PREVENTIVE EFFECT OF ACETOVANILLONE ON THE DEVELOPMENT OF BORDERLINE HYPERTENSION

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Summary

The purpose of this study was to investigate the preventive effect of acetovanillone (4 - hydroxy - 3 – methoxyacetophenone, apocynin), recognized as a specific inhibitor of NAD(P)H oxidase, on the blood pressure (BP) development in borderline hypertensive rats (BHR) in comparison with normotensive Wistar Kyoto rats. Young 6-week-old male BHR (offspring of SHR dams and Wistar Kyoto sires) were treated with acetovanillone in the dose of 30 mg/kg/day for six weeks. Systolic BP was measured by tail-cuff plethysmography. Nitric oxide synthase (NOS) activity was determined by measuring the formation of L-[^{3}H] citrulline from L-[^{3}H] arginine in the left ventricle and kidney. Concentration of conjugated dienes (CD) and expression of nuclear factor NF-kappa B, markers of reactive oxygen species, were detected in the kidney and left ventricle, respectively. Systolic BP of WKY and BHR was 119±2 and 144±3 mmHg, respectively, at the end of experiment. NOS activity was increased in the left ventricle and kidney of borderline hypertensive group and acetovanillone treatment further increased NOS activity in the kidney. Acetovanillone decreased significantly BP rise in BHR (to 131±2 mmHg) and lowered CD concentration and expression of NF-kappa B in the tissues investigated. Yet, acetovanillone without affecting NOS activity in the left ventricle decreased concentration of reactive oxygen species leading to the partial prevention of blood pressure rise in borderline hypertensive rats.

Key Words: acetovanillone, reactive oxygen species, nitric oxide synthase, bordeline hypertension

Introduction

Acetovanillone (4-hydroxy-3-methoxyacetophenone) is a methoxy substituted catechol which has been used by Peruvian Indians as an anti-inflamantory agent. This compound is derived from the rhizome of the medicinal herb *Picrorhiza kurroa* found in the hilly sides of Himalayan highlands. *Picrorhia. kurroa* has been used as a herbal medicine for centuries to treat inflammation and certain infectious diseases (1).

Acetovanillone has been recognized as a specific inhibitor of NAD(P)H oxidase (Griendling et al. 2000). Accumulated data indicate that the NAD(P)H oxidase is the most significant source of superoxide in vascular tissues and seems to be involved in the pathogenesis of atherosclerosis, diabetes, heart failure, restenosis after balloon angioplasty and hypertension (2-4). NAD(P)H oxidase consists of the membrane subunits gp91phox and p22phox and the cytosolic subunits p67phox, p47phox, and the small GTPase rac1. The subunits assemble on activation and form the functional enzyme, which produces superoxide radicals after electron transfer to molecular oxygen (5).

Acetovanillone has been demonstrated to prevent translocation of p47phox and p67phox subunits from cytoplasm to membrane, and is therefore thought to prevent the assembly of NAD(P)H oxidase (6). In this way acetovanillone effectively eliminate the increase in $^{.}O^{2-}$ production. Hamilton et al (7) documented that the acetovanillone decreases $^{.}O^{2-}$ production in rat and human vascular rings, increases NO production in cultured human endothelial cells and improves endothelial function *ex vivo* in human arteries and veins as well as arteries from spontaneously hypertensive stroke prone rats. Furthermore, in aorta from spontaneously hypertensive rats treated with acetovanillone, i.e. under the conditions in which NAD(P)H oxidase-derived $^{.}O^{2-}$ production was inhibited and spontaneous tone was decreased, exogenous addition of $^{.}O^{2-}$ was able to restore the tone (8).

The aim of our study was to investigate the preventive effect of acetovanillone on the blood pressure (BP) development in borderline hypertensive rats and to analyse the mechanism of this prevention.

Methods

Animals and treatment: 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.*

Young 6-week-old male borderline hypertensive rats (offspring of spontaneously hypertensive rats dams and Wistar Kyoto sires) were treated with acetovanillone in the dose of 30 mg/kg/day for six weeks. Age-matched Wistar Kyoto rats were used as a control (n=6 in each group). During the experiment, drinking fluid consumption was controlled and adjusted, if necessary. All animals were housed at a temperature of 22-24 ºC, in individual cages and fed with a regular pellet diet *ad libitum*. Blood pressure (BP) was measured by the non-invasive method of tail-cuff-plethysmography. At the end of experiment total NO synthase (NOS) activity was determined in the left ventricle and kidney. Conjugated diene (CD) concentration was determined in the kidney and nuclear factor NF-κB protein expression was determined in the left ventricle.

Total NO synthase activity: Total NO synthase activity was determined in crude homogenates of the left ventricle and kidney by measuring the formation of $[^3H$]-L-citrulline from $[^3H$]-L-arginine as previously described by Bredt and Snyder (9) with minor modifications (10). 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 $[^3H]$-L-arginine (specific activity 5 GBq/mmol, approx. 100000 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 ºC, the reaction was stopped (by adding 0.02 M Hepes containing 2 mM EDTA, 2 mM EGTA and 1 mM $[^3H]$-L-citrulline), the samples were centrifuged, and supernatants were applied to 1-ml Dowex 50WX-8 columns (Na+ form). $[^3H]$-L-citrulline was eluted with 2 ml of water and radioactivity was determined by liquid scintillation counting. Total NO synthase activity was expressed as pmol/min/mg of proteins.

NF-κB protein expression: Samples of the left ventricle 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 (15000xg, 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 analyzed densitometrically using Photo-Capt V.99 software.

Conjugated diene concentration: The concentration of CD was measured in lipid extracts of the kidney according to Kogure et al. (11). Briefly, after chloroform

evaporation under the inert atmosphere of nitrogene and addition of 2 ml cyclohexane, CD concentration was determined spectrophotometrically ($\lambda = 233$ nm, $\varepsilon = 29000$ l.mol^{-1}.cm^{-1}, Bio-Rad, GBC 911A).

Results

Blood pressure: After six weeks of experiment, blood pressure was 119 ± 2 mmHg in the Wistar-Kyoto rats. Blood pressure in the BHR group was increased significantly compared to Wistar-Kyoto rats to 144 ± 3 mmHg. Acetovanillone decreased significantly blood pressure rise in BHR to 131 ± 2 mmHg.

Total NO synthase activity : The NOS activity was 1.9 ± 0.1 in the left ventricle and 1.2 ± 0.1 pmol/min/mg protein in the kidney of the control Wistar-Kyoto rats. NOS activity was increased significantly in the left ventricle to 3.7 ± 0.3 and kidney to 2.0 ± 0.1 pmol/min/mg protein of BHR in comparison with WKY. Acetovanillone treatment increased significantly NOS activity in the kidney of BHR to $2,4 \pm 0.1$ pmol/min/mg protein while it had no effect on NOS activity in the left ventricle (Fig. 1A, 2A).

NF-κB protein expression: The acetovanillone treatment decreased significantly NF-κB protein expression in the left ventricle by 22 % compared to BHR (Fig. 1B).

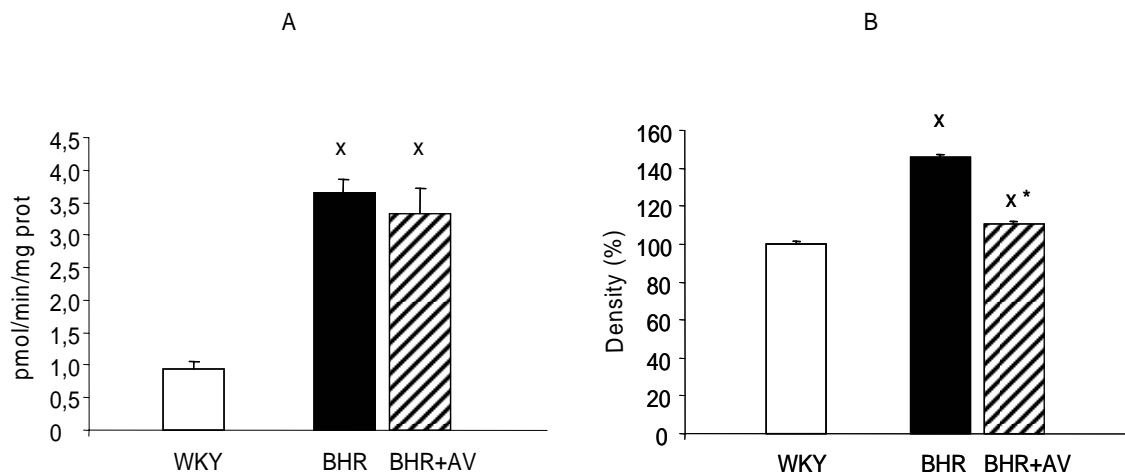A   B

Fig. 1: Effect of acetovanillone (AV) treatment on the nitric oxide synthase activity (A) and expression of nuclear factor NF-kappa B protein (B) in the left ventricle of borderline hypertensive rats (BHR). * $p<0.05$ *vs.* BHR, x $p<0.05$ *vs.* WKY.

Discussion

In the present study we have evaluated the effects of chronic antioxidant treatment by acetovanillone in young borderline hypertensive rats. Acetovanillone significantly decreased blood pressure rise in BHR and lowered CD concentration in the kidney and expression of NF-kappa B in the left ventricle. NOS activity was increased in the left ventricle and kidney of borderline hypertensive group and acetovanillone treatment further increased NOS activity in the kidney. Yet, acetovanillone without affecting NOS activity in the left ventricle decreased concentration of reactive oxygen species leading to the partial prevention of blood pressure rise in borderline hypertensive rats.

Conjugated diene concentration: The CD concentration in the kideney of BHR was significantly decreased by acetovanillone treatment by 15% (Fig. 2B).
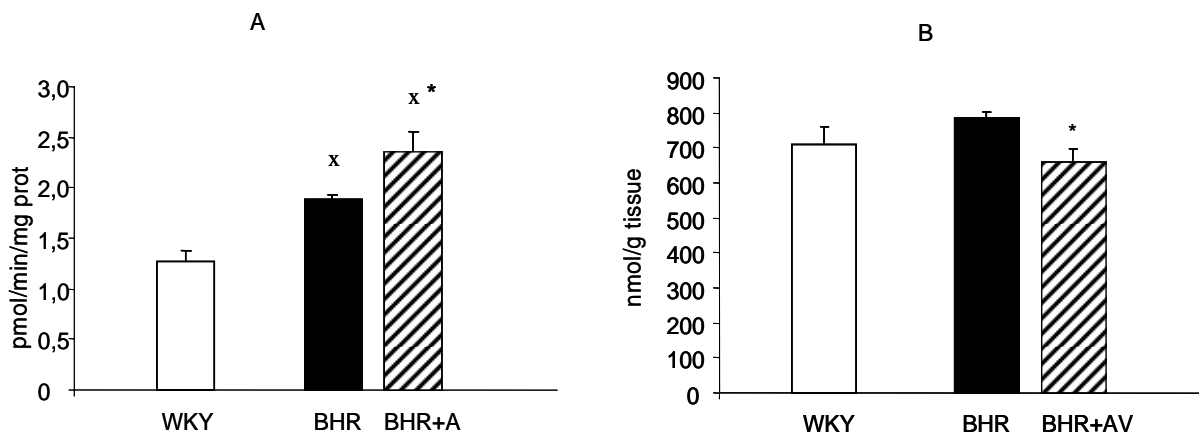

Fig. 2: Effect of acetovanillone (AV) treatment on the nitric oxide synthase activity (A) and concentration of conjugated dienes (B) in the kideny of borderline hypertensive rats (BHR). * $p < 0.05$ *vs.* BHR, x $p < 0.05$ *vs.* WKY.

Our results are in good agreement with the study of Hu et al (12). The authors documented that in male Sprague-Dawley rats, acetovanillone but not L-arginine prevented and reversed Dexamethasone-hypertension, suggesting that NAD(P)H oxidase-mediated superoxide production is important also in this model of hypertension (12). Similarly, excessive renal medullary superoxide production in Dahl-salt sensitive rats contributes to salt-induced hypertension, and NAD(P)H oxidase has been identified as the major source of this excessive superoxide production (13).

Recently it was suggested that NAD(P)H oxidase-derived superoxide enhanced spontaneous tone by inactivating nitric oxide in DOCA-salt hypertensive rats (14). Immunohistochemical analysis revealed a strong increase in p47phox expression in the medial layer of the aorta in spontaneously hypertensive rats (8). The results indicate that enhanced NAD(P)H oxidase activity and, hence, NAD(P)H driven superoxide production, is involved in the spontaneous tone in experimental hypertension. Thus, preventing the decrease of NO level, although without increasing NO synthase activity, may prevent partially the increase of blood pressure. Analogically, in the study of Matsumoto et al (15) incubation of basilar artery rings with acetovanillone improved endothelium-dependent relaxation and acetylcholine-induced cGMP production.

Beltowski et al (16) demonstrated that antioxidant treatment with acetovanillone normalized nitric oxide production, renal sodium handling and blood pressure also in experimental hyperleptinemia. Besides the antioxidant acetovanillone, also vitamins C and E (17), free radical scavengers N-acetylcysteine (18), melatonin (19) and polyphenolic compounds (20) have been shown to posses normalizing effect on endothelial dysfunction through regulation of eNOS and/or NAD(P)H oxidase activities.

In conclusion, acetovanillone yet without affecting NOS activity in the left ventricle decreased concentration of reactive oxygen species leading to the partial prevention of blood pressure rise in borderline hypertensive rats.

Acknowledgement

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