INTRODUCTION

Several epidemiological studies have indicated that regular intake of vegetables, fruit, and beverages such as red wine and green tea is associated with a decrease in global mortality, due to the reduced number of cancer and coronary diseases (1). The protective effect has been attributed to bioflavonoids (2). One of the most abundant bioflavonoids in nature is quercetin, which was shown to have protective effects on the aorta (3), cerebrovascular system (4), the heart (5), kidney (6), etc. Quercetin protects via various mechanisms due to its antioxidative properties (7), as e.g. protection of endothelial function (8), its antihypertensive properties (9-12), decrease of the level of LDH, iNOS (13), decrease of plasma concentration for oxidized LDL (11). Besides the above mentioned positive effects of quercetin, previous in vitro studies demonstrated also negative effects of this flavonoid on some ion transporting ATPases like the Ca-ATPase in sarcoplasmic reticulum (14, 15) and Na,K-ATPase (16-19). The latter plays a crucial role in maintaining the homeostasis of sodium in the organism. Since ion transport mediated by Na,K-ATPase is the major consumer of metabolic energy in the kidney, utilizing about 20-30% of ATP production (20), the enzyme is critically important to renal function. The function and regulation of renal Na,K-ATPase is altered in various forms of spontaneous hypertension (21, 22). Since information on the influence of quercetin on the enzyme in vivo is rather scarce, the present study was focused on the effect of four-week administration of quercetin to normotensive and spontaneously hypertensive rats on functional properties of renal Na,K-ATPase.

MATERIAL AND METHODS

Animals and experimental protocol

Four-week-old male rats were divided in 4 groups: normotensive Wistar controls (AC), normotensive Wistar rats treated with quercetin (AQ), control group of SHR (HC) and SHR treated with quercetin (HQ). The number of animals was the same in each group (n=6). The rats were placed in plastic cages and housed in a room with controlled temperature (22-24°C), a 12-h light-dark cycle and were fed a regular pellet diet. All procedures used in this study were approved by the Veterinary Council of Slovakia (Decree 289, part 139, July 9th 2003). The groups AQ and HQ received quercetin in a dose of 20 mg kg⁻¹·day⁻¹ for 4 weeks. Quercetin was dissolved in a little amount of ethanol, so that the final concentration of ethanol in drinking water was 1% (vol./vol.) and then dissolved in 0.1mol·l⁻¹ phosphate buffer, pH 6.0 to keep maximal stability of quercetin in solution (previous experiments showed maximal stability of quercetin at pH 6.0). The control groups AC and HC received 0.1M phosphate buffer, pH 6.0 with...
1% ethanol as drinking solution. After 4 weeks of treatment, the rats were anesthetized and the excised kidneys were quickly frozen and stored in liquid nitrogen until biochemical analysis.

**Assay of Na,K-ATPase activity**

The plasmalemmal membrane fraction was isolated from the kidney according to Jorgensen (1974) (23). The amount of proteins was determined by the procedure of Lowry et al. (24), using bovine serum albumin as a standard.

All enzyme assays were carried out at 37°C using 10 µg ml⁻¹ of membrane protein. The Na,K-ATPase activity was assessed in an assay buffer containing (in mmol·l⁻¹): 4 MgCl₂, 100 NaCl, 10 KCl and 50 imidazole (pH=7.4). Subsequently, after 20 min of preincubation in substrate-free medium, the enzyme reaction was initiated by adding increasing amounts of ATP in the range of 0.16-8.00 mmol·l⁻¹. The reaction was stopped after further 20 min by adding 12% ice-cold trichloracetic acid. The liberated inorganic phosphorus originating from ATP hydrolysis was determined according to the method of Taussky and Shorr (25). In order to establish the Na,K-ATPase activity, the ATP hydrolysis that occurred in the presence of Mg²⁺ only was subtracted. The enzyme kinetics for sodium activation was determined by the same approach. The concentration of NaCl varied in the range of 2-100 mmol·l⁻¹ and the amount of ATP was constant (4 mmol·l⁻¹).

**Statistical analysis**

All results are expressed as means±S.E.M. The kinetic parameters were evaluated by direct non-linear regression of the obtained data. ANOVA and Bonferroni test were used for statistical analysis. The differences were considered to be significant when the P-value was less than 0.05.

**RESULTS**

**Body weight, kidney weight**

The body and kidney weight did not differ in the normotensive and hypertensive group. Neither did administration of quercetin affect the weight parameters investigated (Table 1).

**Kinetics of Na,K-ATPase**

The comparison of untreated normotensive control rats (AC) with untreated SHR (HC) showed variations in kinetic properties of the Na,K-ATPase molecule. On activating the enzyme with increasing concentrations of ATP, we observed a significant increase of enzyme activity in the HC group throughout the whole concentration range of the substrate. The highest increase observed in the presence of 0.16 mmol·l⁻¹ ATP amounted to 58%. With increasing concentrations of ATP the effect decreased and in the presence of 8 mmol·l⁻¹ ATP the activity was higher by 20% only in the HC group (Fig. 1). Evaluation of the above data by the method of nonlinear regression resulted in a statistically significant increase of Vₘₐₓ by 15% and a significant 30% decrease of the Kₘ value in the HC group, as compared to respective controls. In both groups the effect of increasing enzyme activity due to increased concentrations of the substrate. In normotensive rats the inhibition was in the range of 9-6% and in hypertensive animals it represented 18-8%, with the highest effect in the presence of the lowest concentration of ATP (Fig. 1). Evaluation of the above data by the method of nonlinear regression resulted in a statistically significant increase of Vₘₐₓ by 15% and a significant 30% decrease of the Kₘ value in the HC group, as compared to respective controls. In both groups the effect of increasing concentrations of ATP was significant (Fig. 1).

![Graph showing Na,K-ATPase activity versus ATP concentration](image1)

**Table 1. Weight parameters of normotensive and hypertensive rats after 4 weeks lasting administration of quercetin in a dose of 20 mg.kg⁻¹.day⁻¹. Bw-body weight, Kw-kidney weight (left+right), Kw (L+R)/Bw-kidney weight/body weight ratio**

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Bw [g]</th>
<th>Kw (L+R) [mg]</th>
<th>Kw (L+R)/Bw [mg·g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>243±11</td>
<td>1900±124</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>AQ</td>
<td>250±15</td>
<td>1932±136</td>
<td>7.7±0.3</td>
</tr>
<tr>
<td>HC</td>
<td>240±5</td>
<td>2075±49</td>
<td>8.7±0.3</td>
</tr>
<tr>
<td>HQ</td>
<td>226±5</td>
<td>2000±91</td>
<td>8.9±0.6</td>
</tr>
</tbody>
</table>

Data represent means±S.E.M. at the end of experiment, n=6 in all groups. Normotensive wistar rats treated (AC), normotensive wistar rats treated with quercetin (AQ), control SHR (HC) and SHR treated with quercetin (HQ).

Quercetin decreased the Na,K-ATPase activity in both treated groups. On activating the enzyme with increasing concentrations of ATP, we observed a slight decrease of the enzyme activity in the AQ as well as in the HQ group, as compared to respective controls. In both groups the effect of treatment decreased with increasing concentrations of the substrate. In normotensive rats the inhibition was in the range of 9-6% and in hypertensive animals it represented 18-8%, with the highest effect in the presence of the lowest concentration of ATP (Fig. 1). Evaluation of the above data by the method of nonlinear regression resulted in a statistically significant increase of Vₘₐₓ by 15% and a significant 30% decrease of the Kₘ value in the HC group, as compared to respective controls. In both groups the effect of increasing concentrations of ATP was significant (Fig. 1).
regression resulted in statistically insignificant changes of V_{max} and K_{m} values in both the AQ and HQ group, as compared to respective controls (Fig. 2). On activating the enzyme with increasing concentrations of Na^{+} ions, the inhibitory effect of quercetin varied between 20-7% and 24-8% in the AQ and HQ groups, respectively (Fig. 3). Determination of kinetic parameters resulted in insignificant alteration of V_{max} and in significant increase of the K_{Na} value by respective 22% and 31% in the AQ and HQ groups (Fig. 4).

DISCUSSION

Previous studies documented the involvement of Na,K-ATPase in the development of hypertension. Decreased Na,K-ATPase activity with increased intracellular Na^{+} concentration has been described in erythrocytes, leukocytes, and lymphocytes from hypertensive and from normotensive patients with a family history of hypertension (26-30). Experimental studies documented the importance of Na,K-ATPase during the development of hypertension in various tissues, such as vascular smooth muscle (31-33), cardiac tissue (34, 28, 35) and kidney (21, 22, 36). An interesting finding of the present study involving young rats between their 5th-8th week of age is the observed increased number of active Na,K-ATPase molecules in renal tissue, as indicated by the increased V_{max} value in hypertensive rats. This finding is in disagreement with our previous observations in the kidney of 16-week-old rats (37) and also in the heart (35), documenting a decreased number of active Na,K-ATPase molecules. These differences may be ascribed to a higher synthesis of Na,K-ATPase molecules at various developmental stages of hypertension. It was observed that pre-hypertensive 4-week-old SHR hearts exhibited an approximately 4-fold elevation in alpha1 mRNA levels compared with age-matched control hearts (38). The observed increase in the affinity to the substrate ATP, as indicated by a decreased K_{m} value in our 8-week-old hypertensive rats, was presumably also age dependent. This positive adaptation of young rats to hypertension is probably lost during maturation, as indicated by the lack of protection in 16-week-old hypertensive rats (35).

Besides the previously documented cardioprotective and antihypertensive action of quercetin (9-12, 39), we observed that four weeks lasting in vivo administration of quercetin in the dose of 20 mg.kg^{-1}.day^{-1} induced a significant deterioration in the affinity of renal Na,K-ATPase to sodium, independently of the pathophysiological state of the rats. The difference between in vitro and in vivo studies revealing no change of affinity to sodium in vitro (17) and decrease in affinity to sodium after 4 weeks lasting in vivo administration of quercetin observed in the present study suggests that quercetin influences the enzyme in the vicinity of the Na^{+}-binding site indirectly, probably by modifying the enzyme molecule during its synthesis or maturation. The observed modulatory effect of in vivo administration of quercetin was higher by a half in hypertensive animals, indicating that the sodium binding area of the enzyme may be slightly more sensitive to quercetin treatment, as suggested by the higher inhibitory effect of quercetin on the

Fig. 2. Kinetic parameters of renal Na,K-ATPase during activation with ATP in normotensive control Wistar rats (AC), normotensive Wistar rats treated rats with quercetin (AQ), control SHR (HC) and SHR treated with quercetin (HQ). Data represent means±S.E.M, n=6 in each group. a: p<0.01 as compared to the AC group.

Fig. 3. Activation of renal Na,K-ATPase by low concentrations of cofactor Na^{+} in normotensive control Wistar rats (AC), normotensive Wistar rats treated rats with quercetin (AQ), control SHR (HC) and SHR treated with quercetin (HQ). Insert: activation of the enzyme in the whole investigated concentration range of NaCl.
activity during activation of Na,K-ATPase with increasing concentrations of Na+ ions (as documented by 22% and 31% increase of the $K_{av}$ value in normotensive and hypertensive groups, respectively). Previous in vitro studies clearly showed a concentration dependent manner of action of quercetin on Na,K-ATPase. Quercetin in the presence of 1.5 micromolar concentration enhanced the Na,K-ATPase activity (40). On the other hand, when its concentration exceeded 25 µmol·l⁻¹, quercetin inhibited the enzyme from a variety of tissues (16, 17, 19). In our experiment, the concentration of quercetin in renal tissue was probably in the higher concentration range, as documented by previous studies with in vivo administration of quercetin, showing that this antioxidant and its metabolites were accumulated mainly in kidney and other organs with high metabolic activity (41, 42). This concept is supported by the observation that a shorter, 8 days lasting administration of ten times lower doses of quercetin (2 mg·kg⁻¹·day⁻¹) partially protected Na,K-ATPase against renal dysfunction induced by a combined use of gentamycin and diclofenac (43).

Contrary to qualitative changes of renal Na,K-ATPase implicated by an increased $K_{av}$ value, the quantity of active enzyme molecules remained unchanged, as documented by the stable value of $V_{max}$ in normotensive and hypertensive animals after administration of quercetin.

Concerning the present study with 4-week administration of pure quercetin, an interesting comparison may be made with previous studies where a mixture of red wine polyphenolic compounds (RWPC) was administered to LNAME-hypertensive rats, also for a period of 4 weeks. In 16-week-old animals RWPC protected the Na+-binding site in the kidney (37) and in the heart (44) of hypertensive rats. In 8-week-old animals, pure quercetin impaired the properties of the Na+-binding site, as indicated by an increased value of $K_{av}$. Two possible explanations for the above discrepancy may be relevant. The first may concern the lower proportion of quercetin in RWPC experiments, as indicated by a lower concentration of flavonols amounting approximately to 2% of polyphenols (45, 46). Thus the amount of flavonols, including quercetin, was in RWPC experiments much lower than the 20 mg·kg⁻¹·day⁻¹ of pure quercetin applied in the present study. This finding is supported also by the protective effect of alcohol-free red wine administration on Na,K-ATPase against renal damage induced by oxidative stress or rhabdomyolysis (47-50). The positive effects of other polyphenols, like resveratrol, which are present in red wine in a much higher content, probably block out the negative effect of quercetin. In addition, the effect of quercetin is concentration dependent, inducing at low concentrations a stimulation of Na,K-ATPase, as shown by (40). The second explanation of the above mentioned discrepancy may be ascribed to different ages of the animals. It is possible that Na,K-ATPase may differ in structure and resultant functional properties due to a much higher synthesis of the enzyme molecule, which was documented in premature animals by Tsuruya et al. (38).

In conclusion, quercetin independently of the pathophysiological status of the investigated animals, i.e. normotension or hypertension, elicited an impairment in the affinity of the Na+-binding site of Na,K-ATPase molecules, which may be responsible for the deteriorated enzyme function in the kidney.

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REFERENCES


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