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Susceptibility of Weakly Ouabain-Sensitive Na, K-ATPase Isoform in Ischemic and Reperfused Rat Retinas

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KUBOKI, J., ISHIGURO, S. and TAMAI, M. *Susceptibility of Weakly Ouabain-Sensitive Na, K-ATPase Isoform in Ischemic and Reperfused Rat Retinas.* Tohoku J. Exp. Med., 1999, **187**(4), 353-361 —— It is possible that Na, K-ATPase may play some roles in ischemic damage of nervous tissue. To determine whether Na, K-ATPase is affected in ischemic and reperfused retina, we measured enzyme activities. Retinal ischemia was induced by clamping the optic nerve of female adult Sprague-Dawley (SD) rats for 90 minutes. At 0.5, 2 and 24 hours after reperfusion, rat eyes were enucleated, and the retinas were removed. In addition to unseparated, total ouabain-sensitive Na, K-ATPase activity, we measured weakly ouabain-sensitive (α) and highly ouabain-sensitive ($\alpha[+]$) isoform activities separately by ATP hydrolysis. Total ouabain-sensitive Na, K-ATPase activity, α and $\alpha(+)$ isoform activities showed no significant difference from sham-operated contralateral eyes at 0.5 and 2 hours of reperfusion. After 24 hours of reperfusion, total ouabain-sensitive Na, K-ATPase activity decreased to 63% of the control. The activities of α and $\alpha(+)$ isoforms were 47% and 72%, respectively. The ratios of the α and $\alpha(+)$ isoform activities ($\alpha/\alpha[+]$) significantly decreased at 2 and 24 hours of reperfusion. Activity in α isoform decreased markedly in reperfused rat retinas. This response may be beneficial for reducting the oxidative stress in reperfused retinas. ———— Na, K-ATPase; α isoform; ischemia; oxidative stress

It is well known that the excessive release of glutamate, which is called excitotoxin, plays an important role in ischemic injury of neuronal tissue. Excess glutamate contributes to glutamate receptor activity and leads to neuronal degeneration. Results of experiments in the ischemic retina have shown that excess glutamate was released transiently after reperfusion (Louzada-Junior et al. 1992) and that the innner layer, rich in receptors for glutamate, was more damaged (Hamasaki and Kroll 1968; Kroll 1968; Hayreh and Weingeist 1980; Bresnick 1989; Hughes 1991; Hamassaki-Britto et al. 1993; Brandstatter et al. 1994). Neurons of the inner nuclear layers showed extensive loss and decrease in the thickness of the layer (Hughes 1991). Ischemic damage was prevented by

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pretreatment with an antagonist of glutamate receptors (Yoon and Marmor 1989). It is thought that glutamate participates in the ischemic injury of retina.

Recently, it has been thought that two distinct forms are present in the mechanism of glutamate receptor-mediated ischemic neuronal degeneration (Coyle and Puttfarcken 1993). The acute form is characteristic of neuronal swelling caused by influx of ions through glutamate-gated cation channels and it leads to osmotic lysis of neurons. The delayed form is neuronal degeneration caused by production of oxygen-derived free radicals in neuronal cells. Free radicals are produced by two pathways. Intracellular Ca^{2+} is increased by influx through both voltage-dependent Ca^{2+} channels and glutamate receptor. Arachidonic acid is subsequently produced. Free radicals are generated in the metabolic process of arachidonic acid. Another pathway is present in mitochondria. It produces free radicals when ATP consumption increases.

It is assumed that Na, K-ATPase (EC 3.6.1.37) contributes to delayed ischemic damage by glutamate. Na, K-ATPase is a membrane enzyme that maintains concentration gradients of Na^+ and K^+ by active transport. If Na, K-ATPase is very active to recover the ion gradient and osmotic pressure in the ischemic/reperfused tissues, subsequent increase of ATP consumption by Na, K-ATPase may increase the generation of free radicals in mitochondria (Coyle and Puttfarcken 1993).

Recently, three different Na, K-ATPase isoforms, $\alpha 1$, $\alpha 2$ and $\alpha 3$, have been found in the brain and retina (Stahl and Baskin 1984; McGrail and Sweadner 1986; Schneider et al. 1988a, b; Sweadner 1989; Urayama et al. 1989; McGrail et al. 1991). These isoforms exist in tissue and cell specific patterns of expression and can be classified into two types by differences in sensitivity for the cardiotonic steroid ouabain. The α ($\alpha 1$) is a weakly ouabain-sensitive and $\alpha(+)$ ($\alpha 2$, $\alpha 3$) is a highly ouabain-sensitive isoform. The $\alpha(\alpha 1)$ isoform is ubiquitous and has been designated as the common type or housekeeping isoform, whereas the $\alpha(+)$ ($\alpha 2$, $\alpha 3$) exists typically in the brain and has been designated as the brain type isoform. These Na, K-ATPase isoform activities are known to be affected by the administration of glutamate in the cultured cerebral neurons (Inoue and Matsui 1990).

In the present study, we examined total retinal Na, K-ATPase activity and weakly and highly ouabain-sensitive Na, K-ATPase activities in reperfused rat retinas.

Materials and Methods

Reagents

We obtained phosphorylase *a*, glucose-6-phosphate dehydrogenase and glycogen from Sigma (St. Louis, MO, USA). Phosphoglucomutase was obtained from Boehringer Mannheim (Mannheim, Germany). Prior to use, glycogen was dissolved in deionized water (80 mg/ml) and dialyzed for 24 hours against 1000 ml of

deionized water at 4°C. Phosphorylase *a* was dissolved at the concentration of 10 mg/ml in 0.05 M imidazole buffer (pH 7.0) and stored at −36°C after lyophilization until use. Phosphoglucomutase was centrifuged at 12 000 rpm and 4°C for 5 minutes and used after removal of supernatant fluid. Glucose-6-phosphate dehydrogenase was dissolved (100 units/0.26 ml) in deionized water and stored at −36° C after lyophilization.

Animals

Female adult Sprague-Dawley (SD) rats, weighing 250–300 g each, were anesthetized with intramusculary administered ketamine HCl (66.7 mg/kg) and xylazine (3.9 mg/kg). Left retinal ischemia was induced by clamping the optic nerve for 90 minutes (Tomita et al. 1994). Sham operation was performed in the right eyes. At 0.5 ($n=6$), 2 ($n=7$) and 24 hours ($n=7$) after reperfusion, rat eyes were enucleated and the retinas were removed. All animals in this investigation were treated in compliance with the ARVO (Association for research in vision and Ophthalmology) statement for the Use of Animals in Ophthalmic and Vision Research.

Assay of Na, K-ATPase activity

The retinas were homogenized with 0.5 ml of ice-cold 0.32 M sucrose solution containing 1 mM EGTA (Sigma, St. Louis, MO, USA) and 30 mM histidine (pH 7.0). Sodium deoxicholate (final 0.1%) was added to the homogenates. The samples were centrifuged at 100 000 × g for 60 minutes at 4°C. The precipitate was resuspended with 0.5 ml of ice-cold deionized water by brief sonication and stored at −80°C. Protein assay was carried out by the previous method (Lowry et al. 1951).

Total retinal ouabain-sensitive Na, K-ATPase activity, α and $\alpha(+)$ isoform activities were measured separately by ATP hydrolysis method based on the previous procedures (Petty et al. 1981; MacGregor and Matschinsky 1986; Inoue and Matsui 1990). In brief, dephosphorylation of ATP by Na, K-ATPase reaction was carried out by adding 25 μl of reagent A to 25 μl of retinal samples (about 2.5 μg protein/tube) at 37°C for 15 minutes. Reagent A contained 97 mM Tris-HCl (pH 7.5), 72.5 mM NaCl, 6.25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, and varying concentrations of ouabain from 0.0002 to 2 mM. The enzyme reaction was terminated by heating at 95°C for 2 minutes and then 1 ml of reagent B was added. Reagent B contained 50 mM imidazole-HCl (pH 7.0), 1 mM EGTA, 0.5 mM magnesium acetate, 0.03 mM NADP, 0.01 mM 5′-AMP, 0.08% glycogen, 0.02% BSA, 0.4 units/ml glucose-6-phosphate dehydrogenase, 1.5 units/ml phosphoglucomutase, and 1.0 unit/ml phosphorylase *a*. All tubes were incubated at 37°C for 30 minutes. NADPH was produced from inorganic phosphate (Pi) by two steps of chain (MacGregor et al. 1986). After 1 ml of deionized water was added to each tube, NADPH was measured fluorometrically (Ex: 340 nm, Em:

460 nm). The enzyme activities were expressed as nmol Pi liberated per minutes per mg protein. Statistical significance was calculated using paired t-test.

Results

There was no difference in total ouabain-sensitive Na, K-ATPase activity between reperfused and sham-operated control retinas at 0.5 and 2 hours of reperfusion. After 24 hours of reperfusion, the total ouabain-sensitive Na, K-ATPase activity decreased to 63% of the control ($p<0.01$) (Fig. 1).

To analyze the decreased Na, K-ATPase further, we measured isoform activities of the enzyme in reperfused retinas. On the basis of susceptibility to 3 μM ouabain, α and $\alpha(+)$ isoform activities were separated as shown in Fig. 2 and represented 42% and 58% of the total Na, K-ATPase activity, respectively. The activities of α and $\alpha(+)$ isoforms, which are weakly and highly sensitive to ouabain, showed no difference between ischemic and sham-operated control retinas at 0.5 and 2 hours of reperfusion. After 24 hours of reperfusion, the activities of α and $\alpha(+)$ isoforms decreased to 47% (sham-operated control: 37.5 nmol/min/mg; ischemic/reperfused: 17.7 nmol/min/mg, $p<0.01$) and 72% sham-operated control: 46.9 nmol/min/mg; ischemic/reperfused: 33.8 nmol/min/mg, $p<0.05$), respectively (Figs. 3a and b). The weakly ouabain-sensitive α isoform affeared to be more susceptible to injuries caused by ischemia and reperfusion in the retina.

To confirm the susceptibility of α isoform, we calculated the ratio of α to $\alpha(+)$ isoform activity ($\alpha/\alpha[+]$) in reperfused retinas. At 0.5 hour of reperfusion, the $\alpha/\alpha(+)$ was no different between ischemic and nonischemic retinas

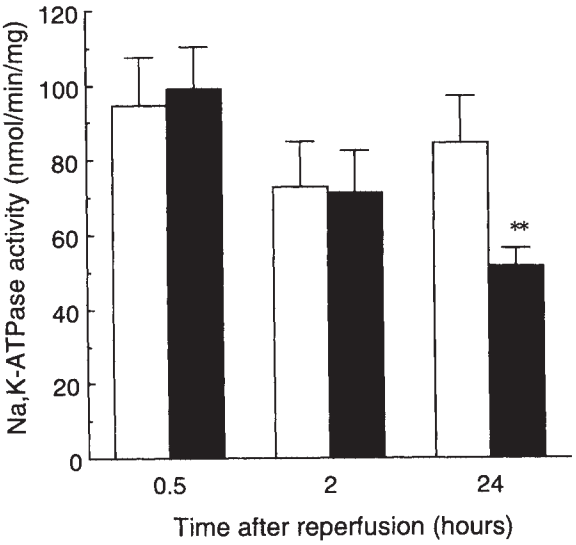

Fig. 1. Change of total ouabain-sensitive Na, K-ATPase activity in reperfused and sham-operated control retinas. No significant difference between ischemic and control retinas was found at 0.5 ($n=6$) and 2 hours ($n=7$) of reperfusion. After 24 hours ($n=7$) of reperfusion, the specific activiy decreased to 63% of the control ($^{**}p<0.01$). Each data shows mean$\pm$S.D. Open colum, sham-operated control retina; closed colum, ischemic/reperfused retina.

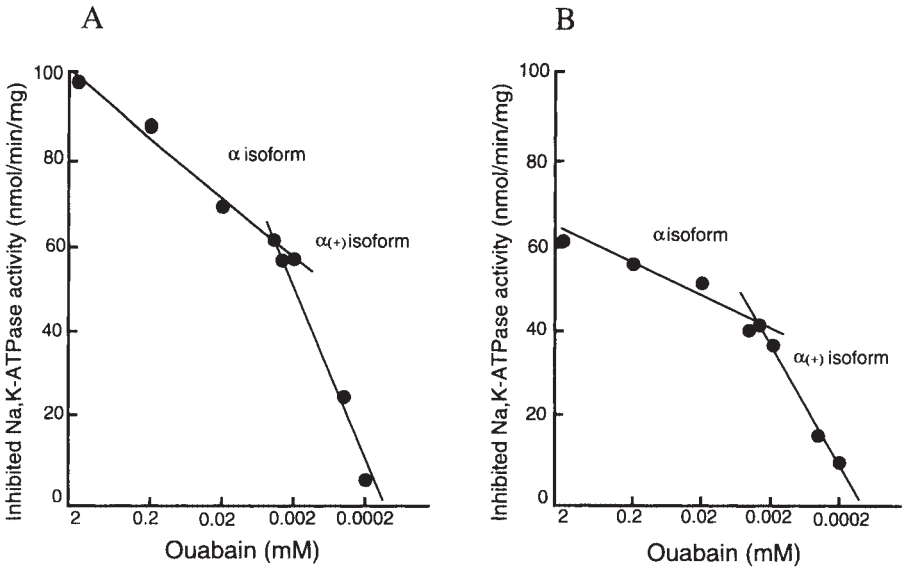

Fig. 2. Dose-response studies for inhibition of Na, K-ATPase activity by ouabain. The total ouabain-sensitive Na, K-ATPase activity was obtained using 2 mM ouabain. The activity of $\alpha(+)$ isoform, which is highly sensitive to ouabain, was obtained with 3 μM ouabain. The activity of α isoform, which is weakly sensitive to ouabain, was calculated by subtracting $\alpha(+)$ isoform activity from the total activity. Reperfused retinas used for this experiments were obtained after 24 hours of reperfusion. A: sham-operated control retina; B: ischemic/reperfused retina.

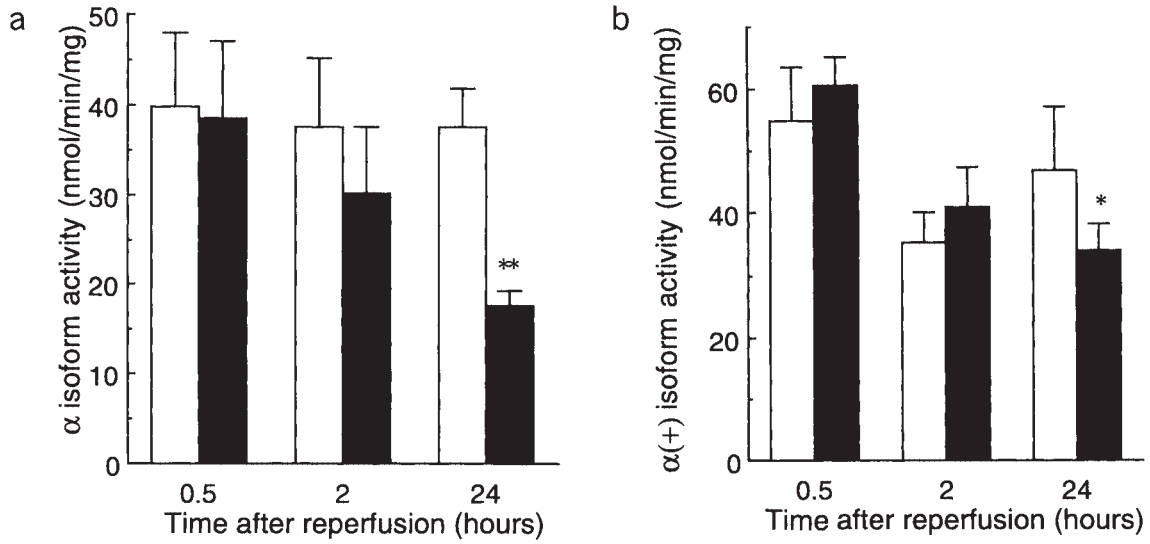

Fig. 3. Weakly(α) and highly ($\alpha[+]$)-ouabain sensitive Na, K-ATPase activities in reperfused and sham-operated control retinas. The activities of α and $\alpha(+)$ isoforms showed no difference between ischemic and sham-operated control retinas at 0.5 and 2 hours of reperfusion. After 24 hours of reperfusion, the activities of α and $\alpha(+)$ isoforms in the ischemic retinas decreased to 47% ($^{**}p<0.01$) (a) and 72% ($^{*}p<0.05$) (b), respectively. Each data shows mean$\pm$S.D. Open colum, sham-operated control retina; closed colum, ischemic/reperfused retina.

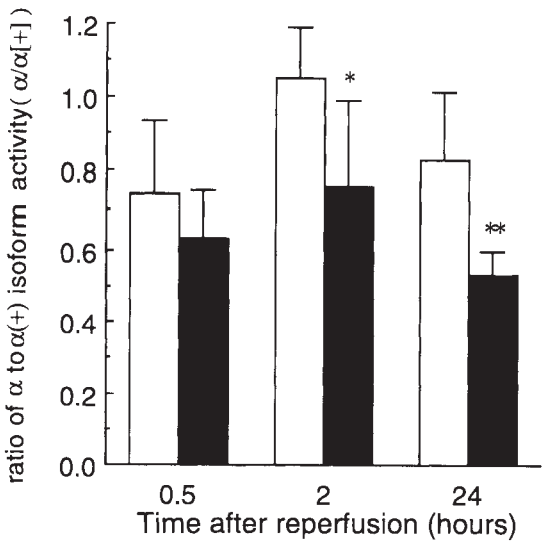

Fig. 4. The ratio of the α to $\alpha(+)$ isoform activity ($\alpha/\alpha[+]$) in reperfused and sham-operated control retinas. No significant difference between ischemic and sham-operated control retinas was found at 0.5 hour of reperfusion. The ratio in the ischemic retinas decreased at 2 ($^*p<0.05$) and 24 ($^{**}p<0.01$) hours of reperfusion. Each data shows mean$\pm$S.D. Open colum: sham-operated control retina; closed colum: ischemic/reperfused retina.

(Fig. 4), whereas the ratio decreased significantly at 2 ($p<0.05$) and 24 ($p<0.01$) hours of reperfusion. The results showed that α isoform activity was affected as quickly as 2 hours after reperfusion.

In consequence of protein assay, total retinal protein increased to about 126% as compared with sham-operated retinas after 24 hours of reperfusion (sham-operated retinas: 2.31 ± 0.37 mg/ml, $n=7$; ischemic/reperfused retinas: 2.90 ± 0.61 mg/ml, $n=7$).

Discussion and Conclusions

In the present study, we found that total ouabain-sensitive Na, K-ATPase activity decreased after ischemia and reperfusion. We measured α and $\alpha(+)$ isoform activities to analyze the response further and demonstrated that α isoform was more susceptible to ischemic injury. Our results suggested that decreased Na, K-ATPase activity reduces oxidative stress in reperfused rat retinas.

After ischemia and 24 hours of reperfusion, total retinal protein increased to about 126% as compared with sham-operated retinas ($n=7$, $p<0.05$). Contamination of red blood cells or other blood components may have contributed to this increase. As red blood cells have very low Na, K-ATPase activity (Bonting et al. 1961), the Na, K-ATPase activity of reperfused retinas is expected to be about 79% of control. After 24 hours of reperfusion, the activities of α and $\alpha(+)$ isoforms decreased to 47% ($p<0.01$) and 72% ($p<0.05$), respectively. The decrease of $\alpha(+)$ isoform activity was not significant.

The decrease of Na, K-ATPase may be explained by toxicity of oxygen free radicals. Previous study (Kim and Akera 1987) have shown that sarcolemmal

Na, K-ATPase activity and Na pump activity decrease during reperfusion of ischemic heart and that the inhibition of these activities is prevented by oxygen radical scavengers. It is believed that oxygen free radicals may play a crucial role in the ischemic damage in the retina and protection of the retina by free radical scavengers has been reported (Nayak et al. 1993; Yamamoto et al. 1994). The mechanism of the toxicity is not fully elucidated but it is likely that Na, K-ATPase is affected by oxygen free radicals after reperfusion in the rat retina.

In the present study, the α isoform activity decreased and the ratio of α to $\alpha(+)$ isoform activity ($\alpha/\alpha[+]$) also decreased in the ischemic retinas after 24 hours of reperfusion. A possible explanation for the susceptibility of α isoform can be made by its distribution in the rat retina. McGrail and Sweadner (1989) demonstrated that the highest levels of Na, K-ATPase were confined to the inner segments of photoreceptor cells and two plexiform layers. The outer plexiform layer stained brightly for $\alpha 1$ and $\alpha 3$, and the inner plexiform layer stained for $\alpha 1$, while the inner segments stained exclusively for $\alpha 3$. Therfore, weakly ouabain-sensitive α isoform ($\alpha 1$) is distributed in the inner retina and highly ouabain-sensitive $\alpha(+)$ isoform ($\alpha 3$) is expressed mainly in the outer retina. As experimental studies of ischemic retina reveal damage of the inner part of the retina (Hamasaki and Kroll 1968; Kroll 1968; Hayreh and Weingeist 1980; Hughes 1991), α isoform activity should be affected strongly and $\alpha(+)$ isoform also can be affected partially during reperfusion.

Properties of Na, K-ATPase isoforms of the retina are similar to those of cerebral neurons with respect to ouabain sensitivity. In the present study, 3 μM ouabain separated weakly and highly ouabain-sensitive isoforms. The results correlated with those described by previous report (Inoue and Matsui 1990) using cultured rat cerebral neurons. In addition, they reported that glutamate decreases the weakly ouabain-sensitive isoform activity of the cerebral neurons. During reperfusion, we also observed decreased activity of weakly ouabain-sensitive α isoform, which is distributed mainly in the inner retina. As glutamate is released by reperfusion in the retina (Louzada-Junior et al. 1992), it is possible that decreased activity of weakly ouabain-sensitive α isoform may be caused by delayed, glutamate-induced injuries. Discrepancy, however, is present with respect to the activity of highly ouabain-sensitive $\alpha(+)$ isoform. Glutamate increases the activity of $\alpha(+)$ isoform in cultured cerebral neurons, while no activation of the $\alpha(+)$ isoform activity was observed in the present study. If glutamate is released during reperfusion and glutamate receptors are present, $\alpha(+)$ isoform activity should be activated. However, as described above, $\alpha 3$ isoform exists mainly in photoreceptor inner segments, where no glutamate receptor is present.

Finally, if reduced Na, K-ATPase activity helps protect retinas from oxidative stress, weakly ouabain-sensitive α isoform may play an important role in reperfused retinas.

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