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European Journal of Pharmacology 335 (1997) 245–254

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Inhibitory effect of 2-phenyl-4-quinolone on serotonin-mediated changes in the morphology and permeability of endothelial monolayers

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Received 17 April 1997; revised 21 July 1997; accepted 25 July 1997

Abstract

The integrity of endothelial cell monolayers, a critical requirement for barrier maintenance, is needed for the prevention of edema formation. To investigate the mechanisms by which 2-phenyl-4-quinolone (YT-1) provided protection against serotonin-induced exudation, rat heart endothelial cell cultures were used. In this study, serotonin and phorbol myristate acetate (PMA) caused endothelial cells to became permeable to macromolecules by causing cell contraction and intercellular gap formation. These responses were attenuated by staurosporine, a protein kinase C inhibitor. Further experiments showed that YT-1 (1) did not alter serotonin-mediated early signal events such as protein kinase C activation, (2) protected against serotonin-induced endothelial barrier dysfunction by increasing intracellular cAMP levels, (3) played a role in regulating adenylate cyclase activity, (4) reversed serotonin-induced permeability to macromolecules, an effect which did not correlate with intracellular cGMP concentrations. This study demonstrates a possible mechanism by which YT-1 protects endothelial function and preserves the microvasculature from pharmacologic injury by vasoactive agents. © 1997 Elsevier Science B.V.

Keywords: 2-Phenyl-4-quinolone; Endothelial cell, rat; 5-HT (5-hydroxytryptamine, serotonin); Gap formation; Permeability; cAMP

1. Introduction

2-Phenyl-4-quinolone (YT-1) is a newly synthesized compound (Kuo et al., 1993). Recently, Wang et al. (1994) reported that YT-1 plays a role in inhibiting local edema formation and in protecting the vasculature against histamine- and serotonin-induced plasma extravasation in vivo. In other studies, YT-1 was also reported to be cytotoxic to several human cancer cell lines (Kuo et al., 1993) and to have a positive inotropic effect in both atrial and ventricular muscle by increasing the slow inward Ca^{2+} current and by inhibiting the transient outward current (Su et al., 1993).

Vascular endothelial cells separate the circulating blood from the surrounding vessel wall and tissue. One of the functions of these cells is to efficiently control the influx of macromolecules into tissues. The intercellular junction may become permeable to macromolecules as a result of a change in endothelial cell shape (retraction) in response to humoral mediators of inflammation (Garcia et al., 1986; DeFouw et al., 1993; Hirata et al., 1995; Ding et al., 1996). This opening of the intercellular junction to macromolecules is thought to result in edema formation (Majno and Palade, 1961).

Reduction of the extravasation of macromolecules may prevent edema formation. Cyclic adenosine monophosphate may be an important regulator of the endothelial barrier. Stimulation of cyclic AMP in endothelial cells in vitro is associated with cell flattening and enhanced cell-to-cell contact, which might make paracellular pathways for macromolecule flux narrower (Bensch et al., 1983). Other studies have shown that the barrier function of endothelial cells can be improved by increasing the cellular adenosine 3′,5′-cyclic monophosphate concentration, and suggested that cAMP might play a role in preventing edema (Laposata et al., 1983; Killackey et al., 1986; Carson et al., 1989; Casnocha et al., 1989; Stelzner et al., 1989; Minnear et al., 1990; Langeler and Van Hinsbergh, 1991; Patton et al., 1991; Buchan and Martin, 1992; Patterson and Garcia, 1994; Imai Sasaki et al., 1995).

Various vasoactive mediators such as serotonin, thrombin, bradykinin and histamine increase endothelial permeability in association with a change in cell shape, causing

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the development of intercellular gaps (Majno and Palade, 1961; Boswell et al., 1992; Garcia et al., 1995). This study examines whether YT-1 attenuates the loss of barrier function in rat heart endothelial cell monolayers challenged with serotonin, and investigates the mechanisms by which YT-1 inhibits the serotonin-induced permeability to macromolecules.

2. Materials and methods

2.1. Materials

All culture media and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY, USA). Endothelial cell growth supplement, bovine serum albumin, serotonin, Evans blue dye, staurosporine, cyproheptadine, phorbol myristate acetate (PMA), forskolin, isobutyl-methylxanthine (IBMX), Trypan blue and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) were purchased from Sigma (St. Louis, MO, USA); Transwells (diameter, 0.65 cm; pore size, 3 μm) were from Corning Costar (Cambrige, MA, USA); anti-Von Willebrand factor, human fibronectin and lactate dehydrogenase (LDH) Detection Kit were from Boehringer-Mannheim (Mannheim, Germany); 9-(tetrahydro-2′-furyl)adenine (SQ 22536) was from Biomolecules (Plymouth Meeting, PA, USA); cyclic AMP EIA kit and cyclic GMP EIA kit were from Amersham (Amersham, UK); sodium nitroprusside was from Merck (Taiwan). Serotonin and sodium nitroprusside were dissolved in Hank's Balanced Salt Solution (HBSS) and other substances were dissolved in less than 0.5% dimethylsulfoxide (DMSO).

2.2. Rat heart endothelial cells (RHEC) culture preparation

RHEC were isolated as described previously (Richards et al., 1986). Briefly, ventricles of 3 hearts were removed from 4-day-old donor rats. This tissue was washed four times with Hanks balanced salt solution (HBSS) and finely minced with dissecting scissors. The tissue was then subjected to four successive trypsinization (10 ml 0.125% trypsin in calcium- and magnesium-free HBSS) steps under stirring in a 50 ml trypsinization flask. After each trypsinization, free cells (myocardial and endothelial) were removed and placed in 2 ml of endothelial cell culture medium consisting of Dulbecco's modified essential medium (DMEM) and 20% fetal bovine serum. The cells were recovered by centrifugation at $1000 \times g$, resuspended in endothelial cell culture medium, and allowed to adhere to the bottom of fibronectin-coated wells in a 24-well tissue culture cluster plate (Falcon) for 90 min. The culture medium was then removed and the culture wells were washed twice with HBSS to remove nonadherent (mostly myocardial) cells. Thereafter, culture medium supplemented with 150 μg of endothelial cell growth supplement and 1000 U penicillin–1000 μg streptomycin per milliliter as well as 20% fetal bovine serum was added to the remaining adherent cells. At 90 min, the adherent cells are primarily endothelial cells (Kasten, 1973), since myocardial cells require more time (approximately 24 h) to adhere to a surface (Kasten, 1973).

2.3. Cytochemical immunofluorescence staining of factor VIII related antigen

Factor VIII-related antigen was assayed by indirect immunofluorescence. The cells to be tested were grown on sterile coverslips in 35 mm dishes in DMEM and 20% FBS in an atmosphere of 5% CO_2 under conditions of 96% humidity. The coverslips containing cells were washed with HBSS, fixed for 10 min with cold acetone, and washed again with HBSS. The cells were incubated with a 1:10 dilution of mouse anti-human factor VIII or the same dilution of goat anti-mouse IgG for 30 min at 37°C. The cells were given two additional washes with HBSS, each for 30 min, and were incubated with FITC-conjugated goat anti-mouse IgG for 45 min at 37°C. The coverslips were again washed, mounted in glycerin, and photographed using a Zeiss photomicroscope III epi-illumination system equipped for excitation and observation of FITC.

2.4. Measurement of RHEC monolayer barrier function

RHEC cultured on filters were used for 3 days after seeding. Exchange of marcromolecules through the RHEC monolayers was investigated by assaying the transfer of Evans blue dye-bound bovine serum albumin (4% final concentration). Passage of EB-BSA through endothelial cell monolayers was monitored as described previously (Garcia et al., 1986) with some modifications. Briefly, endothelial cell monolayers were cultured on 48 Transwell (Costar) polycarbonate membrane assemblies (6.5 mm diameter, 3 μm pore size). The membrane assemblies were then placed in 0.6 ml of DMEM containing 20% FBS in 24-well plates and the cells were allowed to grow to form a tight monolayer. For experimentation, membrane assemblies with cells attached were washed twice by immersion in HBSS and transferred to 24-well plates. Thereafter, 600 μl of HBSS was placed in each of the wells, which formed the lower chamber, and 100 μl of HBSS was placed above the endothelial monolayers. These volumes were chosen so as to avoid creating a hydrostatic gradient across the monolayers. Drugs were then added to the top and bottom chambers and the lower chamber was stirred for rapid mixing. The entire system was kept at 37°C by a thermostatically regulated water bath. At the end of the experiment, a 200-μl aliquot was removed from each of the lower chambers and transfer of EB-BSA across the monolayers was quantified by measuring optical density at 600 nm.

2.5. Measurement of cyclic nucleotides

Cyclic nucleotides were measured by enzyme immunoassay (Amersham) as previously described (Stelzner et al., 1989). Cells were plated onto 24-well Falcon plates at 2×10^5 cells/well and grown to confluence in DMEM with 20% FBS for 3 days prior to cyclic nucleotide determination. Before cyclic nucleotides were measured, the cells were washed twice in HBSS and incubated in HBSS alone or in HBSS containing the agents at 37°C in a 5% CO_2-humidified atmosphere. After incubation for the indicated time, the medium was removed completely and the cells were washed twice with HBSS containing 0.5 mM isobutyl–methylxanthine (IBMX) to inhibit phosphodiesterase and to prevent subsequent breakdown of the cyclic nucleotide during cell solubilization, sample collection, and processing. The HBSS containing IBMX was decanted and the cells were immediately solubilized in 250 μl of ice-cold 0.1 N HCl, and the plates were stored at −70°C. Extracts were removed from the plates and centrifuged at $1000 \times g$ for 10 min. A 200 μl aliquot of the supernatant was acetylated, and the amount of cyclic nucleotide was determined by enzyme immunoassay.

2.6. Assay of lactate dehydrogenase (LDH) release

LDH release from RHEC monolayers was determined. RHEC grown on the plastic tissue culture plates were exposed to various concentrations of reagent in DMSO in HBSS with 0.1% BSA for 30 min. The supernatant was removed and centrifuged at $1000 \times g$ for 10 min. LDH activity in the supernatant was determined with the LDH Detection Kit.

2.7. Morphological investigation

RHEC monolayers grown in 24-well plates were photographed in real time during incubations with serotonin and YT-1 with a Nikon Diaphot TMD-300 phase-contrast microscopy. A field was chosen in the center of each well at approximately the same location for photography.

2.8. Statistical analysis

Results were analyzed for statistical significance by analysis of variance with repeated measures and a Newman–Keuls test. Significance level was taken to be $P < 0.05$.

3. Results

3.1. Effects of staurosporine and YT-1 on the serotonin-induced passage of macromolecules across the RHEC monolayers

To investigate the mechanisms by which YT-1 protects against serotonin-induced exudation, RHEC cultures were used. All cultures demonstrated a typical contact-inhibited cobblestone appearance. Factor VIII-related antigenicity was confirmed by indirect immunofluorescence with human factor VIII antiserum and fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Incubation of RHEC monolayers with serotonin induced concentration- and time-dependent increases in the passage of EB-BSA (data not shown). The serotonin-induced increase in the amount of EB-BSA that passed across the monolayers was significant at 1 mM serotonin and a 30-min incubation period. Therefore, 1 mM serotonin and a 30-min incubation period

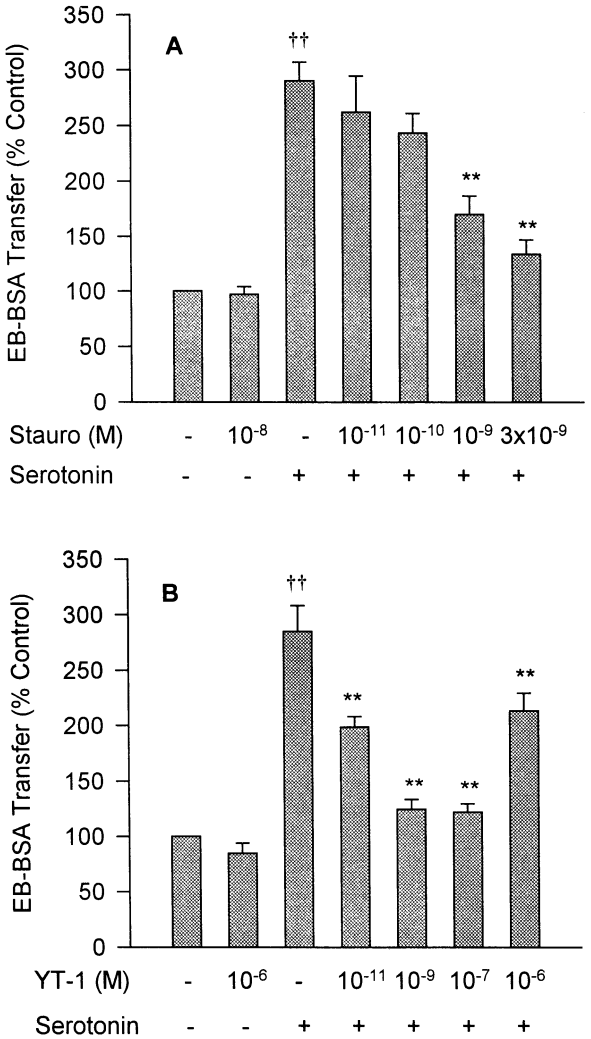

Fig. 1. Effects of staurosporine (Stauro) and YT-1 on serotonin-induced RHEC monolayer barrier dysfunction. (A) RHEC monolayers were pretreated with staurosporine for 10 min and then challenged for an additional 30 min with 1 mM serotonin. Staurosporine alone had no effect on the passage of EB-BSA across the monolayers. (B) The effects of YT-1 (10^{-6} M) and serotonin (1 mM) on the passage of EB-BSA and the effect of YT-1 (10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M) on serotonin-induced increases in the permeability of RHEC monolayers to EB-BSA. Each concentration of YT-1 was added 10 min before serotonin; the incubation lasted 30 min. The results are expressed as the mean % control ± S.E.M. ($n = 20$–25). †† $P < 0.01$ compared to the control values. ** $P < 0.01$ compared to the serotonin-treated monolayers.

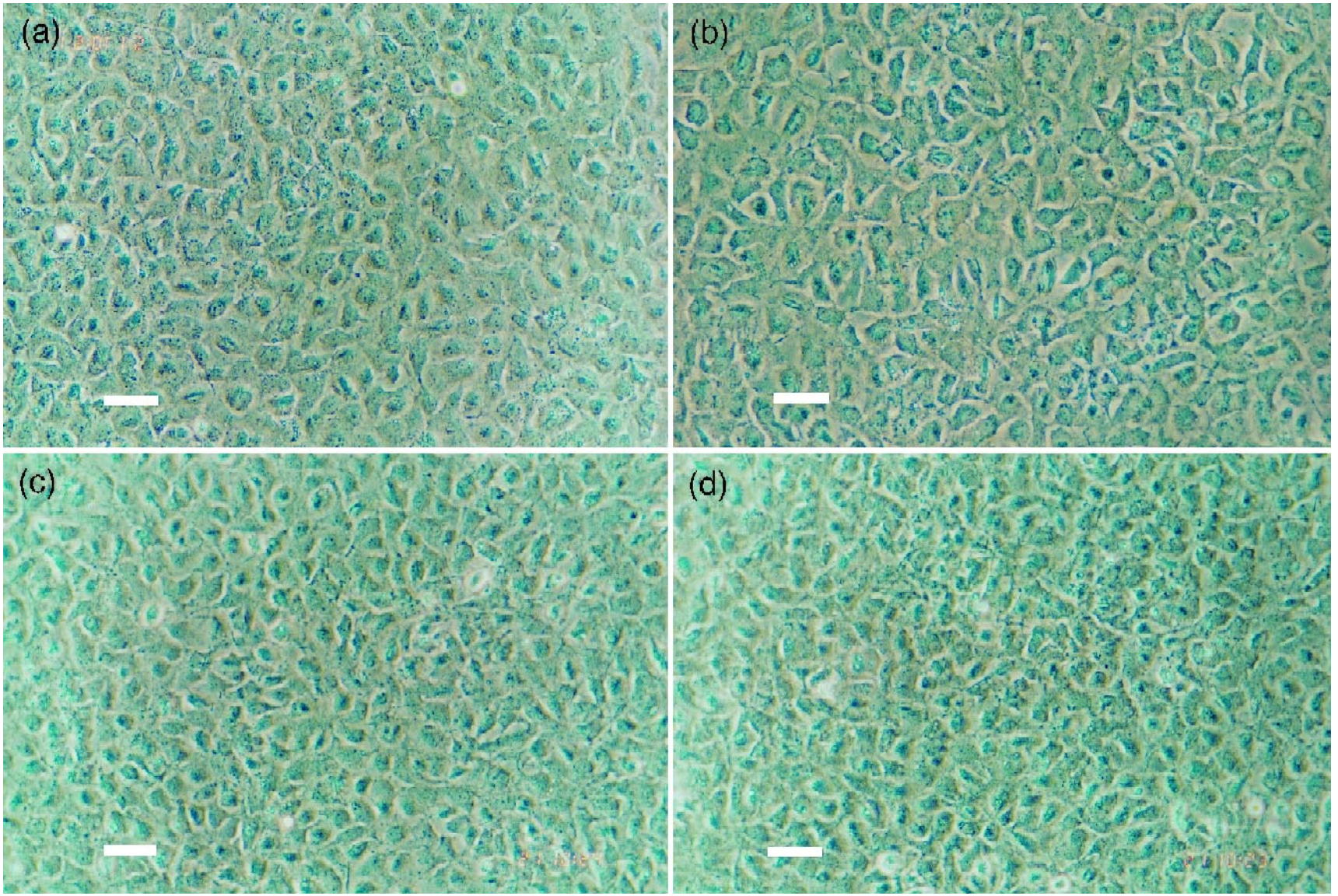

Fig. 2. Morphological analysis by phase-contrast microscopy of RHEC monolayers grown in 24-well plates. (a) Control monolayers show normal cobblestone appearance and no evidence of intercellular gaps. (b) Monolayers incubated for 30 min with serotonin (1 mM). Many cells retracted from one another, resulting in prominent intercellular gaps. (c) Monolayers incubated with 10^{-7} M YT-1 for 30 min. Cells are similar in appearance to those in (a). (d) Confluent monolayers were pretreated with 10^{-7} M YT-1 for 10 min and then challenged for an additional 30 min with 1 mM serotonin. Cells are similar in appearance to those in (c). Intercellular gaps are not evident. Bar = 50 μm.

were chosen for further experiments. Cyproheptadine, a 5-HT_2 receptor antagonist, abolished the serotonin-induced permeability to macromolecules, confirming that serotonin induced the passage of EB-BSA across RHEC monolayers by activating the serotonin receptor (data not shown). The PKC inhibitor staurosporine also caused dose-dependent inhibition of serotonin-induced permeability (Fig. 1A), indicating that PKC-mediated events are important to serotonin-induced permeability. In this study, the effect of YT-1 on the passage of EB-BSA across RHEC monolayers grown on porous filters was examined. YT-1 alone had no effect on the passage of EB-BSA across the monolayers in 30 min; however, serotonin (1 mM) caused an about 3-fold increase. This response was antagonized by 10 min pretreatment of the monolayers with YT-1 (10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M) (Fig. 1B). Examination of the concentration dependency of this inhibitory effect of YT-1 revealed an inverse bell-shaped dose–response curve such that 10^{-7}, 10^{-9} M were more effective than 10^{-11} and 10^{-6} M.

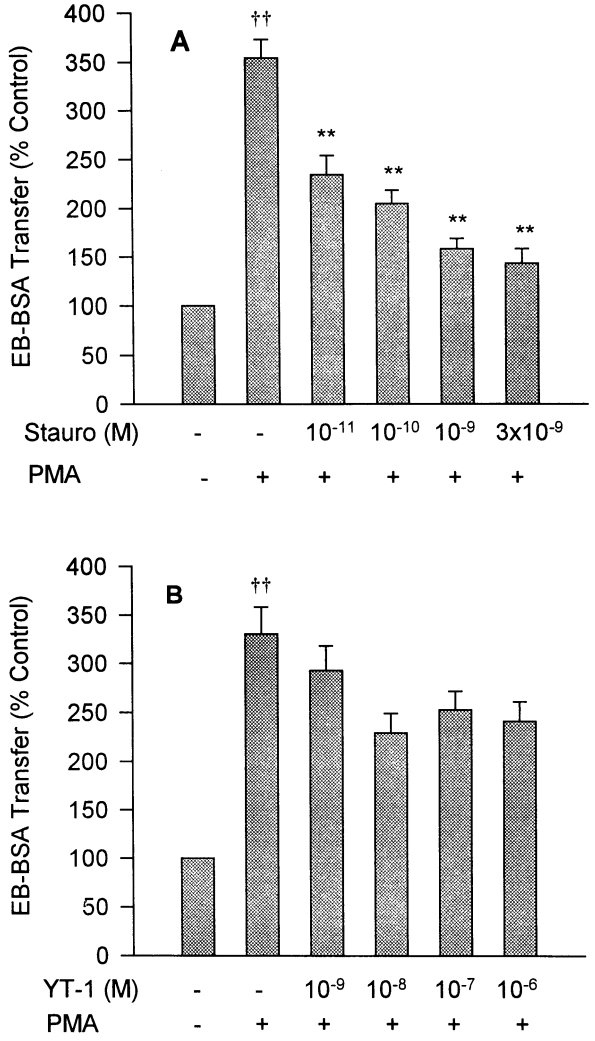

Fig. 3. Effects of staurosporine (Stauro) and YT-1 on PMA-induced RHEC monolayer barrier dysfunction. RHEC monolayers were pretreated with vehicle, staurosporine (A), and YT-1 (B) after 10 min, and the monolayers were challenged with 10^{-5} M PMA for 30 min. Each value represents the mean % control ± S.E.M. for the 15 independent experiments. $^{\dagger\dagger}P < 0.01$ compared to the control values. $^{**}P < 0.01$ compared to the PMA-treated monolayers.

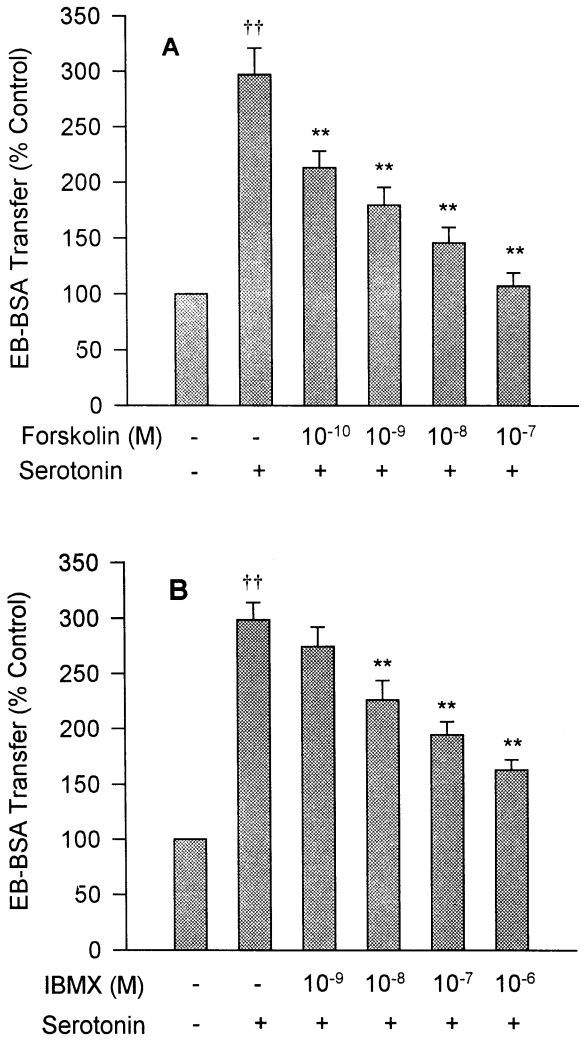

Fig. 4. Effects of increasing concentrations of forskolin (10^{-10}–10^{-7} M) (A) and IBMX (10^{-9}–10^{-6} M) (B) on the passage of EB-BSA through monolayers of RHEC after exposure to 1 mM serotonin. Cells were preincubated for 10 min in HBSS with 0.1% BSA in the presence of forskolin or IBMX and incubated for another 30 min after the addition of 1 mM serotonin. EB-BSA was measured after the monolayers were challenged with serotonin. Values represent the mean % control ± S.E.M. for the 12 independent experiments. $^{\dagger\dagger}P < 0.01$ compared to the control values. $^{**}P < 0.01$ compared to the serotonin-treated monolayers.

3.2. Effect of YT-1 on the serotonin-induced intercellular gap formation in RHEC monolayers

Phase-contrast microscopy showed that RHEC monolayers grown to confluency in plastic wells had a typical cobblestone pattern of contact-inhibited monolayers (Fig. 2a), in which intercellular gaps were not present. After a 30-min incubation with serotonin (1 mM) many cells

retracted, creating intercellular gaps (Fig. 2b). A 30-min incubation with YT-1 (10^{-7} M) did not alter this morphological profile. There were no morphological differences from the control (Fig. 2c). Similar to the cells shown in Fig. 2a, cells pretreated with YT-1 (10^{-7} M) and then challenged with serotonin exhibited a near-normal appearance without evidence of gap formation (Fig. 2d). This result indicates that YT-1 blocked the contractile interactions and prevented the evolution of morphologic changes elicited by serotonin.

3.3. Effects of staurosporine and YT-1 on PMA-induced barrier dysfunction in RHEC monolayers

Phorbol myristate acetate (PMA) is a potent stimulus for PKC activation. Treatment of RHEC monolayers with PMA induced a concentration-dependent increase in the passage of EB-BSA, with a maximal effect observed at 30 min with 10^{-5} M of PMA (data not shown). To further substantiate the role of PKC in PMA-induced barrier dysfunction, RHEC monolayers were pretreated (10 min) with the PKC inhibitor staurosporine (10^{-11}, 10^{-10}, 10^{-9}, 3×10^{-9} M) prior to challenge with PMA (10^{-5} M, 30 min). Staurosporine abolished the PMA-induced barrier dysfunction (Fig. 3A). To determine whether YT-1 affects PMA-mediated permeability of RHEC monolayers to macromoles, monolayers were preincubated with YT-1 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) for 10 min followed by PMA (10^{-5} M) for 30 min. YT-1 had no significant effect on the PMA-mediated passage of EB-BSA across the monolayers (Fig. 3B). These results indicate that YT-1 does not alter PMA-induced PKC activation.

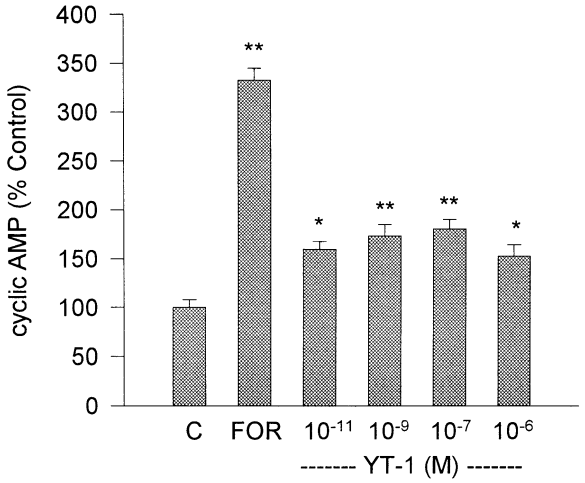

Fig. 5. Effects of increasing doses of YT-1 (10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M) and forskolin (For) (10^{-6} M) on the intracellular cAMP levels of RHEC monolayers ($n = 12$–16). The cells were incubated with YT-1 or forskolin for 10 min in the presence of IBMX, and the cAMP concentration was determined by enzyme immunoassay. Values represent the mean % control ± S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to the corresponding control values.

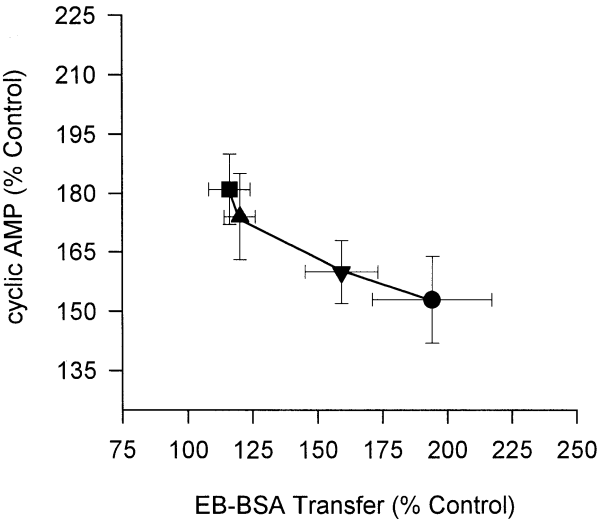

Fig. 6. Relationship between intracellular cAMP concentration and the passage of EB-BSA across RHEC monolayers exposed to (●) 10^{-6} M, (■) 10^{-7} M, (▲) 10^{-9} M, and (▼) 10^{-11} M YT-1. Data for cAMP concentration and endothelial permeability were taken from Figs. 1 and 5. A correlation between cAMP and endothelial permeability was found for YT-1. The reduction in permeability was paralleled by a relative increase in cAMP concentration. Values represent the mean % control ± S.E.M. for the number of experiments mentioned in Figs. 1 and 5, respectively.

3.4. Effects of forskolin and IBMX on the serotonin-induced passage of macromolecules across the RHEC monolayers

The possible role of cAMP as an intracellular mediator of serotonin-induced changes in endothelial permeability was investigated. The effect of agents that stimulate cAMP production at different stages of the cAMP amplification cascade on the permeability of endothelial monolayers was

Table 1
Effects of YT-1 and forskolin on SQ 22536-induced decrease in intracellular cAMP concentration in RHEC

Reagents	Cyclic AMP concentration [a] (% control)
Control (vehicle)	100
SQ 22536 (10^{-4} M)	87 ± 5 [b]
Forskolin (10^{-6} M)	333 ± 12 [c]
SQ 22536 + Forskolin (10^{-6} M)	286 ± 22 [d]
YT-1 (10^{-8} M)	176 ± 8 [c]
SQ 22536 + YT-1 (10^{-8} M)	147 ± 8 [d]
YT-1 (10^{-7} M)	183 ± 8 [c]
SQ 22536 + YT-1 (10^{-7} M)	138 ± 7 [d]

[a] RHEC were cultured to confluence in 24-well plates. The intracellular cAMP concentration was measured after preincubation with SQ 22536 (10^{-4} M, 10 min) prior to treatment with YT-1 or forskolin for 10 min ($n = 12$). The results are expressed as the mean % control ± S.E.M.
[b] $P < 0.05$, compared to the control values.
[c] $P < 0.01$, compared to the control values.
[d] $P < 0.01$, compared to the SQ 22536-treated monolayers.

determined. The addition of the adenylate cyclase activator forskolin caused a dose-dependent decrease in serotonin-induced endothelial permeability (Fig. 4A). The phosphodiesterase inhibitor IBMX also caused a decrease in serotonin-induced macromolecule passage across the endothelial monolayers (Fig. 4B). The results substantiated the involvement of intracellular cAMP in the modulation of RHEC permeability.

3.5. Effects of YT-1 or forskolin on the cAMP concentration in RHEC

To determine the specificity of the cAMP-YT-1 relationship on endothelial permeability, the intracellular cAMP concentration was measured. Confluent monolayers of RHEC were stimulated with forskolin (10^{-6} M, 10 min) and YT-1 (10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M, 10 min). In the presence of IBMX, a phosphodiesterase inhibitor, forskolin and YT-1 induced a increase in the cellular cAMP concentration (Fig. 5). The YT-1-induced change in intracellular cAMP levels was correlated with its effect on endothelial permeability (Fig. 6). There was a relationship between increasing cAMP levels and decreasing paracellular permeability, which suggests a cause-and-effect relationship. To further confirm the role of YT-1 in mediating changes in intracellular cAMP, the effect of the membrane-permeable adenylate cyclase inhibitor SQ 22536 was examined. In this study, SQ 22536 (10^{-4} M, 10 min) caused a significant decrease in endothelial cell cyclic AMP levels compared to those of control cells (Table 1). The endothelial cells were pretreated with SQ 22536 (10^{-4} M, 10 min) and then YT-1 (10^{-8}, 10^{-7} M) or forskolin (10^{-6} M) for 10 min. The response to pretreatment with SQ 22536 and then YT-1 compared with the response to SQ 22536 alone showed that YT-1 significantly reversed the SQ 22536 effect on cAMP concentration, as did forskolin (Table 1). It was also noted that the YT-1- or forskolin-induced increase in cyclic AMP was not significantly greater in the absence of SQ 22536 than that in the presence of SQ 22536. The results indicated that YT-1 reversed the activity of adenylate cyclase after being inhibited by SQ 22536. To investigate the possibility that YT-1 inhibited the increase in endothelial permeability seen after the cAMP content was decreased by inhibition of adenylate cyclase, the ability of YT-1 to affect the SQ 22536-mediated increase in permeability of RHEC to macromolecules was investigated. Pretreatment with YT-1 (10^{-8} M, 10 min) or forskolin (10^{-6} M, 10 min) attenuated the SQ 22536-mediated increase in permeability (Table 2). These data further demonstrate that the YT-1-induced enhancement of endothelial barrier function was correlated directly with increased intracellular cAMP levels, via the YT-1-mediated regulation of adenylate cyclase activity.

Table 2
Effects of YT-1 and forskolin on SQ 22536-induced increase in the permeability of RHEC to macromolecules

Reagents	EB-BSA passage [a] (% control)
Control (vehicle)	100
SQ 22536 (10^{-4} M)	226 ± 25 [b]
Forskolin (10^{-6} M)	89 ± 7
SQ 22536 + Forskolin (10^{-6} M)	101 ± 9 [c]
YT-1 (10^{-8} M)	96 ± 13
SQ 22536 + YT-1 (10^{-8} M)	105 ± 9 [c]
YT-1 (10^{-7} M)	— [d]
SQ 22536 + YT-1 (10^{-7} M)	— [d]

[a] RHEC cultured on filters were grown to confluence. Cells were pretreated with YT-1 or forskolin (10 min) and then with SQ 22536 (10 min). EB-BSA passage was determined at the end of experiment ($n = 12$). The results are expressed as the mean % control ± S.E.M.
[b] $P < 0.01$, compared to the control values.
[c] $P < 0.01$, compared to the SQ 22536-treated monolayers.
[d] —: not determined.

Table 3
Effects of YT-1 and sodium nitroprusside on intracellular cGMP levels in RHEC

Reagents	Cyclic GMP concentration [a] (% control)
Control (vehicle)	100
Sodium nitroprusside (10^{-6} M)	177 ± 5 [b]
YT-1 (10^{-9} M)	65 ± 14
YT-1 (10^{-7} M)	75 ± 9
YT-1 (10^{-6} M)	88 ± 9

[a] RHEC were cultured to confluence in 24-well plates. After a 10-min incubation, the intracellular cGMP level (four wells per treatment, $n = 8$) was assayed by using an enzyme immunoassay as described under Section 2. The results are expressed as the mean % control ± S.E.M.
[b] $P < 0.01$, compared to the corresponding control.

3.6. Effects of YT-1 or sodium nitroprusside on cGMP concentration in RHEC monolayers

This study tested the effect of YT-1 on the intracellular cGMP concentration in RHEC monolayers, on the assump-

Table 4
LDH release from rat heart endothelial cells

Reagents	LDH release (% control) [a]
Serotonin (2 mM)	99.9 ± 0.1
YT-1 (10^{-5} M)	99.9 ± 0.1
Cyproheptadine (3×10^{-5} M)	99.9 ± 0.2
Staurosporine (10^{-6} M)	99.7 ± 0.1
PMA (5×10^{-5} M)	99.8 ± 0.1
IBMX (10^{-5} M)	100.0 ± 0.1
Forskolin (10^{-5} M)	100.0 ± 0.1
SQ 22536 (10^{-4} M)	99.8 ± 0.2

[a] RHEC were cultured in 24-well plates and then incubated with various concentrations of reagent in HBSS with 0.1% BSA for 30 min. LDH activity in the supernatant was determined by using an LDH Detection Kit. The results are expressed as the mean % control ± S.E.M. ($n = 20$).

tion that the cGMP concentration is related to RHEC permeability. Confluent monolayers of RHEC were incubated with SNP (10^{-6} M, 10 min) and YT-1 (10^{-9}, 10^{-7}, 10^{-6} M, 10 min). In the presence of IBMX, SNP induced an increase in the cellular cGMP concentration. However, YT-1 had no effect on the endothelial cGMP concentration (Table 3).

3.7. Cytotoxicity assay

Treatment of RHEC with various agents for 30 min did not increase LDH release (Table 4). This finding indicates that the increase in endothelial permeability was not due to a cytolytic effect of these agents.

4. Discussion

The integrity of the endothelium plays an essential role in controlling vascular permeability. Factors which influence endothelial permeability directly affect the influx of macromolecules and solutes into tissues. Furthermore, endothelial cells are the first cells in the vasculature to be exposed to circulating substances. This study used rat heart endothelial cells to examine the role of YT-1 in the regulation of serotonin-induced permeability of endothelial cell monolayers to macromolecules. Morphological and functional studies of endothelial cells show characteristic similarities in the response of these cells to various inflammatory mediators; (a) a change in the shape of endothelial cells (intercellular gap formation) (Galdal et al., 1983; Baron et al., 1989; Garcia et al., 1995), (b) increase in endothelial permeability to macromolecules (Garcia et al., 1986, 1995; Rotrosen and Gallin, 1986; Brauneis et al., 1992; Patterson et al., 1994), and (c) activation of second messenger systems secondary to the breakdown of membrane phospholipids and generation of inositol phosphates and 1,2-diacylglycerol, and so on (Siess et al., 1984; Buchan and Martin, 1992; Garcia et al., 1995).

Various vasoactive mediators, including thrombin, bradykinin, histamine and serotonin, increase endothelial permeability in many endothelial cell cultures such as bovine pulmonary artery endothelial cells, human umbilical vein endothelial cells, bovine artery endothelial cells, bovine pulmonary microvascular endothelial cells, porcine pulmonary artery endothelial cells (Buchan and Martin, 1992; Schaeffer et al., 1993; Sheldon et al., 1993; Garcia et al., 1995). The data of this study indicate that serotonin caused a significant increase in the permeability of RHEC monolayers and the separation of adjacent cells with the resultant formation of large paracellular openings. The 5-HT_2 receptor antagonist, cyproheptadine, prevented the serotonin-induced permeability of monolayers to macromolecules. This indicates that the serotonin-induced endothelial cell contraction and increased permeability to macromolecules are receptor-mediated events. YT-1 has been previously shown to prevent serotonin-induced plasma extravasation in vivo (Wang et al., 1994). In order to define more exactly the inhibitory effect of YT-1 on serotonin-induced edema formation in vivo, the effects of YT-1 on serotonin-stimulated EB-BSA passage across RHEC was examined. The results of the present study indicate that YT-1 reverses the serotonin-mediated morphologic changes and increased permeability to macromolecules of endothelial cells.

The relationship between the activation of the second messenger pathways and the increase in endothelial permeability remains unclear. The activation of protein kinase C (PKC), which can occur as a result of the generation of 1,2-diacylglycerol (Nishizuka, 1984), decreases endothelial barrier function (Lynch et al., 1990; Stasek et al., 1992; Krizbai et al., 1995; Nagpala et al., 1996), suggesting that second messengers regulate endothelial barrier function via a PKC-dependent pathway. The loss of endothelial barrier function secondary to PKC activation is associated with the phosphorylation of specific cytoskeletal proteins and decreased cell–cell contacts (Stasek et al., 1992). The present study showed that the activation of rat heart endothelial cell PKC by PMA led to the dose-dependent increase in endothelial permeability to albumin, an effect that was inhibited by staurosporine (a PKC inhibitor). Staurosporine also attenuated the serotonin-induced increases in permeability, indicating that PKC-mediated events are important cellular mechanisms leading to serotonin-induced permeability. YT-1 had no effect on PMA-induced passage of macromolecules across the endothelial cell monolayers. It appeared that YT-1 did not directly affect the activation of PKC by serotonin. Therefore, it is likely that the beneficial effect of YT-1 on endothelial barrier function is subsequent to both 1,2-diacylglycerol release/PKC activation and IP_3 release/Ca^{2+} mobilization.

Recent studies have also demonstrated that elevation of the intracellular concentrations of cyclic nucleotides can improve the endothelial barrier function (Stelzner et al., 1989; Loffon et al., 1990; Milton and Knutson, 1990; Minnear et al., 1990; Langeler and Van Hinsbergh, 1991; Westendrop et al., 1994; Garcia et al., 1995). Patterson et al. (1994) have provided evidence that cholera toxin enhances endothelial barrier function through a cAMP-dependent process. Furthermore, other investigators demonstrated that cAMP protection did not alter transmembrane signal transduction prior to phospholipase C-mediated PIP_2 hydrolysis and subsequent Ca^{2+} mobilization (Jaffe et al., 1987; Garcia et al., 1992a,b; Patterson and Garcia, 1994; Patterson et al., 1994). Therefore, the mechanism by which YT-1 modulated endothelial permeability probably involved increases in the intracellular cAMP concentration. In this study, increases in cAMP after stimulation with forskolin and IBMX resulted in significant attenuation of the serotonin-mediated barrier dysfunction. These protective effects of cAMP suggest that the cAMP-dependent

protein kinase, PKA, the only known target of cAMP, modulates the serotonin-induced contraction and barrier dysfunction of endothelial cells. This study also showed that YT-1 increased the cAMP concentration in RHEC. Fig. 6 shows the direct correlation between the YT-1-induced increase in intracellular cAMP levels and the decrease in endothelial paracellular permeability. The present findings suggest that the YT-1 enhancement of endothelial barrier function is mediated in part by intracellular cAMP. This result is consistent with previous observations in which cAMP-enhancing agents enhanced endothelial barrier function (Stelzner et al., 1989; Patterson et al., 1994; Ma and Pedram, 1996). Furthermore, our study investigated the role of YT-1 in mediating the elevation of endothelial cAMP concentration. YT-1 significantly reversed the SQ 22536-mediated decrease in intracellular cAMP concentration and prevented the SQ 22536-induced increase in paracellular permeability. These results indicate that YT-1 may regulate adenylate cyclase activity and cause a decrease in endothelial paracellular permeability via enhancement of cAMP levels in endothelial cells.

Finally, this study investigated whether YT-1, by changing cell cGMP concentrations, attenuated the serotonin-induced permeability to macromolecules. Loffon et al. (1990) and Westendrop et al. (1994) have suggested that an increase in cGMP production prevents thrombin-induced increases in monolayer permeability. In this study, YT-1 had no effect on the intracellular cGMP concentration. It appears that the YT-1 regulation of endothelial permeability is not mediated by increases in intracellular cGMP levels.

In conclusion, in the system YT-1 attenuates the serotonin-indued permeability of RHEC to macromoles in association with elevated cAMP levels. This decrease in endothelial cell permeability may be one of the mechanisms for the protective effects of YT-1 against edema formation in the mouse in response to serotonin in vivo.

Acknowledgements

This study was supported by China Medical College and Taichung Veterans General Hospital.

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