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Changes of Sodium and ATP Affinities of Renal Na,K-ATPase During and After Nitric Oxide-Deficient Hypertension

N. VRBJAR, V. JAVORKOVÁ, O. PECHÁŇOVÁ[1]

Department of Biochemistry, Institute for Heart Research and [1]Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovak Republic

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Summary

The aim of this study was to assess the molecular basis of renal Na,K-ATPase disturbances in response to NO-deficient hypertension induced in rats by NO-synthase inhibition with 40 mg/kg/day N^G-nitro-L-arginine methyl ester (L-NAME) for four weeks. After 4-week administration of L-NAME, the systolic blood pressure (SBP) increased by 30 %. Three weeks after terminating the treatment, SBP recovered to control value. When activating the Na,K-ATPase with its substrate ATP, a 36 % increase in K_m and 29 % decrease in V_{max} values were observed in NO-deficient rats. During activation with Na^+, the V_{max} was decreased by 20 % and the K_{Na} was increased by 111 %, indicating a profound decrease in the affinity of the Na^+-binding site in NO-deficient rats. After spontaneous recovery from hypertension, the V_{max} remained at the level as in hypertension for both types of enzyme activation. However, in the presence of lower concentrations of substrate which are of physiological relevance an improvement of the enzyme activity was observed as documented by return of K_m for ATP to control value. The K_{Na} value for Na^+ was decreased by 27 % as compared to hypertension, but still exceeded the corresponding value in the control group by 55 % thus resulting in a partial restoration of Na^+ affinity of Na,K-ATPase which was depressed as a consequence of NO-dependent hypertension.

Key words

Sodium pump • Nitric oxide • Hypertension • L-NAME • Kidney

Introduction

Multiple mechanisms were proposed to participate in the pathogenesis of genetic or induced forms of hypertension. One of the crucial substances involved in vasodilatation and regulation of systemic blood pressure is the endothelium-derived relaxing factor which was identified as nitric oxide (Ignarro *et al*. 1987, Khan and Furchgott 1987, Palmer *et al*. 1987). It was demonstrated that chronic inhibition of NO synthesis by L-arginine analogues induced sustained hypertension associated with decreased heart rate (Pecháňová and Bernátová 1996, 2000, Halčák *et al*. 2000). Beside hemodynamic changes, structural changes such as fibrosis in the cardiac tissue and wall thickening in the aorta (Babál *et al*. 2000, 2001), hypertrophy and injury of cardiomyocytes together with enhancement of the atrial granules and their translocation into the sarcolemma (Tribulová *et al*. 2000), and induction of angiogenesis (Okruhlicová *et al*. 2000) were also observed in NO-deficient rats. As far as the intercellular signaling in the cardiac tissue is concerned, the NO-deficient

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

hypertension results in remodeling of gap junctions that is associated with higher incidence of hypokalemia-induced arrhythmias (Nováková *et al.* 2001). Another important change in cardiac sarcolemma is connected to altered molecular properties of Na,K-ATPase (Vrbjar *et al.* 1999) which is involved in maintaining the intracellular homeostasis of ions. Although alterations of the cardiovascular system are intensively studied in NO-deficient hypertension, less data are available about renal functions in this model of hypertension. It is known that chronic inhibition of NO synthesis has a great impact on renal hemodynamics, causing proteinuria and glomerular sclerotic injury (Baylis *et al*. 1992, Mandarim-de-Lacerda and Pereira 2001) as well as a reduction of sodium excretion without lowering the filtered sodium load (Majid and Navar 1997). However, the contribution of Na,K ATPase to alterations induced by inhibition of NO synthesis has still not been elucidated satisfactorily. Previous studies using other models of hypertension showed various effects on the renal Na,K-ATPase. This enzyme activity was suppressed in kidneys of young salt-sensitive rats with salt hypertension while this was not true in adult hypertensive animals (Zicha *et al*. 1987). Acute systolic arterial hypertension provokes a rapid decrease in proximal tubule sodium reabsorption and diuresis associated with inhibition of renal cortex Na,K-ATPase activity (Zhang *et al*. 1998).

The present study was designed to investigate the properties of Na,K-ATPase in isolated plasmalemmal membranes from kidneys of rats with developed chronic NO-deficient hypertension and also from kidneys obtained after the recovery from hypertension. Studying the function of Na,K-ATPase we focused our attention on two main issues concerning the response to blood pressure variations, i.e. the energy supply provided by ATP, and the affinity-changes of the Na-binding site in the enzyme molecule.

Methods

Male Wistar Kyoto rats, 15 weeks old (weighing approximately 350 g) were divided into three groups. One group (n=12) served as control. The second group (n=12) was treated with N^G-nitro-L-arginine methyl ester (L-NAME) in a dose 40 mg/kg/day in drinking water for 4 weeks (hypertensive group). The third group (n=12) was treated with the same amount of L-NAME for four weeks, followed by three weeks without any treatment (recovery group). During the experiment, each animal was kept in a separate cage. Every week the systolic blood pressure (SBP) was measured by the noninvasive method of tail-cuff plethysmography. At the end of the experiment the kidneys were quickly excised, aliquots of samples were used for immediate estimation of NO synthase activity and other aliquots were immediately frozen in liquid nitrogen and stored for further investigations of Na,K-ATPase properties.

The calcium and calmodulin-dependent NO synthase activity was determined in crude homogenates of fresh renal tissue by measuring the production of [^{3}H]-L-citrulline from [^{3}H]-L-arginine (Amersham, UK) according to Bredt and Snyder (1990) with a modification as described by Bernátová *et al.* (1996).

The plasmalemmal membrane fraction from kidneys was prepared according to Jorgensen (1974). The protein content was assayed by the procedure of Lowry *et al*. (1951) using bovine serum albumin as a standard.

The substrate kinetics of Na,K-ATPase was estimated measuring the hydrolysis of ATP by 10 μg plasmalemmal proteins at 37 ^{o}C in the presence of increasing concentrations of ATP in the range of 0.16-16.0 mmol/l in a total volume of 0.5 ml of medium containing 50 mmol/l imidazole (pH 7.4), 4 mmol/l $MgCl_2$, 10 mmol/l KCl and 100 mmol/l NaCl. After 15 min of preincubation in the substrate free medium, the reaction was started by addition of ATP and 20 min later it was terminated by 1 ml of 12 % solution of trichloroacetic acid. Verification of the time dependence of ATP-hydrolysis showed that up to 25 min the ATP splitting was linear in the whole ATP concentration range applied. The inorganic phosphorus liberated was determined according to Tausssky and Shorr (1953). In order to establish the Na,K-ATPase activity, the ATP hydrolysis that occurred in the presence of Mg^{2+} only was subtracted.

The Na,K-ATPase kinetics for sodium was determined by the same approach, in the presence of increasing concentrations of NaCl in the range 2.0-100.0 mmol/l, using a constant amount of ATP (8 mmol/l).

The kinetic parameters were evaluated by direct nonlinear regression of the data obtained. All results were expressed as mean $\pm$ S.E.M. The significance of differences between the individual groups was determined with the use of ANOVA and Bonferroni test. A value of $p<0.05$ was regarded as significant.

Results

Daily application of L-NAME (40 mg/kg/day) significantly elevated systolic blood pressure after 7 days.

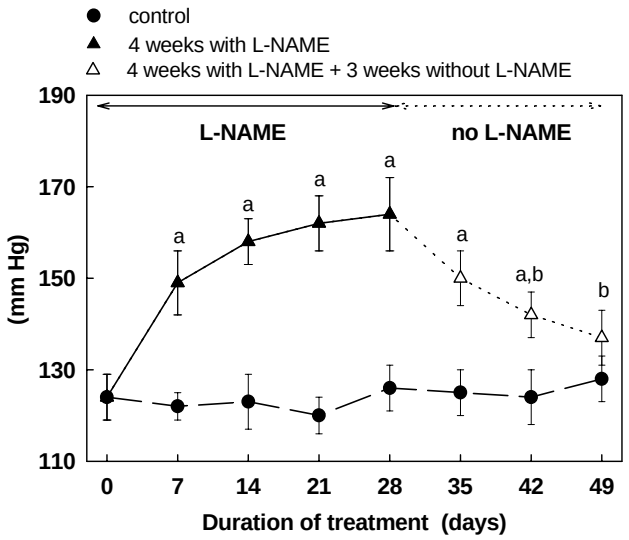

Fig. 1. *Systolic blood pressure in rats during treatment with 40 mg/kg/day of L-NAME and during the following recovery period without treatment lasting three weeks. Data represent means ± S.E.M., a: p<0.05 vs. control, b: p<0.05 vs. L-NAME group.*

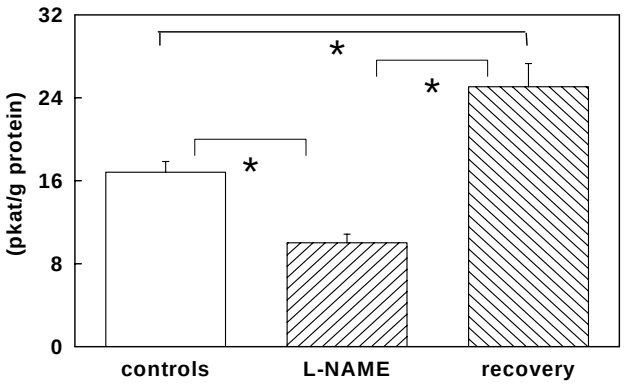

Fig. 2. *Activity of calcium and calmodulin-dependent nitric oxide synthase in kidneys at the end of 4 weeks lasting treatment with 40 mg/kg/day of L-NAME and 3 weeks after terminating the treatment (recovery group). Data represent means ± S.E.M. *P<0.05.*

Continuing the treatment of rats, blood pressure increase persisted and after 28 days it was higher by 30 % as compared to the controls (Fig. 1). In the recovery group, the SBP spontaneously returned to pretreatment values after 3 weeks (Fig. 1).

In the control group, the activity of NO-synthase amounted to 16.82±1.04 pkat/g protein. Chronic administration of L-NAME (40 mg/kg/day) for 4 weeks induced hypertension and significantly inhibited NO-synthase activity (to 60 %). In the group of animals with the recovery period after treatment with L-NAME, the NO-synthase activity was even higher (149 %) as compared to control animals (Fig. 2).

When activating Na,K-ATPase with increasing concentration of ATP, we observed significant inhibition of the enzyme throughout the investigated concentration range in kidneys of animals, which had been treated with L-NAME. At the lowest investigated concentration of ATP (0.16 mmol/l) the inhibition represented 47 %. With increasing concentrations of the substrate, the inhibitory effect gradually decreased to 32 % which we observed in the presence of 16 mmol/l (Fig. 3). Evaluation of the above data by the method of nonlinear regression revealed significant alterations of K_m and V_{max} values. Hypertension induced by the application of L-NAME, resulted in a statistically significant rise of the K_m value by 36 % and a significant depression of the V_{max} value by 29 % as compared to the controls (Table 1).

Withdrawal of the inhibitor L-NAME after its 4-week lasting application, which caused the above mentioned recovery of the blood pressure to control values, also induced significant changes in kinetic behavior of the Na,K-ATPase. In this group, we observed an increase of enzyme activity during the activation with ATP; it was most significant in the presence of lower concentrations of ATP as compared to the hypertensive group. In the presence of 0.16 mmol/l ATP this increase represented 39 %. Elevation of the ATP concentration was followed by a gradual diminution of the above effect and at 16 mmol/l ATP the stimulation was only 7 %. These changes are also reflected in the kinetic parameters of the enzyme. The gradual diminution of the stimulatory effect depending on the growing concentration of ATP resulted in unchanged V_{max} value as compared to the L-NAME group. The significant stimulatory effect observed especially at low ATP concentration seems to be a manifestation of improved enzyme affinity to the substrate as was evident from the significant 27 % decrease of the K_m value (Table 1).

Blood pressure in the recovery group was the same as in the controls, but the Na,K-ATPase in both groups revealed different kinetic properties. When

Table 1. Kinetic parameters of Na,K-ATPase in kidneys from rats during activation with increasing concentrations of ATP and NaCl, respectively.

[ATP] Activation	**V_{max} (µmol P_i/mg prot/h)**	**K_m (mmol ATP/l)**
Controls	25.44±0.65	1.486±0.086
L-NAME	17.95±1.00 [a]	2.016±0.150 [a]
Recovery	18.63±0.74 [a]	1.467±0.145 [b]
[NaCl] Activation	**V_{max} (µmol P_i/mg prot/h)**	**K_{Na} (mmol NaCl/l)**
Controls	26.99±0.91	5.737±0.335
L-NAME	21.55±1.65 [a]	12.099±0.929 [a]
Recovery	18.92±0.60 [a]	8.872±0.500 [a,b]

Number of estimations: control rats (n=12), rats with L-NAME induced NO-deficient hypertension (n=12), and rats with systolic blood pressure recovered to control values (n=12). Data represent means ± S.E.M. a: p<0.005 as compared to the control group, b: p<0.005.as compared to the L-NAME group.

activated with increasing concentrations of ATP at all the investigated concentrations of the substrate, a significant stable inhibition was observed in the recovery group. In the presence of 0.16 mmol/l the inhibition amounted to 26 % and in the presence of 16 mmol/l ATP the inhibition was 27 % (Fig. 3). Evaluation of the observed data by nonlinear regression showed a significant decrease of the V_{max} value by 27 % in the recovery group as compared to the controls, while the K_m value remained unchanged (Table 1).

When activating Na,K-ATPase with increasing concentrations of NaCl, in the L-NAME group there we observed a significant, on Na^+ concentration dependent, decrease of the enzyme activity in the whole concentration range as compared to the controls (Fig. 4). In the presence of the lowest concentration of 2 mmol/l NaCl the inhibition amounted to 56 %, whereas in the presence of the highest 100 mmol/l NaCl the inhibition was 25 % only (Fig. 4). Evaluation by nonlinear regression revealed significant changes of both investigated kinetic parameters. The V_{max} value dropped in the L-NAME group by 20 %. The K_{Na} value increased by 111 % as compared to the controls (Table 1).

After the L-NAME application was discontinued in the recovery group, the increasing concentrations of NaCl induced a biphasic effect on Na,K-ATPase activity

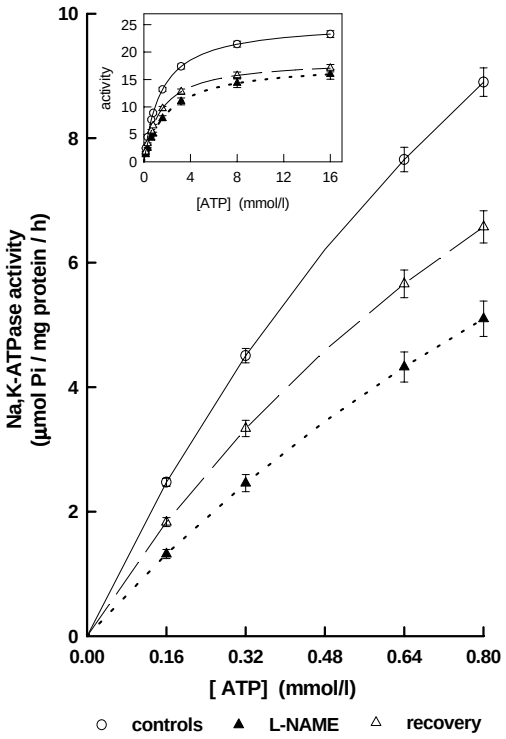

Fig. 3. *Activation of renal Na,K-ATPase by substrate ATP in control rats, in rats with L-NAME induced NO-deficient hypertension, and in rats with systolic blood pressure recovered to control values. Detailed projection of activities in the presence of low concentrations of ATP. Insert: Activation of the enzyme in the whole investigated concentration range of ATP.*

as compared to the hypertensive L-NAME group. At concentrations below 10 mmol/l the enzyme was slightly stimulated (14 % stimulation in the presence of 2 mmol/l NaCl), whereas above 20 mmol/l there begins the inhibition of the enzyme amounting to 10 % loss of the activity in the presence of 100 mmol/l (Fig. 4). These changes were reflected in a non-significant decrease of V_{max} and significant 27 % decrease of the K_{Na} value (Table 1).

When comparing the recovery group with the control group we observed significant inhibition of the enzyme in the whole concentration range of NaCl. The inhibition attained 50 % in the presence of 2 mmol/l NaCl. An additional increase of the NaCl, concentration was followed by a gradual decrease of the inhibitory effect. At the highest investigated concentration (100 mmol/l NaCl), the inhibition was only 32 % (Fig. 4). The above activity changes were also manifested in the alterations of kinetic parameters. The V_{max} value dropped by 30 % and the K_{Na} value increased by 55 % (Table 1).

Discussion

With 4-week lasting administration of L-NAME followed by its elimination for 3 weeks, we were able to introduce conditions with lower and higher synthesis of nitric oxide in rat kidneys. The 49 % increase of calcium and calmodulin-dependent NO-synthase activity in the recovery group can be explained by a hypothesis that at this stage the number of NO-synthase molecules in the renal tissue may be increased. Previous studies showed that 4-week lasting NO-deficient hypertension induced a significant increase of proteosynthesis, as documented by the increased level of DNA, RNA and [^{14}C] leucine incorporation into proteins of the kidney (Bernátová *et al*. 1999). It is possible that during the NO-deficient hypertension, the increased proteosynthesis causes an overexpression of NO-synthase molecules, which, however, cannot exhibit their enzymatic activity due to the presence of NO-synthase inhibitor L-NAME. Three weeks after discontinuing the application of L-NAME NO-synthase molecules may become fully reactivated, so that the final activity of the NO-synthase overexpressed during the NO-deficient hypertension is even higher than in controls. The used experimental model allowed us to study the effect of nitric oxide on the function of renal Na,K-ATPase *in vivo* after the application of L-NAME and its subsequent withdrawal without any other exogenously induced elevation of NO concentration.

As far as Na,K-ATPase is concerned, the aim of the present study was to find out if the hypertension induced by inhibition of NO-synthase is followed by any functional changes of Na,K-ATPase in the kidney and whether the activity of this enzyme is restored or not after the withdrawal of the inhibitor L-NAME. It is known that hypertension is accompanied by increased intracellular concentration of sodium (Jelicks and Gupta 1994) which may be related to functional changes of the Na,K-ATPase. Concerning the function of this enzyme in hypertension the present study deals with the energy supply *via* the hydrolysis of ATP and the affinity-changes of the Na-binding site in the Na,K-ATPase molecule.

It is known that Na,K-ATPase converts 20-30 % of the current ATP production in mammals to active Na,K transport in the kidney, central nervous system, and other cells of the body where Na,K gradients are required for maintaining the membrane potential and cell volume (Jorgensen and Pedersen 2001). Hence, the changes in energy utilization of the Na,K-ATPase which we investigated by activation of the enzyme with increasing

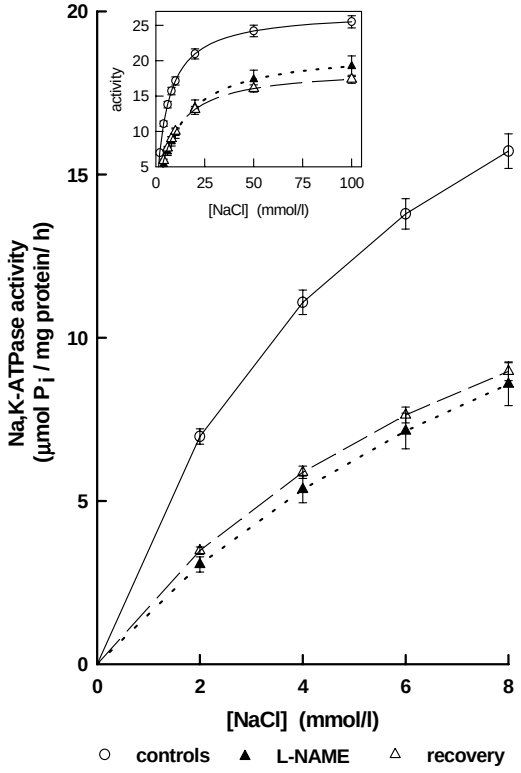

Fig. 4. *Activation of renal Na,K-ATPase by cofactor Na^+ in control rats, in rats with L-NAME induced NO-deficient hypertension, and in rats with systolic blood pressure recovered to control values. Detailed projection of activities in the presence of low concentrations of ATP. Insert: Activation of the enzyme in the whole investigated concentration range of ATP.*

concentrations of substrate (ATP), seem to be very important. The observed increase of the K_m value in the L-NAME group as compared to the controls indicates a deterioration of the affinity of the ATP binding site. Simultaneously, a decrease of V_{max} value was observed. The above data point to a mixed type of inhibition. It is known that this kind of effect is probably caused by the inhibitory influence of reaction products. Therefore one of the possible explanations might be that the inhibition of the Na,K-ATPase by the products of ATP hydrolysis may occur in rats with NO-deficient hypertension. This may suggest that lowered synthesis of NO induces changes in the enzyme molecule resulting in higher sensitivity to the feedback mechanism by ADP or P_i. According to the long-lasting time-course of our experiment during which the expression of the Na,K-

ATPase molecules may be altered, a second plausible explanation may concern the decrease in the number of enzyme molecules in renal plasma membranes of hypertensive rats. Independently of the mechanisms supposed to be responsible for the observed effect, in the presence of lower ATP concentrations the utilization of substrate by the enzyme is limited under the conditions of NO-deficient hypertension.

The withdrawal of L-NAME improved the affinity of the ATP-binding site as is indicated by a significant decrease of the K_m value to the same level as in the controls. Hence, in the recovery group there is a restoration of the ATP-binding site in its ability to bind and to hydrolyze the substrate, especially at low concentrations of ATP. Consequently, the Na,K-ATPase is able to utilize more efficiently the energy derived from ATP-hydrolysis for the transport of excessive Na^+ out from the cells. Our results pointed out a partial reversibility of molecular changes of Na,K-ATPase induced by hypertension as a result of L-NAME application. This reversibility was not manifested at higher concentrations of ATP what resulted in consistently lowered V_{max} value. The still depressed V_{max} value suggests that the number of Na,K-ATPase molecules after the spontaneous restoration of SBP still remains at a lowered level as during hypertension. Our data on positive effect of increased NO synthesis do not seem to be consistent with previously published inhibitory effect of elevated NO concentration on renal Na,K-ATPase. It should be stressed that the stimulatory effect of NO is limited to the amount of NO produced by the calcium and calmodulin-dependent constitutive NO synthase. If the amount of NO increases above this limit, the Na,K-ATPase might be inhibited again, as was demonstrated at very high concentrations of NO generated by the inducible NO-synthase (Guzman *et al*. 1995) or by application of very high concentration of NO donor, sodium nitropruside (Liang and Knox 1999).

Studies of the enzyme activation with increasing concentrations of sodium resulted in a lowered affinity to Na^+ during hypertension as revealed from the increase of the K_{Na} value. Due to this deterioration, the enzyme activity is significantly depressed especially in the presence of lower concentrations of Na^+. It is interesting that the increase of the Na^+ concentration was followed by a stepwise decrease of the inhibitory effect, suggesting that Na,K-ATPase probably adapts by changing the affinity of the Na-binding site accordingly to elevated intracellular concentration of Na^+ during hypertension. The observed diminution of V_{max} in this group during the Na-dependent activation of this enzyme, again indicates a decrease in the number of Na,K-ATPase molecules in renal plasmalemmal membranes during NO-deficient hypertension. This conclusion is in agreement with our previous assumption expressed in the part dealing with the ATP-dependent activation of the enzyme. This similarity in kinetic properties in both types of enzyme activation seems to be a consequence of the well-known fact that both binding sites, for ATP and also for Na^+ are oriented towards the intracellular space.

The withdrawal of L-NAME induced a significant decrease of the K_{Na} value as compared to the L-NAME group indicating an improvement of the affinity to Na^+. This improvement was only partial because the K_{Na} value in the recovery group is still higher by 55 % as compared to the control group. The persisting depression of V_{max} for the Na-dependent activation suggested that the number of Na,K-ATPase molecules still remains reduced as during hypertension. In this respect the functional response of renal Na,K-ATPase to elevated NO synthesis is less effective than in the case of the cardiac enzyme for which complete restoration of Na^+ affinity was documented (Vrbjar *et al*. 1999).

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Reprint requests

N. Vrbjar, Institute for Heart Research, Department of Biochemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 33 Bratislava, Slovak Republic, FAX: +421 2 5477 6637, E-mail: usrdnorb@savba.sk