Antianoxic Action and Active Constituents of Evodiae Fructus

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In order to develop new drugs from natural products, constituents of natural medicines were examined for their effectiveness in the KCN-induced anoxia model in mice. Methanol extract from a Chinese medicine, evodia (fruits of *Evodia rutaecarpa* BENTH. or *E. officinalis* DODE), had a significant effect in the KCN-induced anoxia model in mice and therefore the active constituents were further examined. The results indicated that the antianoxic action of evodia was found in the fraction containing evodiamine. Further analysis of the active constituent indicated that evodiamine and rutaecarpine, indole-alkaloids found in large amounts in the Chinese medicine evodia, were mainly responsible for the antianoxic action.

Keywords KCN-induced anoxia; antianoxic action; Evodiae Fructus; indole alkaloid; evodiamine; rutaecarpine

Introduction

In recent years, with the increase in the propotion of older people in the population, disease and illness due to cerebrovascular circulation disorders, such as stroke, are on the increase. For the improvement of disorders following cerebral injuries due to traffic accidents, cerebral metabolic activators and cerebrovasodilators have been receiving attention. Currently available cerebral metabolic activators and cerebrovasodilators, which are used for the treatment of post disorders of cerebral infarction and cerebral hemorrhage, as well as cerebroarteriosclerosis, are recognized as having antianoxic action which is effective against ischemia.[1-3]

During screening for the development of drugs from natural products, methanol (MeOH) extract of evodia (fruits of *Evodia rutaecarpa* BENTH. or *E. officinalis* DODE) was found to be effective in a KCN-induced anoxia model in mice. Further analysis of the extract indicated strong activity in the fraction containing indole alkaloids, evodiamine and rutaecarpine, and therefore these compounds were individually isolated and confirmed to be the active principles.

Materials and Methods

Fractionation and Purification Evodiae Fructus (2.5 kg), obtained from a local market in Osaka, was extracted with 6 l of MeOH (Wako Pure Chemical) for 1 d at room temperature. The same procedure was repeated twice thereafter. The resultant filtrate was concentrated under reduced pressure at below 40 °C to obtain 356 g of MeOH extract (14.3% recovery rate from the raw materials). Since the MeOH extract was found to be effective against the anoxia model, the extract was fractionated for isolation of the active constituents as shown in Fig. 1.

The evodia MeOH extract (130 g) was fractionated by silica gel column chromatography (Merck, Silica gel 60, 70—230 mesh, elution solvent, chloroform : acetone = 30 : 1) and each fraction was dried under reduced pressure.

Fractions 2, 4 and 5 were found to be effective in the KCN-induced anoxia model in mice. The thin layer chromatography (TLC) patterns of evodia MeOH extract and its fractions, using Dragendorff reagent as the color developing agent, suggested that fractions 2, 4 and 5 contain large amount of alkaloids. Therefore, a further fractionation was carried out aiming at obtaining the alkaloid constituents.

First, fraction 2 contained two types of Dragendorff reagent positive components with *Rf* values of 0.75 and 0.8. Fraction 2 (9.36 g) was therefore subjected to silica gel column chromatography (benzene : acetone = 20 : 1) and the products were purified by recrystallization from chloroform–MeOH to obtain 2 types of yellow crystals. Comparisons of mass spectrum (MS), proton nuclear magnetic resonance (^{1}H-NMR), infrared spectrum (IR) and melting point data of the crystals with those of the standards indicated that the compound with the *Rf* value of 0.75 was evodiamine[4] and the one with the *Rf* value of 0.8 was rutaecarpine.[5]

Fraction 4 (20 g) was similarly separated by silica gel column chromatography (benzene : acetone = 10 : 1) into fractions 4-1 and 4-2. Fraction 4-2 showed a single spot on TLC and reacted with Dragendorff reagent.

Fraction 5 was also separated by silica gel column chromatography (chloroform : MeOH = 3 : 1) into fractions 5-1, 5-2 and 5-3. Each of these fractions contained many alkaloid components, based on TLC, but a further analysis was not performed since each fraction showed only weak activity.

KCN-Induced Anoxia Model in Mice Male ddY mice (Kitayama Labes) weighing 18—20 g were injected with a fatal dose of KCN (3.0 mg/kg) through the tail vein, and the time taken to reach respiratory arrest was recorded as the survival duration together with the death rate. The survival duration was measured for 180 s following the KCN injection. Mice which did not show respiratory arrest during the 180 s were counted as survivors, and their survival duration was recorded as 180 s for the calculation of the mean survival duration. Test drugs were either suspended in 5% arabic gum and purified water and administered orally

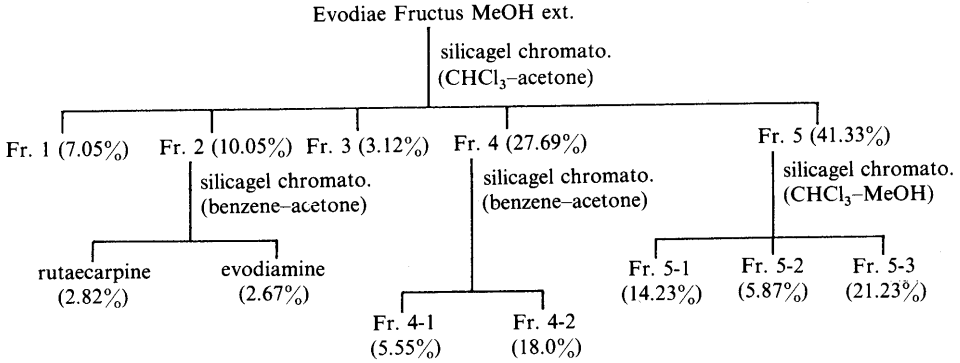

Fig. 1. Flow Diagram of Fractionation of Evodiae Fructus MeOH Extract

1 h before the KCN injection through the tail vein, or suspended in 5% arabic gum and physiological salt solution and injected intraperitoneally 30 min before the KCN injection, and the dosage was determined based on the rate of recovery for each of the fractions. Control groups received a mixture of arabic gum and physiological salt solution 30 min before the KCN injection. Hydergine (Sankyo), used here as a reference drug, was suspended in the same manner as described for the test drugs, and was injected intraperitoneally. The effects of the test drugs were evaluated using Student's *t*-test for the survival duration and Fisher's exact test method for the death rate.

Results

Effects of Extracts All the mice in the control group, which received the KCN injection through the tail vein, had respiratory arrest following about 1 min of repeated convulsive attacks, leading to death. The mean survival duration was 69.5 ± 7.3 s. As shown in Table I, all the mice survived in the presence of evodia MeOH extract at 300 mg/kg, i.p. and at 100 mg/kg, i.p. 8 out of 10 mice survived. At both concentrations, the extract significantly lowered the death rate and prolonged the mean survival duration as compared to the control. Evodia MeOH extract at 3000 mg/kg, *p.o.* was also significantly effective on the death rate and the mean survival duration. Hydergine at 25 mg/kg, i.p., used here as a reference drug, caused 7 out of 8 mice to survive.

Effects of Fraction 1 to 5 As shown in Table II, the mean survival duration of mice in the control group was 57.9 ± 3.9 s and all 9 mice died. In mice treated with fraction 2 at 90.5 mg/kg, i.p., it was 146.8 ± 12.9 s and 6 out of 10 mice survived, while for fraction 4 at 249 mg/kg, i.p., it was 161.8 ± 9.6 s and 7 out of 10 mice survived, and for fraction 5 at 372 mg/kg, i.p., it was 139.9 ± 16.4 s and 5 out of 9 mice survived. Each of these fractions was significantly effective in prolonging life as compared to the control.

Effects of Subfractions of Fraction 2 In mice treated with evodiamine and rutaecarpine, two alkaloids obtained from fraction 2 as described above, both at 50 mg/kg, i.p., there was a significant life-prolonging effect as compared to the control. The mean survival duration was 169.4 ± 10.6 s with the survival rate of 7 mice out of 9 for evodiamine and 142.1 ± 15.7 s with the survival rate of 5 out of 10 for rutaecarpine as compared to the control, whose mean survival duration was 69.4 ± 13.0 s with 1 out of 10 survival rate. The effect of evodiamine was greater than that of rutaecarpine (Table III).

Effects of Subfractions of Fraction 4 The mean survival duration in the control was 65.1 ± 8.1 s and all 9 mice died as shown in Table IV. Fraction 4-2 at 160 mg/kg, i.p.,

Table I. Effects of Evodiae Fructus MeOH Extract on KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg)	Survival time (s)	Prolongation (%)	No. of mice surviving/ No. used (Mortality %)
Control	—	69.5 ± 7.3	—	0/10 (100.0)
Evodiae fructus MeOH ext.	100 (i.p.)	163.4 ± 11.9[b]	135.0	8/10 (20.0)[b]
	300 (i.p.)	180.0 ± 0.0[b]	158.9	10/10 (0.0)[b]
	3000 (*p.o.*)	128.1 ± 17.5[a]	84.3	5/10 (50.0)[a]
Hydergine	25 (i.p.)	169.9 ± 10.1[b]	144.4	7/ 8 (12.5)[b]

Test drugs were administered i.p. 30 min before or orally 1 h before KCN treatment (3.0 mg/kg, i.v.). Each value represents the mean ± S.E. Significantly different from the control at *a*) $p<0.05$, *b*) $p<0.01$.

Table II. Effects of Fractions 1—5 on KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg)	Survival time (s)	Prolongation (%)	No. of mice surviving/ No. used (Mortality %)
Control	—	57.9 ± 3.9	—	0/9 (100.0)
Fraction 1	63.5	98.4 ± 15.0[a]	69.8	2/10 (80.0)[b]
Fraction 2	90.5	146.8 ± 12.9[b]	153.5	6/10 (40.0)[b]
Fraction 3	28.1	72.1 ± 6.2	24.4	0/9 (100.0)
Fraction 4	249.0	161.8 ± 9.6[b]	179.3	7/10 (30.0)[b]
Fraction 5	372.0	139.9 ± 16.4[b]	141.5	5/9 (44.4)[a]
Hydergine	12.5	161.2 ± 9.3[b]	178.3	5/10 (50.0)[b]

Test drugs were administered i.p. 30 min before KCN treatment (30 mg/kg, i.v.). Each value represents the mean ± S.E. Significantly different from the control at *a*) $p<0.05$, *b*) $p<0.01$.

Table III. Effects of Rutaecarpine and Evodiamine on KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg)	Survival time (s)	Prolongation (%)	No. of mice surviving/ No. used (Mortality %)
Control	—	69.4 ± 13.0	—	1/10 (90.0)
Rutaecarpine	50.0	142.1 ± 15.7[a]	104.9	5/10 (50.0)
Evodiamine	50.0	169.4 ± 10.6[a]	144.1	7/9 (22.2)[a]
Hydergine	12.5	174.4 ± 5.6[a]	151.4	8/10 (20.0)[a]

Test drugs were administered i.p. 30 min before KCN treatment (30 mg/kg, i.v.). Each value represents the mean ± S.E. Significantly different from the control at *a*) $p<0.01$.

Table IV. Effects of Fractions 4-1 and 4-2 on KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg)	Survival time (s)	Prolongation (%)	No. of mice surviving/ No. used (Mortality %)
Control	—	65.1 ± 8.1	—	0/9 (100.0)
Fraction 4-1	60	73.7 ± 8.8	1.8	0/9 (100.0)
Fraction 4-2	40	75.2 ± 6.6	15.4	0/10 (100.0)
	160	154.8 ± 13.9[a]	137.8	7/10 (30.0)[a]
Hydergine	10	142.0 ± 20.1[a]	118.1	7/10 (30.0)[a]

Test drugs were administered i.p. 30 min before KCN treatment (30 mg/kg, i.v.). Each value represents the mean ± S.E. Significantly different from the control at *a*) $p<0.01$.

Table V. Effects of Fractions 5-1, 5-2 and 5-3 on KCN-Induced Anoxia in Mice

Treatment	Dose (mg/kg)	Survival time (s)	Prolongation (%)	No. of mice surviving/ No. used (Mortality %)
Control	—	60.7 ± 5.2	—	0/10 (100.0)
Fraction 5-1	128.0	68.9 ± 6.6	13.5	0/10 (100.0)
Fraction 5-2	52.8	70.7 ± 6.0	16.4	0/10 (100.0)
Fraction 5-3	191.0	60.9 ± 3.1	0.3	0/9 (100.0)
Hydergine	12.5	130.5 ± 17.6[b]	114.9	5/10 (50.0)[a]

Test drugs were administered i.p. 30 min before KCN treatment (30 mg/kg, i.v.). Each value represents the mean ± S.E. Significantly different from the control at *a*) $p<0.05$, *b*) $p<0.01$.

significantly increased the mean survival duration to 154.8 ± 13.9 s with survival of 7 out of 10 mice as compared to the control. However, fraction 4-1 at 60 mg/kg, i.p., and fraction 4-2 at 40 mg/kg, i.p. did not have significant life-prolonging effects.

Effects of Subfractions of Fraction 5 As shown in Table V, fraction 5-1 at 128 mg/kg, i.p., fraction 5-2 at 52.8 mg/kg, i.p. and fraction 5-3 at 191 mg/kg, i.p. did not have significant life-prolonging effects.

Discussion

Evodia is the fruits of *Evodia rutaecarpa* BENTH. or *E. officinalis* DODE (Rutaceae). Evodia is one of the herbal drugs described in Shen Nung Pen Tsao Ching, a classic herb book written approximately 1700 years ago. It contains indole alkaloids (evodiamine, rutaecarpine and rhatsine), a quinolon alkaloid (evocarpine), bases (5-methoxy-*N*,*N*-dimethyltryptamine, *N*-methylanthranylamide, synerphrine), a bitter principle (limonin) and a fragrant principle (ocimene).[6,7] There are many Chinese herbal formulas containing evodia and among them, Wu-Chu-Yu-Tang has been used for migraine. Among the pharmacological effects of evodia, its ethanol extract has hypertensive, respiratory stimulation and analgesic effects,[8] its hot water extract has an inhibitory effect on the serotonin-induced contraction of rat isolated uterus,[9] and rutaecarpine and dehydroevodiamine have constricting action on the rat uterus. Recently, we have reported that evocarpine inhibits the K^+-induced contraction, $CaCl_2$-induced contraction and Ca^{2+} uptake in rat and rabbit thoracic aorta.[10]

Brain tissue has a very high oxygen requirement as compared to other tissues and is quite sensitive to low oxygen conditions caused by ischemia. Cyanidine compounds, such as KCN, are known to interfere with cytochrome oxidase in mitochondria, thereby inhibiting cellular respiration.[11] Hydergine,[12-14] which is used for treatment of cerebrovascular disorders and hypertension, and was used as the reference drug in the present study, is a mixture of mesyl salts of 3 types of ergot indole alkaloids in equal amounts. As the main pharmacological effects, it has an improving effect on cerebral metabolism, such as the inhibition of adenosine triphosphate (ATP) catabolism caused by excessive adrenaline stimulation and the increase in intracellular adenosine 3′,5′-cyclic monophosphate (cyclic-AMP), and its action in increasing cerebral circulation has been recognized. In the present study, evodia MeOH extract was shown to have a life-prolonging effect in a KCN-induced anoxia model, but when the route of drug administration was examined, i.p. administration showed a strong effect at less than 1/10 of the amount needed for the *p.o.* administration. Since *p.o.* administration requires a large amount of the test drugs, i.p. administration was mainly employed in the subsequent pharmacological study using fractions of the extract for the analysis of the active constituents.

The results of analysis of the effects of the extract fractions also indicated that evodiamine and rutaecarpine (in fraction 2) had a strong antianoxic action. Although other fractions also had antianoxic action, further fractionations caused them to be less effective. These results suggest that the antianoxic action of evodia MeOH extract is due to the combined effects of evodiamine and other constituents. Although the active ingredients of evodia are not yet fully identified, the results of this study indicated that evodiamine and rutaecarpine have antianoxic action in the KCN-induced anoxia model.

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5) mp: 264.5 °C, MS *m/z*: 287 (M^+), NMR ($CDCl_3$) δ: 3.22 (2H, t, J=6.8 Hz), 7.0—7.8 (7H, m), 8.32 (1H, d, J=7.6 Hz), 9.45 (1H, br s, exchangeable with D_2O), IR (KBr) cm^{-1}: 3345, 1650, 1600.
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