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Decreased protein kinase C activation mediates inhibitory effect of norathyriol on serotonin-mediated endothelial permeability

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Abstract

We examined the mechanisms of norathyriol on the serotonin-induced increased permeability of rat heart endothelial cell monolayers. The present study showed that the activation of rat heart endothelial cell protein kinase C by phorbol myristate acetate led to the dose-dependent increase in endothelial permeability to albumin, an effect that was inhibited by staurosporine (a protein kinase inhibitor). Staurosporine also attenuated the serotonin-induced increase in permeability. Norathyriol abolished both serotonin- and phorbol myristate acetate-induced permeability. We investigated whether norathyriol, by inhibiting protein kinase C activation, attenuated the serotonin-induced permeability. Immunofluorescence studies demonstrated that norathyriol prevented the redistribution of protein kinase C isozymes following stimulation with serotonin. Western blot analysis showed that norathyriol significantly inhibited the serotonin-induced translocation of the α protein kinase C isozyme from the cytosolic to the particulate fraction. In conclusion, norathyriol attenuates the serotonin-induced permeability of rat heart endothelial cells to macromolecules in association with inhibition of protein kinase C activation. This decrease in endothelial cell permeability may be one of the mechanisms for the protective effects of norathyriol against edema formation in response to inflammatory agonists in vivo.

Keywords: Norathyriol; Endothelial cell, rat; 5-HT (5-hydroxytryptamine, serotonin); Permeability; Protein kinase C

1. Introduction

Vascular endothelial cells separate circulating blood from the surrounding vessel wall and tissue. One of the functions of these cells is to 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 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).

Evidence indicates that early signals converge on the family of protein kinase C (PKC) isozymes, which are critical in mediating the increase in transendothelial permeability (Lynch et al., 1990; Lum et al., 1993; Nagpala et al., 1996). Addition of phorbol myristate acetate (PMA), which activates PKC, to endothelial cell cultures disrupted intercellular junctions (Ben-Ze'ev, 1986; Mullin and O'Brien, 1986) and increased endothelial permeability to solutes (Ojakian, 1981; Lynch et al., 1990). Studies have demonstrated that inflammatory mediators which increase endothelial permeability, such as bradykinin, α-thrombin, and H_2O_2, also activate PKC (Lynch et al., 1990; Siflinger-Birnboim et al., 1992; Aschner et al., 1993). PKC inhibitors, such as H-7 or calphostin C, reduce vasoactive agent-induced increases in endothelial permeability (Lynch et al., 1990; Garcia et al., 1991; Siflinger-Birnboim et al., 1992).

Norathyriol (1,3,6,7-tetrahydroxyxanthone), an aglycon of a xanthone glycoside, mangiferin, isolated from the aerial parts of *Tripterospermum lanceolatum* (Hayata) Hara ex Satake (Gentianaceae) (Lin et al., 1982), was found to

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inhibit platelet aggregation (Teng et al., 1991), relax the rat thoracic aorta (Ko et al., 1991), inhibit cutaneous plasma extravasation (Wang et al., 1994a,b), and induce Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle (Kang et al., 1996). Recently, Wang et al. (1997) suggested that norathyriol inhibited the PMA-induced neutrophil respiratory burst and aggregation, and that these effects can be attributed to the suppression of PKC activity through blockade of the catalytic region. We have previously shown that PKC-mediated events are important cellular mechanisms leading to serotonin-induced permeability in rat heart endothelial cells (Lee, 1997). In this experiment, rat heart endothelial cell cultures were used to investigate the effect of norathyriol on edema formation. We demonstrated that the activation of endothelial PKC by serotonin resulted in significant changes in the localization and translocation of PKC isozymes. We also attempted to examine whether norathyriol attenuated the loss of barrier function in rat heart endothelial cell monolayers challenged with serotonin by inhibiting PKC activation.

2. Materials and methods

2.1. Materials

Norathyriol was isolated and purified as previously described (Lin et al., 1982). All culture media and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Bovine serum albumin (BSA), endothelial cell growth supplement, serotonin, staurosporine, phorbol myristate acetate (PMA), forskolin, Evans blue (EB), 3-isobutyl-1-methyl xanthine (IBMX), antipain, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), tris (hydroxymethyl) aminomethane (Tris), dithiothreitol, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) were purchased from Sigma (St. Louis, MO); Transwells (diameter 0.65 cm; pore size 3 μm) were from Corning Costar (Cambrige, MA); anti-Von Willebrand factor, human fibronectin, and lactate dehydrogenase (LDH) Detection Kit were from Boehringer Mannheim (Germany); Immobilon-P Transfer Membrane was from Millipore (Bedford, MA); the monoclonal antibodies against specific PKC isozymes-α, -β, -δ, -ε, -μ, -θ, -ζ and -λ used for immunocytochemistry were purchased from Transduction Laboratory (Lexington, KY); anti-γ PKC antibody was from Gibco; anti-mouse IgG peroxidase-conjugated secondary antibody, enhanced chemiluminescent (ECL) detection reagents and cyclic nucleotides enzyme immunoassay kit were purchased from Amersham (Buckinghamshire); sodium nitroprusside was from Merck (Taiwan). Serotonin was dissolved in Hanks' Balanced Salt Solution (HBSS) and other substances were dissolved in less than 0.5% dimethylsulfoxide (DMSO).

2.2. Rat heart endothelial cell culture preparation

Rat heart endothelial cells were isolated as described previously (Lee, 1997). Ventricles of 3 hearts were removed from 4-day-old donor rats. The tissue was then subjected to four successive trypsinization steps, with stirring, in a 50-ml trypsinization flask. After each trypsinization, free cells were removed and 2 ml of endothelial cell culture medium consisting of Dulbecco's modified essential medium (DMEM) and 20% fetal bovine serum (FBS) was added. 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 non-adherent 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% FBS was added to the adherent cells. All cultures had a typical contact-inhibited cobblestone appearance. Factor VIII-related antigen was confirmed by indirect immunofluorescence with human factor VIII antiserum and FITC-conjugated goat anti-mouse IgG as previously described (Jaffe et al., 1973). All experimental data were obtained from rat heart endothelial cells in their 2nd and 3rd passage.

2.3. Measurement of the barrier function of rat heart endothelial cell monolayers

Rat heart endothelial cells cultured on filters were used 3 days after seeding (1×10^5 cells/well). Exchange of macromolecules through the endothelial cell monolayers was investigated by assaying the transfer of EB–BSA (4% final concentration). Passage of EB–BSA through endothelial cell monolayers was performed as described previously (Lee, 1997). Briefly, endothelial cell monolayers were cultured on 48 Transwell polycarbonate membrane assemblies. 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 attached cells were washed twice by immersion in HBSS and transferred to 24-well plates. Thereafter, 600 μl of the 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 mono-

layers was quantified by measuring optical density at 600 nm.

2.4. Immunolocalization of PKC isozymes

Cells grown on fibronectin-coated coverslips were washed three times with phosphate-buffered saline (PBS) and then treated with various reagents. After treatment, cells were washed once with PBS, fixed for 5 min in a 1:1 mixture of acetone–methanol at −20°C, and subsequently washed twice with PBS. Cells were then incubated at room temperature for 1 h with 1% bovine serum albumin in PBS containing 0.1% Triton X-100 in order to minimize non-specific binding. Cells were then incubated overnight at 4°C with the PKC isozyme-specific antibodies diluted 1:20 in PBS containing 0.1% Triton X-100 and 2% bovine serum albumin. Cells were washed three times with PBS containing 0.1% Triton X-100 and incubated for 2 h with fluorescein-conjugated anti-mouse IgG antibody diluted 1:100 in PBS containing 0.1% Triton X-100. After three washes with PBS, slides were mounted by using glycerin. A Zeiss Axioskop microscope was used to record images on Kodak ISO 400 film with an exposure time of 60 s.

2.5. Western blot analysis

After treatment, endothelial cells were washed twice with HBSS and scraped, on ice, into ice-cold lysis buffer containing 20 mM Tris Cl, pH 8.0, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin, 1 mM dithiothreitol, and 2.5 mM phenylmethylsulfonyl fluoride. The cells were collected and sonicated for 10 pulses. The sonicated samples were centrifuged at $100\,000 \times g$ for 30 min at 4°C and the resulting supernatant was collected as the 'cytosolic' fraction. The pellet was resuspended in lysis buffer plus 0.5% Triton X-100, sonicated, and centrifuged as before. The supernatant was collected as the 'particulate' fraction. Equal amounts of protein (30 μg/lane) were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris, pH 9.0–9.4, 40 mM glycine, 0.375% SDS, 20% methanol) and electrotransferred to Immobilon-P Transfer Membranes. The blot was blocked with a solution containing 5% non-fat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl) with 0.05% Tween 20 (TBST) for 1 h, washed, and incubated with the PKC isozyme-specific antibodies in TBST with 0.5% non-fat dry milk for 1 h. The blot was washed and incubated with an anti-mouse IgG peroxidase-conjugated second antibody. The enhanced chemiluminescent (ECL) detection system was used for immunoblot protein detection.

2.6. Measurement of cyclic nucleotides

Cyclic nucleotides were measured by enzyme immunoassay as previously described (Lee, 1997). 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 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 M 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.

2.7. Assay of lactate dehydrogenase (LDH) release

LDH release from rat heart endothelial cell monolayers was determined. Endothelial cells grown on plastic tissue culture plates were exposed to various concentrations of reagents 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 using a commercial LDH Detection Kit.

2.8. Data analysis

Results were analyzed for statistical significance by analysis of variance with repeated measures and a Newman–Keuls test. A P-value of less than 0.05 was considered significant for all tests.

3. Results

3.1. Effects of norathyriol and staurosporine on the serotonin-induced passage of macromolecules across the rat heart endothelial cell monolayers

To investigate the inhibitory effect of norathyriol on edema formation, a vasoactive agent, serotonin, was added to rat heart endothelial cell cultures. Serotonin induced dose- and time-dependent increases in the passage of EB–BSA across endothelial cell monolayers (data not shown). The serotonin-induced increase in the amount of EB–BSA was significant at 1 mM serotonin and after a 30-min incubation. Therefore, 1 mM serotonin and a 30-min incubation period were chosen for further experi-

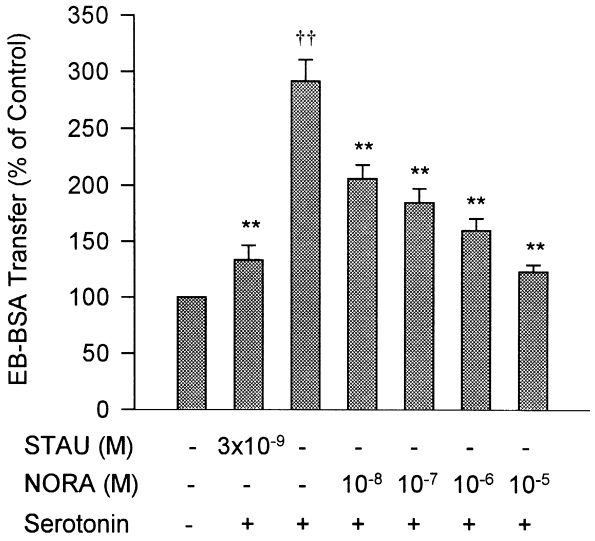

Fig. 1. Effects of staurosporine (STAU) and norathyriol (NORA) on serotonin-induced dysfunction of the rat heart endothelial cell barrier. Endothelial cell monolayers were pretreated with staurosporine (3×10^{-9} M) or norathyriol (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) for 10 min and then challenged for an additional 30 min with 1 mM serotonin. The results are expressed as the mean percentage of control ± S.E.M. ($n = 20$–25). $^{\dagger\dagger}P < 0.01$ compared to the control values. $**P < 0.01$ compared to the values for serotonin-treated monolayers.

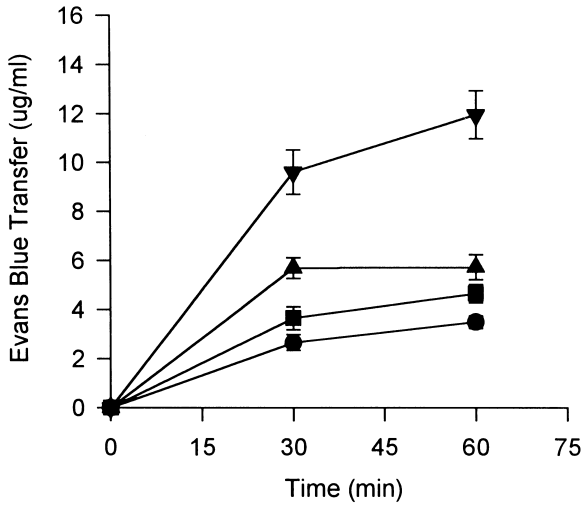

Fig. 2. Effects of PMA on the passage of EB–BSA through rat heart endothelial cell monolayers. Cells were incubated with vehicle (DMSO in HBSS) alone and with indicated PMA concentrations. EB–BSA transfer was measured at 30 and 60 min, respectively. Time course of effect of 10^{-7} M (■), 10^{-6} M (▲), 10^{-5} M (▼) PMA on the passage of EB–BSA. ●, control incubations. Values are means ± S.E.M. for 15 independent experiments.

ments. In this study, the effect of norathyriol on the serotonin-mediated increase in the passage of EB–BSA across endothelial cell monolayers grown on porous filters was examined. Norathyriol (10^{-5} M) alone did not increase the passage of EB–BSA across the monolayers in 30 min (data not shown); however, serotonin (1 mM) caused about a 3-fold increase. This response was antagonized by a 10-min pretreatment of the endothelial cell monolayers with norathyriol (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) (Fig. 1). To further substantiate the role of PKC in serotonin-induced barrier dysfunction, endothelial cell monolayers were pretreated (10 min) with the PKC inhibitor staurosporine (3×10^{-9} M) prior to challenge with serotonin. Staurosporine pretreatment abolished the serotonin-induced barrier dysfunction (Fig. 1).

3.2. Effects of norathyriol and staurosporine on PMA-induced barrier dysfunction in rat heart endothelial cell monolayers

To examine whether norathyriol promotes the barrier function of rat heart endothelial cell monolayers by inhibiting PKC activation, the PMA-induced passage of EB–BSA across the endothelial cell monolayer was investigated. PMA is a potent stimulant of PKC activation. Treatment of endothelial cell monolayers with PMA resulted in significant dose-dependent increases in EB–BSA permeability (Fig. 2). These results suggest that activation of the contractile apparatus, as demonstrated by alteration in endothelial monolayer permeability, may be dependent upon the activation of PKC. Endothelial cell monolayers were pretreated with the protein kinase inhibitor staurosporine (3×10^{-9} M, 10 min) prior to challenge with PMA (10^{-5} M, 30 min). Staurosporine abolished the PMA-mediated barrier dysfunction (Fig. 3). To determine whether norathyriol affects the PMA-mediated permeability of rat

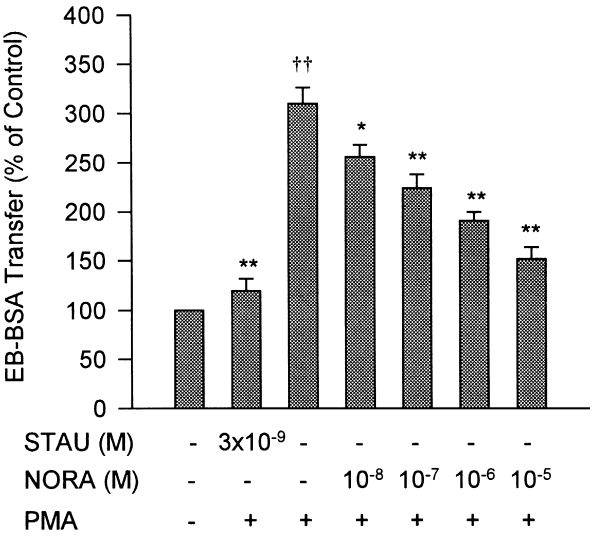

Fig. 3. Effects of staurosporine (STAU) and norathyriol (NORA) on PMA-induced dysfunction of the rat heart endothelial cell barrier. Endothelial cell monolayers were pretreated with vehicle (DMSO in HBSS), staurosporine (3×10^{-9} M), and norathyriol (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) after 10 min, and the monolayers were challenged with 10^{-5} M PMA for 30 min. Each value represents the mean percentage of control ± S.E.M. for 15 independent experiments. $^{\dagger\dagger}P < 0.01$ compared to the control values. $*P < 0.05$ compared to the values for PMA-treated monolayers. $**P < 0.01$ compared to the values for PMA-treated monolayers.

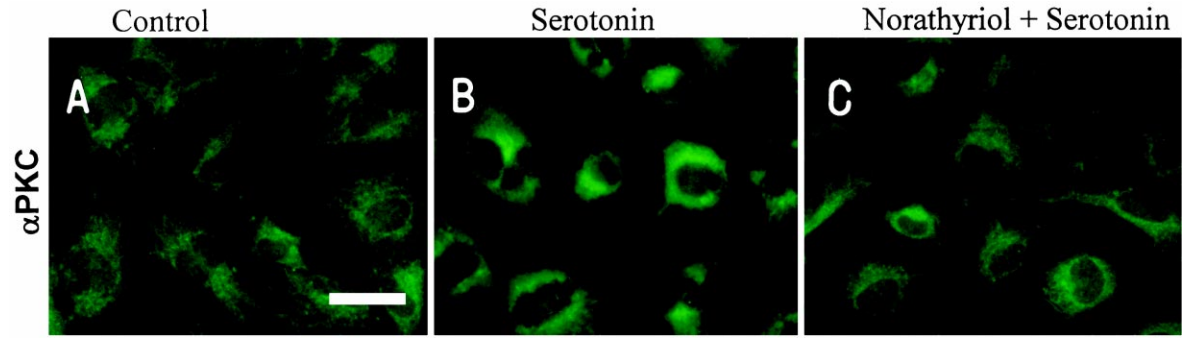

Fig. 4. Immunofluorescence localization of α PKC in rat heart endothelial cells. Cells were fixed and stained with isozyme-specific antibodies as described in Section 2. (A) Control cells (DMSO in HBSS, 10 min; then HBSS, 10 min); (B) serotonin-treated cells (DMSO in HBSS, 10 min; then 1 mM serotonin, 10 min); (C) norathyriol- and serotonin-treated cells (10^{-6} M norathyriol, 10 min; then 1 mM serotonin, 10 min). Results are representative of three independent experiments. Scale bar, 25 μm.

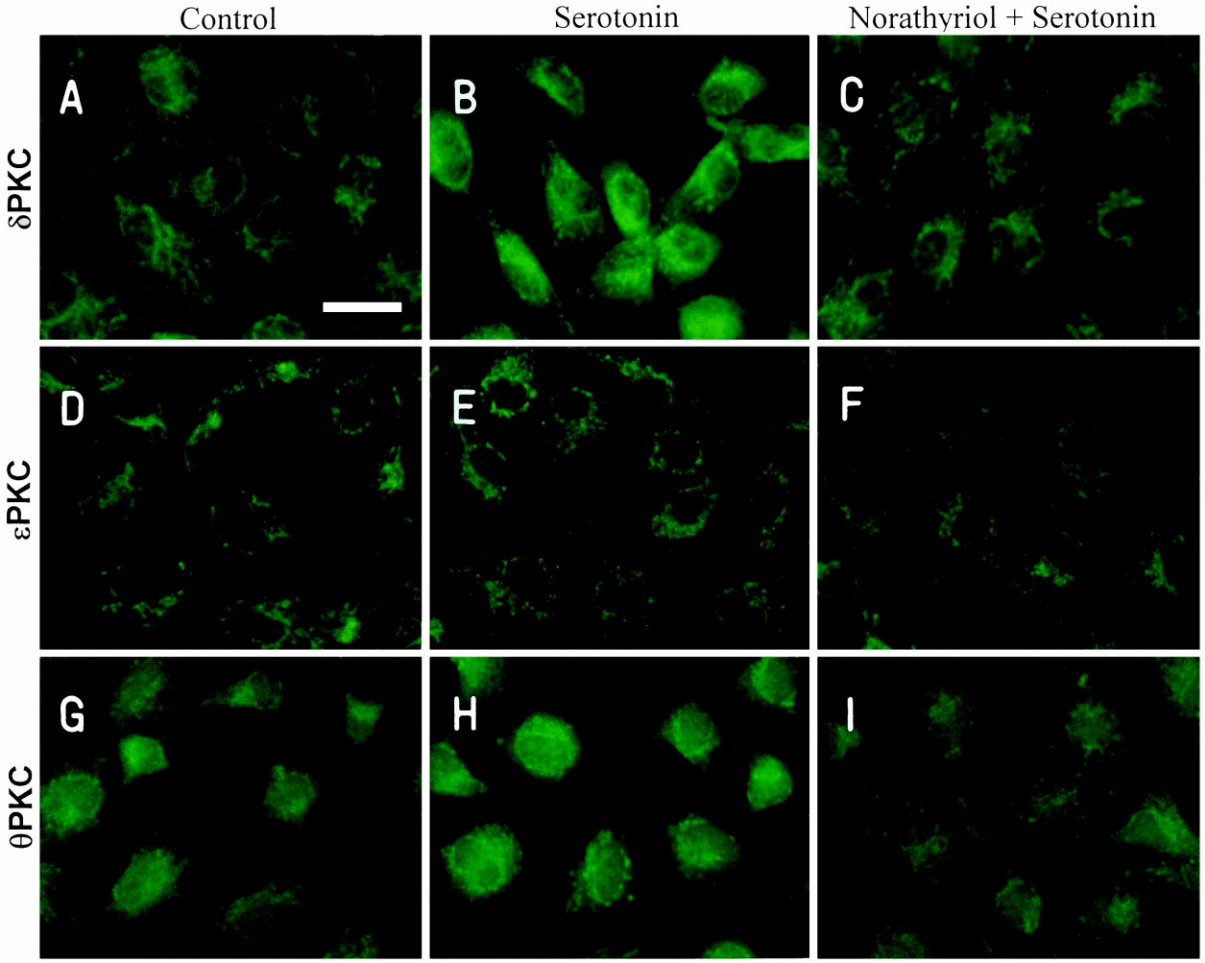

Fig. 5. Immunofluorescence localization of δ, ε, and θ PKC in rat heart endothelial cells. Cells were fixed and stained with isozyme-specific antibodies as described in Section 2. (A, D and G) Control cells (DMSO in HBSS, 10 min; then HBSS, 10 min); (B, E and H) serotonin-treated cells (DMSO in HBSS, 10 min; then 1 mM serotonin, 10 min); (C, F and I) norathyriol- and serotonin-treated cells (10^{-6} M norathyriol, 10 min; then 1 mM serotonin, 10 min). Results are representative of three independent experiments. Scale bar, 25 μm.

heart endothelial cell monolayers to macromolecules, monolayers were preincubated with norathyriol (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) for 10 min followed by PMA (10^{-5} M) for 30 min. Norathyriol had a significant effect on the PMA-mediated passage of EB–BSA across the monolayers (Fig. 3). These results indicated that the norathyriol-induced enhancement of endothelial barrier function was correlated with decreased activation of PKC.

3.3. Effects of norathyriol on the localization of serotonin-stimulated PKC isozymes in rat heart endothelial cells

Since PKC consists of a large family of proteins with multiple isozymes, we characterized the effect of norathyriol on the localization of the various PKC isozymes in rat heart endothelial cells after stimulation with serotonin. To determine the localization of PKC isozymes in rat heart endothelial cells, we performed immunofluorescence studies with isozyme-specific antibodies. Nonstimulated endothelial cells did not stain when the anti-β, anti-γ and anti-μ PKC antibodies were used (data not shown). These findings confirm the Western blot data in which β, γ and μ PKC immunoreactivity was not detected.

The immunoreactivity of α PKC appeared as diffuse staining throughout control cells (Fig. 4A). Incubation of endothelial cells with 1 mM serotonin for 10 min caused intense staining in the cytoplasm (Fig. 4B). As shown in Fig. 4C, 10^{-6} M norathyriol attenuated the serotonin-induced changes in α PKC immunoreactivity.

The δ PKC immunostaining was fibrillar throughout control cells (Fig. 5A). After treatment with serotonin, this pattern of staining was replaced by intense perinuclear staining (Fig. 5B). Monolayers pretreated with norathyriol and then challenged with serotonin showed staining similar to that seen in control cells (Fig. 5C). In the ε PKC staining studies, the immunostaining patterns were similar to those obtained with anti-ε PKC antibody in either nonstimulated (Fig. 5D) or stimulated cells (Fig. 5E,F). Studies with the anti-θ PKC antibody showed that the immunolocalization of θ PKC was primarily fibrillar (Fig. 5G). Serotonin-mediated θ PKC immunolocalization was more intense than that found in untreated and norathyriol-pretreated cells (Fig. 5H,I).

When endothelial cells were treated with anti-ζ PKC antibody, the immunostaining of control cells and norathyriol-pretreated cells was identical, showing a dotted staining pattern (Fig. 6A,C). However, after stimulation with serotonin the dotted staining of ζ PKC was most intense in the cytoplasm (Fig. 6B). With anti-λ PKC antibody the dotted staining was observed in control cells and norathyriol-pretreated cells (Fig. 6D,F). After treatment with serotonin, the filamentous staining in the cytoplasm was increased (Fig. 6E).

3.4. Effects of norathyriol and staurosporine on the expression and translocation of serotonin-stimulated PKC isozymes in rat heart endothelial cells

In this experiment, we examined the effect of norathyriol on the serotonin-induced translocation of the

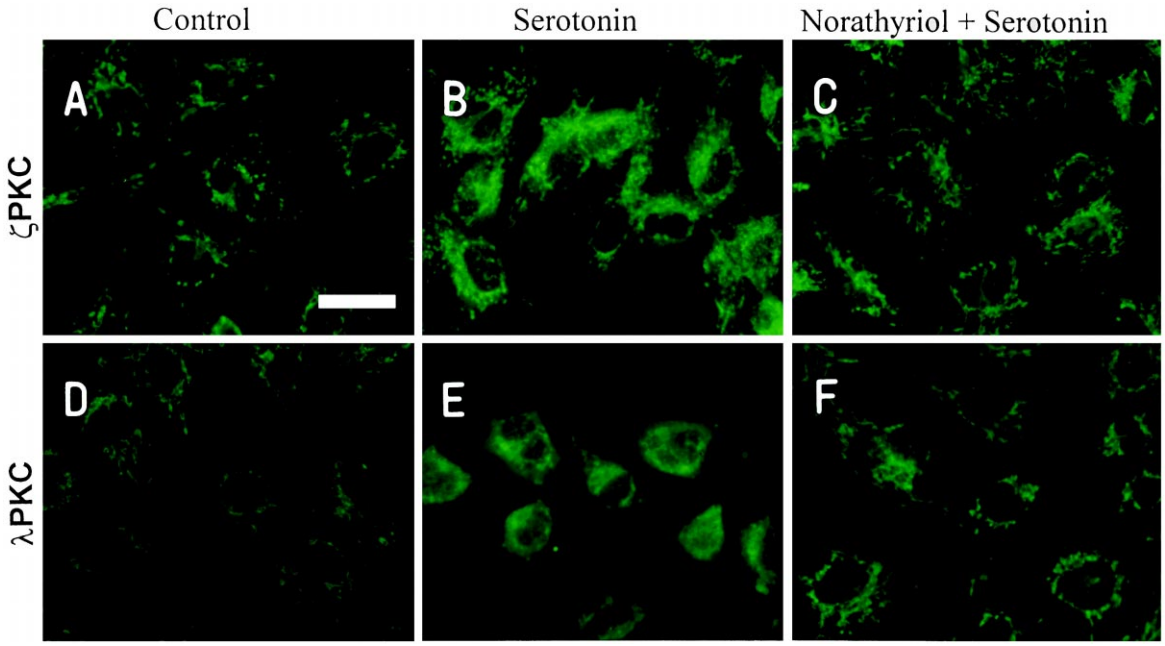

Fig. 6. Immunofluorescence localization of ζ and λ PKC in rat heart endothelial cells. Cells were fixed and stained with isozyme-specific antibodies as described in Section 2. (A and D) Control cells (DMSO in HBSS, 10 min; then HBSS, 10 min); (B and E) serotonin-treated cells (DMSO in HBSS, 10 min; then 1 mM serotonin, 10 min); (C and F) norathyriol- and serotonin-treated cells (10^{-6} M norathyriol, 10 min; then 1 mM serotonin, 10 min). Results are representative of three independent experiments. Scale bar, 25 μm.

various PKC isozymes from the cytosolic to the particulate fraction as another indicator of PKC activation. The expression of PKC isozymes in rat heart endothelial cells was examined by Western blot analysis using isozyme-specific anti-PKC antibodies. β, γ, and μ PKC isozymes were not found in rat heart endothelial cell extracts even when various dilutions of primary and secondary antibodies were used (data not shown). In contrast to these three isozymes, immunoreactive bands of four PKC isozymes, α, δ, ε and ζ, were clearly detected in the cytosolic and particulate fractions, whereas very faint immunoreactive bands of θ and λ PKC were observed (Fig. 7A). Isozymes δ, ε and θ, were mainly detected in the particulate protein fraction, whereas ζ PKC was detected in the cytosolic fraction. Isozymes α, δ, ε, ζ, θ and λ had apparent molecular masses of 82, 78, 90, 72, 79 and 74 kDa, respectively. When endothelial cell were treated with 1 mM serotonin for 10 min, cells exhibited extensive translocation of cytosolic α PKC to the particulate fraction (Fig. 7A). As illustrated in Fig. 7B, α PKC isozyme translocated to the particulate fraction, where levels increased 1.5- to 2-fold, as quantified by densitometric analysis. In addition, no significant changes in the content of other PKC isozymes were noted in either the cytosolic or the particulate fraction after serotonin stimulation (Fig. 7A). Monolayers were preincubated with staurosporine (3×10^{-9} M) for 10 min followed by 1 mM serotonin for 10 min. Staurosporine had no significant effect on the serotonin-mediated PKC translocation (Fig. 7A). However, after pretreatment of endothelial cell monolayers with 10^{-6} M norathyriol for 10 min, the serotonin-induced translocation of α PKC from the cytosolic to the particulate fraction was significantly inhibited (Fig. 7A,B). We also demonstrated the translocation of PKC isozymes in endothelial cells stimulated with 10^{-5} M PMA for 10 min. α PKC was largely found in the cytosolic fraction in unstimulated cells and was found almost entirely in the particulate fraction after treatment with PMA; δ, ε and θ PKC were always found in the particulate fraction whereas ζ PKC was found in both cytosolic and particulate fractions in the unstimulated cells and was unaffected by PMA (Fig. 8).

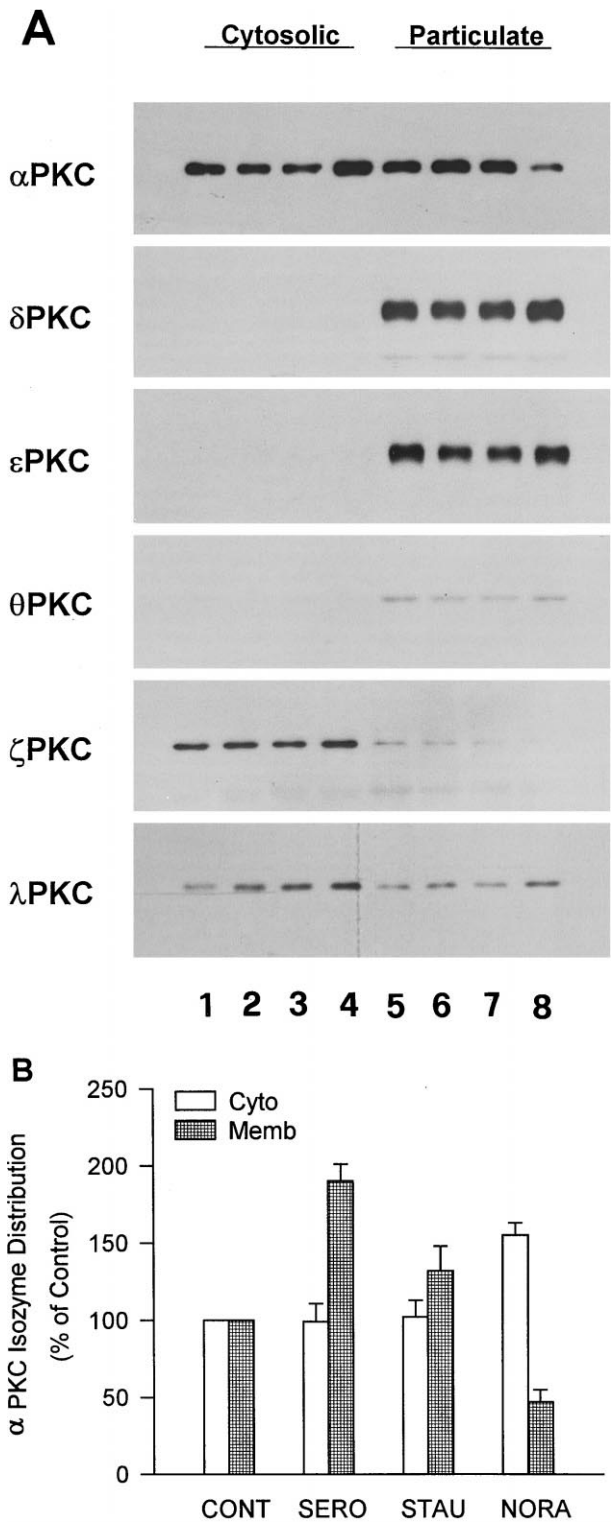

Fig. 7. (A) Western blot showing the effects of norathyriol and staurosporine on serotonin-induced PKC isozyme translocation in rat heart endothelial cells. Cytosolic and particulate fractions were analyzed by 7.5% SDS–PAGE and probed with antibodies against peptides specific for PKC isozymes. Western blot analysis with a mAb to detect PKC isozymes was performed as described in Section 2. Lanes 1 and 5, control cells (DMSO in HBSS, 10 min; then HBSS, 10 min); lanes 2 and 6, serotonin-treated cells (DMSO in HBSS, 10 min; then 1 mM serotonin, 10 min); lanes 3 and 7, staurosporine- and serotonin-treated cells (3×10^{-9} M staurosporine, 10 min; then 1 mM serotonin, 10 min); lanes 4 and 8, norathyriol- and serotonin-treated cells (10^{-6} M norathyriol, 10 min; then 1 mM serotonin, 10 min). Results are representative of three independent experiments. (B) Densitometric analysis of α PKC was carried out on autoradiographs. Data are plotted as percentages of control (absence of serotonin). Results are representative of three independent experiments.

3.5. Effects of norathyriol on cAMP and cGMP concentrations in rat heart endothelial cell monolayers

To further investigate whether the enhancement of endothelial barrier function by norathyriol could be linked to

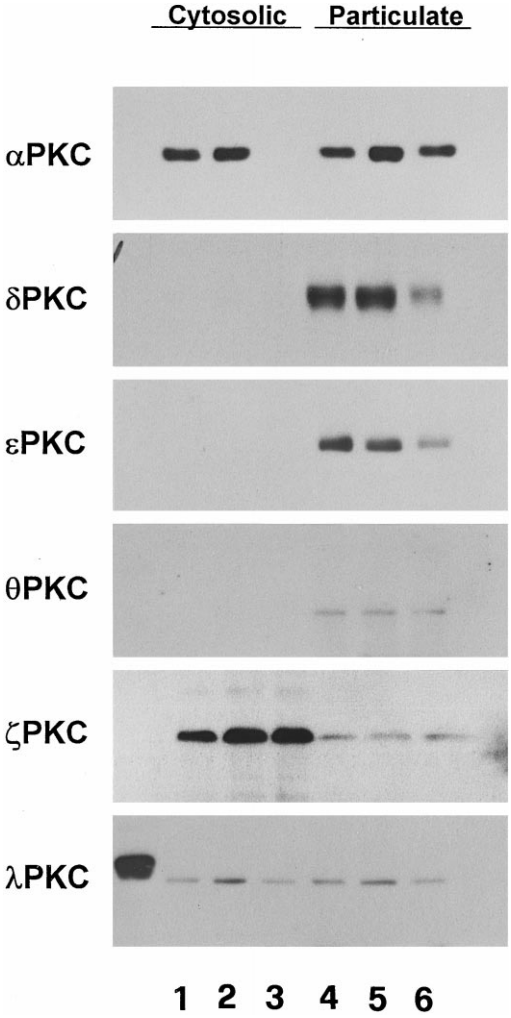

Fig. 8. Western blot showing the effects of 1 mM serotonin and 10^{-5} M PMA on PKC isozyme translocation in rat heart endothelial cells. Cytosolic and particulate fractions were analyzed by 7.5% SDS–PAGE and probed with antibodies against peptides specific for PKC isozymes. Western blot analysis with a mAb to detect PKC isozymes was performed as described in Section 2. Lanes 1 and 4, control cells (DMSO in HBSS, 10 min; then HBSS, 10 min); lanes 2 and 5, serotonin-treated cells (DMSO in HBSS, 10 min; then 1 mM serotonin, 10 min); lanes 3 and 6, PMA-treated cells (DMSO in HBSS, 10 min; then 10^{-5} M PMA, 10 min). Results are representative of three independent experiments.

Table 2
LDH release from rat heart endothelial cells

Reagents	LDH release (% of control)[a]
Control	100
Serotonin (2×10^{-3} M)	99.9 ± 0.1
Norathyriol (10^{-5} M)	99.5 ± 0.1
Staurosporine (10^{-6} M)	99.7 ± 0.1
PMA (10^{-5} M)	99.8 ± 0.1
IBMX (10^{-5} M)	100.0 ± 0.1
Forskolin (10^{-5} M)	100.0 ± 0.1

[a] Endothelial cells 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 percentage of control ± S.E.M. ($n = 20$).

stimulation of endothelial cAMP, we tested the effect of norathyriol on the intracellular cAMP concentration. Confluent monolayers of endothelial cells were stimulated with forskolin, an adenylate cyclase activator (10^{-6} M, 10 min), and norathyriol (10^{-8}, 10^{-6}, 10^{-5}, 10^{-4} M, 10 min). In the presence of IBMX, a phosphodiesterase inhibitor, forskolin induced an increase in the cellular cAMP concentration. Norathyriol had no effect on the endothelial cAMP concentration (Table 1). The effect of norathyriol on the intracellular cGMP concentration in endothelial cell monolayers was also investigated on the assumption that the cGMP concentration is related to endothelial cell permeability. Confluent monolayers of endothelial cells were incubated with sodium nitroprusside, a guanylate cyclase activator (10^{-6} M, 10 min), and norathyriol (10^{-8}, 10^{-6}, 10^{-5}, 10^{-4} M, 10 min). In the presence of IBMX, sodium nitroprusside induced an increase in the cellular cGMP concentration. However, norathyriol had no effect on the endothelial cGMP concentration (Table 1).

3.6. Cytotoxicity assay

Treatment of rat heart endothelial cells with various agents for 30 min did not increase LDH release (Table 2). This finding indicates that the increase in endothelial

Table 1
Effects of norathyriol on intracellular cAMP and cGMP levels in rat heart endothelial cells

Reagents	cAMP concentration[a] (% of control)	cGMP concentration[a] (% of control)
Control	100	100
Forskolin (10^{-6} M)	293 ± 25[b]	–[c]
Sodium nitroprusside (10^{-6} M)	–[c]	233 ± 25[b]
Norathyriol (10^{-8} M)	89 ± 10	102 ± 10
Norathyriol (10^{-6} M)	87 ± 11	101 ± 11
Norathyriol (10^{-5} M)	95 ± 9	106 ± 8
Norathyriol (10^{-4} M)	84 ± 12	103 ± 7

[a] Endothelial cells were cultured to confluence in 24-well plates. After a 10-min incubation, the intracellular cAMP and cGMP levels ($n = 8$) were assayed by using an enzyme immunoassay as described under Section 2. The results are expressed as the mean percentage of the relative control ± S.E.M.
[b] $P < 0.01$, compared to the corresponding control.
[c] Not determined.

permeability was not due to a cytolytic effect of these agents.

4. Discussion

Vascular endothelial cells separate circulating blood from the surrounding vessel wall and tissues. The endothelium functions as a critical and selective barrier to macromolecules, protects the underlying tissues from edema, and preserves organ function. Endothelial cell contraction plays a pivotal role in the increased extravasation of fluid and macromolecules during vascular leakage. Many investigators suggest that PKC activation is an important signal transduction pathway by which extracellular mediators increase endothelial macromolecule transport (Lynch et al., 1990; Stasek et al., 1992; Krizbai et al., 1995; Nagpala et al., 1996). The activation of protein kinase C, which can occur as a result of the generation of 1,2-diacylglycerol (Nishizuka, 1984), is associated with the phosphorylation of specific cytoskeletal proteins and decreased cell–cell contact (Stasek et al., 1992). The relationship between the activation of second messenger pathways and the increase in endothelial permeability remains unclear. The present study showed that the activation of rat heart endothelial cell PKC by PMA led to a dose-dependent increase in endothelial permeability to albumin, an effect which was inhibited by staurosporine (a protein kinase inhibitor). The result is in agreement with other studies, thereby establishing the importance of PKC activation in mediating the increase in endothelial permeability to albumin (Noel et al., 1995; Patterson et al., 1995; Rabiet et al., 1996; Siflinger-Birnboim and Malik, 1996). Staurosporine also attenuated the serotonin-induced increase in permeability, indicating that PKC-mediated events are important cellular mechanisms leading to serotonin-induced permeability. Norathyriol attenuated both serotonin- and PMA-induced permeability. It appeared that norathyriol directly affected the activation of PKC by serotonin.

PKC constitutes a family (α, β, γ, δ, ε, ζ, θ, ι, λ and μ) of related isozymes (Nishizuka, 1988, 1989). They consist of a single polypeptide with an amino-terminal regulatory domain and a carboxy-terminal catalytic domain (Kikkawa et al., 1987; Ono et al., 1988). Differences in tissue distribution, subcellular localization, and translocation following activation have also been demonstrated (Mochly-Rosen et al., 1990; Disatnik et al., 1994). The localization of PKC has been determined in a number of endothelial cell types (Rosales et al., 1992; Zhou et al., 1996). As previously suggested, it is likely that differentially localized isozymes phosphorylate nearby substrates (Mochly-Rosen, 1995). The variety in the expression of PKC isozymes in endothelial cells of different types may reflect differences in functional responsiveness to environmental stimuli. The present study showed that rat heart endothelial cells contain various amount of PKC isozymes α, δ, ε, ζ, θ and λ but not β, γ and μ. It is also possible that other PKC isozymes which were not studied are present. The immunofluorescence studies demonstrated that the localization of the PKC isozymes following stimulation with serotonin differed from that of untreated cells, with the exception of ε PKC isozyme. This is consistent with the notion that not all PKC isozymes in endothelial cells and other cell types such as platelets are translocated after stimulation with an agonist. The explanation for the difference among the various PKC isozymes is not clear but could be multiple, ranging from differences in time of activation and degradation to different efficiencies of the various antibodies. Further studies to identify isozyme-unique substrates at each of these isozyme sites are underway. The serotonin-induced change in PKC localization was prevented by norathyriol. In our studies, the expression of PKC isozymes in endothelial cells was also examined by Western blot analysis with isozyme-specific anti-PKC antibody. PKC is primarily a cytosolic enzyme in quiescent cells (Nishizuka, 1984; Wolfson et al., 1985; Lynch et al., 1990). Upon activation PKC covalently binds to cell membranes, where it exerts its effects (Nishizuka, 1984). Endothelial PKC activation was demonstrated as a characteristic shift in PKC from the cytosol to the cell membrane. In the present study, serotonin caused the translocation of α PKC from the cytosol to the membrane of rat heart endothelial cells. No significant changes in the distribution of other PKC isozymes in either the cytosolic or particulate fraction was seen after serotonin stimulation. The difference in the expression of PKC isozymes between the immunofluorescence studies and the Western blot analysis remains unclear. However, it is interesting to note that the serotonin-induced translocation of the α PKC isozyme from the cytosolic to the particulate fraction was found to be significantly inhibited by norathyriol. From these data, we demonstrated that norathyriol inhibited the activation of the PKC isozymes following stimulation with serotonin.

Finally, the study investigated whether norathyriol, by changing the concentrations of cyclic nucleotides, attenuated the serotonin-induced permeability to macromolecules. Recent studies have demonstrated that elevation of the intracellular concentrations of cyclic nucleotides can improve endothelial barrier function (Stelzner et al., 1989; Loffon et al., 1990; Langeler and Van Hinsbergh, 1991; Westendrop et al., 1994). In this study, norathyriol had no effect on the intracellular cAMP and cGMP concentrations. These results appear to suggest that the regulation of endothelial permeability by norathyriol is not mediated by increases in the levels of intracellular cyclic nucleotides.

In conclusion, this study showed that the translocation of α PKC from the cytosolic to the particulate fraction may be responsible for the increase in endothelial permeability in response to serotonin stimulation. Norathyriol attenuated the serotonin-induced permeability of endothelial cells to macromolecules in association with inhibition of PKC activation. The results support the hypothesis that

norathyriol has an enhancing effect on endothelial barrier function and prevents the disruption of endothelial barrier function by inflammatory agonists such as serotonin. These findings suggest a possible mechanism by which norathyriol protects endothelial cell function and preserves the microvasculature from pharmacologic injury caused by injurious agents.

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