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Inhibition of hind-paw edema and cutaneous vascular plasma extravasation in mice by acetylshikonin

Jih-Pyang Wang [a,*], Shue-Ling Raung [a], Ling-Chu Chang [a], Sheng-Chu Kuo [b]

[a] *Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan*
[b] *Graduate Institute of Pharmaceutical Chemistry, China Medical College, Taichung, Taiwan*

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Abstract

Acetylshikonin, a naphthoquinone isolated from the Chinese herb medicine, tzu ts'ao, was demonstrated to inhibit the polymyxin B-induced hind-paw edema in normal as well as in adrenalectomized mice. Liver glycogen content was increased in adrenalectomized mice pretreated with dexamethasone, but not with acetylshikonin. Like diphenhydramine, methysergide and isoproterenol, acetylshikonin reduced the plasma exudation evoked in dorsal hind-paw skin by antidromic stimulation of the saphenous nerve, and in passive cutaneous anaphylactic reaction, bradykinin-, substance P-, compound 48/80-, histamine- and serotonin-induced ear edema. Indomethacin was ineffective in these respects. Bradykinin- and substance P-induced plasma exudation were also significantly reduced when [Thi^{5,8},D-Phe^{7}]bradykinin and [D-Pro^{2},D-Trp^{7,9}]substance P were coinjected with bradykinin and substance P, respectively. In isolated rat peritoneal mast cell preparation, acetylshikonin produced a concentration-dependent inhibition of histamine and β-glucuronidase release from mast cells challenged by compound 48/80. In compound 48/80-pretreated mice, acetylshikonin and isoproterenol produced significantly more inhibitory effect on bradykinin- and substance P-induced plasma exudation than did diphenhydramine in combination with methysergide. Pretreatment with diphenhydramine/methysergide in compound 48/80-pretreated mice significantly further reduced the bradykinin- and substance P-induced plasma exudation if [Thi^{5,8},D-Phe^{7}]bradykinin and [D-Pro^{2},D-Trp^{7,9}]substance P were coinjected with bradykinin or substance P, respectively. The results suggest that the inhibitory effect of acetylshikonin on the edematous response is due neither to the release of steroid hormones from the adrenal gland nor to the glucocorticoid activity, but probably partly to the suppression of mast cell degranulation and partly to protection of the vasculature from mediator challenge.

Keywords: Acetylshikonin; Hind-paw edema; Cutaneous anaphylaxis, passive; Neurogenic inflammation; Vascular permeability; Compound 48/80; [Thi^{5,8},D-Phe^{7}]Bradykinin; [D-Pro^{2},D-Trp^{7,9}]Substance P

1. Introduction

Tzu ts'ao, the dried purple roots of *Lithospermum erythrorhizon* Sieb. et Zucc., *Arnebia euchroma* (Royle) Johnst, or *Macrotomia euchroma* (Royle) Pauls (*Boraginaceae*), has been used for the relief of wounds, burns, bleeding, dermatitis and constipation since ancient times in the Far East. Chemical investigations of the roots of the former species have led to the isolation of shikonin, acetylshikonin and several other naphthoquinones (Kuroda, 1918; Majima and Kuroda, 1922). Shikonin and acetylshikonin have been found to possess antibacterial, antitumor, antipyretic, analgesic and anti-inflammatory activity (Motohide, 1977; Ushio et al., 1977; Tanaka et al., 1986).

This study investigated the anti-inflammatory effect of acetylshikonin on paw edema, and on plasma extravasation in the passive cutaneous anaphylactic reaction and in neurogenic electrical stimulation. We have also determined the anti-inflammatory activity and the liver glycogen content in acetylshikonin-treated adrenalectomized mice in order to assess the role of glucocorticoid activity. The effect of acetylshikonin on mast cell release reaction in vitro, and on several chemical mediator-induced plasma exudation in normal and in compound 48/80-pretreated mice in vivo, were also tested as part of the investigations into the mechanism of action of acetylshikonin.

* Corresponding author.

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2. Materials and methods

2.1. Materials

Acetylshikonin was isolated and purified from *Lithospermum erythrorhizon* as previously described (Majima and Kuroda, 1922). Sodium pentobarbital, polymyxin B, diphenhydramine, indomethacin, compound 48/80, monoclonal anti-dinitrophenyl antibody (clone no. SPE-7), dinitrophenyl-bovine serum albumin, Evans blue, bovine serum albumin, isoproterenol, histamine, serotonin, bradykinin, [Thi^{5,8},D-Phe^{7}]-bradykinin, substance P and [D-Pro^{2},D-Trp^{7,9}]substance P were purchased from Sigma Chemical Co., St. Louis, USA. Dimethylsulphoxide was obtained from Merck Taiwan, Taiwan. Methysergide was supplied by Sandoz Pharmaceutical, Basle, Switzerland. Acetylshikonin and indomethacin were dissolved in dimethylsulphoxide, other substances were dissolved in normal saline.

2.2. Hind-paw edema

Mice (ICR, 20–25 g) were used in this and other studies. Hind-paw edema was induced as previously described (Wang et al., 1992). Briefly, a single subplantar injection of 5 μl of 0.2% polymyxin B or sterile saline was given in the right and left hind-paw, respectively, of normal or adrenalectomized mice. The volumes of both hind-paws of each mouse were measured with a plethysmometer at the beginning and at various time intervals after induction of edema. Hind-paw swelling was calculated as follows: paw swelling (%) = {[(right paw volume − initial volume)/(right paw initial volume)] − [(left paw volume − initial volume)/(left paw initial volume)]} × 100. The data were analyzed to compare the area under the time-paw swelling curve (AUC) based on the Trapezoidal rule (Tallarida and Murray, 1987).

2.3. Adrenalectomized mouse

Adrenalectomized animals were operated as described (Waynforth, 1980), except that mice were used. Briefly, pentobarbital-anesthetized mice were carefully adrenalectomized bilaterally from the shaved dorsal region. Adrenalectomized mice were given normal physiological saline to drink ad libitum. On the 4th postoperative day, the animals were used for experiments.

2.4. Glucocorticoid activity

On the 4th day after the operation, the adrenalectomized mice were deprived of food and saline for 18 h before intraperitoneal administration of the test drugs, and then 8 h after drug treatment the mice were killed (Schiatti et al., 1986). Liver glycogen was isolated and determined (Good et al., 1933; Fong et al., 1953).

2.5. Ear edema

In the 48-h passive cutaneous anaphylactic reaction, monoclonal antidinitrophenyl antibody 0.1 μg or sterile saline was injected into the right and left ears, respectively, of pentobarbital-anesthetized mice (Wang et al., 1994a). After 48 h, 0.5% Evans blue with 1.5% sodium pentobarbital in saline (4 ml/kg) was intravenously injected followed 5 min later by dinitrophenyl-bovine serum albumin (10 mg/kg i.v.) challenge. In the non-immunological phlogist-induced edema, a single injection of a phlogist (3 μg histamine, 1 μg compound 48/80, 0.3 μg bradykinin, 0.1 μg serotonin or 0.1 μg substance P) or sterile saline into the right and left ears, respectively, at 5 min after the intravenous injection of 0.5% Evans blue and 1.5% sodium pentobarbital in saline (4 ml/kg) (Wang et al., 1994a). The animals were killed 45 min after the induction of edema.

A sample of tissue (9 mm diameter) was punched out from both the right and left ears. Exuded blue dye in the tissue sample was extracted as described (Katayama et al., 1978). Briefly, the tissue sample was soaked in 1 N NaOH at 37°C overnight and the dye was extracted with a mixture of 0.6 N H_3PO_4 and acetone. After centrifugation, blue dye in the supernatant was measured by spectrophotometry at 620 nm. In order to evaluate the volume of exudative plasma, an absorbance-plasma volume standard curve was prepared by measuring the absorbance of different volumes of plasma isolated from mice pretreated with Evans blue. Therefore, the volume of plasma leakage of each tissue sample was calculated by interpolation on this standard curve. The intensity of the inflammatory response was monitored by measuring the difference in the volume of exudative plasma between the two tissue samples from each animal.

2.6. Neurogenic plasma exudation

The saphenous nerve on one side of a pentobarbital-anesthetized mouse was carefully exposed, cut at the thigh, and the distal end was placed on bipolar platinum electrodes (Wang et al., 1993). The nerve was covered with paraffin oil. Evans blue (25 mg/kg i.v.) in saline was given and 5 min afterwards the nerve was stimulated for 10 min (1 V/10 Hz per 20 ms). The animals were killed 30 min after the stimulation, and dorsal skin of the hind-paw was then removed. Exuded blue dye in the skin was extracted as previously described.

2.7. Mast cell release reaction

Rat peritoneal mast cells were prepared as previously described (Wang et al., 1989). Briefly, heparinized Tyrode solution was injected into the peritoneal cavity of exsanguinated rats (Sprague-Dawley, 250–300 g). After abdominal massage, the cells in the peritoneal fluid were harvested and separated in 38% bovine serum albumin in glucose-free Tyrode solution. The cell pellet was washed and suspended in Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 12, NaH_2PO_4 0.3, $MgCl_2$ 1.0, $CaCl_2$ 1.0, dextrose 5.6 and bovine serum albumin 0.1%, to a final concentration of $1–1.5 \times 10^6$ cells/ml.

The cell suspension was preincubated at 37°C with dimethylsulphoxide or acetylshikonin for 3 min, and then the release reaction was triggered by the addition of compound 48/80 (10 μg/ml). The reaction was terminated 15 min later by the addition of ice-cold Tyrode solution and the mix was then centrifuged for 10 min at $1000 \times g$. The contents of histamine and β-glucuronidase in the supernatant were determined by fluorescence spectrophotometry at 350/450 nm (Håkanson and Rönnberg, 1974) and by spectrophotometry at 550 nm (Barrett, 1972), respectively, and expressed as % release of the total content. The total content of histamine and β-glucuronidase was measured after treatment of the cell suspension with Triton X-100.

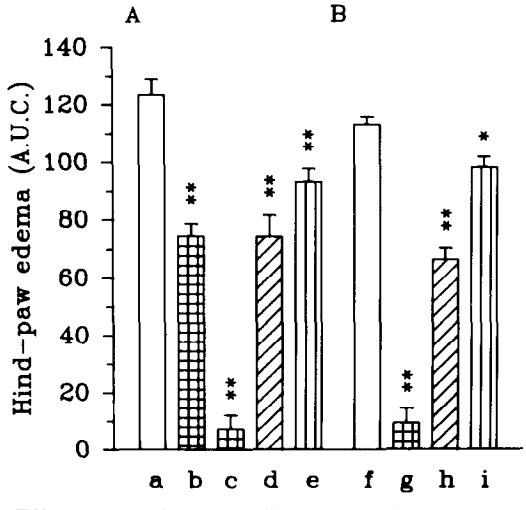

Fig. 1. Effects of acetylshikonin, diphenhydramine and indomethacin on polymyxin B-induced hind-paw edema in mice. (a,f) Control, no pretreatment with anti-inflammatory drugs; acetylshikonin (b) 3 and (c,g) 10 mg/kg; (d,h) diphenhydramine 10 mg/kg; (e,i) indomethacin 3 mg/kg, were given intraperitoneally 30 min before subplantar injection of 10 μg polymyxin B into the right hind-paw of (A) normal or (B) adrenalectomized mice. Responses are presented as the area under the curve (AUC) measured in the 6-h period after induction of paw edema. Values are expressed as the means ± S.E.M. for seven to ten animals. Statistically significant differences from the corresponding control values (a,f) are noted as $^*P < 0.05$, $^{**}P < 0.01$.

2.8. Depletion of histamine and serotonin

Mice were injected with compound 48/80 or sterile saline into the right and left ears, respectively, twice a day for six doses (Wang et al., 1993). The dose of compound 48/80 was 1 μg for the first three injections, and 3 μg for the last three injections. After these treatments, the histamine content of the ear, a measurement based on the method described by Shore et al. (1959), was reduced to about 18% of the control value.

2.9. Statistical analysis

The data are presented as the means ± S.E.M. The statistical significance of changes was analyzed with a one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. P values < 0.05 were considered to be significant. Analysis of the regression line was used to calculated the IC_{50} values.

3. Results

3.1. Hind-paw edema in normal and adrenalectomized mice

A single subplantar injection of 10 μg polymyxin B induced a 28.0 ± 1.0% and 28.2 ± 0.8% hind-paw volume increase in normal and adrenalectomized mice, respectively, at 6 h after induction of the edematous response. These responses were significantly reduced in normal as well as in adrenalectomized mice by pretreatment with diphenhydramine (10 mg/kg i.p.) or indomethacin (3 mg/kg i.p.) (Fig. 1). Intraperitoneal administration of acetylshikonin (3 and 10 mg/kg i.p.) 30 min prior to the injection of polymyxin B into the paw resulted in a dose-dependent inhibition of paw swelling in normal mice (39.6 ± 3.3% and 94.1 ± 3.9% inhibition, respectively, in AUC, $P < 0.01$). Moreover, the inhibitory effect of acetylshikonin 10 mg/kg was also demonstrated in adrenalectomized mice (91.7 ± 4.6% inhibition in AUC, $P < 0.01$) (Fig. 1B). Dexamethasone (0.5 mg/kg i.p.) greatly increased the liver glycogen content (21.80 ± 5.75 vs. 0.66 ± 0.15 mg/g liver in control, $P < 0.01$) in adrenalectomized mice, whereas acetylshikonin (3 mg/kg i.p.) was ineffective in this respect (1.07 ± 0.31 mg/g liver).

3.2. Plasma exudation in immunological and non-peptide phlogist-induced ear edema

In the passive cutaneous anaphylactic reaction, plasma exudation was significantly reduced in mice pretreated with diphenhydramine (10 mg/kg i.p.), methysergide (3 mg/kg i.p.) or isoproterenol (3 mg/kg

i.p.), but not with indomethacin (3 mg/kg i.p.) (Table 1). In addition, mice pretreated with these compounds produced an inhibitory profile for plasma exudation in compound 48/80-induced ear edema similar to that they had in the passive cutaneous anaphylactic reaction. A dose-dependent inhibition of plasma exudation was also observed with acetylshikonin (3 and 10 mg/kg) treatment in the passive cutaneous anaphylactic reaction (47.9 ± 8.8% and 74.6 ± 2.6% inhibition for 3 and 10 mg/kg i.p., respectively, $P < 0.01$) and in compound 48/80-induced ear edema (45.1 ± 6.6% and 62.4 ± 10.9% inhibition for 3 and 10 mg/kg i.p., respectively, $P < 0.01$).

Fig. 2 shows that histamine- and serotonin-induced ear edema were significantly inhibited ($P < 0.01$) by diphenhydramine (10 mg/kg i.p.) and methysergide (3 mg/kg i.p.), respectively. Both histamine- and serotonin-induced edematous responses were also greatly reduced ($P < 0.01$) by isoproterenol (1 mg/kg i.p.) but not by indomethacin (3 mg/kg i.p.). Mice pretreated with acetylshikonin (3 and 10 mg/kg i.p.) had a dose-dependent inhibition of edema formation caused by injection of histamine (56.0 ± 5.9% and 66.1 ± 7.7% inhibition for 3 and 10 mg/kg i.p., respectively, $P < 0.01$) or serotonin (56.3 ± 6.5% and 85.7 ± 5.7% inhibition for 3 and 10 mg/kg i.p., respectively, $P < 0.01$) into the ears.

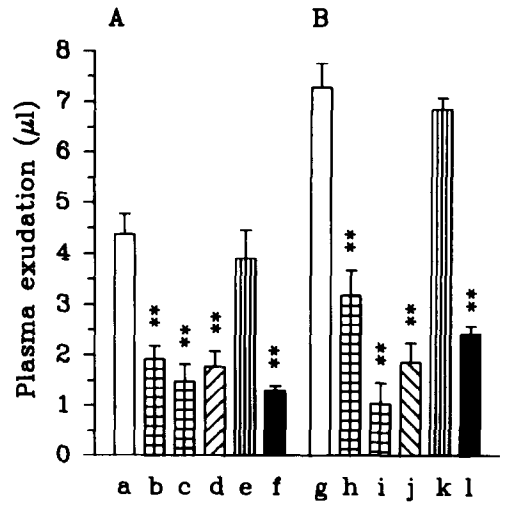

Fig. 2. Effects of acetylshikonin, diphenhydramine, methysergide, indomethacin and isoproterenol on histamine- and serotonin-induced mouse ear edema. (a,g) Control, no pretreatment with anti-inflammatory drugs; acetylshikonin (b,h) 3 and (c,i) 10 mg/kg; (d) diphenhydramine 10 mg/kg; (e,k) indomethacin 3 mg/kg; (f,l) isoproterenol 1 mg/kg; (j) methysergide 3 mg/kg, were intraperitoneally administered 30 min before (A) histamine 3 μg or (B) serotonin 0.1 μg injection into the ears. The animals were killed 45 min after induction of ear edema. Values for plasma exudation are expressed as the means ± S.E.M. for six to eight animals. Statistically significant difference from the corresponding control values (a,g) is noted as $^{**}P < 0.01$.

3.3. Neurogenic plasma exudation

Plasma exudation from the vasculature of dorsal hindpaw skin was evoked by electrical stimulation of the saphenous nerve. Neurogenic plasma exudation was significantly reduced ($P < 0.01$) in mice pretreated with diphenhydramine (10 mg/kg i.p.), methysergide (3 mg/kg i.p.) or isoproterenol (1 mg/kg i.p.), but not indomethacin (3 mg/kg i.p.) (Table 2). Acetylshikonin greatly suppressed the plasma exudation in a dose-dependent manner (70.1 ± 4.5% and 80.4 ± 2.5% for 1 and 3 mg/kg i.p., respectively, $P < 0.01$).

Table 1
Effects of acetylshikonin, diphenhydramine, methysergide, indomethacin and isoproterenol on plasma extravasation in 48-h passive cutaneous anaphylactic reaction (PCA) and in compound 48/80-induced ear edema

Drugs [a]	(mg/kg)	Plasma exudation [b] (μl)	
		PCA	Compound 48/80
Control		9.2 ± 0.6	5.5 ± 0.3
Acetylshikonin	3	4.8 ± 0.8 [d]	3.0 ± 0.3 [d]
	10	2.3 ± 0.2 [d]	2.0 + 0.6 [d]
Diphenhydramine	10	6.2 ± 0.3 [c]	3.7 ± 0.5 [c]
Methysergide	3	2.5 ± 0.3 [d]	2.0 ± 0.2 [d]
Indomethacin	3	8.5 ± 0.8	4.2 ± 0.5
Isoproterenol	3	2.6 ± 0.8 [d]	3.5 ± 0.3 [c]

[a] In the passive cutaneous anaphylactic reaction, anti-dinitrophenyl antibody (0.1 μg) was injected into the ear 48 h prior to dinitrophenyl-bovine serum albumin challenge. Drugs were intraperitoneally administered 30 min before intravenous injection of dinitrophenyl-bovine serum albumin (10 mg/kg) in the passive cutaneous anaphylactic reaction, or 30 min before injection of compound 48/80 (1 μg) into the ear in the compound 48/80-induced ear edema.
[b] Values are expressed as the means ± S.E.M. for six to nine animals. Statistically significant differences from the corresponding control values are noted as [c] $P < 0.05$, [d] $P < 0.01$.

3.4. Plasma exudation in peptide phlogist-induced ear edema

Intradermal injection of substance P and bradykinin into the ears evoked a local plasma exudation at the site of injection. Pretreatment with diphenhydramine (10 mg/kg i.p.), methysergide (3 mg/kg i.p.) or isoproterenol (3 mg/kg i.p.), but not indomethacin (3 mg/kg i.p.), reduced ($P < 0.01$) both the substance P- and bradykinin-induced edematous response (Table 2 and Fig. 3). When [Thi^{5,8},D-Phe^{7}]bradykinin (1 μg) and [D-Pro^{2},D-Trp^{7,9}]substance P (1 μg) were coinjected with bradykinin and substance P, respectively, into the ears, the volumes of exudative plasma were significantly reduced ($P < 0.01$) to 41.6 ± 3.5% and 64.2 ± 4.5% of the control values for bradykinin and substance P, respectively. Acetylshikonin markedly attenuated ($P < 0.01$) the plasma exudation caused by substance P (32.2 ± 6.4% and 71.1 ± 0.9% inhibition for 3

Table 2
Effects of acetylshikonin, [D-Pro^2,D-Trp^{7,9}]substance P, diphenhydramine, methysergide, indomethacin and isoproterenol on plasma extravasation in neurogenic inflammation and in substance P-induced ear edema

Drugs [a]	(mg/kg)	Plasma exudation (μl) [b]	
		Neurogenic	Substance P
Control		5.8 ± 0.2	6.5 ± 0.3
Acetylshikonin	1	1.7 ± 0.2 [c]	n.d.
	3	1.1 ± 0.1 [c]	4.4 ± 0.4 [c]
	10	n.d.	1.8 ± 0.1 [c]
Diphenhydramine	10	3.5 ± 0.2 [c]	4.9 ± 0.4 [c]
Methysergide	3	2.4 ± 0.2 [c]	4.6 ± 0.3 [c]
Indomethacin	3	5.7 ± 0.3	6.8 ± 0.2
[D-Pro^2,D-Trp^{7,9}]substance P		n.d.	4.2 ± 0.3 [c]
Isoproterenol	1	0.8 ± 0.1 [c]	n.d.
	3	n.d.	0.6 ± 0.1 [c]

[a] Drugs, except [D-Pro^2,D-Trp^{7,9}]substance P, were intraperitoneally administered 30 min before electrical antidromic stimulation of saphenous nerve (1 V/10 Hz per 20 ms) for 10 min in neurogenic inflammation, or 30 min before substance P (0.1 μg) injection into the ears in substance P-induced ear edema. [D-Pro^2,D-Trp^{7,9}]substance P (1 μg) was coinjected with substance P. [b] Values are expressed as the means ± S.E.M. for seven to nine animals; n.d., not determined. Statistically significant differences from the corresponding control values are noted as [c] $P < 0.01$.

and 10 mg/kg i.p., respectively, $P < 0.01$) and by bradykinin (44.4 ± 2.6% and 82.1 ± 3.2% inhibition for 3 and 10 mg/kg, respectively, $P < 0.01$).

3.5. Mast cell degranulation

Histamine and β-glucuronidase were released from mast cells stimulated with compound 48/80. Acetylshikonin inhibited compound 48/80-induced mast cell degranulation in a concentration-dependent manner, with IC_{50} values of 11.3 ± 2.2 μM and 11.7 ± 3.7 μM for histamine and β-glucuronidase assay, respectively (Fig. 4).

3.6. Plasma exudation in compound 48/80-pretreated mice

In compound 48/80-pretreated mice, the histamine content of the ear was greatly reduced (0.22 ± 0.03 μg/punched ear sample from compound 48/80-pretreated mice vs. 1.25 ± 0.03 μg/punched ear sample from control mice). Under these conditions, isoproterenol (3 mg/kg i.p.) greatly inhibited, whereas indomethacin (3 mg/kg i.p.) had no effect on, the bradykinin- and substance P-induced ear edema (Fig. 5). Pretreatment with diphenhydramine (10 mg/kg i.p.) in combination with methysergide (3 mg/kg i.p.) did not affect the ear edema caused by bradykinin, whereas a slight but significant ($P < 0.05$) change was observed in response to substance P. Moreover, in these diphenhydramine/methysergide-treated mice, the bradykinin-

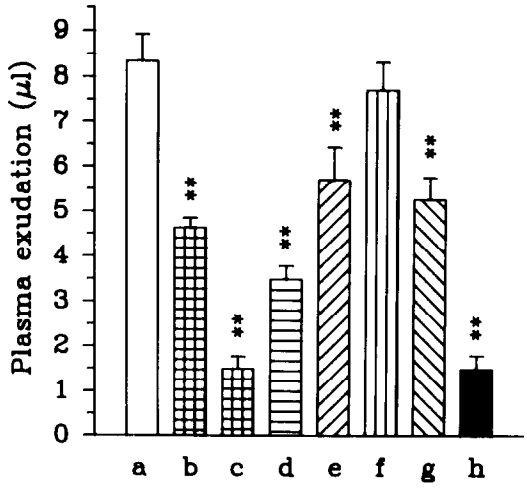

Fig. 3. Effects of acetylshikonin, [Thi^{5,8},D-Phe^7]bradykinin, diphenhydramine, indomethacin, methysergide and isoproterenol on bradykinin-induced mouse ear edema. (a) Control, no pretreatment with anti-inflammatory drugs; acetylshikonin (b) 3 and (c) 10 mg/kg; (e) diphenhydramine 10 mg/kg; (f) indomethacin 3 mg/kg; (g) methysergide 3 mg/kg; (h) isoproterenol 3 mg/kg, were intraperitoneally administered 30 min before bradykinin 0.3 μg injection into the ears; (d) [Thi^{5,8},D-Phe^7]bradykinin 1 μg was coinjected with bradykinin into the ears. The animals were killed 45 min after ear edema induction. Values for plasma exudation are expressed as the means ± S.E.M. for five to six animals. Statistically significant difference from the control value (a) is noted as $^{**}P < 0.01$.

and substance P-induced plasma exudation was further inhibited when [Thi^{5,8},D-Phe^7]bradykinin was coinjected with bradykinin (73.3 ± 2.7% and 91.3 ± 2.9% inhibition for 0.3 and 1 μg [Thi^{5,8},D-Phe^7]bradykinin,

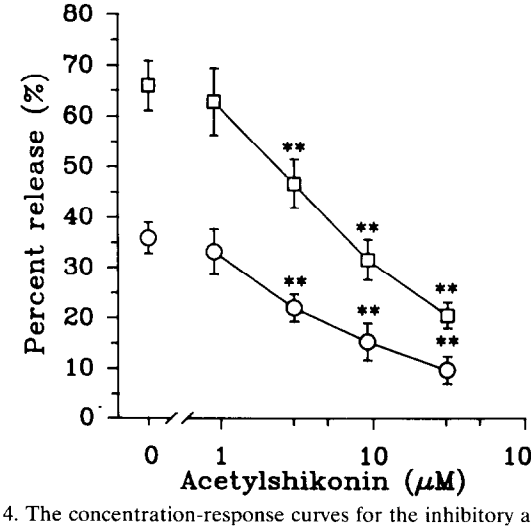

Fig. 4. The concentration-response curves for the inhibitory action of acetylshikonin on the release of histamine (□) and β-glucuronidase (○) from isolated rat peritoneal mast cells stimulated with compound 48/80 10 μg/ml. Values are expressed as the means ± S.E.M. of seven to eight separated experiments. Statistically significant difference from the corresponding control values is noted as $^{**}P < 0.01$.

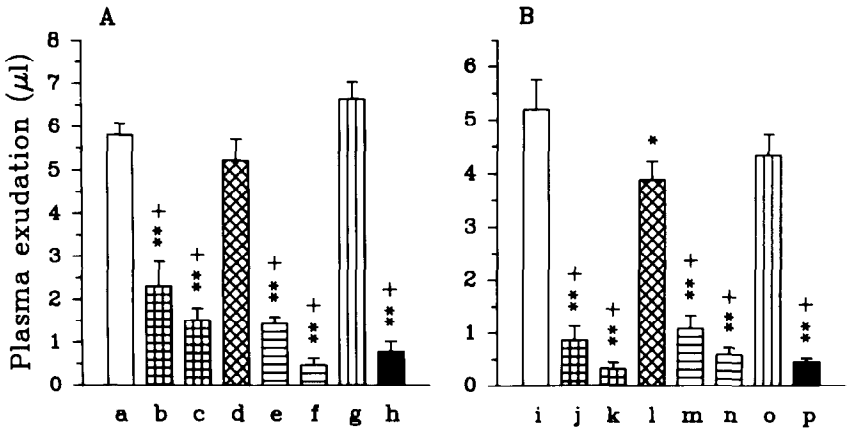

Fig. 5. Effects of acetylshikonin, diphenhydramine, methysergide, [Thi^{5,8},D-Phe^{7}]bradykinin, [D-Pro^{2},D-Trp^{7,9}]substance P, indomethacin and isoproterenol on bradykinin- and substance P-induced ear edema in compound 48/80-pretreated mice. (a,i) Control, no pretreatment with anti-inflammatory drugs; acetylshikonin (b,j) 3 and (c,k) 10 mg/kg; (g,o) indomethacin 3 mg/kg; (h,p) isoproterenol 3 mg/kg, were intraperitoneally administered 30 min before injection of (A) bradykinin 0.3 μg or (B) substance P 0.1 μg into the ear of compound 48/80-pretreated mice. Diphenhydramine 10 mg/kg in combination with methysergide 3 mg/kg was intraperitoneally injected 30 min before intradermal injection of bradykinin 0.3 μg (d) alone or combined with [Thi^{5,8},D-Phe^{7}]bradykinin (e) 0.3 μg and (f) 1 μg, or before intradermal injection of substance P 0.1 μg (l) alone or combined with [D-Pro^{2},D-Trp^{7,9}]substance P (m) 0.3 μg and (n) 1 μg into the ears. The animals were killed 45 min after ear edema induction. Values for plasma exudation are expressed as the means ± S.E.M. for five to seven animals. Statistically significant difference from the corresponding control values (a,i) is noted as $^{**}P < 0.01$, from the diphenhydramine/methysergide-treated values (d,l) is noted as $^{+}P < 0.01$.

respectively, $P < 0.01$), and [D-Pro^{2},D-Trp^{7,9}]substance P was coinjected with substance P (83.4 ± 5.1% and 93.6 ± 2.3% inhibition for 0.3 and 1 μg [D-Pro^{2},D-Trp^{7,9}]substance P, respectively, $P < 0.01$). Acetylshikonin (3 and 10 mg/kg) significantly inhibited the ear edema caused by bradykinin (60.1 ± 9.8% and 73.9 ± 4.8% inhibition for 3 and 10 mg/kg i.p., respectively, $P < 0.01$) and by substance P (83.4 ± 5.1% and 93.6 ± 2.3% inhibition for 3 and 10 mg/kg i.p., respectively, $P < 0.01$) in compound 48/80-pretreated mice. Moreover, acetylshikonin as well as isoproterenol produced significantly more inhibition ($P < 0.01$) of plasma exudation than diphenhydramine/methysergide did.

4. Discussion

Polymyxin B-induced hind-paw edema is mediated by prostaglandins and the mast cell released mediators such as histamine and serotonin (Bertelli and Soldani, 1979; Wang et al., 1992). In the present study, acetylshikonin, indomethacin, a cyclooxygenase inhibitor (Insel, 1990), and diphenhydramine, a histamine antagonist, inhibited the polymyxin B-induced paw edema in normal as well as in adrenalectomized mice. Besides, acetylshikonin did not increase the liver glycogen content as had dexamethasone, which enhanced the processes of gluconeogenesis and glycogenesis (Haynes, 1990). These observations indicate that the anti-inflammatory effect of acetylshikonin was probably mediated neither by glucocorticoid activity nor by the release of steroid hormones from the adrenal gland.

Mast cells release chemical mediators in the passive cutaneous anaphylactic reaction and in compound 48/80 challenge, which in turn increase vascular permeability and then evoke plasma exudation from the nearby vasculature (Maling et al., 1974; Perper et al., 1975; Saria et al., 1983; Saria et al., 1984). Acetylshikonin, like diphenhydramine, methysergide, a serotonin antagonist (Garrison, 1991), and isoproterenol, a β-adrenoceptor stimulant, reduced the plasma exudation in both the passive cutaneous anaphylactic reaction and in compound 48/80-induced ear edema. Indomethacin was ineffective in these respects (Perper et al., 1975; Taira et al., 1988; Wang et al., 1994a; Wang et al., 1994b). β-Adrenoceptor stimulants have been reported to inhibit the mast cell degranulation and the vascular permeability changes probably through an increase in the cellular cAMP level (Assem and Schild, 1969; Winslow and Austen, 1984; Kennedy et al., 1989; Morikawa et al., 1993). If histamine and serotonin, both preformed mediators stored in the secretory granules (Sjoerdsma et al., 1957), were released from mast cells during cell activation, they are proposed to act through the specific receptors on the nearby vasculature and to induce plasma extravasation (Heltianu et al., 1982; Owen, 1987). Mice pretreated with acetylshikonin, diphenhydramine, methysergide, indomethacin or isoproterenol had inhibitory profiles for plasma exudation in histamine- and serotonin-induced ear edema similar to those they had in the passive cutaneous anaphylactic reaction and in compound 48/80-induced ear edema. These results indicate that acetylshikonin may act directly on the vasculature to pre-

vent histamine- and serotonin-induced permeability changes.

Activation of sensory nerves by antidromic electrical stimulation causes vasodilation and an increase of vascular permeability (Jancsó et al., 1967). Substance P, probably with other peptides released from sensory nerve endings mediates this neurogenic inflammation (Lembeck and Holzer, 1979; Gamse et al., 1980). The bradykinin- and substance P-induced plasma exudation is partly through a direct action on the vasculature and partly through the release of chemical mediators from nearby mast cells (Erjavec et al., 1981; Marceau et al., 1981; Saria et al., 1983; Foreman and Jordan, 1984; Wang et al., 1989). Acetylshikonin, like diphenhydramine, methysergide and isoproterenol, reduced the plasma exudation in neurogenic inflammation, and in substance P- and bradykinin-induced edematous responses. Indomethacin was ineffective in these respects (Lembeck and Holzer, 1979; Wang et al., 1993). Recently, the inhibitory effect of β-adrenoceptor stimulants on neurogenic and bradykinin-induced inflammation was also proposed to be exerted partly through the presynaptic inhibition of the release of substance P from sensory nerves (Advenier et al., 1992; Morikawa et al., 1993). The reports that the tachykinin NK_1 receptor is involved in the mediation of substance P-induced plasma extravasation and inflammation (Andrews et al., 1989; Watling, 1992) are in line with our observation that [D-Pro^2,D-Trp^{7,9}]substance P, a tachykinin NK_1 receptor antagonist (Lembeck et al., 1981; Appell et al., 1992), significantly antagonized the substance P-induced ear edema. Moreover, the finding that [Thi^{5,8},D-Phe^7]bradykinin, a bradykinin B_2 receptor antagonist (Regoli et al., 1986), suppressed the bradykinin-induced edematous response is consistent with our previous reports (Wang and Teng, 1988; Wang et al., 1989).

Mast cell degranulation in vitro was examined as part of the investigations into the mechanism of action of acetylshikonin. Acetylshikonin produced a concentration-dependent inhibition of preformed mediators of mast cell release, such as histamine and β-glucuronidase. From this result, it is assumed that the inhibitory effect of acetylshikonin on the passive cutaneous anaphylactic reaction, neurogenic, compound 48/80-, substance P- and bradykinin-induced inflammation is probably partly through the suppression of chemical mediators released from mast cells.

In order to assess whether acetylshikonin also protected the vasculature from the challenge with bradykinin and substance P, plus histamine and serotonin, the compound 48/80-pretreated mice were used. In compound 48/80-pretreated mice, the role of histamine and serotonin in the edema formation is much reduced because of the depletion of mast cells after several doses of compound 48/80 (Wang et al., 1993). In compound 48/80-pretreated mice, the treatment with diphenhydramine and methysergide is expected to minimize the participation of histamine and serotonin released from the residue mast cells during induction of the edematous response. In this situation, bradykinin- and substance P-induced plasma exudation is proposed to occur largely through the activation of the bradykinin B_2 and tachykinin NK_1 receptors, respectively, on the vasculature, since the responses were greatly reduced by [Thi^{5,8},D-Phe^7]bradykinin and [D-Pro^2,D-Trp^{7,9}]substance P, respectively. Like [Thi^{5,8},D-Phe^7]bradykinin, [D-Pro^2,D-Trp^{7,9}]substance P and isoproterenol, acetylshikonin produced significantly more inhibitory effects than diphenhydramine/methysergide did. Isoproterenol has been reported to prevent passage of the large molecules and, therefore, to improve the barrier function of endothelial cells through the increase of cellular cAMP and disappearance of F-actin content (Langeler and Van Hinsbergh, 1991). These observations indicate that acetylshikonin may also suppress the direct action of bradykinin and substance P on the vasculature. However, to assess the action mechanism of acetylshikonin on endothelial cells needs further investigation.

In conclusion, the anti-inflammatory effect of acetylshikonin is exerted neither through the release of adrenal hormones from adrenal gland nor through glucocorticoid activity. It is proposed that acetylshikonin suppressed the plasma exudation partly via the inhibition of preformed chemical mediators released from mast cells near the vasculature and, probably, largely via protection of the vasculature from mediator challenge.

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References

Advenier, C., Y. Qian, J.D. Law Koune, M. Molimard, M.L. Candenas and E. Naline, 1992, Formoterol and salbutamol inhibit bradykinin- and histamine-induced airway microvascular leakage in guinea pig, Br. J. Pharmacol. 105, 792.

Andrews, P.V., R.D. Helme and K.L. Thomas, 1989, NK_1 receptor mediation of neurogenic plasma extravasation in rat skin, Br. J. Pharmacol. 97, 1232.

Appell, K.C., B.J. Fragale, J. Loscig, S. Singh and B.E. Tomczuk, 1992, Antagonists that demonstrate species differences in neurokinin-1 receptors. Mol. Pharmacol. 41, 772.

Assem, E.S.K. and H.O. Schild, 1969, Inhibition by sympathomimetic

amines of histamine release by antigen in passively sensitized human lung, Nature 224, 1028.

Barrett, A.J., 1972, Lysosomes, in: A Laboratory Handbook, ed. J.T. Dingle (Elsevier/Amsterdam) p. 118.

Bertelli, A. and G. Soldani, 1979, Polymyxin B-induced oedema in hind paw of the rat as an assay for antiinflammatory drugs, Arzneim. Forsch./Drug Res. 29, 777.

Erjavec, F., F. Lembeck, T. Florjanc-Irman, G. Skofitsch, J. Donnerer, A. Saria and P. Holzer, 1981, Release of histamine by substance P, Naunyn-Schmied. Arch. Pharmacol. 317, 67.

Fong, J., K.L. Schaffer and P.L. Kirk, 1953, The ultramicrodetermination of glycogen in liver: a comparison of the anthrone and reducing sugar methods, Arch. Biochem. Biophys. 45, 319.

Foreman, J.C. and C.C. Jordan, 1984, Neurogenic inflammation, Trends Pharmacol. Sci. 5, 116.

Gamse, R., P. Holzer and F. Lembeck, 1980, Decrease of substance P in primary afferent neurones and impairment of neurogenic plasma extravasation by capsaicin, Br. J. Pharmacol. 68, 207.

Garrison, J.C., 1991, Histamine, bradykinin, 5-hydroxytryptamine, and their antagonists, in: The Pharmacological Basis of Therapeutics, eds. A.G. Gilman, T.W. Rall, A.S. Nies and P. Taylor (Pergamon Press/New York) p. 575.

Good, C.A., H. Kramer and M. Somogyi, 1933, The determination of glycogen, J. Biol. Chem. 100, 485.

Håkanson, R. and A.L. Rönnberg, 1974, Improved fluorometric assay of histamine, Anal. Biochem. 60, 560.

Haynes, R.C., Jr., 1990, Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones, in: The Pharmacological Basis of Therapeutics, eds. A.G. Gilman, T.W. Rall, A.S. Nies and P. Taylor (Pergamon Press/New York) p. 1431.

Heltianu, C., M. Simionescu and N. Simionescu, 1982, Histamine receptor of the microvascular endothelium revealed in situ with a histamine-ferritin conjugated: characteristic high affinity binding sites in venules, J. Cell Biol. 93, 357.

Insel, P.A., 1990, Analgesic-antipyretics and anti-inflammatory agents: drugs empolyed in treatment of rheumatoid arthritis and gout,in: The Pharmacological Basis of Therapeutics, eds. A.G. Gilman, T.W. Rall, A.S. Nies and P. Taylor (Pergamon Press/New York) p. 638.

Jancsó, G., A. Jancsó-Gabor and J. Szolcsanji, 1967, Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin, Br. J. Pharmacol. Chemother. 31, 138.

Katayama, S., H. Shionoya and S. Ohtake, 1978, A new method for extraction of extravasated dye in the skin and the influence of fasting stress on passive cutaneous anaphylaxis in guinea pigs and rats, Microbiol. Immunol. 22, 89.

Kennedy, T.P., J.R. Michael, J.R. Hoidal, D. Hasty, A.M. Sciuto, C. Hopkins, R. Lazar, G.K. Bysani, E. Tolley and G.H. Gurtner, 1989, Dibutyryl cAMP, aminophylline, and β-adrenergic agonists protect against pulmonary edema caused by phosgene, Am. J. Physiol. 67, 2542.

Kuroda, C., 1918, The coloring matter in *Lithospermum erythrorhizon*, J. Tokyo Chem. Soc. 29, 1051.

Langeler, E.G. and V.W. Van-Hinsbergh, 1991, Norepinephrine and iloprost improve barrier function of human endothelial cell monolayers: role of cAMP, Am. J. Physiol. 260, C1052.

Lembeck, H. and P. Holzer, 1979, Substance P as neurogenic mediators of antidromic vasodilation and neurogenic plasma extravasation, Naunyn-Schmied. Arch. Pharmacol. 310, 175.

Lembeck, F., K. Folkers and J. Donnerer, 1981, Analgesic effect of antagonists of substance P, Biochem. Biophys. Res. Commun. 103, 1318.

Majima, R. and C. Kuroda, 1922, The coloring matter of *Lithospermum erythrorhizon*, Acta Phytochem. 1, 43.

Maling, H.M., M.E. Webster, M.A. Williams, W. Saul and W. Anderson, Jr., 1974, Inflammation induced by histamine, serotonin, bradykinin and compound 48/80 in the rat: antagonists and mechanism of action, J. Pharmacol. Exp. Ther. 191, 300.

Marceau, F., M. Knap and D. Regoli, 1981, Pharmacological characterization of vascular permeability enhancing effects of kinins in the rabbit skin, Can. J. Physiol. Pharmacol. 59, 921.

Morikawa, M., K. Sekizawa and H. Sasaki, 1993, Inhibitory actions of cyclic AMP on neurogenic plasma extravasation in rat airways, Eur. J. Pharmacol. 241, 83.

Motohide, H., 1977, Pharmacological studies on crude plant drugs, Shikon and Tooki. (II). Shikonin and acetylshikonin, Nippon Yakurigaku Zasshi 73, 193.

Owen, D.A.A., 1987, Inflammation-histamine and 5-hydroxytryptamine, Br. Med. Bull. 43, 256.

Perper, R.T., A.L. Oronsky and U. Blamcuzyi, 1975, An analysis of the specificity in pharmacological inhibition of the passive cutaneous anaphylaxis reaction in mice and rats, J. Pharmacol. Exp. Ther. 193, 594.

Regoli, D., G. Drapeau, P. Rovero, S. Dion, P. D'Orleans-Juste and J. Barabé, 1986, The actions of kinin antagonists on B_1 and B_2 receptor systems, Eur. J. Pharmacol. 123, 61.

Saria, A., J.M. Lundberg, G. Skofitsch and F. Lembeck, 1983, Vascular protein leakage in various tissue induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge, Naunyn-Schmied. Arch. Pharmacol. 324, 212.

Saria, A., X. Hua, G. Skofitsch and J.M. Lundberg, 1984, Inhibition of compound 48/80-induced vascular protein leakage by pretreatment with capsaicin and a substance P antagonist, Naunyn-Schmied. Arch. Pharmacol. 328, 9.

Schiatti, P., D. Selva, G. Galliani, E. Baldoli, A. Diena, A. Glässer, M. Leali and E. Toja, 1986, High selective anti-inflammatory and analgesic activity of 3-(1-methyl-ethyl)-2-(4-methoxyphenyl)-3*H*-naphth[1,2-*d*]imidazole, a new non-acidic molecule, Arzneim. Forsch./Drug Res. 36, 120.

Shore, P.A., A. Burkhalter and V.H. Cohn, Jr., 1959, A method for the fluorometric assay of histamine in tissue, J. Pharmacol. Exp. Ther. 127, 182.

Sjoerdsma, A., T.P. Wualkes and H. Weissbach, 1957, Serotonin and histamine in mast cells, Science 125, 1202.

Tallarida, R.J. and R.B. Murray, 1987, Manual of Pharmacologic Calculations with Computer Programs (Springer-Verlag, New York) p. 77.

Tanaka, S., M. Tajima, M. Tsukada and M. Tabata, 1986, A comparative study on anti-inflammatory activities of the enantiomers, shikonin and alkannin, J. Nat. Prod. 49, 466.

Taira, M., S.W. Kohno, H. Yamamura and K. Ohata, 1988, Lack of involvement of leukotriene and platelet activating factor in passive cutaneous anaphylaxis in rats, Agents Actions 24, 189.

Ushio, S., E. Yutaka, M. Terutaka, I. Yasuo, O. Hideaki, S. Shoji, I. Motoko and F. Fumiko, 1977, Antitumor activity of shikonin and its derivatives, Chem. Pharm. Bull. 25, 2392.

Wang, J.P. and C.M. Teng, 1988, Rat paw edema induced by trimucase II, a kinin-releasing enzyme from *Trimeresurus mucrosquamatus* venom, Eur. J. Pharmacol. 157, 61.

Wang, J.P., M.F. Hsu, C. Ouyang and C.M. Teng, 1989, Edematous response caused by [Thi^{5,8},D-Phe^{7}]bradykinin, a B_2 receptor antagonist, is due to mast cell degranulation, Eur. J. Pharmacol. 161, 143.

Wang, J.P., M.F. Hsu, S.L. Raung, C.C. Chen, J.S. Kuo and C.M. Teng, 1992, Anti-inflammatory and analgesic effects of magnolol, Naunyn-Schmied. Arch. Pharmacol. 346, 707.

Wang, J.P., S.L. Raung, C.C. Chen, J.S. Kuo and C.M. Teng, 1993, The inhibitory effect of magnolol on cutaneous permeability in mice is probably mediated by a nonselective vascular hyporeactivity to mediators, Naunyn-Schmied. Arch. Pharmacol. 348, 663.

Wang, J.P., M.F. Hsu, S.L. Raung and S.C. Kuo, 1994a, Suppressive effect of 2-phenyl-4-quinolone (YT-1) on hind-paw edema and cutaneous vascular plasma extravasation in mice, Naunyn-Schmied. Arch. Pharmacol. 349, 324.

Wang, J.P., S.L. Raung, C.N. Lin and C.M. Teng, 1994b, Inhibitory effect of norathyriol, a xanthone from *Tripterospermum lanceolatum*, on cutaneous plasma extravasation, Eur. J. Pharmacol. 251, 35.

Watling, K.J., 1992, Nonpeptide antagonists herald new era in tachykinin research, Trends Pharmacol. Sci. 13, 266.

Waynforth, H.B., 1980, Specific surgical operations, in: Experimental and Surgical Technique in The Rat, ed. H.B. Waynforth (Academic Press/London) p. 124.

Winslow, C.M. and K.F. Austen, 1984, Role of cyclic nucleotides in the activation- secretion response, Prog. Allergy 34, 236.