medulla (nmol/mg protein)</i>	3.33 ± 0.23	3.32 ± 0.25	3.22 ± 0.17
<i>Conjugated dienes in renal cortex (μmol/g tissue)</i>	0.67 ± 0.07	0.66 ± 0.05	0.69 ± 0.07
<i>Conjugated dienes in renal medulla (μmol/g tissue)</i>	0.68 ± 0.07	0.64 ± 0.09	0.65 ± 0.06
<i>Oxidative index in renal cortex (A₂₃₃/A₂₁₅ ratio)</i>	0.54 ± 0.01	0.54 ± 0.01	0.55 ± 0.01
<i>Oxidative index in renal medulla (A₂₃₃/A₂₁₅ ratio)</i>	0.30 ± 0.01	0.31 ± 0.01	0.31 ± 0.01
<i>Thiols in renal cortex (μmol/g tissue)</i>	4.65 ± 0.47	4.47 ± 0.21	5.09 ± 0.49
<i>Thiols in renal medulla (μmol/g tissue)</i>	4.04 ± 0.33	4.40 ± 0.54	4.36 ± 0.36

Data are means ± SEM. Significantly different: *P<0.05 from HanSD rats, [#]P<0.05 TGR rats. HanSD – Hannover Sprague-Dawley control rats, TGR – transgenic rats (Ren-2), LOS – losartan.

Table 4. Oxidative stress in the heart and liver of experimental groups.

	HanSD	TGR	TGR + LOS
<i>Conjugated dienes in the heart (μmol/g tissue)</i>	0.59 ± 0.06	0.60 ± 0.06	0.60 ± 0.07
<i>Thiols in the heart (μmol/g tissue)</i>	2.55 ± 0.10	2.51 ± 0.10	2.53 ± 0.05
<i>Thiols in the liver (μmol/g tissue)</i>	7.35 ± 0.35	7.97 ± 0.26	7.98 ± 0.78

Data are means ± SEM. HanSD – Hannover Sprague-Dawley control rats, TGR – transgenic rats (Ren-2), LOS – losartan.

Discussion

Our present study shows new data on NADPH oxidase-dependent O₂⁻ production and oxidative damage in two parts of the brain, which are essential for BP control, and in renal cortex and medulla of Ren-2 TGR with angiotensin II-dependent hypertension.

We confirmed higher SBP in 3-month-old Ren-2 TGR compared with age-matched normotensive HanSD. We found a good correspondence between SBP elevation in our present study and the earlier studies, which followed Ren-2 TGR over a wide spectrum of age ranging from 15-69 weeks (Kasper *et al.* 2005, Nautiyal *et al.* 2012a). Losartan treatment lowered SBP as has been shown in the previous studies (Stula *et al.* 1998, Teisman *et al.* 1998, Zhou *et al.* 1999, Rakusan *et al.* 2010).

We did not find significantly higher body weight of Ren-2 TGR compared to HanSD as reported by Nautiyal *et al.* (2012a) in 25-week-old animals. We

observed a significant increase of body weight in Ren-2 TGR after LOS treatment that was consistent with valsartan treated 6-week-old Ren-2 TGR (30 mg/kg/day for 21 days) in the study of Whaley-Connell *et al.* (2006). The significantly higher heart-to-body weight ratio in Ren-2 TGR indicates the severity of hypertension.

We did not find any difference in the activity of NADPH oxidase-dependent O₂⁻ production in the brain part A or part B among experimental groups. Nautiyal *et al.* (2012a) described significantly higher NADPH oxidase activity of brain dorsal medullary tissue in 25-week-old Ren-2 TGR compared with age-matched HanSD controls. Thus, the different results of our study could be related to substantially younger age of our animals. Higher NADPH oxidase activity in brain dorsal medullary tissue was significantly reduced by two weeks of intracerebroventricular infusion of ROS scavenger tempol (cell-permeable superoxide dismutase mimetic). On the other hand, the infusion of artificial cerebrospinal fluid or candesartan (angiotensin II type 1 receptor

antagonist) had no significant effect on brain NADPH oxidase activity (Nautiyal *et al.* 2012b).

As far as the kidney is concerned, NADPH oxidase is the primary source of O_2^- under physiological conditions (Kitiyakara *et al.* 2003, Chen *et al.* 2005). We found significant elevation of NADPH oxidase-dependent O_2^- production in both cortex and medulla of Ren-2 TGR. Similarly, the studies in young (6-week-old) TGR described the elevation of renal NADPH oxidase-dependent O_2^- production as compared with age-matched Sprague-Dawley controls (Whaley-Connell *et al.* 2008, Whaley-Connell *et al.* 2009). The increased renal NADPH oxidase activity of Ren-2 TGR was in concert with the enhancement of NADPH oxidase subunit expression and oxidative stress markers. These changes were also associated with structural changes in kidney (Whaley-Connell *et al.* 2006, 2008). Our results of increased NADPH activity in both parts of the kidney in Ren-2 TGR are partially in contrast to our previous results in salt-hypertensive Dahl rats in which NADPH activity was increased only in medulla (Vokurková *et al.* 2015). This contradictory finding could be related to higher RAS-dependent activation of ROS production in Ren-2 TGR, which have angiotensin II-dependent hypertension with increased activity of endogenous RAS.

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Glutathione, a tripeptide present in all tissues, is involved in various detoxification mechanisms. It also serves as a scavenger of free radicals (Sies 1999, Aquilano *et al.* 2014). Intracellular content of GSH was different in the examined tissues but it was not influenced by genotype or LOS treatment.

In summary, the present study shows increased NADPH oxidase activities only in renal cortex and medulla with significantly increased TBARS in renal cortex. No significant changes were detected in the studied brain regions.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The authors would like to thank to Alena Charvátová, Zdeňka Kopecká, Iva Nahodilová and Marie Schützová, for their excellent technical assistance. This work was supported by research grant 304/12/0259 (Czech Science Foundation), 15-25396A by Ministry of Health of the Czech Republic, ERDF, OPVK Biomodels CZ.2.16/3.1.00/24017 and RVO: 67985823 (Institute of Physiology).

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