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The Time-Dependent Effect of Provinols™ on Brain NO Synthase Activity in L-NAME-Induced Hypertension

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Summary

Red wine polyphenols have been reported to possess beneficial properties for preventing cardiovascular diseases but their neuroprotective effects during chronic L-NAME treatment have not been elucidated. The aim of this study was to analyze a time course of Provinols™ effects on brain NO synthase activity and oxidative damage in L-NAME-induced hypertension. Male Wistar rats, 12 weeks old, were divided into six groups: control groups, groups treated with N^G-nitro-L-arginine methyl ester (L-NAME, 40 mg/kg/day) for 4 or 7 weeks and groups receiving Provinols™ (40 mg/kg/day) plus L-NAME for 4 or 7 weeks. At the end of the treatment, marker of membrane oxidative damage – conjugated dienes (CD) in the brain and NO synthase activity in the cerebral cortex, cerebellum and brainstem were determined. L-NAME treatment for 4 or 7 weeks led to the increase in blood pressure, elevation of CD concentration and decrease of NO synthase activity in the brain parts investigated. Provinols™ partially prevented blood pressure rise and elevation of CD concentration. Comparing to the L-NAME treated group, Provinols™ increased NO synthase activity after 4 weeks of treatment. However, the prolonged Provinols™ treatment for 7 weeks had no effect on NO synthase activity decreased by L-NAME treatment. In conclusion, Provinols™ partially prevents L-NAME induced hypertension *via* the different mechanisms depending on the duration of treatment. Prevention of oxidative damage in the brain with modulating effect on NO synthase activity is suggested.

Key words

Red wine polyphenols • Oxidative damage • Nitric oxide • Brain • Hypertension

Introduction

Numerous experimental and epidemiological data have documented that selected natural polyphenols, flavonoids particularly, exert protective action on the cardiovascular system, and they have anticancer, antiviral and antiallergic effects as well (Middleton *et al.* 2000, Rotondo et al, 2000). Many epidemiological studies have shown that regular flavonoid intake is associated with reduced risk of cardiovascular diseases (Middleton *et al.* 2000). In the coronary heart disease, the protective effects of flavonoids include mainly antithrombotic,

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antiischemic, antioxidant, and vasorelaxant activities (Wollny *et al.* 1999, Zenebe and Pecháňová 2002, Curin and Andriantsitohaina 2005, Babál *et al.* 2006).

Besides cardiovascular system, the brain most often suffers from the long-term impact of the increase of reactive oxygen species (ROS) (Schaffer *et al.* 2006). The brain is especially susceptible to oxidative stress because it is not particularly endowed with an antioxidant defense. It has only low catalase activity and moderate levels of the antioxidant enzymes like superoxide dismutase and glutathione peroxidase. The high levels of iron and ascorbate in the brain participate significantly on the catalysis of lipid peroxidation. Finally, many neurotransmitters are autoxidized to generate reactive oxygen species (for review see Lau *et al.* 2005). In agreement with these observations, there is evidence that increased oxidative stress plays an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer and Parkinson diseases (Esposito *et al.* 2002). It has also been shown that modulation of NO availability is an important determinant of ischemic stroke risk (McCarty 2000). Thus optimal nitric oxide/ROS balance in the brain seems to be a crucial parameter in the prevention of brain damage including ischemic stroke as well as neurodegenerative diseases.

Different polyphenolic compounds were shown to have scavenging activity and the ability to activate key antioxidant enzymes in the brain and thus breaking the vicious cycle of oxidative stress and tissue damage (Lau *et al.* 2005, Esposito *et al.* 2002). Moreover, red wine polyphenolic compounds have been documented to increase NO synthase activity in different tissues (Pecháňová *et al.* 2004, Bernátová *et al.* 2002, Sulová *et al.* 2005, Kucharská *et al.* 2005).

The aim of this study was to analyze a time-dependent effect of red wine polyphenols, Provinols^{TM} on brain NO synthase activity and oxidative damage in L-NAME-induced hypertension.

Methods

Chemicals and drugs

All the chemicals used were purchased from Sigma Chemicals Co. (Germany) except of [^{3}H]-L-arginine (Amersham, UK). The Provinols^{TM} is an alcohol-free dry powder extract from red wine (Languedoc-Roussillon regions in the South-East of France). The content of polyphenols in Provinols^{TM} (in mg/g of dry powder) was: 480 proanthocyanidins, 61 total anthocyanins, 19 free anthocyanins, 38 catechin, 18 hydroxycinnamic acids, 14 flavonols, 370 polymeric tanins.

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.

Male Wistar rats, 12 weeks old, were divided into six groups: control groups (age-matched rats to 4 and 7 weeks of treatment), groups treated with N^G-nitro-L-arginine methyl ester (L-NAME, 40 mg/kg/day) for 4 or 7 weeks and groups receiving 40 mg/kg/day Provinols^{TM} plus L-NAME for 4 or 7 weeks (n=6 in each group). L-NAME and Provinols^{TM} were administered *via* the drinking water from the 12th week of age for 4 or 7 weeks. Daily water consumption was estimated individually for every animal one week before the experiment. During the experiment, drinking fluid consumption was controlled and adjusted, if necessary. All animals were housed at a temperature of 22-24 ^{o}C, in individual cages and fed with a regular pellet diet *ad libitum*. Blood pressure (BP) was measured by the non-invasive tail-cuff-plethysmography. Conjugated diene (CD) concentration was determined in brain homogenate, whereas total NO synthase activity was determined separately in the cerebral cortex, cerebellum and brainstem, respectively.

Conjugated diene concentration

The concentration of CD was measured in lipid extract of the brain according to Kogure et al. (1982). Briefly, after chloroform evaporation under the inert atmosphere of nitrogen and after the addition of 2 ml cyclohexane, CD concentration was determined spectrophotometrically (λ = 223 nm, ε=29000 l.mol^{-1}.cm^{-1} , Bio-Rad, GBC 911A).

Total NO synthase activity

Total NO synthase activity was determined in crude homogenates of the brain cortex, cerebellum and brainstem 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 (Pecháňová *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,

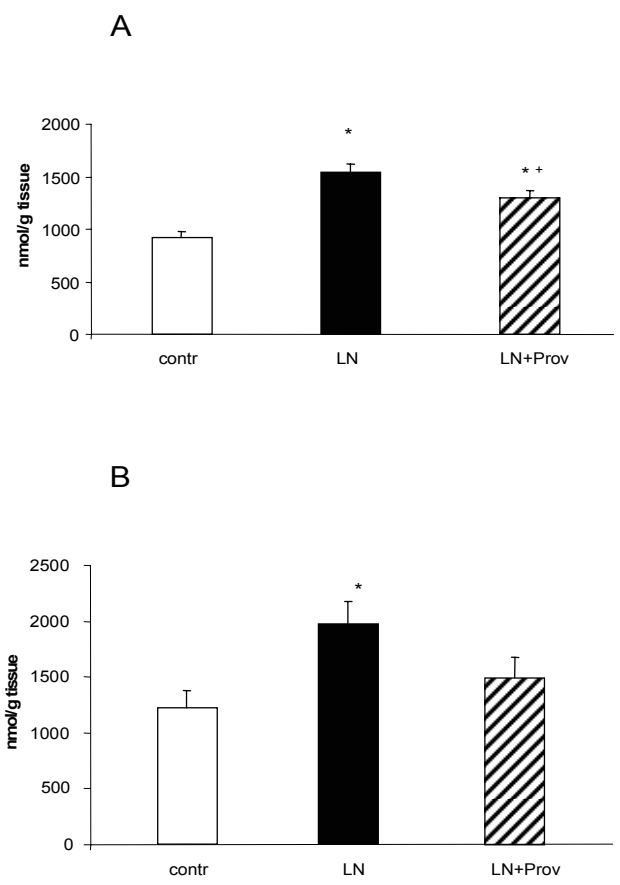

Fig. 1. Effect of 4-week (A) and 7-week (B) L-NAME (40 mg/kg/day) and L-NAME+ Provinols™ (40 mg/kg/day) treatment on the concentration of conjugated dienes (CD). Contr – control, LN – L-NAME, Prov – Provinols™, * P<0.05 compared to control, + P<0.05 compared to the L-NAME group.

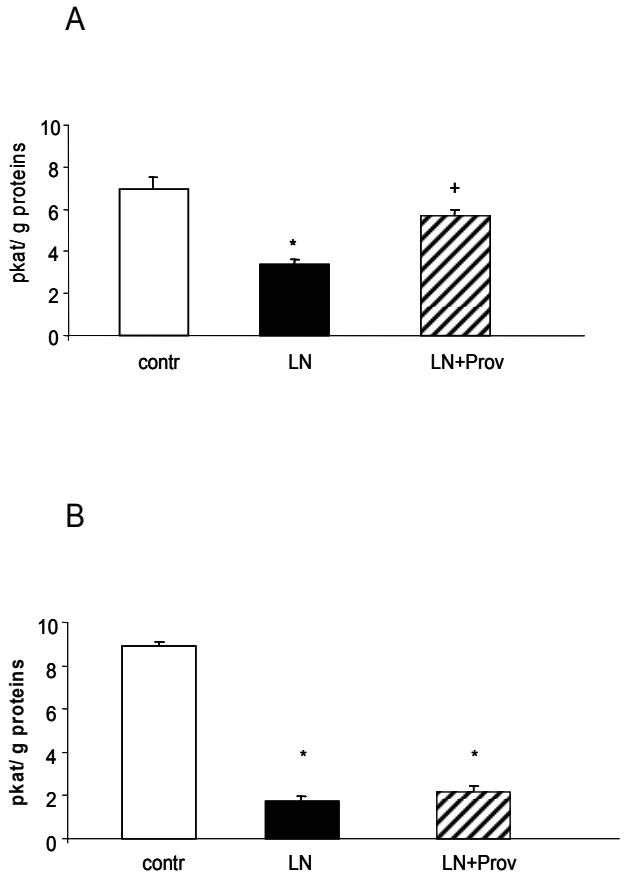

Fig. 2. Effect of 4-week (A) and 7-week (B) L-NAME (40 mg/kg/day) and L-NAME+ Provinols™ (40 mg/kg/day) treatment on NO synthase activity in the cerebral cortex. Contr – control, LN – L-NAME, Prov – Provinols™, * P<0.05 compared to control, + P<0.05 compared to the L-NAME group.

containing 1 µmol/l [^{3}H]-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 [^{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 ± S.E.M. One-way analysis of variance and Bonferroni test were used for statistical analysis. Values were considered to differ significantly if the probability value was less than 0.05.

Results

Blood pressure

BP was not significantly different in the six groups of rats before the beginning of the treatment and represents 112 ± 10 mmHg. In the control groups, BP did not change significantly during the experiment. Treatment of rats with L-NAME (40 mg/kg/day) for 4 and 7 weeks induced a progressive increase in BP (by 39% after 4 weeks and by 41% after 7 weeks of treatment as compared to BP of the control age-matched rats, P<0.05). BP increase induced by L-NAME was significantly lowered by concomitant treatment with Provinols™ (40 mg/kg/day) by 19% after 4 weeks and 17% after 7 weeks of treatment as compared to L-NAME treated age-matched rats, P<0.05.

Conjugated diene concentration

CD concentration in the brain of controls was 920±74 and 1221±120 nmol/g tissue in age-matched rats

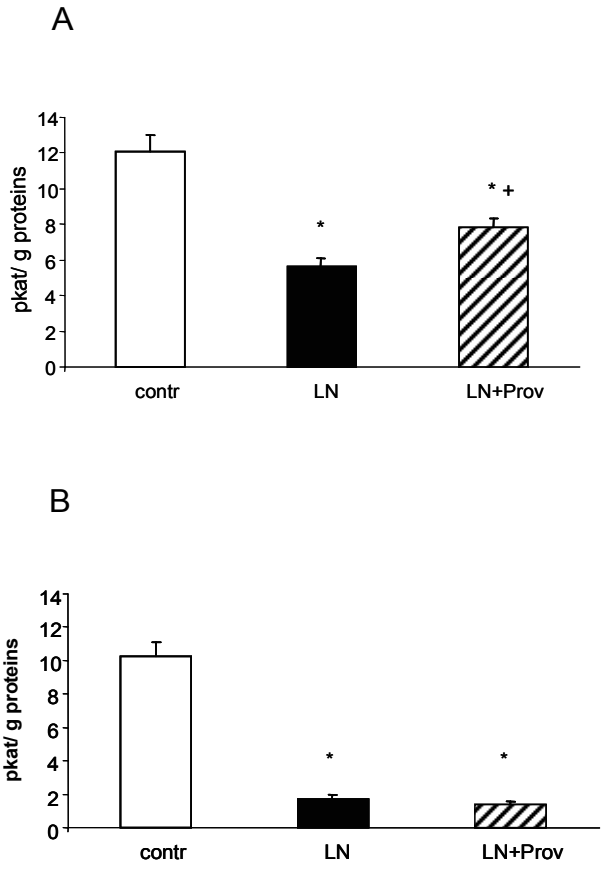

Fig. 3. Effect of 4-week (A) and 7-week (B) L-NAME (40 mg/kg/day) and L-NAME+ Provinols™ (40 mg/kg/day) treatment on NO synthase activity in the cerebellum. Contr – control, LN – L-NAME, Prov – Provinols™, * P<0.05 compared to control, + P<0.05 compared to the L-NAME group.

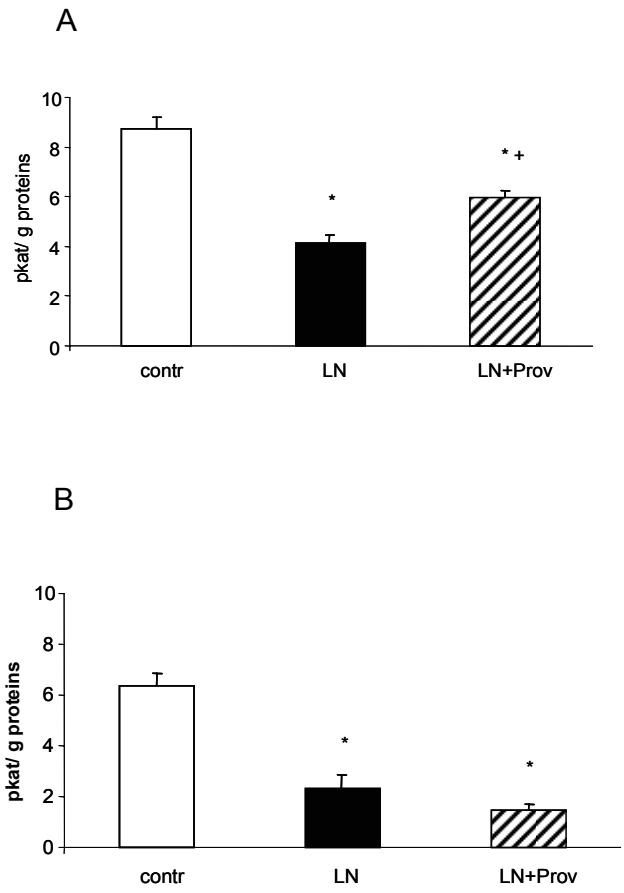

Fig. 4. Effect of 4-week (A) and 7-week (B) L-NAME (40 mg/kg/day) and L-NAME+ Provinols™ (40 mg/kg/day) treatment on NO synthase activity in the brainstem. Contr – control, LN – L-NAME, Prov – Provinols™, * P<0.05 compared to control, + P<0.05 compared to the L-NAME group.

to 4 weeks and 7 weeks of treatment, respectively. L-NAME treatment for 4 or 7 weeks resulted in the increased CD concentration by 67 % and 61 %, respectively (P<0.05). The increase in CD concentration induced by L-NAME was significantly lowered by 4-week concomitant treatment with Provinols™ by 16 % as compared to age-matched L-NAME-treated rats (P<0.05). Simultaneous L-NAME and Provinols™ treatment for 7 weeks kept the CD concentration on the control level (Fig. 1 A,B).

Total NO synthase activity

In the control groups total NO synthase activity in the cerebral cortex, cerebellum and brainstem was comparable. L-NAME treatment for 4 or 7 weeks resulted in the decreased NO synthase activity in the brain parts investigated. Provinols™ treatment for 4 weeks led to the partial increase of NO synthase activity lowered by L-NAME treatment, while Provinols™ treatment for 7 weeks had no effect on NO synthase activity decreased by L-NAME (Fig. 2A,B; 3A,B; 4A,B).

Discussion

In the present study we have demonstrated that 4 and 7 weeks of L-NAME treatment led to the increase in blood pressure, elevation of CD concentration in the brain and decrease of NO synthase activity in the cerebral cortex, cerebellum and brainstem. Provinols™ partially prevented blood pressure rise and elevation of CD concentration. Compared to the L-NAME-treated group, Provinols™ increased NO synthase activity after 4 weeks of treatment. However, prolonged Provinols™ treatment for 7 weeks had no effect on NO synthase activity decreased by L-NAME treatment.

Increased level of the reactive oxygen species in hypertension leads to the oxidative stress with subsequent damage of biological molecules such as proteins, DNA and lipids of cell membranes. Lipid peroxidation *in vivo* involves a radical chain reaction forming lipid

hydroperoxides, which can stimulate vascular cells to produce monocyte-chemotactic and macrophage-stimulating factors resulting in formation of so-called foam cells and atherosclerotic plaques. Oxidized low-density lipoproteins also play a role in thrombus development, since they stimulate procoagulant activities in endothelial cells and monocytes and they inhibit vasodilatation by decreasing the expression of endothelial NO synthase (Halliwell and Chirico, 1993). In the prevention of cardiovascular and neurodegenerative diseases (including ischemic stroke), many of the observed effects of polyphenols can therefore be attributed to their antioxidant and radical scavenging properties which may delay the onset of atherogenesis by reducing chemically and enzymatically mediated peroxidative reactions (Kandaswami and Middleton, 1995). Our observation of Provinols™-induced decrease of CD concentration in the brain of L-NAME-hypertensive rats is in good agreement with the above study. Similarly, Frankel *et al.* (1993) reported that polyphenols like quercetin and *trans*-resveratrol were more effective than α-tocopherol in inhibiting the oxidation of human low-density lipoproteins. The antioxidant activities of flavonoids and their glycosides were even higher than those of vitamin C and E (Rice-Evans *et al.* 1995).

Moreover, we have shown that Provinols™ increased NO synthase activity after 4 weeks of treatment in the cerebral cortex, cerebellum and brainstem. Similarly, Provinols™ increased NO synthase activity in the heart and aorta of L-NAME-treated rats (Pecháňová *et al.* 2004). These data strongly suggest that Provinols™ is a potent activator of NO-synthase activity in both the cardiovascular and nervous systems. The moderate increase of eNOS expression by Provinols™, which was found in the heart and aorta, need not be the only factor responsible for observed high levels of NO-synthase activity. Recently, we have reported that the endothelial NO production caused by Provinols™ was associated with the increase in calcium signaling and the activation of tyrosine kinase pathway within the endothelial cells (Martin *et al.* 2002, Zenebe *et al.* 2003). In L-NAME-hypertensive rats, both the decrease of oxidative load and the increase in NO synthase activity in the brain may contribute to the blood pressure decrease after Provinols™ treatment lasting 4 weeks. Similarly, Diebolt *et al.* (2001) demonstrated a decrease of blood pressure induced by red wine polyphenol treatment of normotensive rats.

Interestingly, prolonged Provinols™ treatment for 7 weeks had no effect on NO synthase activity decreased by L-NAME treatment. Recently, Han *et al.* (2006) suggested that the neuroprotective action of various polyphenols and resveratrol analogues could be mediated by the activation of common "receptor" binding sites particularly present at the level of the cellular plasma membrane in the rat brain. It is hypothesized that binding polyphenols to this receptor may be associated with increased NO synthase activity in the brain. Prolonged action of polyphenols on its receptor could, however, lead to decreased receptor sensitivity and/or increased tolerance. Since Provinols™ used in our study was a composition of different polyphenolic compounds, it is not certain which type of the phenolic components may be responsible for this effect (Andriantsitohaina *et al.* 2005). Despite inhibited NO synthase activity after the prolongation of Provinols™ treatment to 7 weeks, the blood pressure was partially decreased compared to L-NAME-treated rats. Probably, the antioxidant effect of Provinols™ was able to increase the availability of biologicaly active NO resulting in a partial decrease of blood pressure.

We conclude that Provinols™ partially prevents L-NAME-induced hypertension *via* the different mechanisms depending on the duration of treatment. Prevention of oxidative damage in the brain with modulating effect on NO synthase activity is supposed.

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