Effect of Baicalin on Tracheal Permeability in Ovalbumin (OA)-Sensitized Guinea Pigs

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INTRODUCTION

It has been known that allergy-induced (dust, smoke, antigens, etc.) asthma consists of increasing epithelial permeability (epithelial damage) (1–2), which releases a variety of cellular chemotactic mediators, and the sequence produces/enhances mucous secretion (3–5). Boucher *et al.* (2) showed cigarette smoke increases mucosal permeability, which allows penetration of the nerve endings that form the irritant receptors. It was suggested the production of asthmatic hyperactivity is due to the opening of the tight junctions of the bronchial epithelium and the receptors becoming increasingly sensitized from penetrating antigens. Therefore, one proposed asthma treatment is repairing tracheal and bronchial epithelia damage (3,6) or closing these tight junctions of epithelium tissue (7).

Scutellariae radix, the dried root of *Scutellaria baicalensis*, is often used in the treatment of various respiratory inflammatory diseases in traditional Chinese medicine (8–9). Its major component, baicalin, has been reported to possess anti-inflammatory activity (3,9), including protecting asthmatic anaphylaxis in systemic and cutaneous anaphylaxis in guinea pigs, as well as, inhibiting the release of histamine, slow-reacting substances of anaphylaxis (SRS-A) and leukotriene C4 (LTC4). Also, it exhibits direct relaxing effects on the trachea. In addition, by using acetic acid to increase vascular permeability (8), baicalin demonstrated the recovery of membrane permeability in acute paw edema in mice. However, the effectiveness and recovery of junction permeability of asthmatic trachea by baicalin are still unknown. Therefore, the purpose of this project was to evaluate the repair of an asthmatic trachea by baicalin, using radioactive markers in an in vitro guinea pig model of acute antigen OA-enhanced permeability, as well as monitoring the tracheal membrane integrity by electric resistance measurement.

MATERIALS AND METHODS

Animals and Materials

Male Dunkin-Hartley guinea pigs (300–350 g, Animal Center of National Taiwan University) were used. Radioactively labeled compounds were obtained from NEN Life Science Products (DuPont Chemical Corp., Boston, MA). The purity of all radiolabeled compounds was 98–99% as determined by thin layer or high pressure gas chromatography. Baicalin with a purity of 98% was purchased from Nacalai Tesque Inc. (Kyoto, Japan) and OA was commercially obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals were of reagent or analytical grade and were used as received.

Immunization

The procedures of sensitization of guinea pigs followed the methods from previous reports by Chou *et al.* (4) and Takeda *et al.* (5). Male Dunkin-Hartely guinea pigs were first sensitized by the inhalation of aerosolized antigen (10 mg/ml OA) for 30 min daily for 8 d by a DeVilbiss nebulizer (25 × 15 cm chamber, Pulmo Amide 5610D, Somerset, PA) with 0.5 to 5 um particle size range. The second sensitization was performed on the 16th day after the first sensitization by 100 mg/ml OA aerosol for 2 min; the immediate allergic response (IAR) group. The second group only received the first sensitization and was used before the second challenge (BC); and the control group was not sensitized with OA, and served as the non-sensitized group.

In Vitro Perfusion Studies

Guinea pigs were sacrificed with an overdose injection of 3% sodium pentobarbital given via intraperitoneal injection. Tracheas were removed immediately after the injection and were mounted on acrylic rings and placed in perfusion cells (10). Both faces of the trachea were bathed in glutathione bicarbonated Ringer solution (GBR). All experiments were carried out at 37°C with mixture of 95% O_2 and 5% CO_2 and at a pH adjusted to 7.4 with NaOH or HCI. A 0.24 cm^2 area of trachea was exposed to donor (epithelial) and receiver (endothelial) chamber solutions, each containing 7 ml of solution. Each 0.5 ml sample was placed in a liquid scintillation vial, and 2 ml of scintillation cocktail (Ecoscint H. National Diagnostics, Atlanta, GA) was added. Samples were analyzed by counting the total number of disintegrations per minute (DPM) using a Beckman 6500 liquid scintillation counter.

Electrical Measurement

Electrodes were prepared from silver wires (10) and tracheal resistances were measured using an Ag-AgCl four electrode system. Variable current pulses, stepwise up to 15 pA/cm^2 were measured with a digital multimeter, and a second digital multimeter measured the corresponding potential differences. The resistance was calculated from the slope of the applied current and potential difference plot. To correct for the potential drop due to the solution, measurements were carried out in the same bathing solution in the diffusion chamber. The actual tracheal tissue resistance was then calculated by subtracting the resistance determined in the absence of the tracheal tissues from that in its presence.

Assay of Adenosine 3,5-Cyclic Monophosphate (cAMP)

The cAMP assay method (11) was performed using a cAMP assay kit obtained from NEN Life Science Products

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(Boston, MA). Briefly, after IAR tissue was treated with or without 0.5 mM baicalin or the control (0.24 cm^2), the sample was placed in a glass pulverizer at 4°C with 6% trichloroacetic acid (TCA) to make a 1 ml 10% (W/V) homogenate. To determine the recovery of cAMP during extraction, each sample extract was amended with approximately 400 cpm of ^3H cAMP to the TCA extract. After the centrifugation of TCA extracts at 2500 g and 4°C for 15 min, the supernatant was collected and extracted 4 times with 5 volumes of water-saturated ether. Samples were then placed in a water bath at 70–80°C and evaporated to dryness under a stream of air. Using 100 ul of dissolved the residue in assay; cAMP Radioimmunoassay Kit (NEN Life Science Product, Boston, MA) directly performed on the sample.

Inhibition of cAMP Phosphodiesterase by Baicalin

The phosphodiesterase activity method (11) was performed using a phosphodiesterase activity assay kit obtained from NEN Life Science Products Boston, MA). Briefly, the standard reaction mixture (500 μl) contained Tris-HCl (pH 7.5, 50 mM), bovine serum albumin (250 μg), ^3H-cAMP (0.01 mM), beef heart phosphodiesterase (2.25 mU), and a sample (0.5 mM baicalin). The reaction mixture was incubated for 30 min at 37°C, and the test tube was immersed in boiling water for 3 min, stopping the reaction. Snake venom nucleotidase (500 μg) was added to the cooled reaction mixture and incubated for 30 min at 37°C. Then, a 50% suspension of Dowex AGl-XS resin was added to the reaction mixture. The resin, which absorbed unchanged ^3H-cAMP, was precipitated by centrifugation, and the radioactivity of an aliquot of the supernatant containing ^3H-adenosine resulted in the assay reaction, which was measured with a liquid scintillation counter. All assays were performed in triplicate.

Scanning Electron Microscopy

After completion of the diffusion study, the trachea was carefully removed and placed in a fixative solution containing 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3 for 2 h. After washing in the same buffer, the tissues were postfixed in 2% OsO_4 in 0.1 M phosphate buffer solution (pH 7.3) and were dehydrated using a graded series of ethanol solutions at concentrations of 35, 50, 70, 90, 95, and 100%. Prior to viewing, specimens at critical point were dried in a pressure chamber using liquid carbon dioxide. Subsequently, a uniform 100 Å layer of gold was applied to the specimen surfaces by means of a sputter coating. The specimens were finally viewed in a scanning electron microscope.

Apparent Permeability Coefficient Calculation

In vitro apparent permeability coefficients (P) were calculated from:

$$P = V/A \times 1/C \times dC/dt$$

$$= (\text{fraction of dose transported})/dt \times V/A;$$

where the fraction of dose transported through the trachea can be calculated after correction for sampling and solution replacement at each time point. These values were then plotted versus time. Therefore, the apparent permeability coefficient, P (cm/sec), can be calculated from the slope using the receiver compartment volume V (7 ml) and surface area of tissue A (0.24 cm^2).

Data Analysis

The studies for each experiment were repeated in duplicate for 6 experiments. Data from all experiments were pooled to determine the mean and standard error mean (SEM). ANOVA test with Dunnett's multiple comparison tests at 95% confidence levels were used to determine significant differences between each group of experiments.

RESULTS

The paracellular permeability coefficients of the 3 groups (control, BC, and IAR) are shown in Table 1. The ^{14}C-mannitol permeability of the trachea in the IAR and BC groups ([1.92 ± 0.11, and 2.32 ± 0.17] × 10^{-5} cm/sec) were significantly higher (statistically) than the control group ([1.49 ± 0.11] × 10^{-5} cm/sec). There was no significant difference between the BC and IAR groups ($p > 0.05$). In the intracellular pathway-^{14}C-estradiol apparent permeability coefficient, there are no statistical differences among the 3 groups ($p > 0.05$). Figure 1 shows the electrical resistance property profile of the guinea pig tracheas with different treatments by aerosolized OA. The range of electrical resistance of control tracheal tissues was 317~342 Ω.cm^2 within a 5 h period. On the other hand, in the BC and IAR groups, the resistance values decreased to around 201~284 Ω.cm^2, indicating that the junctional integrity of these 2 types of tracheal tissue were influenced by OA challenge.

Table 1 also shows the effects of 4 different concentrations (0.001, 0.01, 0.1, and 0.5 mM) of baicalin for treating IAR tracheal tissues on the permeability of mannitol; the apparent permeability coefficients of mannitol were 1.99 ± 0.11, 1.92 ± 0.23, 1.51 ± 0.13, and 1.50 ± 0.13 × 10^{-5}cm/sec, respectively. Among these 4 treatments, 0.1 and 0.5 mM baicalin have no statistical difference with control tracheal tissues. In addition, in the 0.5 mM baicalin treatment of the control and BC groups, the results showed the BC group could decrease the apparent permeability coefficient to (1.78 ± 0.21) × 10^{-5} cm/sec. However, the recovery of tracheal tissues is not as complete when compared with the control tracheal tissues ($p < 0.01$). There is no influence with or without the 0.5 mM baicalin treatment on the control group. After removing calcium ions from the bathing medium, the apparent permeability coefficient of mannitol was found to be significantly decreased ([1.31 ± 0.09] × 10^{-5} cm/sec), with no difference compared with the control group. A typical resistance-time profile of guinea pig trachea with 0. 5 mM baicalin is illustrated in Fig. 2. The resistance of those samples treated with baicalin increased to around 334~422 Ω.cm^2 compared with the non-treated IAR group (201~230 Ω.cm^2).

Using the TCA extraction method, the cAMP content in IAR tracheal tissues was found after baicalin treatment to be reduced from 94 ± 4 to 54 ± 20 pmole/ml (29%). Although the content of cAMP decreased, it was not completely inhibited after treatment with 0.5 mM baicalin compared with the control group (20 ± 0.7 pmole/ml). In addition, the inhibition of phosphodiesterase activity by 0.5 mM baicalin was also performed, and the effect of this enzyme activity was found to be 13%

Table 1. Effect of Baicalin on the *In Vitro* Tracheal Permeability of Guinea Pigs (n = 6)

	App. perm. coeff[a]		Baicalin treated with App. perm. coeff[a]	
	^{14}C-manntiol	^{14}C-estradiol[b]		^{14}C-manntiol
Control	1.49 ± 0.11	2.23 ± 0.26	Control + 0.5 mM	1.42 ± 0.13[h]
BC	2.32 ± 0.17[e]	2.32 ± 0.32	BC + 0.5 mM	1.78 ± 0.21[g]
IAR	1.92 ± 0.11[d]	1.96 ± 0.26	IAR + 0.001 mM	1.99 ± 0.11
IAR-Ca^{++}[c]	1.31 ± 0.09[f,h]		IAR + 0.01 mM	1.92 ± 0.23
			IAR + 0.1 mM	1.51 ± 0.13 *,[h]
			IAR + 0.5 mM	1.50 ± 0.13 *,[h]

[a] App. Perm. Coeff: apparent permeability coefficient ± SEM ($\times 10^{-5}$ cm/sec).
[b] There was no statistical significance difference among 3 groups.
[c] Medium did not contain calcium concentration on both sides of diffusion chamber.
[d] Denotes a statistical significance at $p < 0.05$, as compared to control mannitol group
[e] Denotes a statistical significance at $p < 0.001$, as compared to control mannitol group.
[f] Denotes a statistically significant decrement at $P < 0.01$, as compared to IAR mannitol group.
[g] Denotes a statistically significant decrement at $P < 0.01$, as compared to BC mannitol group.
[h] Denotes a statistically significant decrement at $P > 0.05$, as compared to IAR mannitol group.

inhibition. Taken together, this suggests the treatment of IAR tracheal tissues by baicalin involved the cAMP second messager pathway. Representative scanning electron micrographs of IAR tracheal tissue are shown in Figs. 3a and 3b. Figure 3a shows tissue samples following second OA challenge and trachea of guinea pigs (IAR) with disruption of the tight cell-cell packing present in normal epithelia. Figure 3b shows a tissue sample following administration of 0.5 mM baicalin. There are no significant morphologic changes in the top layers of the membrane compared with normal tissue by scanning electron microscopy observation.

DISCUSSION

The paracellular permeability coefficients of manntiol in 3 guinea pig trachea fell in the range of $(1.49 \sim 2.39) \times 10^{-5}$ cm/sec. These are similar to the values seen in the Wangensteen group (12) who reported results from their vitro guinea pig trachea study (3×10^{-5} cm/sec). Regarding transcellular permeability, estradiol permeability ability in our *in vitro* tracheal data is also similar with those from Hanafi *et al.* (7), which used the lipophilic marker ^{14}C-antipyrine with permeability coefficients of $(2.2 \pm 0.1) \times 10^{-5}$ cm/sec. Furthermore, comparison of our electrical resistance values of tracheal tissues in guinea pigs, showed they had a similar range with those of Croxton (13) who reported values of 327 ± 30 Ω.cm^2. Thus, our tracheal tissues should fall within this range. On the other hand, we have found that there are no meaningful differences among the 3 groups by ^{14}C-estradiol as a transcellular pathway marker (Table 1). Taken together, this reflects the change in guinea pig tracheal permeability after challenged with aerosolized-OA, which mainly influenced a paracellular (tight junctions), rather than a transcellular pathway.

Up to 0.5 mM baicalin treatment, the IAR tracheal permeability decreased as well as the resistance profile recovered (Table 1 and Fig. 2). In general, membrane resistance indicated membrane permeability of ions and thus, it was used as an

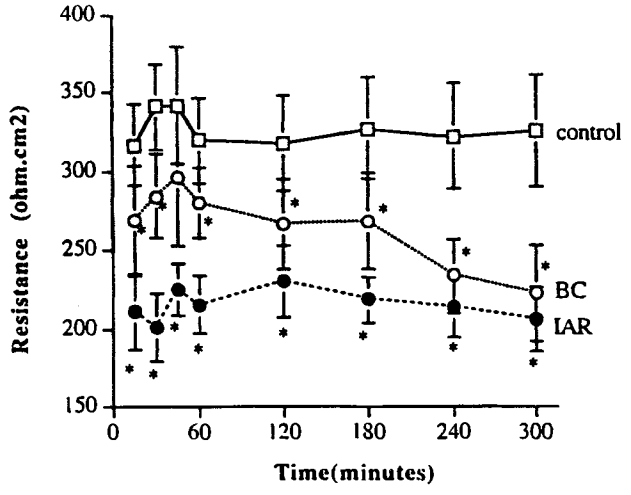

Fig. 1. Typical resistance-time profiles of guinea pig trachea with different sensitization periods by aerosolized OA. Control: without any sensitization, BC: before challenge, IAR: immediate allergic response. Error bars represent 1 SEM; n = 6. *: representative $p < 0.01$ significantly different from the control values.

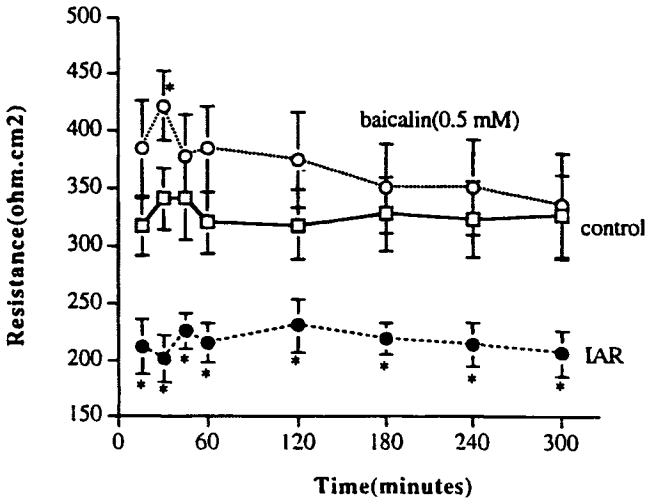

Fig. 2. A typical resistance-time profile of OA-sensitized guinea pig tracheal by 0.5 mM baicalin. Error bars represent 1 SEM; n = 6. *: representative $p < 0.01$ significantly different from the control values.

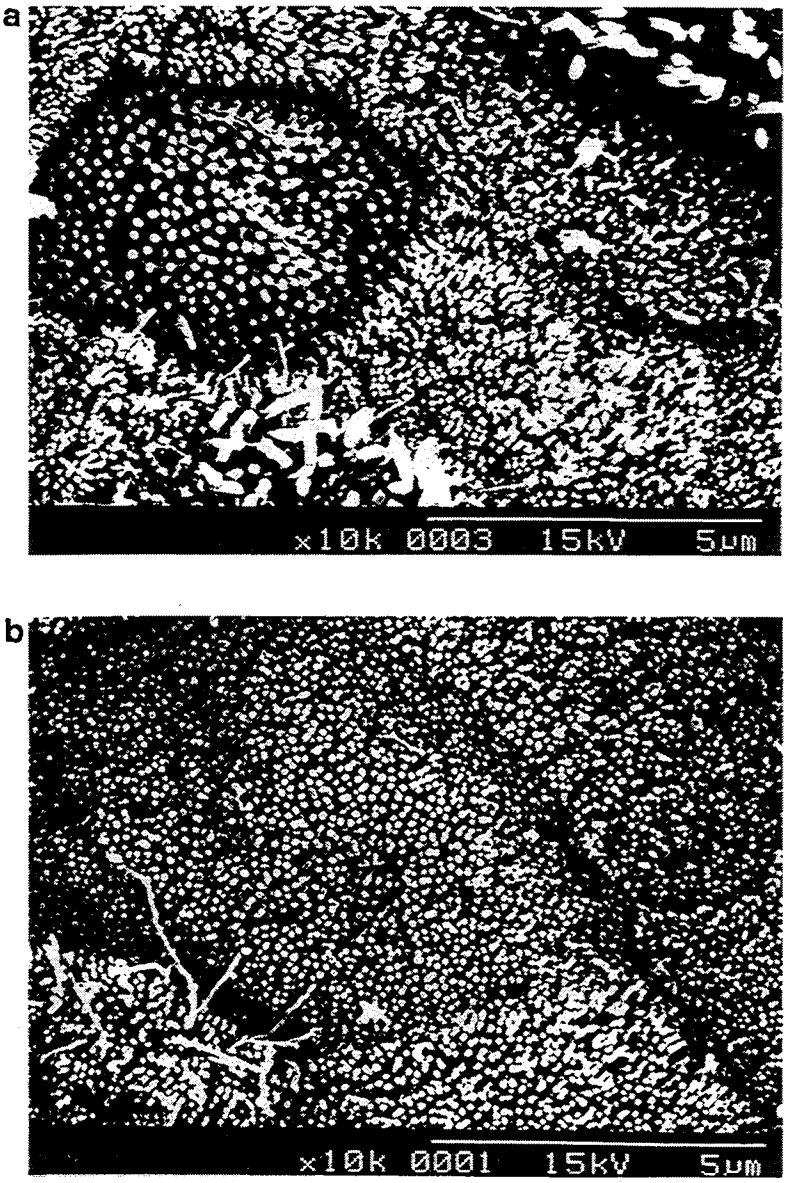

Fig. 3. Effect of aerosolized OA sensitization on guinea pigs by scanning electron microscopy. a. IAR group, note opening of the intercellular junction between 2 cells. Bars indicate 5 um. b. treated with 0.5 mM baicain. Junction opening difficult to see with the scanning microscope.

indication of tissue integrity or damage. Our data indicated that after treatment, baicalin could improve tracheal integrity in the early time (15–30 minutes). Kubo *et al.* (8) observed the main flavonoid components of *Scutellariae Radix*, baicalin, baicalein, and wogonin could inhibit an increase in vascular permeability in mice induced by acetic acid. It was suggested these compounds have anti-inflammatory activity in acute and chronic phases. However, as to the effect of baicalin on BC status of tracheal tissues, 0.5 mM baicalin could only partially repair the integrity of BC tracheal tissues (258 ± 31 $\Omega.cm^2$) as well as decrease 8% the permeability of the BC tracheal.

Recently, Miyamoto *et al.* (3) found baicalein could inhibit the release of SRS-A consisting of LTC4 and D4 from sensitized guinea pig lung after lung challenge. Thus, the repair of the tight junction structure by baicalin may also be involved with arachidonic acid via the 5-lipoxygenasc-pathway. Also, it is known using the cytochalasin D-actin inhibitor (calcium sensitive) could enhance (300%) the paracellular pathway of tracheal epithelia tissues (14). Therefore, when calcium concentration was depleted from the bath medium, the mannitol permeability of IAR tracheal tissues did indeed decrease as low as the control group (Table 1). Taken together with cAMP and enzyme activity, the cellular physiological mechanism of baicalin on OA induced guinea pig trachea should be involved cellular cAMP-Ca^{++} second message and relative to repair the tracheal tight junction.

Using scanning electron microscopy to observe changes in the IAR tracheal tissue, it showed that, following OA challenge, guinea pig trachea exhibited disruption of the tight cell-cell packing present in normal epithelia. This is consistent with the

results of Boucher *et al.* (2) who found that a conducted airway restricts the movement of inhaled cigarette smoke materials into the interstitial and circulatory space. By using transmission electron microscopy and freeze fracture techniques, they found progressive disruption in the tight junctions of tracheal epithelium and suggested cigarette smoke damages the mucosal barrier and disrupts the intercellular tight junctions. Thus, a combination of microscopy, electrical resistance, and permeability studies suggest baicalin can affect the recovery of intercellular pathways of acute asthmatic tracheal tissues in guinea pigs. In conclusion, using OA-sensitized guinea pigs, the IAR tracheal tissues increased permeability. The repair ability of 0.5 mM baicalin on tracheal tissues in OA-sensitized guinea pigs could decrease the paracellular transport mechanism and recover the integrity of tight junctions.

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