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Antiallergic Activities of Rabdosiin and its Related Compounds: Chemical and Biochemical Evaluations

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Abstract—We examined the effects of caffeic acid-containing compounds such as chlorogenic acid, rosmarinic acid and rabdosiin on anti-allergic activities involving active oxygens scavenging activity as well as inhibitory activities of hyaluronidase and β-hexosaminidase release. Rabdosiin exhibited the highest hyaluronidase-inhibitory activity and scavenging activities against active oxygens species such as superoxide anion radicals and hydroxyl radicals among the tested compounds. Both rabdosiin and caffeic acid inhibited β-hexosaminidase release from cultured cells more than 90% at 2 mM. The inhibition by rosmarinic acid and chlorogenic acid were weaker than that of rabdosiin. From these results, rabdosiin has been proposed to possess anti-allergic activity.

Introduction

Rabdosiin, a tetramer of caffeic acid with a lignan skeleton, was isolated from the stem of *Rabdosia japonica* Hara.[1,2] The pharmacological activities of rabdosiin have recently been described to show anti-human immunodeficiency virus (HIV) activity[3] and inhibition on DNA topoisomerase.[4]

Caffeic acid related compounds such as chlorogenic acid, rosmarinic acid, and rabdosiin has one, two and four caffeoyl groups, respectively, in their structures (Figure 1). Among these compounds, caffeic acid, chlorogenic acid and rosmarinic acid were reportedly to have antioxidative activities as well as antiallergic activities.[5–7] However, there is no report of the effects of rabdosiin on antioxidative and antiallergic activities. On the other hand, it has been proposed that active oxygen species induce histamine release from mast cells.[8–11]

In order to elucidate the relationship between the chemical structure and the biochemical activities of rabdosiin and its related compounds in respect to the anti-allergic activities in vitro, we examined the following points: (1) The scavenging activities of rabdosiin and its related compounds against active oxygen species such as superoxide anion radicals and hydroxyl radicals by ESR (electron spin resonance) spin trapping method. (2) Inhibitory effect of those compounds on hyaluronidase, which is known as one of the enzymes involved in allergic effects,[12] migration of cancer cells,[13] inflammation[14,15] and the increase in permeability of vascular system.[16,17] (3) Inhibitory effect of those compounds on release of β-hexosaminidase, as an index of chemical mediators,[18,19] from rat basophilic leukemia (RBL-2H3) cells.[20]

Results

Active oxygen scavenging activities

Scavenging activities of rabdosiin and its related compounds against active oxygen species such as superoxide anion radicals and hydroxyl, radicals were evaluated by the ESR spin-trapping method. The results in terms of IC_{50}-value are summarized in Table 1. Ascorbic acid was used as a positive control in each evaluation system. In superoxide anion scavenging, rabdosiin and its

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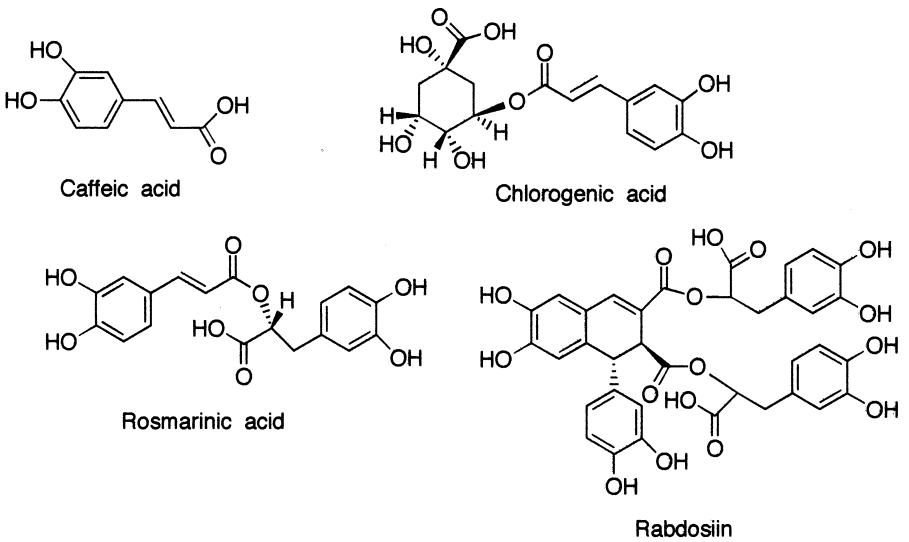

Figure 1. Structures of rabdosiin and its related compounds.

Table 1. Active oxygens scavenging activities of rabdosiin and its related compounds estimated by ESR-spin trapping method

Compd	IC_{50} value (μM)	
	$\cdot O_2^-$	·OH
Rabdosiin	0.53 ± 0.02	7.40 ± 0.59
Rosmarinic acid	$0.62 \pm 0.01^{**}$	$14.55 \pm 3.95^{*}$
Chlorogenic acid	$2.71 \pm 0.22^{**}$	$12.48 \pm 4.87^{*}$
Caffeic acid	$2.88 \pm 0.08^{**}$	$22.40 \pm 3.90^{**}$
Ascorbic acid	$11.66 \pm 2.99^{**}$	$12.70 \pm 4.33^{*}$

Each datum represents the mean ± standard deviation from four repeated experiments. Significance versus rabdosiin $^{**}p < 0.01$; $^{*}p < 0.05$.

related compounds were more active than ascorbic acid. Rabdosiin ($IC_{50} = 0.53 \pm 0.02\,\mu M$) was found to be the most active among the compounds examined. The scavenging activities seemed to increase in the number of caffeoyl groups involved in the compounds.

In hydroxyl radical scavenging, rabdosiin exhibited the highest activity among the compounds as evaluated by IC_{50} value ($IC_{50} = 7.40 \pm 0.59\,\mu M$). No significant difference in the abilities due to rosmarinic acid, chlorogenic acid and ascorbic acid was found. Caffeic acid showed the weakest activity among the test compounds.

Effects of rabdosiin and its related compounds on the activation of hyaluronidase

Inhibitory effects of rabdosiin and its related compounds on the activation of hyaluronidase were examined and the results are shown in Figure 2. Rabdosiin and its related compounds inhibited the activation of hyaluronidase in dose- and caffeoyl number-dependent manners. In addition, the inhibitory effect of rabdosiin was stronger than that of DSCG, which is a clinically-used anti-allergic drug.[21] Rosmarinic acid inhibited hyaluronidase as strong as DSCG. While, chlorogenic acid and caffeic acid, which have one caffeoyl group, showed low inhibitory effects.

Effects of rabdosiin and its related compounds on β-hexosaminidase release from RBL-2H3 cells

Inhibitory effects of rabdosiin and its related compounds on β-hexosaminidase release from RBL-2H3 cells were examined and the results are summarized as in Figure 3. β-Hexosaminidase release induced by antigen (DNP-BSA) was $30.5 \pm 5.2\%$ for the cell content of 2×10^5 cells/well. The spontaneous β-hexosaminidase release was $3.5 \pm 1.2\%$ for the cell content. Rabdosiin and its related compounds inhibited the enzyme release in a dose-dependent manner. Among the test compounds, rabdosiin exhibited the highest inhibitory effect of β-hexosaminidase release, being 90% inhibition. at 2 mM of the compound. Rosmarinic acid and chlorogenic acid also significantly inhibited, the β-hexosaminidase release, but their maximum inhibition effects were less than that by rabdosiin. In contrast, the effect of caffeic acid was comparable to that of rabdosiin. In addition, these compounds did not inhibit β-hexosaminidase activity itself. In fact, when RBL-2H3 cells were degranulated with the stimulator alone and the extracellular fluid was added to these compounds, the enzyme activity was not altered (Figure 4).

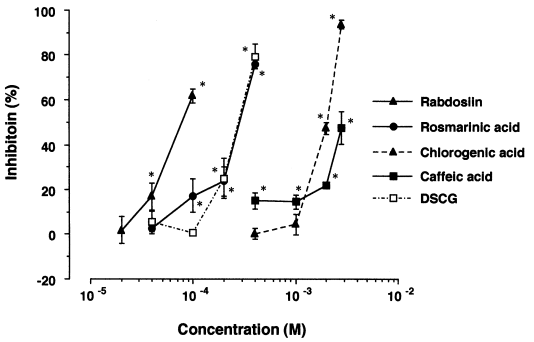

Figure 2. Effect of rabdosiin and its related compounds on hyaluronidase activity: Each point represents the mean ± standard deviation from four experiments. *: significantly different from the control (without the test compounds) at $p < 0.01$.

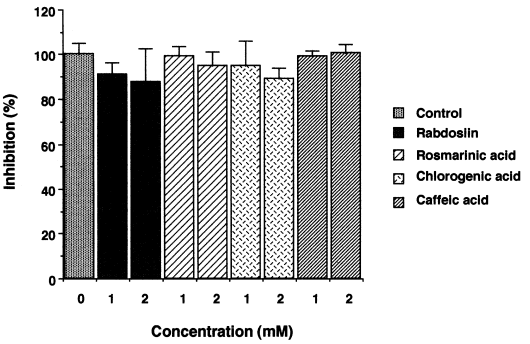

Figure 4. Effect of rabdosiin and its related compounds on β-hexosaminidase activity.

Discussion

According to the recent papers, antioxidants are useful for treating allergic disease,[22,23] since active oxygen species such as superoxide anion radicals and hydroxyl radicals induce histamine release from mast cells.[9,11] Therefore, the prevention of allergic actions could be achieved by both scavenging of active oxygen species and suppression of chemical mediators from mast cells.

The purpose of this study was to assess the relationship between the chemical structures and the biochemical activities of caffeic acid-containing compounds in respect to the anti-allergic activities in vitro. Our results demonstrate that rabdosiin has potent superoxide anion radical and hydroxyl radical scavenging activities (Table 1). The superoxide anion radical scavenging activity increased with the number of caffeoyl groups in the compounds, but the hydroxyl anion radical scavenging activity did not, indicating the important role of catechol moiety in scavenging the active oxygens species.

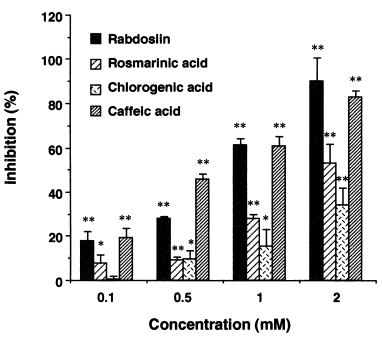

Figure 3. Effect of rabdosiin and its related compounds on β-hexosaminidase release from RBL-2H3 cells. Each column represents the mean ± standard deviation from four experiments. *: significantly different from the control (without the test compounds) at $p < 0.05$. **: significantly different from the control (without the test compounds) at $p < 0.01$.

Hyaluronidase, which is one of the mucopolysaccharide-splitting enzymes, has been known to relate to allergic reaction, migration of cancer cells,[13] inflammation[14,15] and the increase in permeability of the vascular system.[16,17] Kakegawa et al. have reported a good correlation between the inhibitory effect of hyaluronidase and that of histamine release from mast cells in acidic anti-allergic agents.[24] RBL-2H3 cells release histamine, serotonin and β-hexosaminidase when exposed to various stimuli in vitro.[25,26] RBL-2H3 cells have the same function as mast cells and basophils, which concern allergic type I reaction.[25,27] Therefore, in the present study, we examined the effect of rabdosiin and its related compounds on the inhibition of hyaluronidase activity as well as β-hexosaminidase release, since a good correlation has been found between the amounts of histamine and β-hexosaminidase released from human and rat mast cells.[18,19]

We found that rabdosiin and its related compounds have both the highest hyaluronidase-inhibitory activity and β-hexosaminidase release-inhibitory activity (Figures 2 and 3). Particularly, rabdosiin was demonstrated to have the highest hyaluronidase inhibitory activity among the test compounds (Figure 2). Similarly to the observation on the superoxide anion scavenging activity of the compounds, the hyaluronidase-inhibitory activity increased with the number of caffeoyl groups of the compounds. As shown in Figure 5, the superoxide anion radical scavenging activity of the compound correlates linearly to the hyaluronidase-inhibitory activity.

DSCG is a major and active component of anti-allergic drug, Intar®. While, DSCG is an effective inhibitor of hyaluronidase activity[28] and is known to inhibit the release of chemical mediators from mast cells induced

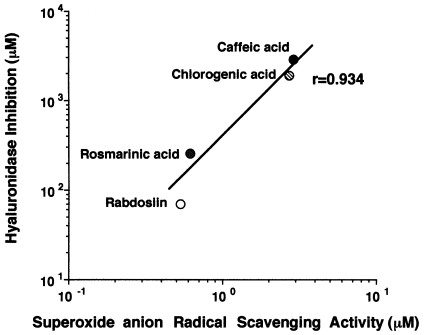

Figure 5. Correlation of the effect of rabdosiin and its related compounds between superoxide anion radical scavenging activity and inhibition of hyaluronidase activity.

by the antigen–IgE antibody reaction.[21] Although RBL-2H3 cells closely resemble to the mucosal mast cells, secretion of chemical mediators is not inhibited by treatment with 10 mM of DSCG.[29] Rat intestinal mucosal mast cells were also found to be non-responsive to the drug.[30] In support to the observations, we have also confirmed that DSCG dose not inhibit the release of β-hexosaminidase during the present investigation (data not shown). However, as shown in Figure 3, both rabdosiin and caffeic acid inhibited β-hexosaminidase release more than 80% at 2 mM. These results suggest that the action mechanism of rabdosiin and caffeic acid for β-hexosaminidase might be different from that of DSCG. More detailed experiments will be needed.

From these observations, we propose that rabdosiin is the most potent and active compound for exhibiting anti-allergy relating activities involving active oxygen scavenging activities, hyaluronidase-inhibitory activity and β-hexosaminidase release-inhibitory activity. On the basis of the results, we speculate that the number of caffeoyl groups is important for giving the activities, but the mechanism of the inhibition of caffeic acid on β-hexosaminidase release is yet unknown. Many of anti-allergic drugs have amphiphilic properties suggesting that their effects on membrane structures are responsible for the inhibition of mediator release.[31–33] Among the test compounds, the inhibition of β-hexosaminidase release by caffeic acid may relate to its lipophilic property. Experiments on the inhibitory effect by caffeic acid and its related compounds are now under way.

In conclusion, rabdosiin has been found to be a potent scavenger of active oxygen species, such as superoxide anion radicals and hydroxyl radicals, as well as possible inhibitors of both hyaluronidase and β-hexosaminidase release. We proposed here that rabdosiin is a candidate for the therapeutic agent for allergy.

Experimental

Chemicals and reagents

Rabdosiin was isolated from *Rabdosia japonica* Hara according to the method of Agata et al.[12] Rosmarinic acid was purchased from Funakosi Co., Ltd (Tokyo, Japan). Chlorogenic acid, caffeic acid and hypoxanthine (HPX) were obtained from Wako Pure Chemical Co., Ltd (Osaka, Japan). Diethylenetriamine-*N*,*N*,-*N*′,*N*″,*N*″pentaacetic acid (DTPA) was from Dojindo laboratories (Kumamoto, Japan). 5, 5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec Co., Ltd (Tokyo, Japan). Hyaluronic acid sodium salt was obtained from Seikagaku Kogyo Co., Ltd (Tokyo, Japan). 2,4-Dinitrophenol (DNP)-conjugated bovine serum albumin (BSA) was obtained from Calbiochem® (San Diego, CA, USA). Xanthine oxidase (XOD), hyaluronidase (from bovine testis, type IV-S), *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, anti-DNP IgE mouse monoclonal antibody and disodium cromoglycate (DSCG) were obtained from Sigma Chemical Co. (Saint Louis, MO, USA).

Superoxide anion scavenging activity estimated by ESR spin trapping

Superoxide anion scavenging activity of the compounds was estimated by ESR spin-trapping according to the method of Kitagawa et al.[34] Briefly, HPX (0.4 mM). DTPA (0.7 mM) and various concentrations of the test compounds were dissolved in 100 mM sodium phosphate buffer (pH 7.4). Then XOD (0.12 units/ml) was added to the above solution. Almost simultaneously, DMPO was added to the solution at the final concentration of 90 mM. After mixing for 1 min, ESR spectra were recorded using a JOEL RE1XG (X-band) spectrometer at 100 kHz field modulation frequency and 1 G modulation amplitude at an output power of 5 mW. Mn(II) doped in MgO was used as standard. All experiments were carried out at room temperature (21 °C). To evaluate the ability of the test compounds, ascorbic acid, which is a typical and water-soluble antioxidant, was used as a positive control. The scavenging activity of a compound is expressed as the IC_{50} value, which is the 50% inhibition concentration of a compound for superoxide anion radicals generated in the HPX-XOD system at pH 7.4.

Hydroxyl radical scavenging activity estimated by ESR spin-trapping

Hydroxyl radical scavenging activity of the test compounds was estimated by ESR spin-trapping using the Fenton system ($Fe(II) + H_2O_2$) according to the method of Masaki et al.[35] In brief, $FeSO_4$, (22.5 μM), various

concentrations of the test compounds and DMPO (90 mM) were mixed. The reaction was started by addition of H_2O_2 (22.5 μM). After mixing for 40 s, ESR spectra were recorded under the same conditions as described above. As a positive control, ascorbic acid, a typical water soluble antioxidant, was used. The scavenging activity of a compound is expressed as the IC_{50} value, which is the 50% inhibition concentration of a compound for hydroxyl radicals generated in the Fenton reaction.

Assay of hyaluronidase activity

Hyaluronidase activity was determined by the Morgan-Elson method[36] as modified by Davidson, et al.,[37] in which incubation of hyaluronidase (200 units/mL) with hyaluronic acid sodium salt (0.4 mg/mL) was performed at 37 °C for 40 min in 0.1 M acetate buffer (pH 4.0). Calcium chloride (2.5 mM) was used as an activator of hyaluronidase.

Inhibitory effect of the compounds on activation of hyaluronidase

Inhibitory effect of the test compounds on activation of hyaluronidase was determined by the method as described above.[36,37] Hyaluronidase was preincubated with the test compounds at 37 °C for 20 min in 0.1 M acetate buffer (pH 4.0). After preincubation for 20 min, calcium chloride (2.5 mM) was added to the above solution and incubated at 37 °C, for 20 min. Then the reaction was started by adding hyaluronic acid sodium salt (0.4 mg/mL) to each reaction mixture by incubating at 37 °C for 40 min. As a control, test compounds were replaced by the buffer solution. Absorbance was measured at 585 nm. The percent inhibition was calculated as follows:

$$\text{Inhibition (\%)} = [(A-B)-(C-D)]/(A-B) \times 100$$

A: Absorbance of control, B: Absorbance of blank (without hyaluronidase) for the control, C: Absorbance involving test compounds, D: Absorbance of test compounds alone.

Cell culture

RBL-2H3 cells (JCRB 0023), from Health Science Research Resource Bank (HSRRB) (Osaka, Japan), were maintained in MEM (minimum essential medium, Nissui. Tokyo, Japan) with 15% heat-inactivated fetal bovine serum (Gibco-BRL, NY, USA) in a humidified atmosphere of 5% CO_2. Cells were removed from the plate by dissociating with a cell scraper, and subcultured three times each week.

Cell stimulation and assay for β-hexosaminidase release

Cells were plated at 2×10^5 cells/400μL/well in 24-well culture plates and were cultured overnight with DNP-specific IgE (0.5 μg/mL). The supernatants were discarded, and the cells were washed three times with Pipes-buffered saline (25 mM Pipes, pH 7.2, 125 mM NaCl, 2.7 mM KC1, 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA). Then the cells were preincubated at 37 °C for 15 min in the presence of various concentration of test compounds. Next they were stimulated by addition of 10 ng/mL DNP-BSA for 20 min. Aliquots (10 μL) of the medium and cell lysate, which was obtained by addition of 200 μL of 0.1% Triton X-100, were incubated with 10 μL of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) at 37 °C for 1 h. At the end of the incubation, 250 μL of carbonate buffer containing 0.1 M, Na_2CO_3 and 0.1 M $NaHCO_3$ (pH 10) was added and then absorbance due to the formation of *p*-nitrophenol was measured at 405 nm. The net percent release of β-hexosaminidase was calculated using the following equation:

$$\text{Net \% release} = [(A-C)/(B-C)] \times 100$$

A: Amount of β-hexosaminidase in the extracellular fluid, B. Total content of β-hexosaminidase in the cells, C. Amount of β-hexosaminidase in the extracellular fluid from the non-stimulated cells.

$$\text{Inhibition (\%)} = [1-(\text{Net \% release involving test compounds}/\text{Net \% release of the stimulated cells}]\times 100$$

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