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Article

Proteoliposome-Based Capillary Electrophoresis for Screening Membrane Protein Inhibitors

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A method for screening of monoamine oxidase (MAO) inhibitor was carried out using capillary electrophoresis (CE) based on the interaction of MAO and its substrate kynuramine (Kyn). Bioactive proteoliposome was reconstituted by liposome and MAO and then was applied as the pseudostationary phase (PSP) of CE to mimic the interaction between the enzyme and its substrate. *N*-prolmrgyl-R-2-heptylamine (R-2-HPA) and rasagiline [*N*-propargyl-1-(R)-aminoindan], which are two kinds of MAO inhibitors, were added into the running buffers containing proteoliposome. The results showed that the relative migration time ratio (RMTR $\times 10^{-1}$) values of Kyn were enhanced from 8.88 to 9.31 with an increase of the concentrations of rasagiline from 10^{-6} to 1 mM. However, the RMTR values of Kyn were enhanced from 8.83 to 9.14 with an increase of the concentrations of R-2-HPA from 10^{-6} to 1 mM. The RMTR value of Kyn in the presence of rasagiline was larger than that in the presence of R-2-HPA when rasagiline and R-2-HPA were at the same concentration. The results indicated that the interaction between Kyn and MAO was weakened with the increase of the inhibitors. In addition, the results of offline incubation showed that the inhibitions of rasagiline were 100.0, 72.1, 51.8 and 5.4% at the concentration of 1, 10^{-2}, 10^{-4} and 10^{-6} mM; moreover, the inhibitions of R-2-HPA were 70.0, 44.9, 4.1 and 0.9% at the concentrations of 1, 10^{-2}, 10^{-4} and 10^{-6} mM. The inhibition efficiency of rasagiline was stronger than that of R-2-HPA at the same concentration. Additionally, the interaction between Kyn and liposome was also investigated. This newly developed method might provide a potential tool for screening MAO inhibitor.

Introduction

Parkinson's disease (PD) is a kind of neuron-degenerative disease, which results from the dopaminergic neuron loss in substantia nigra pars compacta (SNpc). Recent studies found that a variety of neurologic diseases were closely related to abnormal monoamine oxidase (MAO) activity, such as PD (1) and Alzheimer's disease (AD) (2). MAO is a kind of flavin-binding protein that is located in the mitochondrial outer membrane and catalyzes the oxidative deamination of monoamine neurotransmitters. MAO inhibitor can not only lighten the PD symptoms, but also has the capability of neuro-protective effects. Therefore, it is important to establish rapid and simple screening methods for MAO inhibitors.

Several methods have been developed for screening of MAO inhibitors, such as high-performance liquid chromatography (HPLC) (3), radiochemical analysis (4), and fluorometric assay (5). HPLC and radiochemical methods are more expensive and time-consuming. Fluorometric assay is simple and sensitive, but it is susceptible to interference. Compared to other techniques, the volume consumption of capillary electrophoresis (CE) is smaller and the analytical speed is quicker (6). It is widely used in the study of molecule interactions (7–10) and screening enzyme inhibitors (11, 12). In this study, the screening method was carried out using CE based on the interaction between enzyme and substrate.

Capillary electrochromatography (CEC) is usually performed using packed, open tubular or monolithic columns. Pseudostationary phase (PSP) can be used, in which the particles are suspended in the electrolyte, and is called PSP–CEC (13). Liposome is the suitable model for studying biomembrane, which has been used as PSP in CEC to investigate the interaction between drug and biomembrane (14, 15) and to separate compounds (16, 17). Many enzymes are membrane proteins, and when these proteins perform physiological functions, the membrane may also play an important role in the processes, such as drug absorption, distribution and metabolism. In this study, to simulate the interaction between an enzyme and its substrate as naturally as possible, the bioactive proteoliposome was reconstituted by MAO and liposome.

Different from other screening methods, this method was based on the enzyme-substrate interaction and possesses the advantages that it is simple, consumes fewer enzymes,and is not susceptible to the interference of non-target molecules.

Experiment

Instrumentation

The CE experiments were performed on an Agilent HP3DCE system (Agilent, Waldbronn, Germany). Fused-silica capillaries with a dimension of 50 μm i.d. (370 μm o.d.) × 43.5 cm (35 cm to detection window) were purchased from Yongnian Optical Fiber Co. (Hebei, China). Ultrasonication was carried out by Sonication apparatus (Sonics & Materials Newtown, PA).

Reagents and materials

Kynuramine dihydrobromide (Kyn), 4-hydroxyquinoline (4-HQ) and soybean phosphatidylcholine were purchased from Sigma-Aldrich (Steinheim, Germany). Cholesterol was obtained

from Beijing Shuangxuan Microbe Culture Medium Products Factory (Beijing, China). Dimethyl sulfoxide (DMSO) was from Amersco (Cleveland, OH). TEBA was purchased from Sinopharm Chemical Reagent Beijing Co. (Beijing, China). Rasagiline mesylate was from Beijing HuaFeng United Technology Co. (Beijing, China), Tris-HCl buffer was prepared with a concentration of 10 mM (pH 7.4). The enzyme catalytic reaction stopping solution was composed of 1 mM perchloric acid, 0.25 mM EDTA and 0.1 mM $Na_2S_2O_5$.

Proteoliposome preparation

MAO preparation

Fresh brains from Wistar rats were homogenized in Tris-HCl buffer (10 mM, pH 7.4), and centrifuged at 1,000 × g for 30 min. The supernatant was collected and centrifuged at 17,000 × g for 30 min and then the pellet was resuspended in Tris-HCl buffer. This procedure was repeated twice. The suspension containing MAO was finally collected; the protein concentration was measured by the Bradford method and the final working concentration was 4 mg/mL.

Liposome preparation

Phosphatidylcholine and cholesterol (3:1, w/w) were mixed in hexane in a glass flask. Vacuum rotary evaporation was applied for treatment of the mixture under reducing pressure to form a thin lipid film. Then the film was hydrated in Tris-HCl buffer (10 mM, pH 7.4) and ultrasonicated for 30 min in a bath sonicator to yield multilamellar vesicles with the lipid concentration at 4 mg/mL.

Proteoliposome preparation

The suspension of MAO was mixed with liposome in the ratio 1:1 (v/v). The mixture was ultrasonicated on an ice bath: sonication 1 s, interval 6 s, and the total sonication time was 1 min. The final protein concentration of the proteoliposome was 2 mg/mL.

Biological activity assay of proteoliposome and inhibition effect of N-prolmrgyl-R-2-heptylamine and rasagiline offline

Kyn can be converted into 4-HQ under the catalysis of MAO (Figure 1) and the reaction can be abolished by the MAO inhibitors, such as *N*-prolmrgyl-R-2-heptylamine (R-2-HPA) and *N*-propargyl-1-(R)-aminoindan (rasagiline). The biological activity of proteoliposome was determined according to the mechanism of those reactions described previously, and the procedure was as follows: firstly 100 μL proteoliposome was mixed with Tris-HCl (10 mM, pH 7.4) and gradients of R-2-HPA and rasagiline in the same volume for 20 min, respectively. Then, 200 μL Kyn (800 μM) was added into the mixture. After incubation at 37°C for 1 h, 50 μL 0.1M perchloric acid (0.25 mM EDTA, 0.1 mM $Na_2S_2O_5$) was added to stop the reaction for 5 min, and then the suspension was centrifuged at 17,000 × g for 10 min. The supernatant was collected and injected into CE (50 mbar, 5 s) for analysis. The running buffer was Tris-HCl buffer (10 mM, pH 7.4). The voltage was 15 kV and detection was carried out by monitoring ultraviolet (UV) absorption at 214 nm. The peak areas of the reaction product (4-HQ) were integrated and the inhibition rates of MAO activity

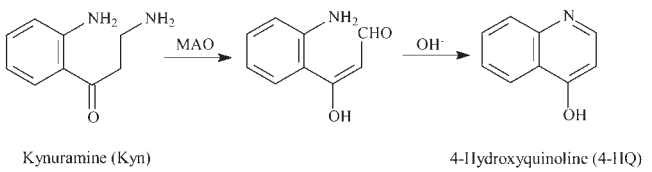

Figure 1. Reaction of Kyn catalyzed by MAO.

by different concentrations of inhibitors were calculated using the following equation:

$$I = (A_0 - A)/A_0$$

where I represents the inhibition, A and A_0 represent the peak areas of product 4-HQ in the system in the presence and absence of inhibitor, respectively.

Proteoliposome capillary electrophoresis

The proteoliposome in the absence and presence of different concentrations of inhibitors were added in the running buffer of CE as pseudo-stationary phases. A sample containing 120 μM Kyn, 2 mM TEBA and 0.1% (v/v) DMSO was injected at 50 mbar 5 s by multiple-plug technique that is expeditious and much faster than previously reported modes of CE (18). The injection was performed three times. After the first injection, the interval time was 0.75 min of each injection. After all the procedures, the electrophoresis was stopped until all the contents of the sample eluted out. The voltage and detection was as same as those described previously.

Liposome capillary

To investigate whether there was interaction between Kyn and MAO, the electrophoresis was carried out using Tris-HCl buffer (10 mM, pH 7.4) containing gradient concentrations of liposome (125–1,000 μg/mL). The sample containing 120 μM Kyn, 2 mM TEBA and 0.1% (v/v) DMSO was pressure-injected into the capillary for analysis. The CE condition was as same as those described previously.

Relative migration time ratio calculation

Mito *et al.* used a two-marker system to analyze the interaction of carbonic anhydrase B and arylsulfonamides in CE (19). The parameter of relative migration time ratio (RMTR) was used to investigate the interaction. The result showed that RMTR is more reliable than electrophoretic mobility μ_R and mobility ratios. The calculation is as follows (n = 3):

$$RMTR = \frac{t_r - t_s}{t_{s'} - t_s}$$

where t_r, t_s and $t_{s'}$ represent the measured migration times of the target molecule (Kyn), the electroosmotic flow (EOF) marker (DMSO) and the non-interacting standard (TEBA), respectively.

Results and Discussion

Reconstitution of proteoliposome

Several studies have developed methods for the reconstitution of membrane protein and liposome, such as organic

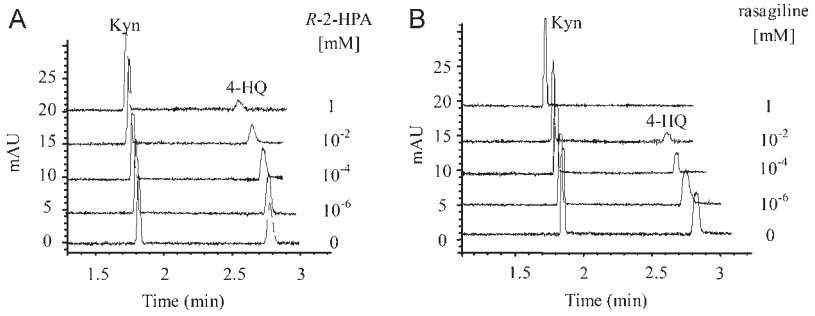

Figure 2. Electropherogram for inhibition effects on bioactive proteoliposome of gradients of: R-2-HPA (A); rasagiline (B). CE conditions: background electrolyte solution, Tris-HCl (10 mM, pH 7.4); applied voltage, 15kV; detection, UV detection at 214 nm.

solvent-mediated reconstitutions (20, 21), detergent mediated reconstitution (22–24) and sonication (25, 26). Organic solvent-mediated strategies that lead to protein inactivation have been limited. The detergent-mediated method is time consuming, and many hours' dialysis of detergent will cause protein to be inactivated. In our study, the sonication method was selected to reconstitute proteoliposome. The whole procedure is very fast. However, this method is not suitable for those proteins that will be sensitive to sonication. Therefore, after reconstitution, the biological activity of the proteoliposome has to be further measured.

Because of the difference of structures between Kyn and 4-HQ, Kyn is positively charged and 4-HQ is not charged at pH 7.4. The substrate Kyn and product 4-HQ can be separated by CE when the pH of the background electrolyte solutions is 7.4. The results (Figure 2) showed that the reconstituted proteoliposome still possessed biological activity after sonication and that the reconstitution method was feasible.

Inhibition of R-2-HPA and rasagiline by offline incubation

The inhibition effects of the two kinds of inhibitors were assayed outside the column. The inhibition effects of R-2-HPA and rasagiline on MAO activity are shown in Figures 2A and 2B, respectively. Increasing the inhibitor concentrations will decrease the amount of the product (4-HQ). In the case of rasagiline, no product can be detected at the concentration of 1 mM. The inhibitions were 100.0, 72.1, 51.8 and 5.4%, respectively, with the concentrations ranging from 1 to 10^{-6} mM. However, for R-2-HPA, there was still product generated at the concentration of 1 mM and the inhibitions were 70.0, 44.9, 4.1 and 0.9%, respectively, with the concentrations ranging from 1 to 10^{-6} mM. A significant difference was found in the inhibition rates of R-2-HPA and rasagiline (Figure 3); the inhibition effect of rasagiline is stronger than that of R-2-HPA at the same concentration.

Comparison of two kinds of inhibitors by proteoliposome CE

The proteoliposome with or without different concentrations of inhibitors were added in the running buffer of CE as pseudo-stationary phases to validate the interaction between Kyn and MAO. TEBA was used as the non-interacting standard and DMSO was used as the EOF marker. The electropherogram

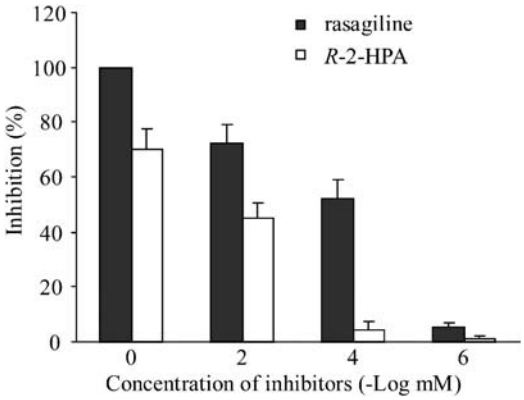

Figure 3. The inhibition rate of gradients of R-2-HPA and rasagiline on bioactive ($n = 3$).

of Kyn and TEBA showed that increasing the concentrations of inhibitors will cause the peak of Kyn to migrate close to the peak of TEBA (Figure 4). It was demonstrated that when the concentrations of the inhibitors increase, the amount of MAO that was inhibited by inhibitors also increases, and the interaction between MAO and Kyn is weakened. Therefore, the peak of Kyn migrated close to the peak of TEBA.

The RMTR values of Kyn with two inhibitors in the running buffer were compared (Figure 5). Our data showed that for both R-2-HPA and rasagiline, the RMTR values of Kyn increased with an increase of the inhibitor concentrations. The RMTR values of Kyn were 8.88×10^{-1}, 9.05×10^{-1}, 9.23×10^{-1} and 9.31×10^{-1} with respect to the rasagiline concentrations of 10^{-6}, 10^{-4}, 10^{-2} and 1 mM. Moreover, the RMTR values of Kyn were 8.83×10^{-1}, 8.86×10^{-1}, 9.03×10^{-1} and 9.14×10^{-1} with respect to the R-2-HPA concentrations of 10^{-6}, 10^{-4}, 10^{-2} and 1 mM. When R-2-HPA and rasagiline were at the same concentration, the RMTR value of Kyn in the presence of rasagiline was larger than that in the presence of R-2-HPA. The RMTR can reflect the inhibition of MAO by the inhibitors. If the value of RMTR is larger, the inhibition of MAO is stronger.

In Figure 4A, the peak of Kyn and TEBA shifted back and forth. This was because the EOF was not steady. Proteoliposome, which can dynamically absorb on the inner surface of the fused silica capillary, will sometimes lead to the instability of EOF. It was demonstrated that RMTR does not require a stable EOF and the data can be analyzed from

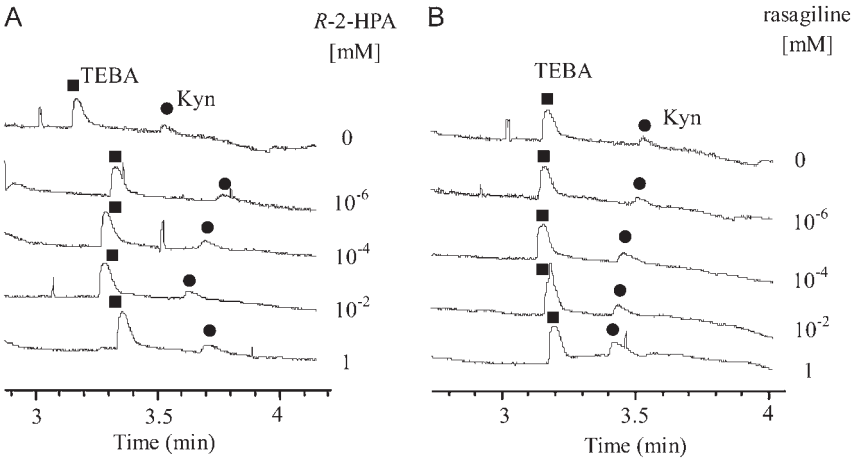

Figure 4. A representative set of electropherograms of Kyn and TEBA in the background electrolyte solutions containing proteoliposome with or without gradients of: R-2-HPA (A); rasagiline (B). CE conditions are the same as those described in Figure 2.

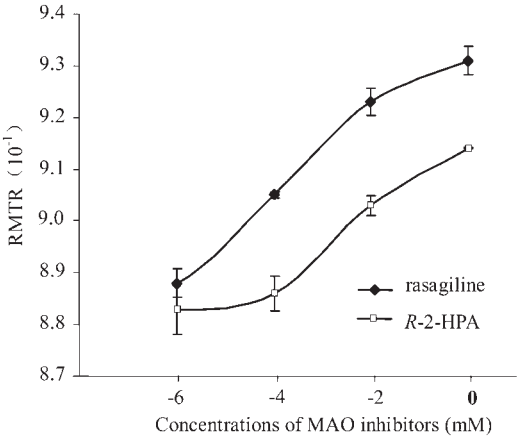

Figure 5. RMTR of Kyn in the background electrolyte solutions containing proteoliposome with or without gradients of R-2-HPA and rasagiline ($n = 3$).

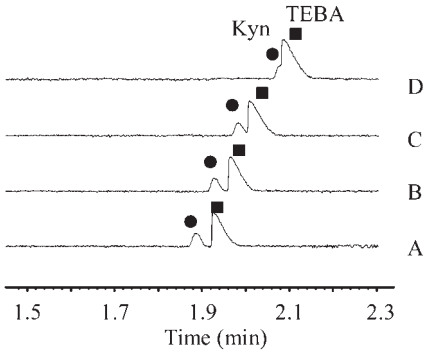

Figure 6. A representative set of electropherograms of Kyn and TEBA in the background electrolyte solutions containing gradient concentrations of liposome. The lipid concentrations in background electrolyte solutions are: 125 μg/mL (A); 250 μg/mL (B); 500 μg/mL (C); 1,000 μg/mL (D). CE conditions are the same as those described in Figure 2.

multiple experiments conducted at varying times (19). Therefore, the parameter of RMTR was used to investigate the interaction in this study.

Liposome capillary

Gradient concentrations of liposome were added into the running buffers to investigate the electrophoretic behavior of Kyn. The eletropherogram (Figure 6) shows that the peak of Kyn migrated to that of TEBA gradually with an increase of liposome. RMTR of Kyn in different concentrations of liposome was calculated (Figure 7). The results show that RMTR of Kyn decreased with an increase of liposome, which indicated that there was an interaction between Kyn and lipsome. This might be due to the hydrogen bond between the phosphate group of liposome and the amino group of Kyn. Liposome is the ideal membrane model. MAO is a membrane protein possessing the transmembrane domain. Substrate entry into the active sites of MAO is proposed to occur near the intersection of the enzyme with the surface of the membrane (2). The membrane may

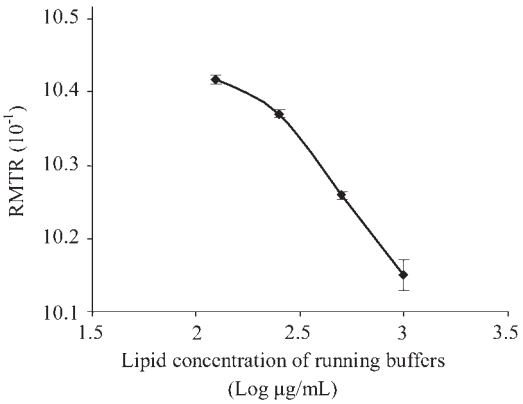

Figure 7. RMTR of Kyn in the background electrolyte solutions containing gradient concentrations of liposome ($n = 3$).

play a role in increasing the local substrate concentration at the active site of MAO (27). This may be caused by the interaction between Kyn and membrane.

Conclusions

Research of interactions between biomolecules may supply the base for disease diagnosis and drug screening. In this study, the bioactive proteoliposome was reconstituted to simulate the interaction between Kyn and MAO by CE. Two different inhibitors were compared by utilizing this specific interaction. It was shown that proteoliposome CE is a useful method for studying molecule interaction and screening inhibitors with rapid analysis speed. It is a potentially analytical tool for protein–ligand interaction and screening enzyme inhibitor and active constituents from natural medicine, with further development.

Acknowledgments

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